

Edited by

GABOR KOVACS
ANTHONY RUTHERFORD
DAVID K. GARDNER

HOW TO PREPARE THE EGG AND EMBRYO TO MAXIMIZE IVF SUCCESS

Physiology of Ovulation

W. Colin Duncan

1.1 The Follicle

At the time of menstruation there are numerous antral follicles in the ovaries measuring 4–8 mm in diameter. These follicles have developed from primordial follicles through gonadotrophin-independent and gonadotrophin-sensitive phases of growth. It is difficult to determine how long this process takes *in vivo*, but evidence from tissue transplantation studies suggests that it is longer than three months. These antral follicles are gonadotrophin-dependent and will not grow further without gonadotrophin stimulation.

The rate of gonadotrophin-dependent follicle growth can vary from cycle to cycle and woman to woman. When the lead follicle reaches 12 mm in diameter, generally about day 9 or 10 of the menstrual cycle, it then grows at an average of 2 mm in diameter each day. This growth is associated with rapidly increasing oestradiol concentrations. Oestradiol stimulates endometrial growth and exerts negative feedback on the pituitary to reduce follicle stimulating hormone (FSH) secretion.

Follicles more than 12 mm in diameter will already be developing LH receptors on granulosa cells and luteinizing hormone (LH) will maintain follicle growth and function in the presence of declining FSH concentrations. Growth of smaller follicles will not progress when FSH concentrations decline. This mechanism is responsible for follicular selection to promote the selection of a single dominant follicle.

Clinical Correlation

Using the developing LH receptors on granulosa cells as a marker of the ability of a follicle to respond to the LH surge: follicles of 12 mm diameter may ovulate, and follicles reaching 14 mm or more generally will ovulate in response to an LH surge.

1.1.1 Follicular Formation

The primordial follicles represent the quiescent gamete stores within the ovary. They consist of an oocyte surrounded by a flattened layer of somatic pre-granulosa cells that reside within the ovarian cortex. They are formed before birth and peak in number in the mid-gestation fetal ovary [1].

The fetal gonad starts to develop around the fourth week of gestation. Extraembryonic primordial germ cells from the yolk sac enter the embryo along the hindgut and migrate to the developing ovary. They proliferate extensively, and by ten weeks of gestation there are

around 250,000 germ cells in the ovaries. These germ cells differentiate into oogonia and undergo further mitosis within germ cell clusters. These become oocytes that enter meiosis, which arrests at prophase I. The germ cell clusters break down and there is a marked loss of oocytes. The remaining oocytes associate with a single layer of somatic cells to form primordial follicles. The first follicles in the fetal ovary can be detected from around 16 weeks of gestation.

Although oogonial stem cells in adult ovaries have been described in women, there is no compelling evidence for clinically significant follicle formation in postnatal life. All the follicles that will be ovulated are formed in fetal life and can stay quiescent for over 50 years [2].

Clinical Correlation

New follicles cannot be made in women with a depleted ovarian reserve as a consequence of genetics, age or iatrogenic insult.

1.1.2 Follicular Activation and Growth

From mid-gestation and throughout postnatal and reproductive life some primordial follicles will activate and start growing. Once follicular growth begins the follicle has one of two fates: ovulation or atresia. At birth there are approximately 1 million follicles within the ovaries and by the time of menarche half of these will have activated and have undergone atresia. Overall, very few follicles will ovulate: just over 400 in an uninterrupted reproductive lifespan.

The first stage of follicular growth is the transition of a primordial follicle into a primary follicle. This is characterized by enlargement, then proliferation of the somatic cells, and an increase in the size of the oocyte. When the follicle has acquired three to six layers of granulosa cells, some stromal cells near the basal lamina become aligned parallel to each other around the primary follicle. These fibroblast-like cells change into epithelioid-like cells, capable of steroidogenesis, and stratify into the theca cell layers. At this stage the follicles develop an independent blood supply. This is the preantral stage of follicular development [3].

The appearance of the antral cavity starts with the development of small fluid-filled spaces, within the granulosa cell layer, that coalesce to form the antrum. This is the secondary follicle and from this point onwards there is a specific group of granulosa cells that surround the oocyte. These granulosa cells form the cumulus cells that have cytoplasmic connections to the oocyte. There appears to be a stratification of the rest of the granulosa cells, as those nearest the basement membrane become more columnar in shape. At this stage the follicles measure 180–250 µm in diameter.

Through the accumulation of fluid in the antral cavity and the proliferation of granulosa and theca cell layers, the follicles continue to grow until they measure between 2 and 5 mm in diameter. This process takes at least three months *in vivo*, and these small antral follicles can be found at all stages of the ovarian cycle. The oocyte enlarges over this time from 30 µm to 120 µm in diameter as RNA is accumulated and protein is synthesized. In addition, at this stage the zona pellucida, a thick glycoprotein surrounding the oocyte, becomes fully developed (Figure 1.1).

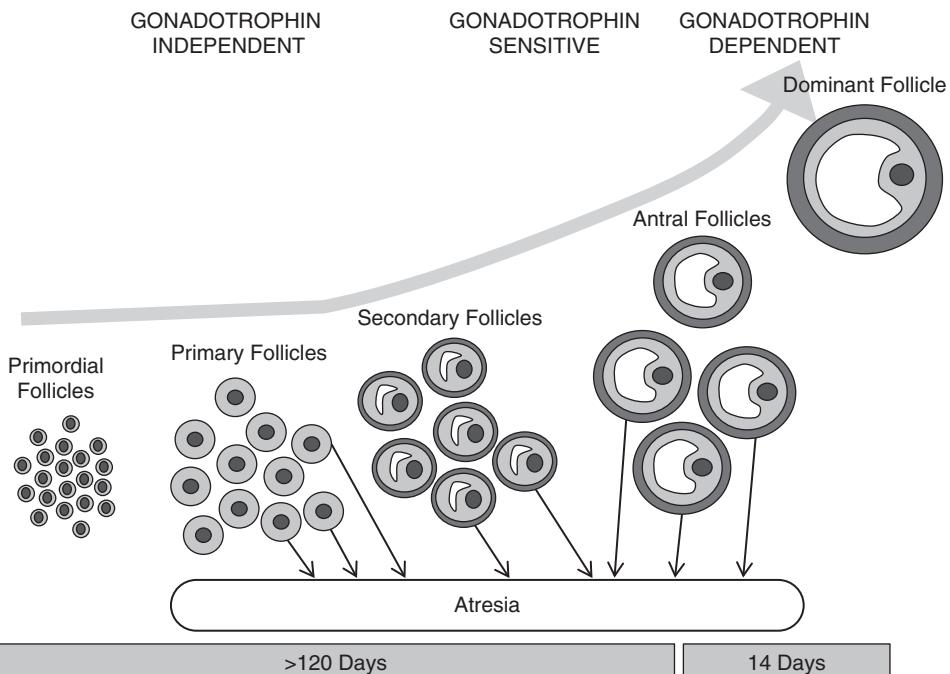


Figure 1.1 Illustration of follicular activation and growth in women. After activation the fate of primordial follicles is either atresia or ovulation. Over more than three months the follicles develop through gonadotrophin-independent, sensitive and dependent stages. The final gonadotrophin-dependent stage is only around two weeks in length and a fraction of the time follicles are growing

Clinical Correlation

Small antral follicles identified in the early follicular phase by ultrasonography have been growing for at least three months.

1.1.3 Regulation of Follicular Activation and Growth

Each day up to 30 primordial follicles are recruited into the pool of growing follicles. It is not entirely certain what causes this activation, but it is clear that it is pituitary independent. Follicular recruitment, and growth to the antral phase, occurs in the absence of gonadotrophins and is seen in fetal life, prepubertally, during pregnancy and while breast feeding, as well as when on hormonal contraception.

Follicular activation is controlled by autocrine/paracrine mechanisms. Whether it is induced by a stimulator or the loss of an inhibitor is uncertain; however, primordial follicles undergo rapid recruitment when removed from the ovary and cultured in vitro, and the chance of activation is increased when there is a reduction in the density of neighbouring follicles. These observations support the theory that activation is by removal of local inhibition [4].

The nature of the inhibitor(s), however, is uncertain. In rodents there is evidence for a role of anti-Müllerian hormone (AMH) in the initiation of follicular activation, but in larger mono-ovulatory animals AMH seems to slow down the growth of follicles rather than

activate them. Certainly there is a role for PTEN, which suppresses the PI3K-AKT-mTORC1 pathway. Inactivation of PTEN promotes activation, but how this is regulated in vivo is not known [5].

Once activated, follicular growth is regulated by multiple local factors. The rate of growth depends on the balance between factors that promote and factors that inhibit growth. In vitro it appears that some inhibitory ones are lost and follicular growth is faster. Multiple intraovarian molecules and pathways have been shown to regulate the initial stages of follicular growth, and these include the insulin growth factor (IGF) and transforming growth factor (TGF) β families as well as many other growth factors produced by ovarian somatic cells, stromal cells, vascular and neural cells or the oocyte. The complex interplay between local factors involved in the activation and initial growth of the follicle remains inadequately understood [3,4].

Clinical Correlation

As yet we don't have the capacity to manipulate the pathways involved in the initiation of follicular activation and growth in vivo, and mimicking them in vitro remains experimental.

1.1.4 Function of Small Growing Follicles

These small growing follicles are not steroidogenically active, but they do synthesize and secrete autocrine, paracrine and endocrine molecules. These molecules are generally local regulators of follicular development and growth. AMH is one of these molecules that is of particular importance. While the physiological role of AMH remains unclear, it has particular utility as a biomarker of ovarian reserve.

AMH is made in the granulosa cells of growing preantral and small antral follicles. As this growth is not dependent on gonadotrophins AMH will generally remain constant throughout the menstrual cycle and still be made when gonadotrophins are suppressed. Quantification of AMH in the serum correlates with the number of small growing follicles and can be used as a biomarker of ovarian reserve, ovarian morphology and capacity to respond to exogenous stimulation [6].

Small growing antral follicles also secrete inhibin B. This will feedback to lower FSH concentrations in the early follicular phase and it is the reason why FSH increases, despite no alteration in oestradiol concentrations, when women age. It also functions as a biomarker of ovarian reserve and is more consistent than measurement of FSH. However, it does vary across the menstrual cycle, and as it is made of two subunits, which can contribute to other molecules, it is more challenging to measure. Clinically it is inferior to AMH as a marker of ovarian reserve and is not routinely used [7].

Clinical Correlation

AMH is not a marker of the primordial follicle pool. A treatment, such as some chemotherapy regimes, that depletes the number of growing follicles but not the primordial pool would involve a decline and then recovery of AMH.

1.1.5 Gonadotrophin Sensitivity

When the follicles reach around 3–5 mm in size they become dependent on gonadotrophins for their further growth and development. However, both LH and FSH receptors can be

detected on follicles much smaller than this. That means that there is a stage where follicles are sensitive to LH and FSH but not dependent on them for their survival. It is likely however that gonadotrophins can promote growth in the smaller follicles before gonadotrophin dependence. There is a role for gonadotrophins in the growth and development of follicles before they become entirely dependent on them for survival [3].

Clinical Correlation

In prolonged gonadotrophin-releasing hormone (GnRH)-induced down-regulation, or in women without a functioning pituitary, gonadotrophins are required for a longer duration to stimulate follicular growth.

1.1.6 Gonadotrophin dependence

The small antral follicles present on the ovary at the time of menstruation express both functional FSH and LH receptors. These follicles require gonadotrophin stimulation to grow further. During the preceding luteal phase progesterone, oestrogen and inhibin A cause negative feedback to lower gonadotrophins. This prevents follicular growth beyond the small antral phase and eventual atresia of a cohort of follicles. The fall in steroids and inhibin A at menstruation increases gonadotrophins and stimulates the growth of a cohort of follicles that enter the gonadotrophin-dependent phase of growth [8].

Clinical Correlation

The antral follicle count (AFC) gives an indication of the maximal potential response to ovarian stimulation with exogenous FSH.

1.1.7 Gonadotrophin Regulation of Follicular Growth

The size of the follicle destined for ovulation increases greatly during the follicular phase of the ovarian cycle by cellular multiplication and accumulation of follicular fluid. This process requires trophic stimulation by FSH. The diameter of the preovulatory follicle increases from 6.9 ± 0.5 mm in the early follicular phase to 18.8 ± 0.5 mm in the late follicular phase. During this period, the mean number of granulosa cells increases from approximately 2–5 million in the early follicular phase to 50–100 million at the time of ovulation. This growth occurs during the follicular phase of the ovarian cycle and is completed within 15 days.

There is a direct correlation between the size of the follicle and its blood supply. The preovulatory follicle becomes a highly vascular structure because of active endothelial cell proliferation in the theca cell layers. The granulosa cells remain separated from the theca cells by a basement membrane and remain avascular. The granulosa cells obtain their nutrients by diffusion, and the thickness of the granulosa cell layer in the dominant follicle is, therefore, limited to a maximum of seven cells [9].

FSH binds to its cell surface receptor on granulosa cells and activates adenylyl cyclase. As well as stimulating cell growth, FSH induces proteins involved in steroidogenesis, such as aromatase (CYP19). This means that there is a marked increase in steroidogenic capacity

as the follicle matures. It has become increasingly clear that the ovarian cellular responses to gonadotrophins can be modified by factors that are produced and have actions within the follicle. Many of the disparate actions of gonadotrophins on the follicle are transduced by multiple regulatory factors in a local paracrine fashion. The local regulators that have generated the most interest are steroids, the IGFs and the inhibin/activin family of proteins. It is clear that the development of the vasculature around the growing follicle involved angiogenesis and that vascular endothelial growth factor (VEGF) is the primary angiogenic factor in this process [10].

Clinical Correlation

A dominant follicle will have a clear vascular network visible on colour Doppler assessment, but this is all in the theca cell layer, as the granulosa cells have no direct contact with the microvasculature.

1.1.8 Follicular Steroidogenesis

The granulosa cells and the theca cells of the antral follicle are steroidogenic in nature. Studies using labelled precursors revealed that the isolated granulosa cells were capable of producing oestrogens only when precursor hormones were present. They could not synthesize the androgens that are the immediate precursors of oestrogens in the steroidogenic pathway. In contrast, isolated theca cells produced progesterone and androgens. Localization of steroidogenic enzymes within the follicle has demonstrated that theca cells express 17 α -hydroxylase (CYP17), the enzyme responsible for androgen synthesis. They do not express aromatase (CYP19), the enzyme responsible for aromatization of androgens to oestrogens. In contrast, granulosa cells express CYP19 but do not have CYP17 activity. Follicular oestrogen biosynthesis therefore requires the co-operation between granulosa cells and their thecal neighbours. LH stimulates the formation of androgens in the theca cells and FSH stimulates the conversion of androgens into oestrogens in the granulosa cells (Figure 1.2) [11].

As well as acting on multiple tissues including the uterus, hypothalamus and pituitary, ovarian steroids also act on the follicle itself. Oestrogen receptors are found on granulosa cells and they may augment FSH action. Some women with hypogonadotropic hypogonadism have normal FSH concentrations but very low LH and oestradiol concentrations. These women have multicystic ovaries, but follicular growth tends not to progress beyond 8–9 mm despite normal FSH concentrations.

Androgen receptors are also present on granulosa cells. High androgen concentrations can cause atresia of larger antral follicles, pause the growth of small antral follicles and potentially promote the growth of preantral follicles [12]. The net effect of increased androgens is a build up of paused small antral follicles and the development of a polycystic ovary [13].

Gonadotrophin-induced steroidogenesis is modified by other local and endocrine factors. Insulin, IGFs, inhibin, activin and other members of the TGF β superfamily, as well as disparate growth factors and cytokines, all can affect ovarian steroidogenic function. Some of these are regulated by gonadotrophins and others are not. The paracrine regulation of

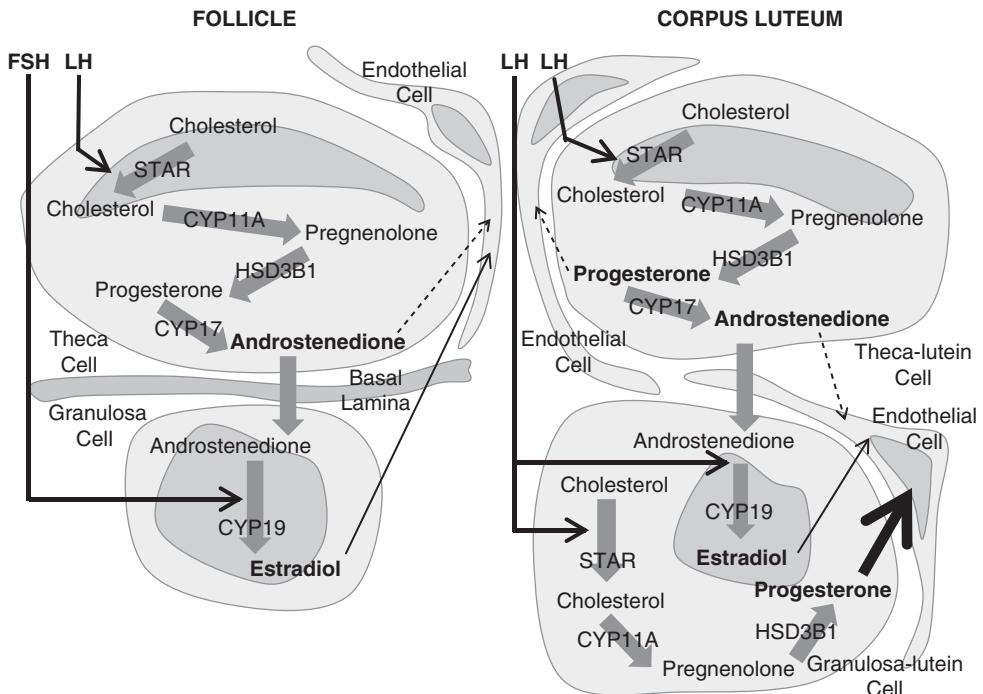


Figure 1.2 Illustration of steroidogenesis in the follicle and corpus luteum. In the follicle oestriadiol synthesis required both LH and FSH and theca cells and granulosa cells. In the corpus luteum both the theca-lutein cells and the granulosa-lutein cells can secrete progesterone. The theca-lutein cells continue to secrete androgen and the granulosa-lutein cells continue to secrete oestrogen, but only LH is required for luteal steroidogenesis

follicular function remains complex and not fully understood. Manipulation in vitro however remains controversial, although there are studies on the effect of androgens and insulin modification through metformin as adjuvant therapy [14].

Clinical Correlation

There is a local role for oestrogen as well as FSH in the growth and development of a dominant follicle. A polycystic ovary is a biomarker of increased intraovarian androgen concentrations.

1.1.9 Negative Feedback on Gonadotrophins

As follicles grow and mature under the influence of FSH, oestrogen secretion, regulated by both FSH and LH, increases. The FSH will stimulate the growth of small antral follicles at various stages of development within the ovary. The increasing oestriadiol concentrations feedback on the hypothalamus and pituitary to decrease FSH secretion (Figure 1.3). As follicle survival is dependent on endocrine trophic support, the fall in FSH concentrations will cause atresia of these growing follicles (Figure 1.1). However, larger follicles are less sensitive to the decline in FSH concentrations.

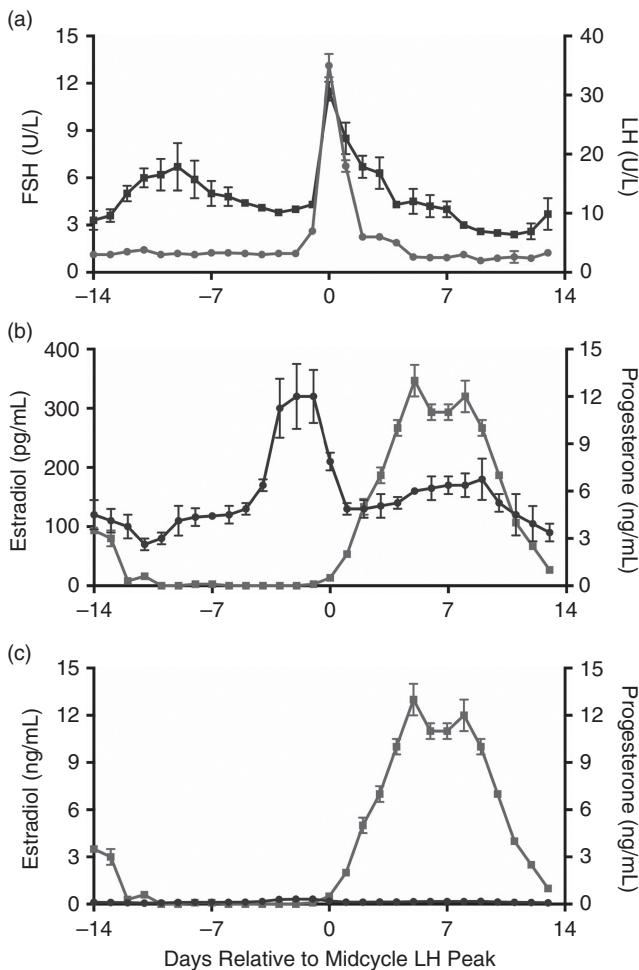


Figure 1.3 Hormone profile of the menstrual cycle of women. (a) Concentrations of FSH and LH. (b) Concentrations of oestradiol and progesterone. (c) Concentrations of oestradiol and progesterone plotted on the same scale highlighting the dominance of progesterone. Data reproduced from [25] and adapted from [27].

Clinical Correlation

When exogenous FSH is used in the absence of down-regulation, endogenous FSH is still present and subject to negative feedback so that total FSH will fall while exogenous FSH concentrations are maintained. Exogenous FSH therefore does not need to be reduced to ensure unifollicular development in gonadotrophin ovulation induction.

1.1.10 Follicular Selection

During the late follicular maturation of the granulosa cells, FSH induces the expression of LH receptors that are also coupled to adenylyl cyclase. Consequently, in the preovulatory

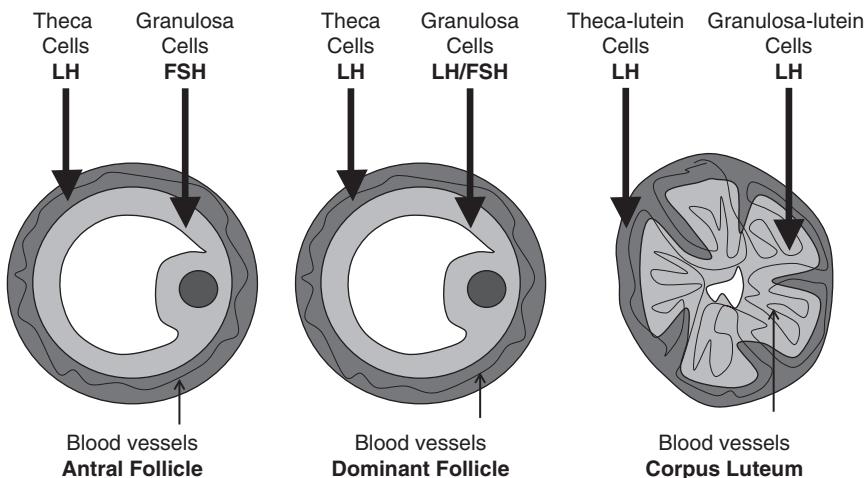


Figure 1.4 Changing requirements for gonadotrophins as the follicle matures. In the dominant follicle both LH and FSH regulate granulosa cell function. In the granulosa cells of smaller antral follicles FSH regulates granulosa cell function while after ovulation LH regulates granulosa-lutein cell function. After ovulation the avascular granulosa cell layer becomes highly vascular

follicle, LH can regulate both androgen synthesis (in theca cells) and aromatization of androgen (in granulosa cells). This increases the responsiveness of the follicle in the face of FSH levels that are declining. This FSH-dependent maturation of granulosa cells during follicular development is associated with increases of messenger ribonucleic acids (mRNAs) for the LH receptor and steroidogenic enzymes (Figure 1.4).

Unlike subordinate follicles, the dominant follicle continues to grow despite low FSH levels because it acquires the capacity to (1) express LH receptor in its granulosa cells and (2) produce large amounts of growth factors such as IGF1 and oestradiol which (a) allow the dominant follicle to respond to LH in addition to FSH and (b) provide higher sensitivity to FSH and LH, respectively. The role of the IGF system in the dominant follicle can be seen during in vitro exposure of granulosa cells to IGF-1 that induces secretion of oestrogen, which in turn stimulates proliferation of granulosa cells, expansion of the antrum and formation of gap junctions [8,11].

In addition, at this stage of development, the granulosa cells of the follicle have matured to acquire the capacity to respond to the ovulatory LH surge. When the dominant follicle is fully mature, the secreted oestrogen has prepared the endometrium for pregnancy. The oocyte is now ready for release and therefore the next process to be considered is ovulation.

Clinical Correlation

In hypogonadotropic hypogonadism, where there are low LH concentrations, dropping exogenous FSH can result in atresia of dominant follicles, as the LH might not be enough to maintain their growth.

1.2 Ovulation

Increasing oestradiol secretion from the dominant follicle promotes a switch to positive feedback at the hypothalamus and pituitary that results in the LH surge. At this stage serum oestradiol

concentrations are usually 740–1250 pmol/l and the dominant follicle will generally measure between 17 mm and 23 mm in diameter. The LH surge usually occurs on day 14 of a normal menstrual cycle and it induces the three separate components involved in the ovulatory process.

One component of ovulation is reactivation of oocyte maturation where the oocyte, which has been maintained in the diplotene stage of prophase, progresses to the metaphase of the second meiotic division. Another component is luteinization of the granulosa cells where they develop the enzymatic machinery to synthesize progesterone. The third and final component of the ovulatory response is follicular rupture, which is an acute inflammatory process involving the breakdown of the apical follicle wall.

Clinical Correlation

Even when follicular rupture is not required, the LH surge is needed to initiate oocyte maturation as well as the transition to progesterone secretion, which promotes endometrial receptivity.

1.2.1 The LH Surge

The gonadotroph cells in the anterior pituitary gland respond to GnRH pulses from the hypothalamus by secreting FSH and LH. While GnRH facilitates FSH synthesis and secretion in a constant manner, its effects on LH are different. LH is sequestered into granules within the gonadotrophs and only some granules release their content with each GnRH pulse, giving characteristic LH pulses. Most LH remains stored in granules for release during the LH surge.

High circulating oestradiol has effects on the hypothalamus. It stimulates a GnRH surge that markedly increases both FSH and LH secretion (Figure 1.3). Although a surge of GnRH can cause an LH surge, there is a direct effect of oestrogens on gonadotrophin secretion in the absence of any alteration in GnRH concentrations. The negative feedback induced by oestrogen during follicular growth and the positive feedback of oestrogen at the time of ovulation are seen in women with hypogonadotropic hypogonadism treated with pulsatile GnRH, where pulses of GnRH are consistent. Thus, women, deficient in GnRH, on exogenous fixed pulsatile GnRH concentrations will have an ovulatory LH surge. This suggests that GnRH is needed to facilitate the oestradiol-induced LH surge but that a surge of GnRH is not obligatory [15].

The evidence for functional oestradiol receptors on GnRH neurons isn't very convincing and there must be other oestrogen-responsive neural networks that regulate GnRH secretion. It is now clear that kisspeptin neurons stimulate GnRH secretion from GnRH neurons, and this is facilitated by neurokinin B (NKB). Treatment with kisspeptin will stimulate GnRH secretion while NKB antagonism will suppress GnRH secretion. Unlike GnRH administration, which produces a relatively normal LH surge, a kisspeptin injection produces an attenuated LH surge with a reduction in amount and duration of LH bioavailability [16].

The concentration of LH increases one hundred fold and is maintained during the surge that lasts up to 48 hours. LH itself has a very short half-life, of around 20 minutes, and an injection of LH is cleared too quickly to mimic the effects of an LH surge. However, hCG, which acts through the LH receptor and has the same action as LH, has a half-life of more

than 24 hours. This means that a single injection of hCG not only mimics the LH surge but it remains in the circulation beyond the normal duration of the LH surge, with ongoing bioactivity.

Clinical Correlation

HCG administration will replicate an enhanced LH surge while Kisspeptin administration will produce an attenuated LH surge.

1.2.2 Oocyte Maturation

LH acts through LH/hCG receptors to primarily increase cAMP, although other messenger systems such as IP₃, DAG, Ca²⁺, protein kinases and phospholipases are also stimulated. At this stage LH/hCG receptors are found on both the theca and granulosa cells of the follicle, while FSH receptors remain on the granulosa cells. As FSH induces similar intracellular signalling pathways to LH and can independently induce the ovulatory process, albeit at pharmacological rather than physiological concentrations, the granulosa cells seem to be the main target for the LH surge.

The oocyte does not express gonadotrophin receptors, so its reactivation in response to the LH surge must depend on signals from the granulosa cells. Oocytes mature spontaneously when removed from granulosa cell contact. This maturation can be prevented by addition of cAMP or adenylyl cyclase to the oocyte. As the oocyte contains little natural adenylyl cyclase activity, the source of the cAMP required to delay maturation is likely to be the granulosa cells through their direct cytoplasmic connections. The high granulosa cell cAMP stimulated during the LH surge breaks down intracellular connections between the granulosa cells and the oocyte. The exposure of oocytes to cAMP therefore falls, altering protein phosphorylation and stimulating reentry into the cell cycle [17].

Clinical Correlation

A reduced capacity to respond to the LH surge, such as in smaller follicles where granulosa cell LH receptor expression is not fully developed, or a reduced LH surge can adversely affect oocyte maturation.

1.2.3 Luteinization

Luteinization is the process where the granulosa cells of the pre-ovulatory follicle acquire the machinery to produce progesterone. Until the LH surge, the steroidogenic role of the granulosa cells is limited to the aromatization of the androgen products from the theca cells. The LH surge induces the expression of the cholesterol transporter STAR and the steroidogenic enzymes CYP11A and HSD3B1, which catalyze the synthesis of progesterone from cholesterol (Figure 1.2). This process can be mimicked by the introduction of high concentrations of cAMP into granulosa cells.

Granulosa cells will spontaneously luteinize and produce progesterone in culture. This means that the follicular environment has an inhibitory effect on luteinization that is removed by the LH surge. Luteinization induced by removing the granulosa cells from

the follicular environment does not involve large increases in intracellular cAMP and cannot be prevented by incubating the cells with follicular fluid. The breakdown of granulosa cell communications with each other, induced hormonally by the LH surge and mechanically during removal from the follicle, seems to regulate luteinization.

The granulosa cells change their appearance during this process. They become larger (increasing in size by 250 percent), develop intracellular granules and become rich in lipid, smooth and rough endoplasmic reticulum, and mitochondria with tubular cristae. These luteinized granulosa cells are able to synthesize and secrete progesterone, as well as synthesize oestrogens by aromatization of androgens from the remaining theca cells. They lose their FSH receptors and their steroidogenic function is now controlled entirely by LH (Figures 1.2 and 1.4). In addition, they become terminally differentiated and cannot divide further [18].

Clinical Correlation

Ovarian progesterone secretion during and after ovulation is dependent on LH acting through LH/hCG receptors on the steroidogenic cells.

1.2.4 Follicular Rupture

There are five layers of tissue between the oocyte–cumulus complex and the abdominal cavity. These have to be breached during the process of follicular rupture. On the surface of the ovary there is a layer of cuboidal epithelium covered with microvilli. Deep to these cells is the tunica albuginea layer that is five to seven cells thick. Meshed into this layer, and difficult to differentiate from it, is the outermost layer of the follicle wall called the theca externa. This consists of connective tissue containing fibroblasts five to seven cells deep. Below this layer lies the theca interna, which represents steroidogenic cells in a vascular capillary bed, around two cells thick. Separated from these cells by a basement membrane are the avascular granulosa cells, five to seven cells deep, with the oocyte–cumulus complex attached to a random point.

The exact mechanisms of follicular rupture are not fully understood, but cellular changes occur in cell layers that do not express LH receptors. This suggests that the steroidogenic cells respond to the LH surge by influencing factors that act on the outer layers of the follicle. The net result is to stimulate and augment an acute inflammatory reaction in the follicle wall. The first response seen in the follicle wall is hyperaemia with an increased blood flow and increasing permeability of the vessels, which results in some oedema. As this can be blocked by administration of antihistamines, locally released histamine is one of the earliest effectors of the LH surge.

The fibroblasts in the follicle wall become more active and the connective tissue around these cells becomes looser. Collagenases are synthesized and activated and the follicular wall softens at the stigma. As the wall becomes softer, fibroblasts become more motile and migrate away from the stigma. The follicle wall reduces to 20 percent of its initial thickness and becomes more avascular. There is some retraction of the tunica and theca cells around the stigma, resulting in a thin translucent area through which ovulation will occur. The epithelial cells over this area die and slough off, and the wall balloons due to its reduced strength and ruptures, allowing the escape of the follicular fluid containing the cumulus–oocyte complex. The process of follicular rupture takes many hours to be completed. The

interval from the start of the LH surge to follicular rupture in the human takes an average of 38 hours.

The initiation of ovulation is associated with an increase in the concentrations of prostaglandins and other products of the cyclooxygenase pathway. Indomethacin and other non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit cyclooxygenase, block ovulation. Injection of indomethacin or prostaglandin antiserum into single follicles inhibits the release of these follicles but not that of neighbouring follicles. Local prostaglandin production is involved in follicular rupture, and granulosa cells can respond to LH by increasing prostaglandin synthesis. However, oocyte maturation, cAMP production, luteinization and steroidogenesis are unaffected by indomethacin, suggesting that prostaglandins are primarily involved in follicular rupture. NSAIDs do not block ovulation if given in the last 20 percent of the ovulatory process, suggesting that prostaglandins initiate a process that eventually does not require further exposure to eicosanoids to be completed [19].

During ovulation the local steroid production changes markedly, with a transition from oestradiol to progesterone dominance. Epostane, an inhibitor of progesterone synthesis, will inhibit ovulation, even in the presence of an artificial LH surge, which can be overcome by the addition of exogenous progesterone. Manipulation of the oestradiol levels by maintaining high levels of oestradiol or blocking oestradiol production has no effect on follicular rupture. Progesterone receptors are found on the steroidogenic cells as well as the surrounding fibroblasts and epithelial cells. Progesterone synthesis can be inhibited during the second half of the ovulatory process without blocking follicular rupture, suggesting an early role for local progesterone. The post-ovulatory follicle develops into the highly steroidogenic corpus luteum (Figures 1.2 and 1.3) [20].

Clinical Correlation

After 35 or 36 hours the processes of oocyte maturation and luteinization are complete but follicular rupture has not yet occurred. The use of potent anti-inflammatory agents in the periovulatory phase can prevent follicular rupture. This results in trapping the oocyte within the ovary, while progesterone concentrations rise normally. This is known as luteinized unruptured follicle syndrome.

1.3 The Corpus Luteum

The corpus luteum produces large amounts of progesterone to promote the establishment of pregnancy. To do this it has large, metabolically active, steroidogenic cells surrounded by an extensive capillary network, which is required to deliver trophic molecules, nutrients and building blocks, and disseminate secreted products. The corpus luteum is formed from the remaining cells of the dominant follicle after ovulation. Its formation is induced by the LH surge and its lifespan is fixed. During the non-fertile menstrual cycle the corpus luteum will lose functional and structural integrity after two weeks. When conception and pregnancy follow ovulation, the interval of luteal function is extended. The continued function of the corpus luteum in early pregnancy is maintained until the placenta assumes endocrine activities, around eight weeks of gestation.

Clinical Correlation

Unlike the follicular phase, which can be variable in timing, the luteal phase has a fixed lifespan of 12–14 days. Variation in the length of the menstrual cycle in women relates to differences in the follicular phase.

1.3.1 Follicular-Luteal Transition

The follicle has now grown, matured and ruptured, releasing a fertilizable oocyte. The collapsing follicle still contains an avascular layer of granulosa cells surrounded by highly vascular theca cells. These follicular remnants are transformed into the corpus luteum. After ovulation, rupture of the dominant follicle is associated with shrinking of the follicular area during the folliculo-luteal transition. However, in one in four to five cycles the corpus luteum does not shrink and may expand to become cystic. It is likely that these represent increased haemorrhage within the corpus luteum rather than luteinized unruptured follicles, as haemorrhagic luteal cysts are seen in 29 percent of patients in early pregnancy.

Clinical Correlation

A cystic corpus luteum may be a variation of normal and does not suggest luteinized unruptured follicle syndrome.

1.3.2 Formation of the Corpus Luteum

Following the LH surge, the basement membrane between the theca interna and the granulosa cells begins to break down. The luteinizing steroidogenic cells become terminally differentiated and do not further divide. However, both the theca and granulosa cell layers undergo hypertrophy. There is extravasation of red blood cells into the antral cavity and this fills with blood. The theca begins to form invaginations into the avascular granulosa cells. Blood vessels from the theca invade the luteinizing granulosa cell layer and, under the influence of angiogenic factors, more extensive vascular structures develop (Figure 1.4). The theca-lutein cells form small clumps at the periphery of the granulosa-lutein cells [20,21]. The steroidogenic cells are surrounded by a dense fibrous stroma containing blood vessels, fibroblasts, immune cells and extracellular matrix (ECM). Eventually each granulosa-lutein cell abuts a vascular endothelial cell and there are as many endothelial cells as steroidogenic cells.

That means that the corpus luteum is highly vascular; indeed, it has been estimated that the corpus luteum has up to eight times the blood supply per unit mass than the kidney. As well as having a marked Doppler signal showing increased flow to larger blood vessels in the periphery of the corpus luteum, contrast ultrasound shows intense microvascular perfusion throughout the corpus luteum. This intense angiogenesis is a key and defining feature of the corpus luteum [21].

The primary angiogenic molecule in the human corpus luteum is VEGF [22]. VEGF is secreted from luteal steroidogenic cells and acts on receptors on the endothelial cells. Luteal VEGF secretion initially is under both hormonal and hypoxic regulation enhanced by the increased metabolic activity of the steroidogenic cells. VEGF is

responsible for the luteal angiogenesis response and the establishment of the microvascular network in the early to mid-luteal phase. When VEGF is inhibited during luteal development a poorly functioning corpus luteum with a markedly reduced vascular network is noted. In the mid-to-late luteal phase VEGF has a role in maintaining endothelial cell survival and luteal cell integrity. Inhibition of VEGF in the post-angiogenic phase results in increased cell death of the endothelial cells, followed by cell death of steroidogenic cells. VEGF is not just a growth factor that regulates angiogenesis; it also regulates vascular permeability by down-regulating endothelial cell-tight junctions [23].

The corpus luteum has a marked blood flow as demonstrated by colour Doppler analysis, sometimes referred to as a 'ring of fire' around the corpus luteum. The resistance index of blood vessels, measured by power Doppler, decreases from ovulation to the mid-luteal phase, with vascular flow $3.1 \times$ higher during the mid-luteal phase (seven days after ovulation) than around the time of the dominant follicle before ovulation. This high blood flow to the corpus luteum is important in luteal function and is maintained in the presence of severe peripheral vascular disease. Serum progesterone concentrations are negatively correlated to the resistance index of luteal vessels.

Clinical Correlation

The development corpora lutea involves intense angiogenesis regulated by the angiogenic and vascular permeability factor VEGF. VEGF is very high when multiple corpora lutea are developing and has a key etiological role in ovarian hyperstimulation syndrome (OHSS).

1.3.3 Regulation of Corpus Luteum Function

The primary function of the granulosa-lutein and theca-lutein cells is steroidogenesis (Figure 1.3). These cells contain the steroidogenic enzymes necessary to synthesize progesterone (Figure 1.2). Granulosa-lutein cells continue to express CYP19 and theca-lutein cells continue to express CYP17, and so the corpus luteum makes both androgen and oestrogen. In addition, the steroidogenic cells secrete peptide hormones such as inhibin A, relaxin and oxytocin. The granulosa lutein and theca-lutein cells express LH receptors but not FSH receptors, and the function of the corpus luteum is dependent on LH. Removal of LH using a GnRH antagonist results in rapid loss of function, and replacement of LH or hCG will maintain normal luteal function. In addition, LH or hCG can directly increase progesterone output from isolated luteal cells and luteal tissue.

Although LH is absolutely required to maintain luteal function, circulating LH levels are low throughout the luteal phase and do not parallel the output of progesterone. It is therefore likely that the action of LH on steroidogenic luteal cells is also modulated in a paracrine fashion by local regulators of steroidogenesis. Just as local paracrine regulators of steroidogenesis have been described in the ovarian follicle, they have been described in the corpus luteum. Inhibins and activins, cytokines, growth factors, eicosanoids, steroids and other molecules have been reported as potential local regulators of luteal function. IGF-1 stimulates progesterone production from isolated luteal cells, and this effect seems to be mediated by specific IGF-1 receptors [24].

Clinical Correlation

In the absence of LH the corpus luteum will undergo luteolysis. While an ovulatory injection of hCG, with a long half-life, can maintain luteal integrity until endogenous hCG is present when GnRH agonist is used to stimulate an LH surge, with a shorter half-life, luteal integrity can be compromised before endogenous hCG is present.

1.3.4 Function of the Corpus Luteum

The human corpus luteum secretes huge amounts of progesterone (Figure 1.3). At its peak of activity it produces up to 25 mg of progesterone each day. Progesterone concentrations in excess of 30 nmol/l (9.4 ng/ml) are used to determine the presence of a mid-luteal corpus luteum and thus confirm ovulation. The building block for progesterone and other steroid hormones is cholesterol. Although the corpus luteum can synthesize cholesterol de novo, the available evidence suggests that luteal cholesterol is derived directly from the plasma and the mobilization of intracellular stores. Cholesterol is transported in the plasma as lipoprotein complexes. Although cholesterol is present in both low-density lipoproteins (LDL) and high-density lipoproteins (HDL), in the human ovary it seems that the main source of cholesterol is LDL. The cholesterol is either used immediately for steroidogenesis or esterified and stored in lipid droplets within the cell responsible for the characteristic colour and texture of the corpus luteum.

Cholesterol is acted on by specific steroidogenic enzymes acting in series. The first step is the transport of cholesterol from the cytoplasm to the inner cytoplasmic membrane of the mitochondria. STAR is the transport protein, stimulated by LH, which is the rate-limiting stage of steroidogenesis. Cholesterol side-chain cleavage (CYP11A) converts cholesterol to pregnenolone, which is converted into progesterone by the action of 3 β hydroxysteroid dehydrogenase (HSB3B1). Progesterone itself can be converted into oestradiol via an androgen intermediate. The enzyme CYP17 converts progesterone into androstenedione, which in turn is converted to oestradiol by CYP19 (Figure 1.2).

There are some additional systemic effects of progesterone outside its role in uterine function. First, there is an increase in basal body temperature of 0.5°C. Also, progesterone is able to relax smooth muscle and women are more likely to be constipated in the luteal phase of the cycle and during pregnancy. This effect, as well as the fact that it will displace cortisol from cortisol-binding globulin, means that asthma may improve after ovulation and deteriorate in the late luteal phase and during menstruation. In addition, progesterone is involved in lobular differentiation in breast tissue, and it also has a proliferative role, meaning that breast tenderness is common after ovulation. There are progesterone receptors in numerous areas of the brain and there is no doubt that changes in sex hormones can affect mood in some women. Bloating, mood swings and an increase in irritability are common, and in around 5 percent of women these symptoms are bad enough to interfere with their ability to live a normal life [25].

As well as secreting progesterone, the primate corpus luteum also secretes oestrogen. Oestrogen synthesis in the corpus luteum appears to involve the same ontogeny as oestrogen biosynthesis in the follicle (Figure 1.2). Both granulosa-lutein cells and theca-lutein cells are involved in its synthesis. Like the follicle, the theca-lutein cells are responsible for the

androgen substrates and the granulosa-lutein cells the aromatization to oestrogens, but LH drives the effect in both cells and there is no role for FSH in luteal function.

In addition to steroid hormones, the corpus luteum also synthesizes and releases a variety of hormones, including relaxin, oxytocin, inhibin A and activin, with likely endocrine effects. With steroids, luteal inhibin A secretion will suppress FSH secretion in the luteal phase to prevent follicular growth. Inhibins and activins may have a local paracrine effect on the corpus luteum. These molecules have been shown to have direct effects on steroidogenesis and tissue remodelling [20,24].

Clinical Correlation

Although the corpus luteum secretes disparate hormones, its function can be replicated by progesterone alone. Other luteal products either enhance progesterone action or are involved in the regulation of luteal integrity.

1.3.5 Structure of the Corpus Luteum

The mature corpus luteum varies in size and appearance, but it will usually measure between 10 mm and 35 mm in diameter and can clearly be visualized on an ultrasound scan *in vivo*. At ovulation there is collapse of the follicle and the corpus luteum is usually smaller than the dominant follicle. The mean follicle volume at ovulation, measured by 3D ultrasound, was 6.94 cm³ while the mean mid-luteal corpus luteum volume was 4.87 cm³.

Another group of cells in the corpus luteum are those whose primary function is likely to be structural. These will include fibroblasts and pericytes in the gland capsule and around blood vessels. There are the migratory cells such as macrophages, monocytes and leucocytes. Their presence has been clearly described in corpora lutea and they have been implicated in the regulation of luteal integrity. All these cells are contained within the ECM. This serves as the glue that holds the corpus luteum together. However, as well as having a major structural role, the ECM probably contributes essential elements to the functional activity of the gland.

Clinical Correlation

The corpus luteum can be seen on an ultrasound scan. This can be used to correlate scan findings with the stage of the cycle. It is also helpful in locating an ectopic pregnancy, as the ectopic is on the side of the corpus luteum in more than 75 percent of cases.

1.3.6 Luteolysis

In women formation of the corpus luteum sets in place an inherent process that results in inevitable luteolysis after 14 days in a non-conception cycle. The steroidogenic cells of the corpus luteum have one fate: death when their function is served. Historically, corpus luteum regression, or luteolysis, has been divided into two distinct stages: functional and structural luteolysis. Functional luteolysis refers to the increasing failure of progesterone synthesis and secretion. Structural luteolysis refers to the processes involved in the dissolution and resorption of the gland that are classically thought to follow functional luteolysis. In the rodent, where much research on the corpus luteum is done, functional luteolysis is

different from structural luteolysis. Thus it can be confusing when reading the many reviews on the CL that mix species. In the human there appears to be no clear temporal and endocrinological distinction between these stages of luteolysis [24].

Progesterone synthesis and secretion by the corpus luteum is dependent on the action of the trophic hormone, LH, from the pituitary gland. Withdrawal of LH using GnRH antagonists during the luteal phase results in a rapid loss of both the functional and structural integrity of the primate corpus luteum. In fact, pituitary LH secretion does change as the luteal phase progresses. There is a progesterone-mediated reduction of the frequency of LH pulses from approximately one pulse per hour during the early to mid-luteal phase to one pulse every four to six hours from the mid-luteal to the late luteal phase.

However, luteolysis occurs in the presence of maintained LH concentrations, confirming that luteolysis is inherent to the corpus luteum itself and not a response to an altered endocrine environment. Expression of LH receptors is maintained across the luteal phase and luteolysis is initiated, and progesterone concentrations begin to fall, in the presence of LH receptors, and all the elements of the progesterone production cellular machinery. It seems that luteal steroidogenic cells are programmed to become incrementally less sensitive to LH and increasingly cannot maintain normal luteal function and progesterone production. During the late luteal phase serum progesterone falls and luteal vascular resistance index increases.

Luteolysis involves changes to non-steroidogenic cells, such as endothelial cells, which proliferate and then regress; macrophages that accumulate during luteolysis; and fibroblasts that secrete growth factors and enzymes involved in connective tissue remodelling. This highlights the role for paracrine molecules from the steroidogenic cells, regulated by the LH receptor, in the regulation of disparate cell function in the human corpus luteum. There are luteolytic roles for some paracrine factors such as connective tissue growth factor (CTGF), the Slit/Robo system, activin, bone morphogenic proteins, galectins, matrix metalloproteinases and endothelin [24].

During the mid-luteal phase, at the peak of its functional activity in a non-conception cycle, the corpus luteum is clearly visible on the surface of the ovary. During the early follicular phase of the next cycle it is difficult to identify that corpus luteum on the ovary macroscopically. Microscopically it has become a small, relatively avascular fibrous remnant [25].

Clinical Correlation

The corpus luteum is a highly regulated structure that is of fundamental importance to fertility and there are no significant disorders of luteal function. The inadequate corpus luteum is a misnomer: impaired luteal function is a consequence of impaired follicular development, a suboptimal LH surge or pituitary down-regulation.

1.3.7 Maternal Recognition of Pregnancy

In a conception cycle, maternal recognition of pregnancy involves the early conceptus sending a trophic signal to the corpus luteum to rescue it from luteolysis. That signal is human chorionic gonadotropin (hCG), which functions as a long-acting LH-like molecule to maintain the structural and functional integrity of the corpus luteum. It binds to and signals through the LH receptor. In early pregnancy removal of the corpus luteum will

induce miscarriage and ongoing luteal function is required to maintain pregnancy. At around eight weeks of gestation the luteo-placental shift occurs, as the placenta produces enough progesterone to support pregnancy, and lutectomy has no effect on the continuation of the pregnancy.

The beta subunit of hCG is similar to that of LH but with differences in size and glycosylation pattern. The hCG beta subunit shares the amino acid sequence with LH beta subunit but has an additional 30 amino acids at the carboxy terminal. The additional amino acids and the thus different glycosylation patterns make hCG, and LH has different properties in the circulation. The plasma half-life of hCG is substantially longer (36 hours) compared to that of LH (approximately 20 minutes) (24).

It requires logarithmically increasing concentrations of hCG to maintain stable, and then declining, luteal steroidogenic cell function during early pregnancy. We do not fully understand the molecular basis for the loss of LH receptor function as the corpus luteum ages. In women hCG can first be detected in the circulation nine to ten days after the LH surge. This rises logarithmically to peak levels at 8–12 weeks of gestation, and then declines to lower but detectable levels throughout the remainder of pregnancy. The role, if any, of hCG after the luteoplacental shift is not known.

VEGF expression is LH dependent and LH is required to form the corpus luteum and maintain its structural integrity. In simulated early pregnancy, hCG promotes additional VEGF synthesis and there is further luteal VEGF secretion and a second wave of angiogenesis. There are luteotrophic roles for other factors such as cortisol, prostaglandin E, progesterone, insulin-like growth factor and VEGF. There are less defined roles for other molecules such as nitric oxide (NO), various cytokines and prostaglandin F_{2α}, in the absence of prostaglandin E withdrawal. It is likely, however, that alterations in these important effector molecules are consequences of differential LH action and do not represent the initial step on the pathway to luteolysis.

There seems to be a window where hCG is able to rescue the corpus luteum. Exogenous hCG could stimulate progesterone secretion in the early luteal corpus luteum and the late luteal corpus luteum, but the effects were much less marked than that seen in the mid-luteal corpus luteum, when normal luteal progesterone secretion is at its peak [26].

Clinical Correlation

There is ongoing VEGF and a second wave of luteal angiogenesis stimulated by hCG in early pregnancy and it is responsible for late onset OHSS.

1.3.8 The Luteo-Placental Transition

Oophorectomy or lutectomy prior to the development of sufficient placental steroidogenesis causes abortion. The principal hormone secreted by the corpus luteum to maintain pregnancy is progesterone. Progesterone supplementation will maintain an early pregnancy after lutectomy. Lutectomy after six or seven weeks of pregnancy does not result in abortion. At this stage the placenta secretes enough progesterone to maintain pregnancy. At this stage the corpus luteum is essentially functionally redundant. This transition is known as the luteo-placental shift. Thus, the corpus luteum of a fertile cycle must function for a further four weeks to ensure continued pregnancy.

Luteal function is required to promote secretory and decidual changes in the endometrium to facilitate implantation and pregnancy maintenance for the first 8–10 weeks of gestation. Although in addition to progesterone the corpus luteum secretes several different peptide and steroid hormones, including relaxin, inhibin A, prokineticin, VEGF, androgen and oestrogen, it is progesterone that is the key luteal product in the establishment and maintenance of pregnancy. Although progesterone alone can promote implantation and maintain pregnancy there is some evidence that the addition of oestrogen may increase the effectiveness. The mechanism of action of the additional oestradiol is not clear. Oestradiol has a role in uterine and endometrial vascularization and it may be that this is involved. However, oestradiol has been shown to up-regulate the progesterone receptor and its role may be to further promote progesterone action.

In early pregnancy the resistance index of the luteal vessels remains at the mid-luteal level until eight weeks of pregnancy, when the levels are equivalent to the late luteal phase, and the vascular flow to the corpus luteum is greatest at five weeks gestation. Circulating progesterone concentrations will decline before this shift and reach a nadir at seven weeks gestation. This is despite increasing hCG concentrations, as this peaks around eight weeks gestation. At this time the volume of the corpus luteum is decreasing and rising concentrations of the placental protein PAPP-A can be detected in the serum, suggesting increasing placental function. The corpus luteum loses its functional integrity between 8 and 10 weeks gestation, while placental steroids and inhibin prevent ovarian follicular activity during pregnancy. Although the corpus luteum is non-functional for the remainder of the pregnancy, and it undergoes marked involution, it can sometimes still be identified as a small remnant on the ovary at the time of birth [25,26].

Clinical Correlation

In the absence of a functioning corpus luteum, exogenous progesterone supplementation is required for the first eight weeks of pregnancy.

Summary

An awareness of basic ovarian physiology is important. This facilitates normalization of ovarian function in ovulation induction. It also informs protocols in the manipulation of ovarian function using ovarian stimulation during assisted reproduction.

References

- Smith P, Wilhelm D, Rodgers RJ. Development of mammalian ovary. J Endocrinol. 2014; **221**: R145–61.
- Kerr JB, Myers M, Anderson RA. The dynamics of the primordial follicle reserve. Reproduction 2013; **146**: R205–15.
- Gougeon A. Human ovarian follicular development: from activation of resting follicles to preovulatory maturation. Ann Endocrinol (Paris) 2010; **71**: 132–43.
- Hsueh AJ, Kawamura K, Cheng Y, Fauser BC. Intraovarian control of early folliculogenesis. Endocr Rev. 2015; **36**: 1–24.
- Adhikari D, Liu K. Molecular mechanisms underlying the activation of mammalian primordial follicles. Endocr Rev. 2009; **30**: 438–64.
- Dewailly D, Andersen CY, Balen A et al. The physiology and clinical utility of anti-Mullerian hormone in women. Hum Reprod Update 20; 370–85. Erratum in: Hum Reprod Update 2014; **20**: 804.

7. Makanji Y, Zhu J, Mishra R et al. Inhibin at 90: from discovery to clinical application, a historical review. *Endocr Rev.* 2014; **35**: 747–94.
8. Macklon NS, Fauser BC. Regulation of follicle development and novel approaches to ovarian stimulation for IVF. *Hum Reprod Update* 2000; **6**: 307–12.
9. Baerwald AR, Adams GP, Pierson RA. Ovarian antral folliculogenesis during the human menstrual cycle: a review. *Hum Reprod Update* 2012; **18**: 73–91.
10. Oktem O, Urman B. Understanding follicle growth in vivo. *Hum Reprod* 2010; **25**: 2944–54.
11. Palermo R. Differential actions of FSH and LH during folliculogenesis. *Reprod Biomed Online* 2007; **15**: 326–37.
12. Lebbe M, Woodruff TK. Involvement of androgens in ovarian health and disease. *Mol Hum Reprod.* 2013; **19**: 828–37.
13. Duncan WC. A guide to understanding polycystic ovary syndrome (PCOS). *J Fam Plann Reprod Health Care* 2014; **40**: 217–25.
14. Nardo LG, El-Toukhy T, Stewart J, Balen AH, Potdar N. British Fertility Society Policy and Practice Committee: adjuvants in IVF: evidence for good clinical practice. *Hum Fertil (Camb).* 2015; **18**: 2–15.
15. Plant TM. A comparison of the neuroendocrine mechanisms underlying the initiation of the preovulatory LH surge in the human, Old World monkey and rodent. *Front Neuroendocrinol.* 2012; **33**: 160–8.
16. Skorupskaite K, George JT, Anderson RA. The kisspeptin-GnRH pathway in human reproductive health and disease. *Hum Reprod Update* 2014 **20**: 485–500.
17. Coticchio G, Dal Canto M, Mignini Renzini M et al. Oocyte maturation: gamete-somatic cells interactions, meiotic resumption, cytoskeletal dynamics and cytoplasmic reorganization. *Hum Reprod Update* 2015; **21**: 427–54.
18. Stouffer RL, Xu F, Duffy DM. Molecular control of ovulation and luteinization in the primate follicle. *Front Biosci.* 2007; **12**: 297–307.
19. Richards JS, Russell DL, Ochsner S, Espey LL. Ovulation: new dimensions and new regulators of the inflammatory-like response. *Annu Rev Physiol.* 2002; **64**: 69–92.
20. Devoto L, Fuentes A, Kohen P et al. 3rd. The human corpus luteum: life cycle and function in natural cycles. *Fertil Steril.* 2009; **92**: 1067–79.
21. Duncan WC. The human corpus luteum: remodelling during luteolysis and maternal recognition of pregnancy. *Rev Reprod.* 2000; **5**: 12–17.
22. Fraser HM, Duncan WC. Vascular morphogenesis in the primate ovary. *Angiogenesis* 2005; **8**: 101–16.
23. Fraser HM, Duncan WC. SRB reproduction, fertility and development award lecture 2008. Regulation and manipulation of angiogenesis in the ovary and endometrium. *Reprod Fertil Dev.* 2009; **21**: 377–92.
24. Duncan WC, Myers M, Dickinson RE, van den Driesche S, Fraser HM. Luteal development and luteolysis in the primate corpus luteum. *Animal Reprod.* 2009; **6**: 34–46.
25. Duncan WC. The Corpus Luteum and Women's Health. 2017. pp. 249–75. In: The Life Cycle of the Corpus Luteum Ed Meidan R, London: Springer International Publishing.
26. Stouffer RL, Bishop CV, Bogan RL, Xu F, Hennebold JD. Endocrine and local control of the primate corpus luteum. *Reprod Biol.* 2013; **13**: 259–71.
27. Groome NP, Illingworth PJ, O'Brien M et al. Measurement of dimeric inhibin B throughout the human menstrual cycle. *J Clin Endocrinol Metab.* 1996; **81**: 1401–5.

In Vivo Oocyte Development

Helen M. Picton

2.1 Introduction

The development of successful strategies for the treatment of female infertility is dependent on the production of highly specialised oocytes that have the capacity to undergo meiosis and complete a programme of cellular and molecular events that support fertilisation and early embryo development. Oocyte growth and development in all mammalian species, including humans, is a protracted process that requires the timely coordination of oogenesis and folliculogenesis (Figure 2.1). Follicles provide the niche that supports oocyte survival, nutrition and growth and the regulation of meiosis. In return, oocytes direct follicle development, and there is metabolic cooperation between the two cellular compartments. Uncoupling of this vital symbiotic relationship has a profound and detrimental impact on oocyte health and subsequent developmental competence (quality). A clear understanding of the processes of oogenesis and folliculogenesis and their interdependence is therefore of great importance to assisted reproduction technology (ART) as this biology underpins the

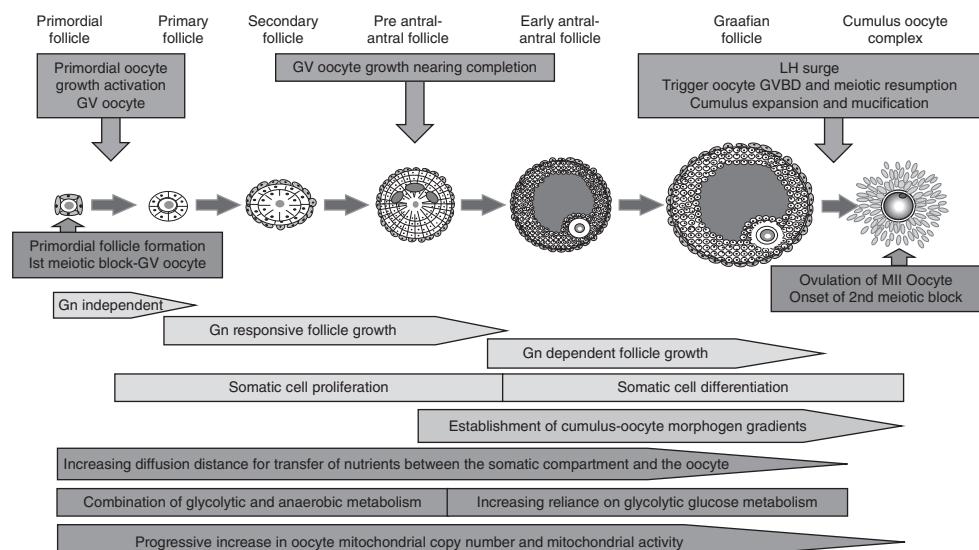


Figure 2.1 Overview of the interaction between oogenesis and folliculogenesis detailing the meiotic checkpoints and cellular, molecular and metabolic events governing the onset of mammalian oocyte growth and the production of a mature metaphase II oocyte in vivo. Adapted from Picton [1] with permission (Gn-gonadotrophin).

production of good-quality oocytes. This chapter will provide an overview of current understanding of the biology of oogenesis with particular reference to human oocytes and will track oocyte development from primordial follicles to the ovulation of a mature, fertile, metaphase II (MII) oocyte *in vivo*.

2.2 Characteristics of Oocytes

The life history of human oocytes begins early in fetal life and is coincident with the initiation of meiosis in the oogonia and the assembly of these cells into spheroidal/elliptical primordial follicles. Oocyte entry into meiosis is immediately followed by meiotic arrest at the diplotene phase of prophase I of meiosis I, which constitutes the first meiotic checkpoint. Each primordial follicle consists of a centrally located germinal vesicle (GV) stage oocyte surrounded by a single layer of squamous pre-granulosa cells derived from the ovarian surface epithelium and enclosed within a thin basal lamina. Human primordial follicles are mainly located in the outermost layer of ovarian cortex; their number constitutes the ovarian reserve and ultimately defines the reproductive longevity of an individual. In women, oocytes can remain arrested in primordial follicles for up to the fifth decade when menopause occurs. The development of oocytes from the primordial to the preovulatory stage of maturity takes approximately six to nine months to complete [2]. This transition in humans is matched by a threefold increase in oocyte diameter from ~35–40 µm in primordial GV oocytes to ~93–120 µm in fully grown GV oocytes [3,4]. The increase in oocyte size is accompanied by an increase in follicle diameter from <40 µm to 17–20 mm. Primordial oocyte survival and retention in the ovarian reserve, or growth activation and maturation, are dependent upon the balance between stimulatory growth factors (as reviewed by Zhang and Liu [5] and growth inhibitors and particularly the anti-Müllerian hormone [6] derived from follicular somatic cells. The coordinated interaction between these genes and transcription factors regulates the reproductive lifespan of women, and many of these factors are biological candidates for the investigation of reproductive disorders such as premature ovarian failure and infertility, as discussed elsewhere in this book.

The cytoplasm of oocytes from primordial follicle stages onwards typically contains a cluster of organelles including mitochondria, Golgi apparatus (GA), endoplasmic reticulum (ER) and ribosomes [3]. The GA conducts post-transcriptional modification, sorting and packaging of macromolecules such as the oocyte-specific cortical granules for delivery to other compartments within the cell. Cortical granules are present in human oocytes from the secondary follicle stage onwards [7] and are continuously formed as the GA breaks into flattened sacs in the cortex. They are then packaged by the coalescence of smaller vesicles into mature membrane-bound granules of 0.2–0.6 µm diameter [4]. Cortical granules contain carbohydrates, proteinases, ovoperoxidase, calreticulin and N-acetylglucosaminidase. They are evenly distributed throughout the cytoplasm of unfertilised oocytes until the time of ovulation when they migrate using the microfilaments of the cell cytoskeleton to the perimeter of the oolemma in readiness for release to prevent polyspermy. The ooplasm also contains a honeycomb of ER that is responsible for fulfilling the protein synthesis and assembly and lipid synthesis needs of the oocyte. In oocytes the ability of the ER to store and release Ca²⁺ is of fundamental importance to oocyte developmental competence. Finally, the subcortical region of oocytes is rich in structures such as cytoplasmic lattices that are thought to be the storage site for mRNAs

and ribosomes as well as key elements of the translational machinery of the cell, including the recently identified subcortical maternal complex (SCMC). The SCMC contains key proteins [8] encoded by maternal effect genes including *MATER*, *KHDC3L*, *PADI6*, *TLE6* and *OOEP* which are abundant in oocytes. The SCMC acts as putative RNA binding proteins as well as plays a role in the regulation of transcription and translation in the oocyte and early embryo and DNA mismatch repair.

The energy needs of oocytes at all stages of development are supplied by the mitochondria. Human primordial oocytes are thought to contain between 6,000 and 10,000 mitochondria, but these numbers are increased to 100,000–400,000 in mature gametes [9]. Mitochondria in primordial oocytes are small and spherical with few cristae and have a predominantly perinuclear location [10]. At this stage of oocyte development mitochondria have a relatively sluggish metabolic activity and, along with other organelles, are briefly clustered around the nucleus forming the ‘Balbiani body’ [3]. Following the activation of oocyte growth and up to the secondary follicle stage, mitochondria become progressively more elongated, which is indicative of their increased activity, and their number and distribution in the ooplasm alter to reflect the changing energy demands of the cell and its specialist functions. While some mitochondria retain their perinuclear distribution in mature oocytes, others colonise the periphery of the cell or form clusters in subcortical locations in readiness for energy-demanding processes such as spindle, polar body and pronuclei formation and cortical granule relocation and/or release. Low oocyte mitochondrial activity is increasingly being linked to poor oocyte and embryo quality [10]. The absolute reliance of the oocyte upon mitochondrial energy production ensures that oocytes acquire developmental competence only if they carry an active mitochondrial load that produces sufficient energy above the threshold needed for survival and fertility. While a threshold of mitochondrial activity is necessary, excessive activity is disadvantageous, and if such is the case in healthy oocytes, the production of destructive reactive oxygen species can be held in check by active cellular antioxidant defence systems that include enzymes, vitamins and metabolites.

2.3 Oocyte Growth

The activation of primordial oocyte growth and accompanying primordial follicle growth occurs independently of gonadotropin stimulation and is characterised by the enlargement of the oocyte, the rounding up of the flattened pre-granulosa cells of the primordial follicles into cuboidal granulosa cells (GCs), followed by the proliferation of these cells to form multiple avascular layers. Follicle and oocyte diameter, as well as GC number, increase dramatically during follicle growth. From primary follicle stages onwards and following the induction of GC receptors for the binding of the pituitary gonadotrophin follicle-stimulating hormone (FSH), follicles become increasingly responsive to FSH stimulation. The point at which the oocyte starts to grow can be linked to the number of GCs in the largest cross-section of the follicle. This cell number is thought to be species-specific and approximates to 14–15 cells in humans [11]. Concomitant with GC replication and progression of preantral follicle growth through the primary and secondary stages of development, the oocyte increases in volume as it progressively acquires the proteins and organelles needed to complete its life cycle. The proliferation of GCs in response to FSH is moderated by oocyte- and somatic cell-derived growth factors that have autocrine and paracrine actions such as the insulin-like growth factors and their

binding proteins, activin and inhibin and numerous oocyte-derived growth factors including members of the transforming growth factor beta (TGF- β) superfamily such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) (as recently reviewed by Chang et al. [12]).

Oocyte growth in vivo is marked by the synthesis and accumulation of rRNA and tRNA and the synthesis and storage of key proteins that support the specialist structural architecture of the cell and cellular function for growth as well as for the completion of meiosis in mature oocytes and early embryo development [13]. A critically important event that occurs early during oocyte growth is the deposition of the glycoprotein coat that forms the zona pellucida (ZP) within the perivitelline space. Human ZP deposition begins in primary-staged follicles and by the transitional stage the ZP proteins have coalesced to form a continuous layer that surrounds the oocyte. Cytoplasmic processes traverse the ZP to enable the oocyte to remain coupled to the GCs so facilitating bidirectional communication between the oocyte and its supporting follicular cells throughout development and maturation [10]. The composition of the ZP is species-specific, ensuring that only sperm from the correct species can fertilise the oocyte. In humans the ZP is composed of four sulphated glycoproteins (ZP1, ZP2, ZP3 and ZP4) and is permeable to relatively large macromolecules [14]. Glycoproteins ZP2 and ZP3 form heterodimers that are cross-linked by ZP1. In the human zona ZP1, ZP3 and ZP4 facilitate sperm binding and the induction of the acrosome reaction [14], whereas ZP2 acts as a secondary sperm receptor. The transcription factor, Factor in the germline- α (Fig- α), has been implicated in the formation of the human ZP [15]. While nutrients and signalling molecules can diffuse across the porous ZP, as follicle development advances and the follicle structure becomes more complex, more sophisticated transport mechanisms are needed to support oocyte and follicle development. Cytoplasmic continuity and bidirectional transport of small molecules between the oocyte and GCs must therefore be facilitated via trans-zonal projections (TZPs) and heterologous gap junctions. The homologous and heterologous gap junctions that connect the cumulus/GCs and the cumulus cells and the oocyte in humans are composed of connexins 43 and 37, respectively [16], and the junctions form at the contact points between the TZPs from cumulus GCs and the oocyte membrane or microvilli. Although the precise mechanism of effect is unknown, FSH appears to affect the density of the TZPs [17]. Mitochondria, endosomes and lysosomes can be moved bidirectionally through the TZPs.

At the transitional stage of follicle development a theca layer begins to form outside the basal lamina of the follicle, from the fibroblast-like precursor cells present in the ovarian stroma. The proliferation of GCs continues and once the follicle has developed to the secondary stage it will become fully enclosed by a full theca layer that will ultimately differentiate into two distinct populations – the steroidogenic and vascularised theca interna layer [2] and the non-steroidogenic, connective tissue layer that forms the theca externa. Throughout the process of early follicle development, up to antral cavity formation, the oocyte increases dramatically in size [18]. Oocyte growth is achieved via the accumulation of ions, water and lipids and is accompanied by large-scale cytoplasmic rearrangement as organelles are redistributed and new organelles and vesicles are formed.

2.4 Antral Follicle Development

Major morphological changes that are key to the life cycle of the oocyte occur as follicle development progresses through antral cavity formation. While follicle growth continues, the GCs differentiate into two discrete populations – the cumulus granulosa that enclose the oocyte and the columnar, mural GCs that line the perimeter of the antral cavity [19]. The major phase of oocyte growth is complete by the time of antrum formation [18]. Antrum formation begins when the secondary follicle reaches a size of approximately 0.2–0.4 mm diameter in humans [2], and it serves to increase the available surface area of the follicle for diffusion of nutrients to the oocyte while supporting the continuing increase of follicular mass. The later stages of preovulatory follicle growth are due to follicular fluid accumulation rather than GC proliferation. Antral cavity formation is promoted by the pituitary gonadotrophins (luteinising hormone (LH) and FSH) and is manifest by the accumulation of GC-derived follicular fluid in multiple lacunae, which eventually expand and coalesce into one large antrum [7]. Fluid accumulation occurs as a result of glycoprotein production by the GCs. Glycoproteins such as GC-derived hyaluronan, inter- α trypsin inhibitor and versican are secreted into the follicular fluid (FF) and they bind/cross-link to each other and so are retained. The presence of these glycoproteins produces an osmotic gradient that causes further fluid to enter the developing antral cavity from the thecal vasculature [20]. This process requires the remodelling of cell-cell junctions, as well as the theca layer and ovarian stroma. The gap junctional contacts extending from the somatic cells through the ZP to the oolemma continue to facilitate the transfer of growth factors between the different cell types. The number and nature of these junctional contacts alter throughout follicular development and are mediated by external factors such as FSH [21]. The follicular fluid acts as an important source of blood and follicular cell-derived substances, including gonadotrophins, steroids, growth factors, enzymes, proteoglycans and lipoproteins, that are required for the regulation of folliculogenesis and the support of oogenesis. Preovulatory follicle growth is marked by the differentiation of GC steroidogenic capacity from a predominantly androgenic to oestrogenic environment following the induction of receptors for the pituitary gonadotrophin LH on the GCs when follicle diameter reaches ~8–9 mm in humans. From this stage of development onwards, follicle growth and survival are dependent on gonadotrophin support. Progression of secondary follicles to preovulatory size of 17–20 mm diameter takes around 85 days to complete in humans [2]. Although oocytes are usually able to resume meiosis once follicular antrum formation has occurred, they have yet to acquire the molecular and cytoplasmic machinery needed to complete nuclear maturation and to progress to MII.

2.5 Oocyte-Derived Factors and the Regulation of Oogenesis and Folliculogenesis

Oocyte survival is dependent upon the establishment and maintenance of cell-cell interactions with the follicular GCs, and vice versa. The somatic cells provide the oocyte with sufficient nutrients, amino acids, growth factors and oxygen to support gamete growth and maturation. Conversely, oocytes secrete an array of growth factors and peptides that promote GC differentiation and regulate GC function so as to provide the oocyte with sufficient substrates for optimal growth and development (Figure 2.1). Oocyte-derived

transcription factors, genes and gene pathways are now recognised as playing central role(s) in oocyte survival and follicle assembly; the activation and maintenance of follicle and oocyte growth; and the acquisition of oocyte developmental competence. The functional significance of oocyte-secreted proteins and transcriptional regulators in intra ovarian cell-cell communication changes as oocyte development progresses. The role played by key oocyte-derived GDF9 and BMP15 has recently been reviewed by Chang and colleagues [12]. Each oocyte is therefore effectively regulating its own microenvironment [22] as well as that of its host follicle. Disruption of these genes and pathways is associated with pathologies such as premature ovarian failure and infertility in humans [5].

2.6 Oocyte Nutrient Supply and Metabolism

The nutrient supply needed to support oocyte growth and maturation is provided by the follicle. *In vivo*, growing follicles are exposed to tissue fluid which is composed of plasma transudate, physiological concentrations of nutrients, oxygen and macromolecules such as albumin. The outer layers of healthy, growing follicles are vascularised such that follicles failing to develop an adequate blood supply become under-oxygenated, which has serious consequences for oocyte energy production and health and can lead to cytoplasmic and nuclear abnormalities [23]. As follicle development progresses nutrients and oxygen are required to diffuse a greater distance through the GCs from the blood supply before reaching the oocyte. Differential concentrations of electrolytes also exist between the plasma and follicular fluid compartments, with the follicle accumulating salts and water which follows osmotically [24]. As the GCs metabolise glucose and amino acids and produce pyruvate and lactate and other amino acids, a marked change in the nutrients and oxygen concentration develops in the follicular fluid as compared to blood, such that follicular fluid glucose concentrations are markedly lower than plasma concentrations, while pyruvate and lactate levels are much higher. Thus, each cumulus-oocyte complex present in a Graafian follicle *in vivo* is bathed in a unique nutrient environment that meets the oxygen and energy requirements needed to complete oocyte growth and maturation.

It is becoming increasingly clear that metabolic processes and particularly energy metabolism play important roles in the production of healthy, fertile oocytes both *in vivo* and *in vitro* during ART. Indeed, nutrient supply and metabolic processes throughout oogenesis appear to play key roles in determining the developmental capacity, and ultimately the fertility, of oocytes. While shared metabolic pathways exist between different cell types, there are recognised differences between metabolism and substrate preferences in oocytes compared to follicle and somatic cells. Key to oocyte metabolism is the supply of oxygen, as oocytes are obligatorily aerobic, and their preferred energy substrate as supplied by the cumulus cells and via the follicular fluid is pyruvate [25]. Although pyruvate is the preferred substrate of oocytes, the machinery for glycolytic metabolism is still present and both pyruvate- and glucose carrier-mediated uptake have been demonstrated in human oocytes [25]. In contrast, glucose is the pivotal substrate metabolised by the cumulus-oocyte complexes and follicular cells by glycolysis, the pentose phosphate pathway (PPP), the hexosamine biosynthesis pathway (HBP) and the polyol pathway [26]. Glucose is taken up by the follicular somatic cells via facilitated transport systems that are

sensitive to substrate availability and are mediated by endocrine stimuli such as FSH and insulin levels. Metabolic needs and processes change as follicle and oocyte development progress in concert. At the primary and early secondary stages of preantral follicle development glucose is predominantly metabolised via the glycolytic energy pathway. As follicle growth progresses, some glucose is used for glycogen synthesis, but as oxygen is in plentiful supply the majority is used for oxidative energy production. However, as follicle size increases further, the distance between the oocyte and the follicle surface extends and the oxygen content of the follicle becomes limiting. At this point the oocyte becomes a major driving force in the differentiation of the GC metabolic phenotype and the follicle switches to a metabolic strategy that is more reliant upon glycolytic glucose breakdown and inhibition of oxidative metabolism in the cumulus cells [27], thereby conserving the oxygen supply. In addition to their consumption of glucose, ovarian follicles produce large quantities of lactate, especially during the peri-ovulatory period. Oocytes themselves may promote cumulus cell uptake of certain amino acids including glutamate, histidine and alanine [28].

Pyruvate and oxygen metabolism progress in tandem in developing oocytes and peak during the pre-ovulatory period *in vivo* (Figure 2.2). The high demand for pyruvate and oxygen during oogenesis is needed to facilitate synthesis of cytoplasmic organelles, proteins and RNA needed not only for growth but also for the synthesis and sequestration of molecules in preparation for meiotic maturation, fertilisation and early embryo development. The stage of oocyte meiosis – whether prophase I, metaphase I or metaphase II – therefore affects the capacity of the oocyte for pyruvate, oxygen and amino acid consumption in many species, including humans. Upon resumption of meiosis, an increase in the oocyte's consumption of oxidisable energy substrates occurs that is coincident with the activation of energy-requiring processes such as spindle formation, chromosome condensation and segregation, protein synthesis and polar body formation. Pyruvate consumption and the metabolic needs of oocytes appear to drop once MII is reached. Glucose plays multiple metabolic roles in the oocyte–cumulus complex and is essential for both maintenance of meiotic arrest in the oocyte and for the completion of hormone-stimulated meiotic maturation [27]. Indeed, glucose metabolism through the cumulus PPP is necessary for the generation of the precursor of phospho-ribosyl-pyrophosphate which is an essential metabolite required for *de novo* purine synthesis. In support of this idea, the meiosis-suppressing purines adenosine and hypoxanthine have been identified in follicular fluid (for review, see Downs [29]). The synthesis of hyaluronic acid from glucose metabolites by cumulus cells facilitates cumulus expansion and so helps break down the gap junctional contacts that link the oocyte to its cumulus compartment. After the preovulatory LH surge, cumulus PPP activity promotes meiotic resumption in the oocyte. Secretion of pyruvate by cumulus cells is also stimulated by gonadotrophins, and this serves to increase pyruvate availability in the immediate vicinity of the oocyte. Metabolites such as pyruvate and amino acids may also serve many subcellular roles such as free radical scavengers, osmolytes, toxin chelators and regulators of intracellular pH.

The final class of nutrients that are critical to oocyte development and fertility are lipids. Lipids have diverse roles providing oocytes with energy and signalling molecules as well as contributing to structural components of the cell and to organelle membranes [30]. The fatty acid composition of oocytes appears to be discretely

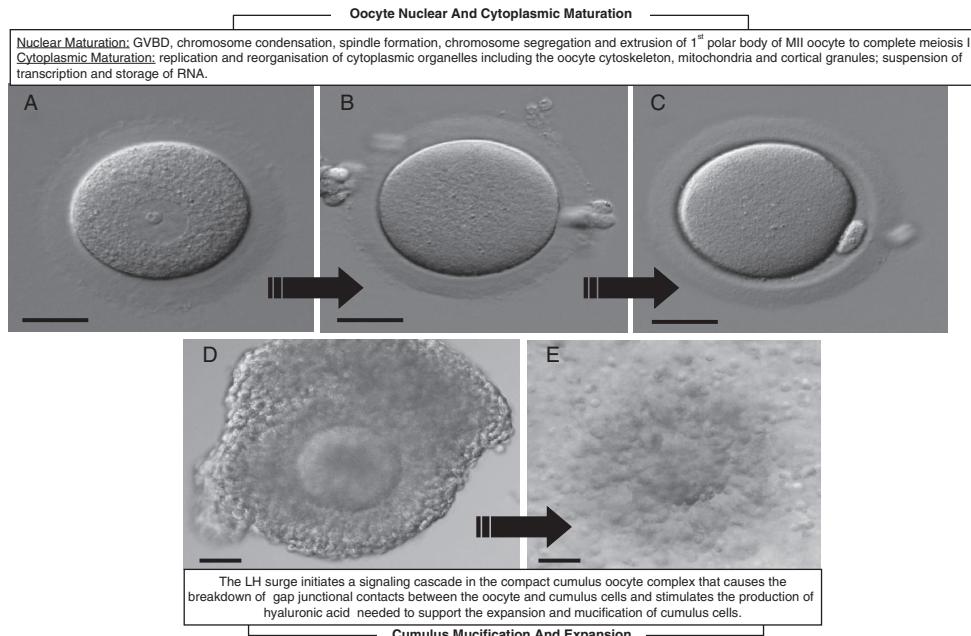


Figure 2.2 Morphological changes of human oocytes and cumulus mass associated with the progression of nuclear and cytoplasmic maturation as exemplified by breakdown of the GV nucleus shown in (a), progression through metaphase I (b) and the extrusion of the first polar body and the formation of an MII oocyte that marks the completion of meiosis I (c). Simultaneously, the morphology of the cumulus–oocyte complex transitions from that of a compact cumulus mass illustrated in (d) to an expanded and mucified complex as shown in (e). Scale bar represents 50 µm

different from that of adipose or follicular somatic tissues. Intracellular triacylglycerol in oocytes and cumulus cells is stored within lipid droplets. Triglycerides act as a metabolic fuel that is hydrolysed by lipases in cumulus cells and oocytes and the fatty acids so released then undergo β -oxidation in the mitochondria to produce adenosine triphosphate (ATP) that is used to support oocyte growth and maturation. Cumulus cells may directly influence oocyte triacylglycerol and/or fatty acid deposition as well as control oocyte cholesterol content [27]. Lipoproteins, particularly high-density lipoproteins (HDL), are the predominant source of cholesterol that is an essential substrate for GC and theca steroidogenesis. Oocyte lipoproteins and fatty acids may also have a cytoprotective role and/or scavenging properties that may protect the cells from oxidative stress. Fatty acids have also been shown to play important roles in supporting embryogenesis and embryo health [31]. The metabolic pathway of β -oxidation of lipids is likely to play an important role in the acquisition of oocyte developmental competence and hence female fertility.

2.7 Oocyte Maturation

Oocyte maturation is defined as the process that leads to the production of a mature gamete *in vivo* following ovulation (Figure 2.2). The long and complex process of oocyte

development confers the competencies required for the cell to complete meiosis and undergo fertilisation and embryogenesis. The morphology of fully grown GV and MII human oocytes is a relatively poor indicator of oocyte health. That said, large cytoplasmic inclusions, central granularity, aggregates of ER and excessive vacuolation are all considered to be detrimental to oocyte developmental competence, but more systematic research is needed to confirm this [3]. There is also recent evidence from murine studies that the eccentric positioning of the nucleus in fully grown GV oocytes is associated with a higher density of TZPs and that this enrichment of cell contacts may be critically important to the positioning and size of the meiotic spindle required for chromosome segregation and the successful completion of an asymmetric meiotic division [4]. In contrast, central positioning of the GV nucleus is associated with the formation of a larger meiotic spindle, increased levels of aneuploidy and the production of a larger-than-normal first polar body. The resulting reduced cytoplasmic mass of the MII oocyte may have a detrimental impact on subsequent embryo development. While the relevance of GV asymmetry and associated cellular behaviour to human oocyte quality is unproven, the sequence of events followed during oocyte maturation is thought to be relatively constant between mammalian species, and it is clear that in all mammalian species cumulus-oocyte complexes housed in preovulatory follicles are bathed in an oestrogenic follicular fluid microenvironment that is rich in secreted growth factors, cytokines and nutrients, including pyruvate and glucose, ATP, lipoproteins, triglycerides and free fatty acids, electrolytes, purines and pyrimidines [26,30].

Oocyte maturation from the GV stage of maturity is triggered by the preovulatory gonadotrophin surge, and it ends with the production of a mature human MII oocyte some 36 hours later. Throughout its development the maintenance of meiotic arrest in the oocyte requires high levels of the second messenger molecule 3',5'-cyclic adenosine monophosphate (cAMP), resulting in G-protein-coupled receptor (GPR) 3 and/or GPR12 activation of adenylate cyclase. As reviewed by Downs [29], it is generally accepted that high levels of cAMP maintain oocyte meiotic arrest by inducing oocyte, protein kinase A (PKA)-dependent phosphorylation of cyclin-dependent kinase 1 which suppresses the activity of meiosis-promoting factor (MPF; Adhikari and Liu [32]). The levels of 3',5'-cyclic guanine monophosphate (cGMP) in mural GCs are increased through the interaction of natriuretic peptide precursor C with its receptor NPR2. During GV arrest in oocytes, the diffusion of cGMP into the oocyte inhibits phosphodiesterase 3A (PDE3A) activity and prevents the hydrolysis of cAMP. For resumption of meiosis to occur a rapid and profound decrease in cGMP is needed, as well as closure of gap junctions to prevent its further diffusion and/or transfer of other signals to the oocyte so disrupting the equilibration between cGMP in the oocyte and somatic compartments in the preovulatory follicle. The LH surge rapidly and simultaneously inactivates NPR2 and activates cGMP PDE5 in the GCs so causing a reduction in oocyte cGMP levels. These alterations in intracellular messengers in turn either directly or indirectly activate downstream kinases, including MPF, mitogen-activated protein kinase (MAPK), PKA, and PKC and AKT, ultimately resulting in the resumption of meiosis [32].

The preovulatory surge of LH activates a cascade of signalling events in the preovulatory follicle which are propagated through both the mural GCs and cumulus cells and which alter the spatial architecture of the cells and promote resumption of meiosis and ovulation of a mature MII oocyte. Oocytes do not possess LH receptors, so the binding of LH to its G-protein-coupled receptor in mural GC effects its action via the generation of

increased levels of the second messenger cAMP which in turn, in humans, stimulates GC and cumulus production of the epidermal growth factor (EGF)-like peptide amphiregulin (AREG) and the associated transactivation of the EGF-receptor (In brief, AREG production and receptor binding activate a large network of genes in the GC and cumulus cells that ultimately lead to cumulus expansion and the resumption of meiotic maturation and ovulation [22]. The up-regulation of AREG occurs prior to GV breakdown in the oocyte. The LH surge causes a rapid decrease in follicular cGMP synthesis via a combination of LH-dependent EGF-receptor activation, NPR2 desensitisation and by cGMP degradation. Evidence now suggests that AREG and other members of the EGF family of peptides may recapitulate many of these effects of LH, including gap junction closure and associated decrease in cGMP, as well as resumption of oocyte meiotic maturation, cumulus expansion and activation of extracellular signal-related MAPK signalling.

The resumption of meiosis is intrinsic to the process of oocyte maturation. The sequence of spatially and temporally choreographed events include condensation of the dispersed nuclear chromatin in the GV nucleus into discrete chromosomes; breakdown of the GV nuclear membrane; formation of the meiotic spindle from the microtubule organising centres; segregation of the chromosomes between the oocyte and the first polar body; and polar body extrusion. Formation of the first polar body marks the completion of meiosis I and heralds the onset of the second meiotic block in the oocyte. This checkpoint remains in place until meiosis II is initiated following sperm fusion with the oocyte membranes.

Cytoplasmic maturation of the presumptive gamete occurs in parallel with oocyte nuclear maturation and is equally fundamental to the production of a fertile MII oocyte. Recent molecular genetic and quantitative live cell imaging experiments have revealed new insights into the relevance of oocyte–somatic cell communication during this process and have re-evaluated the dynamic role played by cytoskeleton-based events that drive the oocyte to maturity [33]. Extensive reorganisation of cytoplasmic organelles occurs during oocyte maturation, including the relocation and replication of mitochondria, the relocation of ER and the migration of the cytoplasmic cortical granules to a sub-cortical location [34]. The self-organisation of microtubules and motor proteins such as actin filaments directs meiotic spindle assembly and chromatin interaction in order to both finalise chromosome segregation and move the meiotic spindle to the periphery of the oocyte. The proximity of meiotic chromatin to the oocyte cytoplasm is thought to trigger cytoplasmic streaming events through the creation of Ran-GTPase gradients [33]. This generates the mechanical forces needed to move cellular organelles around the ooplasm. The proximity of the chromosomes to the oolemma is increasingly thought to stimulate assembly of the actomyosin cap that is essential for the formation and extrusion of the polar body. Both the peripheral positioning of the meiotic spindle and the presence of the chromosomes themselves therefore help to orientate the cleavage plane of the oocyte and enable polar body extrusion to be accomplished with minimal loss of cytoplasm so retaining the majority of the cytoplasmic organelles within the oocyte. Changes in the microtubule cytoskeleton between the GV and MII stages of oocyte facilitate the relocation of both the ER and cortical granules. Cytoplasmic reorganisation in MII oocytes produces large ER clusters in the vicinity of the MII spindle [35]. This ER movement anticipates the oocyte's need for phasic release of Ca^{2+} from ER stores following fertilisation [4]. Migration of cortical granules to a sub-oolemma location prepares the oocyte for blocking polyspermy. Finally, cytoplasmic reorganisation during oocyte maturation is associated with a surge of mitochondrial

replication and relocation so that in human MII oocytes an increased proportion of functionally competent mitochondria, as demonstrated by their higher inner membrane potential, is relocated to the immediate vicinity of the meiotic spindle and to the periphery of the oocyte beneath the oolemma [36]. Changes in mitochondrial amplification and function during oocyte maturation are strongly linked to oocyte quality as the major changes in the oocyte cytoskeleton and cytoplasmic reorganisation described can only be completed if there is sufficient ATP, mainly derived by mitochondrial oxidative phosphorylation, to meet the increased energy demands of the cell (see below).

All of the nuclear and cytoplasmic processes detailed above require energy, and the capacity of human oocytes to undergo maturation is closely correlated to follicular size and maturity, and the follicular environment mirrors the maternal metabolic state, such that over- or under-nutrition can influence oocyte developmental competence and subsequent embryo health. During the course of oocyte maturation the intracellular lipid stores undergo dramatic changes such as structural reorganisation and central aggregation [30]. The bidirectional paracrine and gap junctional communication between the oocyte and its cumulus compartment remains critically important during maturation as significant amounts of glucose are metabolised by the cumulus cells via the glycolytic pathway to provide sufficient metabolic intermediates such as pyruvate to meet the oocytes' increased demand for energy. Glucose is also the substrate for the regulation of nuclear maturation and redox state via the PPP and for the synthesis of substrates of extracellular matrices (cumulus expansion) and O-linked glycosylation (cell signalling) via the HBP [26]. Follicular EGF-like peptides and oocyte-derived growth factors such as GDF9 and BMP15 may moderate the metabolism and behaviour of the COC through increased oxidative phosphorylation and activation of the HBP and/or via an increase in oocyte mitochondrial membrane potential [37]. Indeed, glucose metabolism by the cumulus cells is critical to the completion of oocyte nuclear and cytoplasmic maturation such that the glycolytic pathway in cumulus cells for energy production is needed to support cellular homeostasis and nuclear maturation of the oocyte; the cumulus HBP major fuel sensing pathway is used for the production of extracellular matrix and specifically hyaluronic acid production for cumulus expansion and signalling; the limited amount of glucose metabolised by the PPP in cumulus cells impacts on GV breakdown and the progression of all stages of oocyte meiosis through the provision of substrates for purine synthesis and intra-oocyte redox state; and the polyol pathway in cumulus facilitates the oxidation of glucose to sorbitol and fructose so providing the oocyte with alternative substrates for energy production [37].

Fully grown, GV oocytes are transcriptionally quiescent having undergone a period of active transcription during growth in which they have amassed a stockpile of mRNA and proteins, as well as stores of nutrients and organelles, including the large numbers of functional mitochondria needed to sustain the subsequent transcriptionally silent phase of oocyte maturation and fertilisation [38]. Indeed, a hallmark of the regulation of meiotic progression in mature oocytes is the utilisation of these stored maternal RNA and proteins rather than de novo transcription [13]. Maturing oocytes therefore depend on the post-transcriptional regulation of the stored maternal transcripts for protein synthesis. This process is mediated by the translational repression and de-adenylation of transcripts followed by the spatio-temporal recruitment of specific transcripts for translation during oocyte maturation and early embryo development [39]. With regard to the completion of meiosis,

significant advances have been made in our understanding of the mechanisms regulating chromosomal segregation [40]. Although the majority of studies have been conducted on animal oocytes grown *in vivo*, a limited number of studies have also been conducted on human oocytes using cells derived following ART. While a detailed review of all these important molecular events is beyond the scope of this chapter, overviews of the current understanding of the molecular mechanisms regulating meiosis, polar body extrusion and the mechanism of aneuploidy in oocytes are provided by Webster and Schuh [41] and Touati and Wassmann [42]. Insight into the full inventory of genes and regulatory pathways that characterise the transcriptional and epigenetic framework of mature GV and MII oocytes and their cumulus cells is provided through recent RNA sequencing studies in animals and humans (for example, [43,44]) with evaluations being conducted on different cell types in order to identify the molecular signatures associated with oocyte quality. Recently, microRNAs have been implicated as transcriptional controllers in the processes of oocyte translational repression and mRNA decay. Finally, recent research has highlighted the developmental importance of maternal effect genes [8] as well as the cellular and molecular machinery needed for selective polyadenylation and decapping that regulate the recruitment and stability of dormant maternal RNAs so protecting the oocyte and early embryos against RNA degradation during the period of transcriptional silence that accompanies meiotic completion, fertilisation and early embryo development [13].

2.8 Summary

In summary, human oogenesis is inexorably linked to folliculogenesis. The production of a fertile MII oocyte *in vivo* cannot progress without folliculogenesis and follicles cannot form, develop or ovulate without the presence of healthy oocytes. Oocytes drive their own timely progression through a series of key milestones that are essential for the production of a fertile MII gamete. Metabolic processes play diverse and important roles throughout oocyte growth and maturation *in vivo*, and the energy-generating capacity and distribution of mitochondria appear to significantly contribute to oocyte developmental competence. Most importantly, the key biological events in oocyte development *in vivo* detailed in this chapter must be replicated during the controlled ovarian stimulation regimens used in ART in order to maximise oocyte production and quality during assisted conception cycles.

References

1. Picton HM. Metabolism of the follicle and oocyte *in vivo* and *in vitro*. In “Biology and Pathology of the Oocyte” Ed. RG Gosden, A Trounson, U Eichenlaub Ritter. Cambridge, UK: Cambridge University Press, 2013;pp. 200–211.
2. Gougeon A. Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr Rev*. 1996;17:121–155.
3. Gosden R, Lee B. Portrait of an oocyte: our obscure origin. *J Clin Invest*. 2010;120:973–983.
4. Coticchio G, Dal Canto M, Renzini MM et al. Oocyte maturation: gamete-somatic cells interactions, meiotic resumption, cytoskeletal dynamics and cytoplasmic reorganisation. *Hum Reprod Update*. 2015;21:427–454.
5. Zhang H, Liu K. Cellular and molecular regulation of the activation of mammalian

- primordial follicles: somatic cells initiate follicle activation in adulthood. *Hum Reprod Update.* 2015;21:779–786.
6. Weenen C, Laven JSE, von Bergh ARM et al. Anti-Mullerian hormone expression patterns in the human ovary: potential implications for initial and cyclic follicle recruitment. *Mol Hum Reprod.* 2004;10:77–83.
 7. Baca M, Zamboni L. The fine structure of human follicular oocytes. *J Ultrastruct Res.* 1967;19:354–381.
 8. Bebbere D, Masala L, Albertini DF, Ledda S. The subcortical maternal complex: multiple functions for one biological structure? *J Assist Reprod Genet.* 2016;33:1431–1438.
 9. Steuerwald N, Barritt JA, Adler R et al. Quantification of mtDNA in single oocytes, polar bodies and subcellular components by real-time rapid cycle fluorescence monitored PCR. *Zygote.* 2000;8:209–215.
 10. Makabe S, Van Blerkom J. An Atlas of Human Female Reproduction: Ovarian Development to Embryogenesis In Vitro. 2014;London, UK: Taylor and Francis Books Ltd.
 11. Gougeon A, Chainy GB. Morphometric studies of small follicles in ovaries of women at different ages. *J Reprod Fertil.* 1987;81:433–442.
 12. Chang, H-M, Qiao J, Leung CK. Oocyte-somatic cell interactions in the human ovary- novel role of bone morphogenetic proteins and growth differentiation factors. *Hum Reprod Update.* 2017;23:1–18.
 13. Susor A, Jansova D, Anger M, Kubelka M. Translation in the mammalian oocyte in space and time. *Cell Tiss Res.* 2016;363:69–84.
 14. Gupta SK, Bhandari B. Acrosome reaction: relevance of zona pellucida glycoproteins. *Asian J Androl.* 2010;13:97–105.
 15. Huntriss J, Gosden R, Hinkins M et al. Isolation, characterization and expression of the human Factor. In the Germline alpha (FIGLA) gene in ovarian follicles and oocytes. *Mol Hum Reprod.* 2002;8:1087–1095.
 16. Mora JM, Fenwick MA, Castle L et al. Characterization and significance of adhesion and junction-related proteins in mouse ovarian follicles. *Biol Reprod.* 2012;86:153, 1–14.
 17. Combelles CM, Carabatsos MJ, Kumar TR, Matzuk MM, Albertini DF. Hormonal control of somatic cell oocyte interactions during ovarian follicle development. *Mol Reprod Dev.* 2004;69:347–355.
 18. Cotterill M, Harris SE, Fernandez EC et al. The activity and copy number of mitochondrial DNA in ovine oocytes throughout oogenesis in vivo and during oocyte maturation in vitro. *Mol Hum Reprod.* 2013;19:444–450.
 19. Baerwald AR, Adams GP, Pierson RA. Ovarian antral folliculogenesis during the human menstrual cycle: a review. *Hum Reprod Update.* 2012 January–February;18 (1):73–91.
 20. Rodgers RJ, Irving-Rodgers HF. Formation of the ovarian follicular antrum and follicular fluid. *Biol Reprod.* 2010;82:1021–1029.
 21. Anderson O, Heasman J, Wylie C. Early events in the mammalian germ line. *Int Rev Cytol.* 2001;203:215–230.
 22. Richani D, Gilchrist RB. The epidermal growth factor network: role in oocyte growth, maturation and developmental competence. *Hum Reprod Update.* 2017;1–14.
 23. Van Blerkom J. Epigenetic influences on oocyte developmental competence: perifollicular vascularity and intrafollicular oxygen. *J Assist Reprod Genet.* 1998;15:226–234.
 24. Gosden RG, Hunter RH, Telfer E, Torrance C, Brown N. Physiological factors underlying the formation of ovarian follicular fluid. *J Reprod Fertil.* 1988;82:813–825.
 25. Harris SE, Maruthini D, Tang T, Balen AH, Picton HM. Metabolism and karyotype analysis of oocytes from patients with PCOS. *Hum Reprod.* 2010;25:2305–2315.

26. Sutton-McDowall ML, Gilchrist RB, Thompson JG. The pivotal role of glucose in determining oocyte developmental competence. *Reproduction*. 2010;139:685–695.
27. Su YQ, Sugiura K, Wigglesworth K et al. Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. *Development*. 2008;135:111–121.
28. Eppig JJ, Pendola FL, Wigglesworth K, Pendola JK. Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: amino acid transport. *Biol. Reprod.* 2005;73:351–357.
29. Downs SM. Nutrient pathways regulating the nuclear maturation of mammalian oocytes. *Reprod Fert Dev*. 2015;27:572–582.
30. Dunning KR, Russell DL, Robker RL. Lipids and oocyte developmental competence: the role of fatty acids and B oxidation. *Reproduction*. 2014;148:R15–27.
31. McKeegan PJ, Sturmy RG. The role of fatty acids in oocyte and early embryo development. *Reprod Fert Dev*. 2011;24:59–67.
32. Adhikari D, Liu K. The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes. *Mol Cell Endocrinol*. 2014;382:480–487.
33. Li R, Albertini DF. The road to maturation: somatic cell interaction and self-organisation of the mammalian oocyte. *Nat Rev Mol Cell Biol*. 2013;14:141–152.
34. Coticchio G, Dal Canto M, Fadini R et al. Ultrastructure of human oocytes after in vitro maturation. *Mol Hum Reprod*. 2016;22:110–118.
35. Mann JS, Lowther KM, Mehlmann LM. Reorganization of the endoplasmic reticulum and development of Ca²⁺ release mechanisms during meiotic maturation of human oocytes. *Biol Reprod*. 2010;83:578–583.
36. Van Blerkom J. Mitochondrial function in the human oocyte and embryo and their role in developmental competence. *Mitochondrion*. 2011;11:797–813.
37. Gilchrist RB, Luciano AM, Richani D et al. Oocyte maturation and quality: role of cyclic nucleotides. *Reproduction*. 2016;152R: 143–R157.
38. Clift D, Schuh M. Restarting life: fertilisation and the transition from meiosis to mitosis. *Nat Rev Mol Cell Biol*. 2013;14:549–562.
39. Reyes JM, Ross PJ. Cytoplasmic polyadenylation in mammalian oocyte maturation. *WIREs RNA* 2016;7: 71–89.
40. Capalbo A, Hoffmann ER, Cimadomo D, Ubaldi FM, Rienzi L. Human female meiosis revised: new insights into the mechanisms of chromosome segregation and aneuploidies from advanced genomics and time-lapse imaging. *Hum Reprod Update*. 2017; 1–17.
41. Webster A, Schuh M. Mechanisms of aneuploidy in human eggs. *Trends Cell Biol*. 2017;27:55–68.
42. Touati SA, Wassmann K. How oocytes try to get it right: spindle checkpoint control in meiosis. *Chromosoma*. 2016;125:321–335.
43. Svoboda P, Franke V, Schultz RM. Sculpting the transcriptome during the oocyte-to-embryo transition in mouse. *Curr Top Dev Biol*. 2015;113:305–349.
44. Ernst EH, Grøndahl ML, Grund S et al. Dormancy and activation of human oocytes from primordial and primary follicles: molecular clues to oocyte regulation. *Hum Reprod*. 2017;32: 1684–1700.

Assessment of Ovarian Reserve

Scott M. Nelson, Reshef Tal, and David Seifer

3.1 Introduction

Women are born with a maximum quota of primordial follicles, which constitute the true ovarian reserve. This reserve contains all of the oocytes potentially available for fertilization throughout her fertile lifespan. All growing follicles that subsequently either ovulate or (mostly) die emanate from this pool of primordial follicles, which declines with age. However, the size of the ovarian reserve can vary dramatically between women, such that although the average number of primordial follicles at birth is 295,000, this can range from 34,800 to 2,508,000 primordial follicles [1]. The subsequent continual attrition of this initial ovarian reserve is unrelenting, with an increasing rate of depletion across a woman's reproductive lifespan, such that by the age of 30 only 12 per cent of the original number of primordial follicles present at birth remain and by the age of menopause less than 1,000 follicles remain. The clinical impact of this variation is that women who are born with a lower ovarian reserve/fewer primordial follicles or women who have a compromise in their ovarian reserve due to surgery or gonadotoxic therapy will enter the menopause at an earlier age. Assessment of the ovarian reserve at any age therefore pertains to the evaluation of the quantity of remaining oocytes rather than oocyte quality, for which age still remains the best predictor. The purpose of this chapter is to summarize the state of the art of ovarian reserve testing.

3.2 What Makes a Good Ovarian Reserve Test?

The ideal ovarian reserve test should be readily accessible, cost-effective, minimally invasive and rapidly interpretable [2]. It should also be reproducible and display minimal variability within the menstrual cycle and between cycles. It should be able to detect the decline in ovarian reserve at an early enough stage such that timely interventions could be pursued if desired. Most importantly, it should have validity, i.e., good sensitivity and specificity, and be able to accurately identify women at risk of an abnormally low or excessive response to ovarian stimulation. A test that exhibited all of these characteristics would enable accurate patient-specific counselling, such that individualized treatment plans can be discussed and realistic expectations set prior to commencing treatment.

Over the last three decades, concomitant with the development of assisted conception and the need to accurately predict ovarian response to superovulation and the chance of success with treatment, there has been a continuous evolution of ovarian reserve tests. From the initial introduction of the day-3 follicle-stimulating hormone (FSH) (1988), followed by clomiphene citrate challenge test (CCCT) (1989), gonadotrophin-releasing hormone (GnRH) agonist (1989), inhibin B (1997), antral follicular count (AFC) (1997) and anti-

Müllerian hormone (AMH) (2002), all have been assessed with respect to their ability to predict ovarian response, assign risk and enable stratification of individuals to the appropriate intervention. Most of these initial measures have however been abandoned, due to either poor predictive value, requiring multiple visits, or exhibiting substantial intercycle variation. Today, the markers most often used in clinical practice are the basal tests; FSH, AFC and AMH.

3.3 Early Follicular Follicle-Stimulating Hormone

The use of early follicular phase (basal) FSH as a marker of ovarian reserve was proposed almost 30 years ago, as a tool to predict ovarian response to in vitro fertilization (IVF). This test is an indirect assessment of ovarian reserve and is based on the feedback inhibition of FSH pituitary secretion by ovarian factors. At the beginning of the menstrual cycle, oestradiol and inhibin B levels reach a nadir, offering a glimpse to the un suppressed hypothalamus-pituitary-ovarian axis before levels of these ovarian hormones rise and inhibit FSH secretion. Women with normal ovarian reserve have sufficient production of ovarian hormones at this early stage of the menstrual cycle to maintain FSH levels within normal range. In contrast, elevation of FSH at this stage of the menstrual cycle indicates poor production of ovarian hormones by diminishing the ovarian follicular pool consistent with diminished ovarian reserve (DOR). However, basal FSH testing has several major limitations, including significant intercycle and intracycle variability that limits its reliability, it requires a functional hypothalamus-pituitary-ovarian axis, and it is not adequately sensitive for clinical utility—only elevations carrying significance.

Despite its limitations, FSH is commonly used as an ovarian reserve test, and high values have been associated with both poor ovarian response and failure to achieve pregnancy. FSH has particularly high specificity (45–100 per cent) for predicting poor response to ovarian stimulation (usually defined as ≤ 3 retrieved oocytes) using multiple cutoff points >10 IU/L (10–20 IU/L), but its sensitivity is generally poor (11–86 per cent) and decreases with increasing FSH cutoff points [3]. In terms of predicting failure to conceive, FSH testing is still specific (50–100 per cent) but much less sensitive (3–65 per cent) using similar cutoffs (Table 3.1). The clinical utility of FSH, as compared to AMH and AFC, is primarily in those women with short or irregular cycles, as an abnormally elevated FSH result is almost synonymous with late DOR (high positive predictive value), but the majority of women who are tested (including those with DOR) will have a normal test result (low negative predictive value). Moreover, a single abnormal FSH value in a woman <40 years of age may not predict a poor response to stimulation or failure to achieve pregnancy, and should prompt repeat testing. In terms of OHSS, FSH has no predictive value for this complication.

3.4 Anti-Müllerian Hormone

AMH is a glycoprotein that belongs to the transforming growth factor- β superfamily and is produced in the female exclusively by granulosa cells of small and large preantral and small antral follicles [4]. AMH acts as a leading negative paracrine regulator of early folliculogenesis as it inhibits recruitment of primary follicles from the primordial pool, prevents selection of follicles by FSH, and inhibits aromatase (Figure 3.1). The observation that AMH-deficient mice demonstrated accelerated atresia and premature depletion of primordial follicles led to AMH being considered as an ovarian reserve marker. In humans the

Table 3.1 Comparison of technical and clinical characteristics of the three common ovarian reserve tests

Test	Basal FSH	AFC	AMH
Timing	Day 2–5 of menstrual cycle	Day 2–5 of menstrual cycle	Any day
Temporal change indicating ovarian aging	Latest	Early	Earliest
Intracycle variability	Clinically significant	Clinically significant	Minimal
Intercycle variability	Clinically significant	Minimal	Minimal
Methodology	Automated	Ultrasound	ELISA/automated
Advantages	Widespread use	Immediate results; good predictive value for stimulation ovarian response, including predicting OHSS	Reliable; high sensitivity; good predictive value for stimulation ovarian response, including predicting OHSS
Limitations	Reliability; low sensitivity; dependent on functional HPO axis; less precision due to intercycle and intracycle variability; does not predict OHSS	Interobserver variability (sonographer-dependent); requires cost of ultrasound technician and availability of ultrasound machine; significant intercycle variation in overweight and obese	Lack of international standardized assay; requires careful sample preparation and storage if ELISA being used
Cutoffs used for determining sensitivities and specificities	10–20 IU/L	<3–4 follicles (total)	0.1–1.66 ng/mL or <0.1–<0.3 ng/mL
Sensitivity for poor response (%)	11–86	9–73	44–97
Specificity for poor response (%)	45–100	73–97	41–100
AUC for poor response	0.68 (95% CI 0.61–0.74)	0.76 (95% CI 0.70–0.82)	0.78 (95% CI 0.72–0.84)

AMC, antral follicular count; AMH, anti-Müllerian hormone; AUC, area under the curve; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; FSH, follicle-stimulating hormone; HPO, hypothalamus-pituitary-ovarian; OHSS, ovarian hyperstimulation syndrome.

ovary begins producing AMH in utero at about 36 weeks of gestation; its levels rise throughout childhood and peak at about 25 years of age, then gradually decline until reaching undetectable levels a few years prior to the menopause.

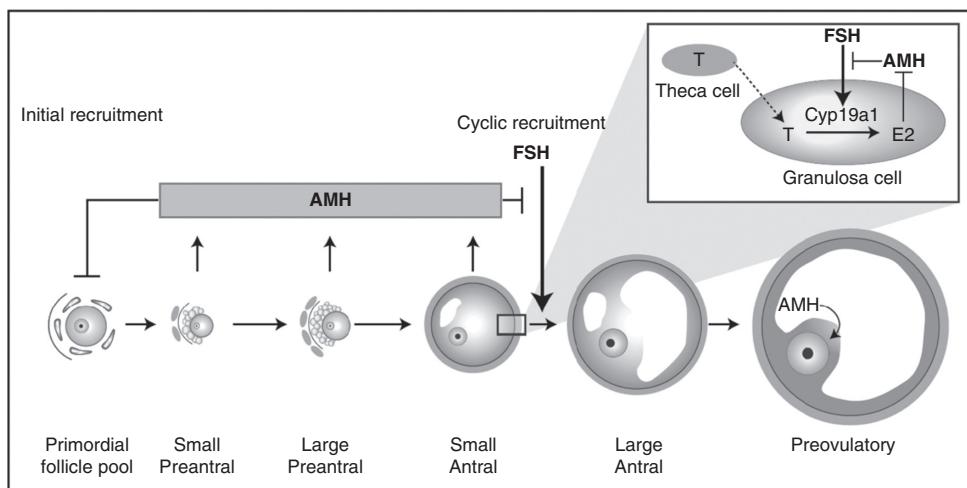


Figure 3.1 Schematic model of AMH actions in the ovary. AMH, produced by the granulosa cells of small growing follicles, inhibits initial follicle recruitment and FSH-dependent growth and selection of pre-antral and small antral follicles. In addition, AMH remains highly expressed in cumulus cells of mature follicles. The inset shows in more detail the inhibitory effect of AMH on FSH-induced CYP19a1 expression leading to reduced oestradiol (E2) levels, and the inhibitory effect of E2 itself on AMH expression. T, testosterone; Cyp19a1, aromatase.

Since AMH is expressed during normal early folliculogenesis (primarily secreted by early follicles up to 8 mm), it is relatively independent of gonadotrophins circulating at physiologic levels and allows for testing anytime throughout the cycle. While several earlier studies suggested that AMH is relatively stable throughout the menstrual cycle in normo-ovulatory women, other studies noted significant fluctuations within one menstrual cycle. While this issue remains highly debated, evidence suggests that if significant fluctuations in AMH levels do occur, they have limited clinical relevance [5]. Moreover, the random and non-cyclic fluctuations in AMH indicate that measuring the hormone on a fixed day of the menstrual cycle would not yield any advantage to random assessment. Furthermore recent trials using individualized AMH-driven gonadotrophin doses utilized random AMH measurements [6]. Collectively these analyses suggest that repeat measurements of AMH during subsequent cycles are not necessary for accurate patient assessment.

3.4.1 Sensitivity and Specificity

Among all ovarian reserve tests, AMH is considered the earliest and most sensitive. It correlates strongly with the primordial follicle pool, has an inverse correlation with chronologic age in adults, reliably predicts ovarian response in ART and is predictive of the timing of the onset of menopause. In a systematic review of studies in women undergoing controlled ovarian stimulation with gonadotrophins, low AMH cutoff points (0.1–1.66 ng/mL) have been found to have sensitivities ranging between 44 and 97 per cent and specificities ranging between 41 and 100 per cent for prediction of poor ovarian response. In a meta-analysis that included 28 studies, AMH was found to have good predictive ability for poor ovarian response, with an area under the curve (AUC) of 0.78 [7]. Moreover, AMH has remarkable utility in predicting ovarian hyperstimulation to

gonadotrophin stimulation, with sensitivities ranging from 53 to 90.5 per cent and specificities ranging from 70 to 94.9 per cent when using cutoff values of 3.36–5.0 ng/mL. However, despite its strong correlation with ovarian response to stimulation in ART, AMH is a poor predictor of non-pregnancy with sensitivities between 19 and 66 per cent, and specificities between 55 and 89 per cent when using cutoffs ranging from <0.1 to 1.66 ng/mL (Table 3.1). Notably, a recent study of the Society for Assisted Reproductive Technology database found that while women with ultralow AMH (<0.16 ng/mL) had 54 per cent cycle cancellation rate, the overall live birth rate per cycle start was 9.5 per cent, supporting the notion that denying infertility treatment solely on the basis of undetectable AMH is not advisable. Similarly, AMH is a poor predictor of pregnancy and live birth following ART, with two recent meta-analyses showing the AUC for AMH as predictor of clinical pregnancy and live birth to be 0.63 and 0.61, respectively [5]. The sensitivities and specificities for AMH in predicting clinical pregnancy ranged from 34.4 to 86.2 per cent and 26 to 78.5 per cent, respectively, with cutoffs ranging between 1.0 and 3.22 ng/mL (Table 3.1). Moreover, AMH values were not associated with fecundability in unassisted conceptions, consistent with AMH being a predictive marker of oocyte quantity but not quality.

3.4.2 Limitations

The main limitations of the AMH test relate to the lack of a universal standard and the different assays that are available. Since the original reports of measurement of serum AMH in 1990, there has been continual development of the immunoassay by a variety of companies, utilizing different antibody pairs. At present four manual enzyme-linked immunosorbent assays are available. Although these assays exhibit well within laboratory reproducibility, they display substantial variability between laboratories, due to the lack of automation and site-specific processes. These manual assays have now been complemented by three fully automated assays, with significantly better performance characteristics, including greater precision (fourfold), faster turnaround time (18 minutes versus 6 hours) and greater sensitivity (tenfold) compared to current enzyme-linked immunosorbent assay-based assays. However, several studies have demonstrated intraassay/interassay calibration differences that continue to limit clinical interpretation if the assay used is not known.

3.4.3 Age-Specific AMH Values

Age-specific AMH values have been primarily derived from the population of women presenting to fertility clinics; however, it is clear that these women have a lower ovarian reserve and thereby AMH than fertile women in the general population. As a general guideline we consider the median of age-appropriate serum AMH values for healthy fertile women using the Roche Elecsys AMH assay for the following in five-year age intervals to be approximately 0.2 ng/mL for 45 years, 1.0 ng/mL for 40 years, 2.0 ng/mL for 35 years, 2.7 ng/mL for 30 years and 3.5 ng/mL for 25 years. So, in practice, if a 35-year-old woman presents with an AMH of 1 ng/mL this may be of concern as one would have conservatively expected an AMH value of at least 2 ng/mL. An AMH of 1 ng/mL is what one might conservatively expect from a 40-year-old woman. Such a lower observed value in the context of the rest of a patient's history (i.e., smoking or early onset of menopause of her mother) may

point to taking a more expeditious approach to her fertility treatment depending on her expectations for conceiving more than one child in the future.

3.4.4 Factors Affecting AMH Results

In addition, when interpreting a patient's AMH test results it is important for the clinician to consider the effects of possible influencing factors to avoid inaccurate assessment of ovarian reserve. Table 3.2 summarizes the biological, reproductive and environmental/lifestyle factors on anti-Müllerian hormone

Table 3.2 Effect of biological, reproductive and environmental/lifestyle factors on anti-Müllerian hormone

Potential factor	Effect on AMH levels
Biological characteristics	
Race and ethnicity	No effect when adjusted for confounders
Systemic illness (e.g., Crohn's, SLE)	Decrease
BRCA1 carrier	Decrease
FMR1 premutation	Decrease
Reproductive factors	
Ovarian suppression (OCPs, GnRH agonists)	Decrease
Polycystic ovarian syndrome	Increase
Current pregnancy	Decrease
Parity	No effect
History of ovarian surgery	Decrease
Endometriosis	Decrease
Granulosa cell tumour	Increase
Environmental/lifestyle	
Body mass index (obesity)	Inconsistent – large studies suggest no change
Socioeconomic status	No effect
Past smoking	Minimal decrease
Current smoking	Decrease
Chemotherapy	Decrease
Low vitamin-D level	Decrease
Alcohol use	No effect
Physical exercise	No effect

AMH, anti-Müllerian hormone; BRCA-1, breast cancer gene-1; FMR1, fragile X mental retardation 1; GnRH, gonadotrophin-releasing hormone; OCPs, oral contraceptive pills; SLE, systemic lupus erythematosus.

lifestyle factors suggested to affect AMH levels. For example, polycystic ovary syndrome (PCOS) is associated with elevated AMH levels, while ovarian suppression related to oral contraceptive pills or GnRH agonist administration can decrease AMH levels, with AMH levels generally returning to baseline within three to four months of oral contraceptive discontinuation. Among environmental/lifestyle factors, current smoking and low vitamin-D levels have been associated with lower AMH levels. Obesity and ethnicity have not been shown to affect AMH levels when values are adjusted for confounders.

3.5 Antral Follicular Count

AFC is the sum of follicles in both ovaries as observed on ultrasound in the early follicular phase (day 2–4) of the menstrual cycle. Antral follicles are defined as those measuring 2–10 mm in largest mean diameter on two-dimensional plane. AFC is easy to carry out, provides an immediate result and has good intercycle reliability and good interobserver reliability when measured in experienced centres using a minimal number of sonographers. Its precision is compromised with overweight and obese individuals or when using multiple sonographers. As suggested by a meta-analysis, a low AFC is associated with poor ovarian response to ovarian stimulation during IVF, but has poor predictability for pregnancy. Across general IVF study populations of patients at both low and high risk of DOR, low AFC cutoff points of 3–4 follicles (both ovaries combined) are highly specific (73–97 per cent) for predicting poor ovarian response (<3–4 oocytes, cycle cancellation) but have low sensitivity (9–73 per cent) [8]. In terms of non-pregnancy prediction, AFC is still specific (64–98 per cent) but much less sensitive (7–34 per cent).

There are several limitations to AFC measurement. It must be carried out at the beginning of a cycle due to intracycle variation. In addition, AFC has inherent variability related to technology and interobserver variability. Significant variation in AFC has been observed both between and within centres. This variation may be caused by differences in operator training, number of sonographers, methodology as well as criteria for measuring antral follicles, and differences in ultrasound technology (e.g., resolution of ultrasound, two-versus three-dimensional). In addition, AFC has a tendency to overestimate the number of FSH-sensitive follicles and oocytes retrieved since it inevitably also measures atretic follicles of the same size. Moreover, greater intracycle and intercycle variation has been observed in overweight and obese women, limiting the predictive value of AFC in this subpopulation of women, which is ever increasing in industrial nations. In terms of OHSS prediction, both AFC and AMH demonstrate strong predictive value for predicting those at greatest risk for OHSS.

3.6 Which Ovarian Reserve Test Should I Choose?

A large body of evidence has demonstrated greater clinical value of AMH and AFC compared to FSH. However, AMH and AFC have often been considered as interchangeable biomarkers for the prediction of ovarian response prior to commencement of ovarian stimulation. Observational cohort studies comparing the performance of AFC and AMH levels have generally yielded similar predictive value for ovarian response and outcome in three meta-analyses. However, in marked contrast to these reports, four recent large, prospective, multicentre trials in IVF/intracytoplasmic sperm injection patients consistently concluded that AMH was a better predictor of the number of oocytes retrieved as well as categorization of low and high responders than AFC. Figure 3.2 shows the mean

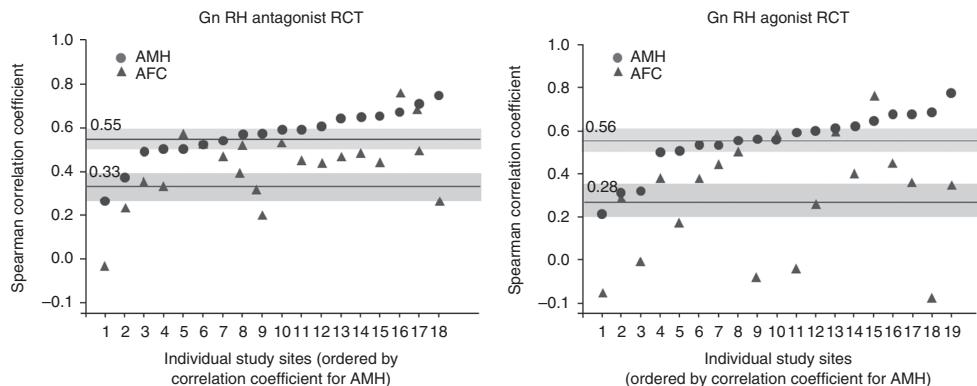


Figure 3.2 Correlation of AMH and AFC at each investigation site with number of oocytes retrieved. The mean correlation coefficient of AMH and AFC with number of oocytes retrieved across all centres is shown by solid line, with the 95% confidence intervals indicated by the shading. Each individual clinic's correlation coefficient is shown by the points. Data derived from Nelson SM et al. Fertil Steril 2015;103:923–930.

correlation between AMH and AFC at each of the centres with the number of eggs retrieved for two of these trials. Due to the limitations of AFC in terms of sonographer-dependent variability and technical aspects of ultrasound equipment and the increasing advantages of AMH testing in terms of patient convenience and assay robustness, AMH is more and more being recognized as the preferred biomarker of ovarian response to controlled ovarian stimulation. A comparison among the three ovarian reserve tests is summarized in Table 3.1.

3.7 What to Do in Case of Discordance between Ovarian Reserve Tests?

Since information about more than one ovarian reserve test is often available for infertility patients, it is not uncommon to have discordant test results that may complicate patient counselling and decision-making regarding the most appropriate treatment. In a large study of 5354 women that examined discordance between AMH and FSH results obtained by a single reference laboratory, one in five women were found to have discordant AMH and FSH values defined as AMH <0.8 ng/mL (concerning) with FSH <10 IU/L (reassuring) or AMH ≥0.8 ng/mL (reassuring) with FSH ≥10 IU/L (concerning). Of the women with reassuring FSH values ($n = 4469$), the concerning AMH values were found in one in five women in a highly age-dependent fashion with increasing frequency as women were ≥38 years old. This is not surprising, since AMH is more sensitive than FSH in diagnosing DOR and would normally reach abnormal levels before FSH levels become abnormal. On the other hand, of the women with reassuring AMH values ($n = 3742$), one in 18 had concerning FSH values, a frequency that did not vary significantly by age. Another study investigated AMH and FSH values in 366 infertility patients with DOR undergoing their first IVF cycle, finding discordance between age-specific values in 38.6 per cent of women. The authors found that at age 34–42 years, normal AMH carried better prognosis than normal FSH in terms of oocyte yield, while at age >42 years normal FSH was associated with better prognosis than normal AMH. For both age ranges, the group with both normal AMH

and FSH always had the best prognosis while the one with both values abnormal had the worst prognosis.

A different study assessing discordance between AMH and AFC in patients undergoing IVF found that 32.3 per cent of women had discordant results. The discordant groups with low to normal ovarian reserve had ovarian responsiveness intermediate between those with concordantly low and concordantly normal AMH and AFC. Likewise the discordant groups with normal to high ovarian reserve had ovarian responsiveness intermediate between those with concordantly normal and concordantly high AMH and AFC. In addition, within each AFC category, those with higher AMH had significantly higher number of oocytes retrieved. Therefore, when ovarian reserve tests fall into discordant categories, it would be reasonable to consider the ovarian reserve to be an intermediate between the two tests. For purpose of IVF stimulation dosing, for example, this would mean choosing an intermediate dose of gonadotrophin as compared to those with concordant AMH and AFC categories on either end.

3.8 Who Should Get Ovarian Reserve Testing and How to Use It in Clinical Practice?

Historically, ovarian reserve testing was used for prediction of ovarian response to controlled ovarian stimulation in ART, helping to identify patients who are more likely to have a poor response or hyper-response to gonadotrophins. Over the past decade, since the discovery of AMH as an ovarian reserve marker, it has been increasingly recognized that AMH testing may have utility for a variety of other clinical applications in reproductive medicine (Box 3.1).

Box 3.1 Indications for ovarian reserve testing

Women undergoing infertility evaluation/treatment

Individualization of assisted reproductive technology ovarian stimulation protocol and dosing

History of premature ovarian failure (insufficiency) or early menopause

Polycystic ovarian syndrome

Women considering elective (social egg) freezing

Oocyte donors

Fertility preservation before and after gonadotoxic treatment

Preoperative prior to ovarian surgery in reproductive-age women

Diagnosis and recurrence surveillance for granulosa cell tumours

Perimenopause

Women with BRCA-1 or FMR1 premutation

BRCA-1, breast cancer gene-1; *FMR1*, fragile X mental retardation 1.

The usefulness of AMH in predicting ovarian response to controlled ovarian stimulation in ART has led to AMH-based pretreatment counselling and individualization of ART stimulation protocols, including choice of regimens and dose adjustments. The following general guidelines are useful for clinical practice:

- AMH <0.5 ng/mL predicts poor ovarian response in IVF with yield of four or less oocytes. In such cases, a discussion with the patient about the short window of

opportunity to conceive seems warranted. Ovarian stimulation protocols in these patients should include those designed to maximize follicular recruitment.

- AMH level ≥ 1.0 ng/mL but ≤ 3.5 ng/mL if age appropriate is consistent with normal ovarian response to ovarian stimulation. IVF protocols for these patients should include standard GnRH agonist or antagonist, with adjustments based on age-specific values.
- AMH >3.5 ng/mL predicts hyper-response to ovarian stimulation and is associated with significantly higher risk of OHSS. Such patients would benefit from mild lower dose regimens and protocols that minimize OHSS risk (e.g., GnRH antagonist with GnRH agonist trigger).

3.9 Menopause

Several studies have suggested that AMH in conjunction with age may be able to predict the timing of menopause with reasonable accuracy in late reproductive-age women. A recent report from the Study of Women's Health Across the Nation provided data on the use of AMH in predicting the final menstrual period (FMP) using the ultra-sensitive picoAMH assay, enabling especially accurate measurements at the lower end of detection. At baseline, women aged 42–52 years had blood collected serially until the first visit after FMP. AMH was measured in a total of 1,560 women. Women whose AMH values were 5–10 pg/mL had a 75 per cent chance of having their FMP within 24 ± 3 months, while women with AMH levels of over 25 pg/mL had less than a 2.5 per cent chance of having their FMP within two years. However, in a Dutch cohort study that included women of younger reproductive age (range 21–46 years), while AMH was predictive for age at natural menopause, the prediction intervals were broad and extreme ages at menopause could not be predicted. The limited predictive ability of AMH for menopause in the general population may make this marker currently unsuitable for individualized counselling of women regarding their reproductive lifespan. Research is ongoing in pursuit of developing more accurate biomarker-based formulas for prediction of menopause timing.

3.10 Fertility Preservation

With rising numbers of young girls and women being successfully cured by life-saving, but often gonadotoxic treatments, subsequent reproductive health has become a major quality-of-life issue, and the ability to predict which patients may lose their fertility or ovarian function as a result of treatment has become of increasing importance. AMH testing in young cancer patients both pretreatment and post-treatment has become a useful tool for assessment of iatrogenic damage to the ovarian follicular reserve inflicted by gonadotoxic chemotherapy agents or pelvic irradiation, and may help in fertility preservation counselling and strategies. Chemotherapy and radiotherapy can both have deleterious effects on ovarian function and consequently AMH levels, with the extent of injury dependent on the patient's age, type of treatment and treatment dose. Studies have demonstrated that women with higher pretreatment AMH levels have higher post-chemotherapy levels and display faster recovery rate in AMH levels once treatment has been completed. Importantly, two studies involving breast cancer patients provided prognostic tools by which clinicians can inform patients more precisely regarding ovarian function after chemotherapy using pre-treatment AMH levels, with other important variables. The first prognostic tool

demonstrated that all women, regardless of age, with AMH levels of <0.54 ng/mL will have amenorrhea post-treatment. The second tool is a prognostic scoring system based on a patient's age, pretreatment AMH levels and body mass index with one point awarded for each of age <40 years, AMH level >0.7 ng/mL and body mass index >25 kg/m 2 . The likelihood of, and time to, recovery of ovarian function after completion of chemotherapy improves with increasing number of points. For example, menses returned in 75 per cent of women with three points by 160 days whereas only 25 per cent of women with zero points had a return of ovarian function by 221 days. Since both of these prognostic systems were developed in breast cancer patients, and the systemic effects of differing forms of cancer and their treatment vary, it is not possible to generalize the results above to all cancers. Therefore, further research into different types of malignancies and treatment regimens is required to build up a panel of prognostic tools. Moreover, it should be emphasized that since the primary outcome of the research thus far has been post-treatment amenorrhea/premature ovarian insufficiency, there is not yet direct evidence that pretreatment AMH levels can predict subsequent fertility. This important outcome should be the focus of future investigations in this field.

3.11 Polycystic Ovarian Syndrome

It has been suggested that AMH plays an important role in the pathogenesis of PCOS. Substantial evidence indicates that AMH correlates strongly with the severity of various hallmarks of PCOS, including polycystic ovarian morphology, hyperandrogenism and oligo/anovulation. Moreover, elevated serum AMH concentrations are predictive of poor response to various treatments of PCOS, including weight loss, ovulation induction and laparoscopic ovarian drilling, while improvement in various clinical parameters following treatment is associated with serum AMH decline, further supporting an important role for AMH in the pathophysiology of this syndrome. A cutoff of AMH >6.2 ng/mL using the Elecsys assay has been shown to be highly diagnostic of PCOS (AUC 0.93, sensitivity 74 per cent, specificity 92 per cent), and suggested to be incorporated as a diagnostic criterion for this syndrome. Thus, AMH testing could be considered in the routine workup of PCOS patients and could aid in establishing the diagnosis, provide insight to the severity and potential treatment resistance, as well as alert physicians to the increased risk of OHSS if ovarian stimulation is being considered.

3.12 Ovarian Reserve Screening for the General Reproductive-Age Female Population?

It is estimated that approximately 10 per cent of the general female population will undergo accelerated loss of ovarian reserve leading to loss of fertility from their mid-30s and early menopause by age 45 years. In the past, this was of minimal concern from a public health standpoint since most women had completed their family plans by their mid-30s. However, over the past two decades the average age of a mother at her first birth has steadily increased and is currently 30 years of age in the Western world, with a further one in five women not having commenced pregnancy attempt by 35 years of age. While some of these women have made a conscious decision not to have children, the majority still do want children but have simply not begun trying for a baby, often due to gaps of knowledge about age-related fertility decline. Importantly, recent studies have shown that information about one's

ovarian reserve would lead individuals to modify life choices. One study showed that among healthcare workers, if testing of the individual or individual's partner indicated DOR, 48 per cent would try to have a child sooner, 21 per cent would opt for oocyte cryopreservation, 7 per cent would try to find a partner sooner, 7 per cent would pursue adoption and 3 per cent would select embryo cryopreservation. Only 14 per cent would not actively pursue treatment or make lifestyle changes.

Despite the consensus that AMH is an excellent marker of ovarian reserve, there is currently no agreement on the use of AMH to screen for ovarian reserve in the general, non-infertile population. Concerns raised by opponents of such testing are that a poor result does not definitively mean diminished chances of natural conception, while an abnormally low value may also lead to substantial anxiety with multiple potential negative consequences (e.g., premature termination of education and career development to have children, seeking motherhood outside of a stable relationship). However, contrary to this argument, a recent study showed that bankers and non-bankers of oocytes have a surprising congruent relational status and reproductive choices, indicating that freezing oocytes does not appear to influence the life choices of the women. The study provides insights into the important psychological aspect of reassurance associated with preventive oocyte banking, expressed by high satisfaction after banking in combination with a decreased intention of ever using the eggs. An important ethical consideration, however, is that oocyte banking is costly and may not be affordable to many women identified as having low ovarian reserve by AMH screening.

Proponents of ovarian reserve screening of the general population of reproductive-age women argue that it may offer several advantages. First, women identified as having low ovarian reserve are thought to be at increased risk of early loss of fertility potential in the longer term. However, evidence in support of this argument is conflicting as one study found that women with low AMH (<0.7 ng/mL) have significantly decreased fecundability after adjusting for age, while a more recent study reported that lower (<1.0 ng/mL) and higher (>3.5 ng/mL) AMH values were not associated with fecundability in unassisted conceptions in a cohort of fecund women with a history of one or two losses. Second, women often disregard generic advice to avoid delaying conception at greater than 30 years of age, yet studies suggest that personalized risk assessment tools such as ovarian reserve testing can actually affect an individual's family planning. Finally, it is reasonable to argue that women have a right, based on the ethical concept of autonomy, to be made aware of ovarian reserve screening, so that they themselves can determine if ovarian reserve testing is useful in assisting them with reproductive life planning. However, before firm recommendations can be made, large longitudinal studies are needed to determine whether such screening strategies may be beneficial from a population health perspective.

3.13 Conclusions and Recommendations

treatment of ovarian reserve can help identify patients who will have poor response or excessive response to ovarian stimulation for ART and individualize treatment protocols to achieve optimal response while minimizing safety risks. It may inform patients regarding their reproductive lifespan and menopausal timing, and also aid in counselling and treatment strategy planning of young female cancer patients receiving gonadotoxic therapy. In addition, it may aid in establishing the diagnosis of PCOS and provide insight into disease severity. Finally, ovarian reserve testing may be considered as a screening

tool in selected populations of women for assisting in their reproductive life planning. The ideal ovarian reserve test should be convenient, reproducible, display little if any intracycle and intercycle variability and demonstrate high specificity to minimize the risk of wrongly diagnosing women as having DOR and accurately identify those at greatest risk of developing OHSS prior to fertility treatment. While there is currently no perfect ovarian reserve test, both AFC and AMH level have good predictive value and are superior to day-3 FSH. The convenience of untimed sampling, age-specific values, availability of an automated platform and potential standardization of AMH assay make this test the preferred biomarker for the treatment of ovarian reserve in most women. It is important to consider age-specific values when interpreting results and to remember that conditions such as PCOS and hormonal suppression can affect the values obtained.

References

1. Wallace WH, Kelsey TW. Human ovarian reserve from conception to the menopause. *PLoS One* 2010;5:e8772.
2. Tal R, Seifer DB. Ovarian reserve testing: a user's guide. *Am J Obstet Gynecol*. 2017 August;217(2):129–140.
3. Broekmans FJ, Kwee J, Hendriks DJ et al. A systematic review of tests predicting ovarian reserve and IVF outcome. *Hum Reprod Update* 2006;12:685–718.
4. Dewailly D, Andersen CY, Balen A et al. The physiology and clinical utility of anti-Müllerian hormone in women. *Human Reproduction Update* 2014;20:370–385.
5. Iliodromiti S, Kelsey TW, Wu O et al. The predictive accuracy of anti-Mullerian hormone for live birth after assisted conception: a systematic review and meta-analysis of the literature. *Hum Reprod Update* 2014;20:560–570.
6. Nyboe Andersen A, Nelson SM, Fauser BC et al. Individualized versus conventional ovarian stimulation for in vitro fertilization: a multicenter, randomized, controlled, assessor-blinded, phase 3 noninferiority trial. *Fertil Steril* 2017;107:387–396.
7. Broer SL, van Disseldorp J, Broeze KA et al. Added value of ovarian reserve testing on patient characteristics in the prediction of ovarian response and ongoing pregnancy: an individual patient data approach. *Hum Reprod Update* 2013;19:26–36.
8. Broer SL, Mol BWJ, Hendriks D, Broekmans FJM. The role of antimullerian hormone in prediction of outcome after IVF: comparison with the antral follicle count. *Fertility and Sterility* 2009;91:705–714.

Natural Cycle and Minimal Stimulation IVF

Jacqueline R. Ho and Richard J. Paulson

4.1 Introduction

The first attempts at in vitro fertilization (IVF) were in stimulated cycles, which ultimately resulted in a tubal gestation. Steptoe and Edwards subsequently attempted IVF using a purely natural ovulatory cycle. Laparoscopic egg retrieval was timed on the basis of the endogenous surge of luteinizing hormone (LH). The subsequent embryo transfer (ET) led to a successful pregnancy and the first IVF live birth (Louise Brown), which was reported in 1978. While this was a positive outcome, the retrieval of only one egg was felt to offer too small of a margin for error when performing a laparoscopic egg retrieval. Some type of ovarian stimulation was the logical answer. Trounson et al. investigated the use of clomiphene citrate to increase oocyte yield, as well as hCG trigger to precisely time laparoscopic retrieval. Successful IVF outcomes were achieved using this protocol [1]. Jones et al. then reported successful use of stimulated controlled ovulation with the use of human menopausal gonadotropin (hMG) and hCG trigger to time laparoscopy [2]. The continued fine-tuning of cycle monitoring and stimulation protocols led to the current utilization of controlled ovarian hyperstimulation (COH), which has since become standard practice.

Stimulation protocols now incorporate gonadotropins as well as GnRH analogues to prevent premature ovulation and allow maximal control of the cycle. Ovarian stimulation certainly has advantages as it increases the number of growing follicles, and thus the number of oocytes that are retrieved. Multiple embryos allow for embryo selection and cryopreservation of supernumerary embryos for additional opportunities for the establishment of pregnancies.

While this is successful for the majority patients, COH does have some drawbacks. For example, some patients do not respond well to high doses of gonadotropins. For certain patients, unstimulated and minimal stimulation protocols may be equally efficacious as standard COH, while avoiding the higher costs of the stronger stimulation.

4.2 Natural and Modified Natural Cycle IVF

Natural cycle IVF (nIVF) refers to oocyte retrieval from the dominant follicle formed during a woman's spontaneous cycle and subsequent fertilization and culture *in vitro*. In the era of laparoscopic oocyte retrieval, the promise of a single oocyte, which may or may not be successfully recovered, led clinicians to attempt protocols which would increase the yield of oocytes obtained by this relatively traumatic procedure. Subsequently, COH became the norm and natural cycles were temporarily abandoned. However, in the late 1980s, the process for oocyte retrieval transitioned from laparoscopy to transvaginal ultrasound guidance. The minimally invasive nature of this procedure made it a reasonable option to

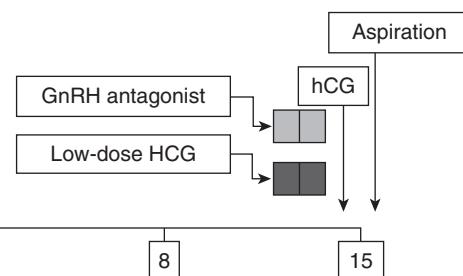


Figure 4.1 Diagram for modified natural IVF cycle

- Cycle monitoring via ultrasound and serum E2
- Ganirelix acetate 0.25 mg SC qd and HCG 200 IU SC qd started when lead follicle size ≥ 14 mm and serum E2 > 100 pg/mL

attempt oocyte retrieval for single follicles, and the natural cycle became a reasonable alternative once again.

4.2.1 HCG Trigger and Cancellation Rates

In order to improve the timing of oocyte retrieval, clinicians began to use the hCG trigger, as in common practice with COH. This type of cycle was no longer purely natural, and thus the designation ‘unstimulated’ was added. Whereas egg retrieval was now much more predictable, patients could still ovulate prior to the hCG trigger with the occurrence of a spontaneous LH surge. In an early series in 1989, Foulot et al. reported a cancellation rate of approximately 15 percent, with only 68 of 80 cycles reaching retrieval in spite of hCG trigger usage [3]. Paulson et al. reported a cancellation rate of 23 percent in 101 unstimulated IVF cycles, with 78 cycles reaching oocyte retrieval [4]. Since then, in spite of further advances, cancellation rates reported in case series and cohort studies still range from 15 to 71 percent [5].

4.2.2 Further Modifications

In the early 1990s, GnRH antagonists first became available for clinical trials. Their ability to acutely stop gonadotropin secretion and thus prevent a premature LH surge was immediately appreciated and they were tried in the context of COH. It was also logical to apply these to unstimulated IVF cycles in order to further decrease the risk of premature ovulation [6,7]. Since GnRH antagonists not only stop the LH surge but also disrupt further FSH stimulation of the growing follicle, some form of ‘add-back’ had to be applied, either in the form of hMG or pure FSH [6,7]. These types of cycles, utilizing GnRH antagonists and gonadotropin add-back, became known as modified natural IVF (mnIVF) (Figure 4.1), and most modern series use this type of protocol.

More recently, it has been demonstrated that low-dose hCG alone can complete follicle maturation in stimulated cycles [8]. HCG binds LH receptors, in the late follicular phase, allowing for continued follicular growth and maturation in spite of pituitary down-regulation. A Cochrane review comparing the use of low-dose hCG and FSH in the late follicular phase of COH cycles concluded that its use does not reduce chances of clinical

pregnancy and likely results in an equivalent number of retrieved oocytes [9]. We reasoned that low-dose hCG could be applied to mnIVF in place of hMG, in a manner analogous to that of COH. Most recently, we have reported live births with the use of low-dose hCG (200 IU daily) in an mnIVF cycle in lieu of gonadotropins for follicular support [10]. The most obvious benefit of using low-dose hCG in place of hMG is a significant decreased cycle cost. It is not yet clear if its use may improve cycle pregnancy outcomes or merely provide a suitable alternative to hMG.

4.2.3 Secondary Follicles and In Vitro Maturation

One limitation of mnIVF is the low oocyte yield in comparison with COH cycles. However, transvaginal aspiration of secondary follicles may lead to retrieval of additional mature oocytes or immature oocytes that may be cultured in vitro to become mature oocytes. In a large retrospective study of mnIVF cycles by Teramoto et al., over 25 percent of oocytes aspirated from a non-dominant follicle were MIIs and 85.6 percent of these were fertilized [11]. The reported live birth rate was 8.6 percent per oocyte from non-dominant follicles and 19.3 percent per oocyte in dominant follicles, showing that secondary follicles in natural cycles can lead to successful pregnancies [11]. For immature oocytes retrieved, in vitro maturation (IVM) is well described in the literature and has led to successful pregnancies [12]. IVM involves collection of secondary follicles and culture of oocytes until maturation to metaphase II (MII). They can then be fertilized using co-incubation with sperm or intracytoplasmic sperm injection (ICSI). Whereas most experience with IVM comes from polycystic ovarian syndrome (PCOS) patients, it is interesting to note that it can be applied to mnIVF cycles in order to substantially increase the oocyte yield and overall chances of success [11–13]. IVM provides a safe and feasible technique for producing embryos from immature oocytes that may be derived from secondary follicles in minimal stimulation or modified natural cycle protocols.

4.2.4 Practical Considerations

During an mnIVF cycle, patients undergo serial monitoring during their follicular phase. We monitor both follicle size and oestradiol (E_2) levels, although it is not clear that E_2 levels are absolutely necessary for cycle success. It is important to emphasize that in an mnIVF cycle, the frequency of ultrasounds may be similar to that of a COH cycle, particularly in poor responders who may ovulate at unpredictable times and at smaller follicle sizes.

When the dominant follicle reaches approximately 14 mm in average diameter, a GnRH antagonist (Ganirelix or Cetrotide) 0.25 mg is typically administered. This is continued once daily until ovulation is triggered with 10,000 IU of hCG. Supplemental gonadotropins are administered daily while GnRH antagonist is used. This may include low-dose FSH 150 IU, HMG 150 IU, or hCG 200 IU per day. Since E_2 assays are highly variable, criteria for ovulation triggering need to be established by each individual clinic. The triggering criteria used at the University of Southern California are listed in Table 4.1.

Follicle aspiration and ET are performed in standard fashion identical to that of COH cycles. The aspiration is scheduled for 34 hours after hCG trigger. Despite fewer follicles being aspirated, we recommend that the level of analgesia and anaesthesia administered should be similar to that of COH cycles. Any movement in reflex to pain may result in a failure to retrieve the oocyte from the dominant follicle. During the retrieval, all visible follicles are aspirated, including secondary follicles in anticipation of IVM.

Table 4.1 Criteria for hCG trigger. Egg retrieval is scheduled for 34 hours after hCG

Follicle size (mm)	Oestradiol level (pg/mL)
20	200
18	250
15	300

Table 4.2 Serum progesterone levels in patients undergoing unstimulated versus COH IVF

	Day of hCG	Follicle aspiration
Unstimulated	0.5±02	0.5±0.1
COH	1.1±0.6*	8.5±2.2*

* p<0.05

Adapted from Kolb BA, Paulson RJ. The luteal phase of cycles utilizing controlled ovarian hyperstimulation and the possible impact of this hyperstimulation on embryo implantation. Am J Obstet Gynecol. 1997 June;176(6):1262–7.

4.2.5 In Vitro Culture and Embryo Transfer

Mature oocytes (MII) are inseminated on the day of follicle aspiration with co-incubation with sperm or ICSI. Immature oocytes (MI) are cultured overnight and checked the next day for maturation – and all mature oocytes at that time undergo insemination or ICSI. Oocytes with germinal vesicles may be cultured for up to 72 hours in vitro. Embryos may be cultured to the blastocyst stage; although given the limited number of embryos typically produced in a natural cycle, it may be judicious to transfer embryos at the cleavage stage. Supernumerary embryos may be cryopreserved for future use.

One major advantage of mnIVF is the avoidance of endometrial-embryo dyssynchrony associated with COH, and particularly with elevated progesterone levels prior to hCG trigger. The presence of only a single (or double) dominant follicle appears to obviate the possibility of premature progesterone rise (Table 4.2). It is unclear if luteal support is needed in mnIVF cycles. However, if desired, it may be achieved with vaginal or intramuscular progesterone starting the day after the oocyte retrieval. It is currently our practice to use 200 mg of progesterone vaginally twice daily.

4.2.6 Pregnancy Outcomes and Patient Selection for mnIVF

Pregnancy rates are highly variable among studies examining mnIVF. In comparison to standard COH, an overall lower reported pregnancy rate per cycle with mnIVF might be attributed to the higher cancellation rates prior to embryo transfer. However, in patients who reach the embryo transfer stage, implantation rates have been reported to be similar to or higher than those of COH [14]. As seen with stimulated IVF, there is an age-related decline in fertility, which is reflected in mnIVF outcomes. Thus, on a per cycle basis, mnIVF

appears to be less efficient than COH. However, because of its lower costs, and no time interval between cycles, it may prove more efficient on a cost basis, or even on the basis of time to pregnancy.

4.2.6.1 Normal Responders

The ideal patient for mnIVF is an ovulatory woman with normal ovarian reserve. Retrospective studies of good responder patients have demonstrated good pregnancy rates per cycle and per embryo transfer. A retrospective review of 1,508 fresh mnIVF cycles by Shaulov et al. reported a clinical pregnancy rate per cycle of 14.5 percent in patients ≤ 35 and 10.2 percent in ≥ 36 years old. The pregnancy rates per ET were 34.5 percent in women under 35 and 23.5 percent in women 36 years or older, reflecting the reality that many patients did not reach the ET stage [15]. In those who are at elevated risk for OHSS due to either their ovarian reserve markers or a history of severe OHSS after COH, mnIVF may be an excellent option as OHSS has not been reported in these modified natural cycles.

4.2.6.2 Poor Responders

Patients who do not respond to COH are logical candidates for mnIVF. Two randomized controlled trials (RCT) have addressed the use of mnIVF in poor responders in comparison to COH. Morgia et al. compared mnIVF versus COH (microdose agonist flare protocol) in poor responders with a history of one cycle with ≤ 3 oocytes retrieved or a previous cancelled cycle [16]. They found that pregnancy rates per cycle and embryo transfer were not significantly different between treatment groups across age strata and concluded that mnIVF is a viable treatment alternative in this cohort, with younger patients (≤ 35) having a better prognosis [16]. Kim et al. compared outcomes between mnIVF and COH in poor responders (history of ≤ 3 follicles with prior IVF) [17]. Clinical pregnancy rates per cycle and per ET were similar between mnIVF and standard groups. Live birth rates per ET were also not significantly different, at 13.5 percent for mnIVF versus 16.7 percent for conventional protocols. The authors concluded that mnIVF was as efficacious as IVF using standard COH protocols, and provided potential benefit to low responders by reducing the amount of gonadotropins required and stimulation length [17]. In 2009, Shimberni et al. reported a series of 500 cycles in poor responders; pregnancy rates per embryo transfer were 29.2 percent in patients under 35 years of age, 20.6 percent for women 36–39 and 10.5 percent in women over 40 [18]. A summary of embryo transfers, clinical pregnancy rates and live birth rates is detailed in Table 4.3.

Patients most likely to benefit from mnIVF are younger, good prognosis patients who produce high-quality embryos and may potentially experience delay in transfer due to OHSS. MnIVF may be a good second-line option for poor responders who have suboptimal responses with standard COH protocols using aggressive doses of gonadotropins. Couples with religious or ethical preferences may also choose mnIVF to avoid embryo cryopreservation. Since mnIVF is substantially less expensive due to the use of minimal medication and decreased laboratory work, it may help increase access to IVF in low-income areas. Patients who would not make good candidates for mnIVF are anovulatory or oligo-ovulatory patients who may not spontaneously form a dominant follicle. Other less ideal candidates are those who would benefit most from COH, particularly women over 40, whose low embryo implantation rates may be countered with multiple embryo transfer.

Table 4.3 Summary of embryo transfer, clinical pregnancy and live birth rates for modified natural cycle IVF in women ≤ 35 years of age [14–16,18, 30, 31]

	CP/initiated cycle (%)	CP/ET (%)	LB/initiated cycle (%)	LB/ET (%)
Normal responders	14.5–19.2	30.6–35.9	15.2	19.9
Poor responders	2.5–18.1	6.1–29.6	2.5	6.1

Higher rates per ET reflect a lower proportion of patients reaching ET per initiated cycle. This number varies from 54 to 61.8 percent for normal responders and 41.8–57.5 percent for poor responders.

4.2.7 Perinatal Outcomes

Favorable perinatal outcomes have been reported from births derived from mnIVF cycles. Most recently, a retrospective cohort study by Mak et al. compared birth outcomes in 190 fresh mnIVF versus 174 stimulated IVF cycles [19]. The average birthweight was statistically significantly higher in the mnIVF group. Very preterm births were also much lower in the mnIVF group (0.52 versus 6.3 percent, $p<0.005$). However, these differences were largely due to earlier delivery after COH, because after controlling for gestational age at delivery, analyses demonstrated no significant differences in birth outcomes between mnIVF and standard IVF [19].

4.3 Mild or Minimal Stimulation IVF

Mild or minimal stimulation IVF (mIVF), which lies between mnIVF and COH, is another alternative to traditional COH protocols. The International Society for Mild Approaches in Assisted Reproduction (ISMAAR) published proposed terminology to define natural cycle, modified natural cycle, mild or minimal stimulation, and standard COH protocols [20]. The use of these definitions may help providers interpret studies to accurately counsel potential candidates regarding the evidence surrounding natural and minimal stimulation IVF.

MIVF protocols as defined by ISMAAR guidelines include oral agents only, a sequential regimen of oral agents and gonadotropins, and low-dose gonadotropins only – and are characterized by a daily dose of 150 IU or less per day. In comparison to a goal of retrieving eight or more oocytes per cycle, the minimal stimulation protocol has a goal of retrieving between 2 and 7 oocytes. Unlike COH, there is no pretreatment with oral contraceptive pills or GnRH agonist. The monitoring may include less frequent ultrasounds (Figure 4.2). Protocols involving gonadotropins require a GnRH antagonist, and trigger criteria are similar to those used in COH. Follicle aspiration, retrieval, luteal support and embryo transfer are performed in a fashion identical to that of COH.

There are five RCTs comparing outcomes in mIVF protocols versus COH. One of these was a non-inferiority RCT that focused on normal responders. The authors found that cumulative live births after a year of treatment were not significantly different between patients undergoing minimal stimulation and COH IVF [21]. The other RCTs examining differences in outcomes of mIVF and COH in ‘expected poor responders’ or poor

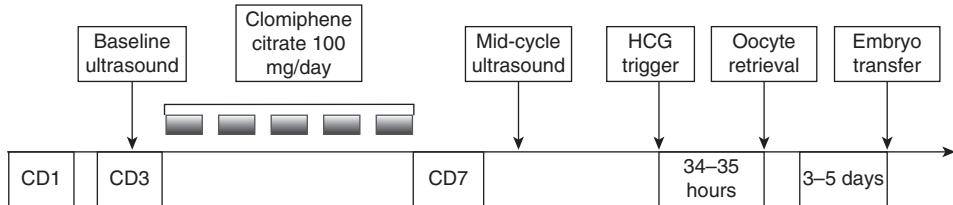


Figure 4.2a Minimal stimulation IVF with oral agent

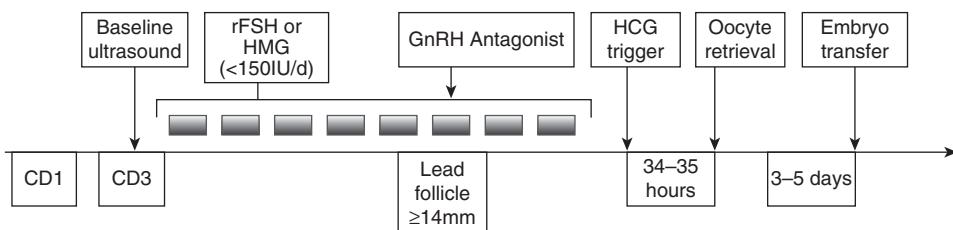


Figure 4.2b Minimal stimulation IVF with low dose gonadotropins and GnRH antagonist

responders concluded that pregnancies were similar between groups. The conclusions are somewhat limited by heterogeneous definitions of mIVF, control group protocol types and primary outcomes. The most recent RCT by Youssef et al. in 2016 [22] was a multicenter randomized non-inferiority trial of 394 poor responders. The authors found that ongoing pregnancy rates were not significantly different between standard COH and mIVF protocols, with the non-inferiority margin set at 10 percent [22]. The minimal stimulation group used less gonadotropin dose and had a shorter duration for their stimulation cycle.

4.4 Discussion

Protocols for COH were developed to maximize oocyte yield during the IVF cycle. Having more mature oocytes increases the probability of successful fertilization with insemination or ICSI, and subsequently supernumerary embryos from which to choose for embryo transfer. Additionally, some studies have suggested that live birth rates are correlated with ovarian response [23]. The ability to transfer more than one embryo can increase the probability of pregnancy, as each embryo has an individual likelihood of implanting.

While transferring more than one embryo may increase live birth rates, this practice has been found to also lead to higher rates of multiple gestation and subsequently higher rates of adverse obstetrical outcomes. Higher order multiple gestations carry significant obstetric risks, and selective reduction may be necessary. Another complication specific to COH includes ovarian hyperstimulation syndrome (OHSS). Patients with OHSS are advised to delay fresh transfer and may require culdocentesis/paracentesis, as well as hospitalization. Other individuals may not be ideal candidates for COH if they have reservations surrounding embryo cryopreservation. COH may not be successful in poor responders, which includes women who do not develop more than three follicles for retrieval despite maximum doses of medication, have low ovarian reserve markers or are ≥ 40 years old. These patients may develop a single dominant follicle or no follicles after down-regulation with

GnRHa, and subsequently will have no or few embryos to transfer. There are also concerns that pregnancy rates may be adversely affected by premature luteinization of the endometrium and the effects on endometrial receptivity associated with premature elevation of progesterone in conjunction with high gonadotropin doses in standard COH protocols [24].

In addition to potentially affecting endometrial receptivity, there is also evidence of diminishing returns after a certain oocyte yield is achieved. One study from the United Kingdom of 400,135 fresh IVF cycles found the ideal number of oocytes retrieved to achieve a live birth was approximately 15, after which an increase was not associated with higher live birth rates [25]. Similar findings were confirmed in a retrospective study of 256,381 cycles from the Society of Associated Reproductive Technology database [26]. Further evidence to support this comes from a recent study analysing data in 650,000 fresh IVF cycles in the United States, which showed that increasing gonadotropin dose was negatively correlated with live birth rates, even when adjusting for age and stimulation protocol [27].

It has been suggested that high-dose gonadotropins might adversely affect oocyte quality and subsequently embryo quality [28]. One proposed mechanism of action suggests that higher doses of gonadotropins may recruit poorer quality oocytes that may not otherwise have been selected in a natural or minimal stimulation cycle [28]. However, studies examining embryo quality and stimulation dose are confounded by the observation that older patients tend to receive higher doses of stimulation, and the premature luteinization of the endometrium seen with aggressive stimulation may adversely affect pregnancy outcomes in fresh IVF cycles. Most recent data do not show differences in aneuploidy if more versus fewer embryos are produced and no difference in rates when comparing unstimulated versus stimulated cycles [29].

4.5 Conclusion

Modified natural cycle IVF produces acceptable pregnancy rates and implantation rates similar to those seen in standard stimulated cycles. It is associated with lower cost and almost 0 percent risk of OHSS. Minimal stimulation IVF also provides similar benefits, with slightly higher costs as more medications are used. It is reasonable to offer these as a first-line option for patients who are good responders, as well as a second-line option for poor responders who do not respond well to standard COH protocols.

References

1. Trounson AO, Leeton JF, Wood C, Webb J, Kovacs G. The investigation of idiopathic infertility by in vitro fertilization. *Fertil Steril*. 1980 November;34(5): 431–8.
2. Jones HW, Jones GS, Andrews MC et al. The program for in vitro fertilization at Norfolk. *Fertil Steril*. 1982 July;38(1):14–21.s
3. Foulot H, Ranoux C, Dubuisson JB et al. In vitro fertilization without ovarian stimulation: a simplified protocol applied in 80 cycles. *Fertil Steril*. 1989 October;52(4): 617–21.
4. Paulson RJ, Sauer MV, Francis MM, Macaso TM, Lobo RA. In vitro fertilization in unstimulated cycles: the University of Southern California experience. *Fertil Steril*. 1992 February;57(2): 290–3.
5. Pelinck MJ, Hoek A, Simons AHM, Heineman MJ. Efficacy of natural cycle IVF: a review of the literature. *Hum Reprod Update*. 2002;8(2): 129–39.
6. Meldrum DR, Rivier J, Garzo G et al. Successful pregnancies with unstimulated cycle oocyte donation using an antagonist of gonadotropin-releasing hormone. *Fertil Steril*. 1994;61: 556–7.

7. Paulson RJ, Sauer MV, Lobo RA. Addition of a gonadotropin releasing hormone (GnRH) antagonist and exogenous gonadotropins to unstimulated in vitro fertilization (IVF) cycles: Physiologic observations and preliminary experience. *J Assist Reprod Genet.* 1994;11:28–32.
8. Filicori M, Cognigni GE, Gameberini E et al. Efficacy of low-dose human chorionic gonadotropin alone to complete controlled ovarian stimulation. *Fertil Steril.* 2005;84:394–401.
9. Martins WP, Vieira AD, Figueiredo JB, Nastri CO. FSH replaced by low-dose hCG in the late follicular phase versus continued FSH for assisted reproductive techniques. *Cochrane Database Syst Rev.* 2013;3: CD010042.
10. Paulson RJ, Chung K, Quaas AM et al. Low dose HCG alone can complete follicle maturity: successful application to modified natural cycle IVF. *Fertil Steril.* 2016 May;105(5): 1228–31.
11. Teramoto S, Osada H, Sato Y, Shozu M. Nondominant small follicles are a promising source of mature oocytes in modified natural cycle in vitro fertilization and embryo transfer. *Fertil Steril.* 2016 July;106(1): 113–18.
12. Tang-Pedersen M, Westergaard LG, Erb K, Mikkelsen AL. Combination of IVF and IVM in naturally cycling women. *Repro Biomed Online* 2012 January;24(1):47–53.
13. Thornton MH, Francisco MM, Paulson RJ. Immature oocyte retrieval: lessons from unstimulated IVF cycles. *Fertil Steril.* 1998 October;70(4): 647–50.
14. Gordon JD, DiMattina M, Reh A et al. Utilization and success rates of unstimulated in vitro fertilization in the United States: an analysis of the Society for Assisted Reproductive Technology database. *Fertil Steril.* 2013 August;100(2): 392–5.
15. Shaulov T, Velez MP, Buzaglo K, Phillips SJ, Kadoch IJ. Outcomes of 1503 cycles of modified natural cycle in vitro fertilization: a single-institution experience. *J Assist Reprod Genet.* 2015;32: 1043–8.
16. Morgia F, Sbracia M, Schimberni M et al. A controlled trial of natural cycle versus microdose gonadotropin-releasing hormone analog flare cycles in poor responders undergoing in vitro fertilization. *Fertil Steril.* 2004 June;81(6): 1542–7.
17. Kim CH, Kim SR, Cheon YP et al. Minimal stimulation using gonadotropin-releasing hormone (GnRH) antagonist and recombinant human follicle-stimulating hormone versus GnRH antagonist multiple-dose protocol in low responders undergoing in vitro fertilization/intracytoplasmic sperm injection. *Fertil Steril.* 2009 December;92(6): 2082–4.
18. Shimberni M, Morgia F, Colabianchi J et al. Natural-cycle in vitro fertilization in poor responder patients: a survey of 500 consecutive cycles. *Fertil Steril.* 2009 October;92(4):1297–1301.
19. Mak W, Kondapalli LA, Celia G et al. Natural cycle IVF reduces the risk of low birthweight infants compared with conventional stimulated IVF. *Hum Reprod* 2016;31(4):789–94.
20. Nargund G, Fauser BC, Macklon NS et al. Rotterdam ISMAAR consensus group on terminology for ovarian stimulation for IVF. *Human Reprod.* 2007;22: 2801–4.
21. Heijnen EM, Eijkemans MJ, De Klerk C et al. A mild treatment strategy for in-vitro fertilization: a randomized non-inferiority trial. *Lancet* 2007 March;369(9563): 743–9.
22. Youssef MA, van Wely M, Al-Inany H et al. van der Veen F. A mild ovarian stimulation strategy in women with poor ovarian reserve undergoing IVF: a multicenter randomized non-inferiority trial. *Hum Reprod.* 2017 January;32(1): 112–18.
23. Drakopoulos P, Blockeel C, Stoop D et al. Conventional ovarian stimulation and single embryo transfer for IVF/ICSI. How many oocytes do we need to maximize cumulative live birth rates after utilization of all fresh and frozen embryos? *Human Reprod.* 31(2): 370–6.
24. Paulson RJ, Sauer MV, Lobo RA: Embryo implantation after human in vitro

- fertilization: Importance of endometrial receptivity. *Fertil Steril.* 1990;53:870.
25. Sunkara SK, Rittenberg V, Raine-Fenning N et al. Association between the number of eggs and live birth in IVF treatment: an analysis of 400,135 treatment cycles. *Hum Reprod.* 2011;26(7): 1768–74.
26. Steward RG, Lan L, Shah AA et al. Oocyte number is a predictor for ovarian hyperstimulation syndrome and live birth: an analysis of 256,381 in vitro fertilization cycles. *Fertil Steril.* 2014 April;101(4): 967–73.
27. Baker VL, Brown MB, Luke B, Smith GW, Ireland JJ. Gonadotropin dose is negatively correlated with live birth rate: analysis of more than 650,000 assisted reproductive technology cycles. *Fertil Steril.* 2015 November;104(5): 1145–52.
28. Baart EB, Martini E, Eijkemans MJ et al. Milder ovarian stimulation for in-vitro fertilization reduces aneuploidy in the human preimplantation embryo: a randomized control trial. *Hum Reprod.* 2007 April;22(4): 980–8.
29. Labarta E, Bosch E, Alamá P et al. Moderate ovarian stimulation does not increase the incidence of human embryo chromosomal abnormalities in in vitro fertilization cycles. *J Clin Endocrinol Metab.* 2012 October;97(10): E1987–94.

GnRH Agonists and Antagonists

Which, When and How?

Georg Griesinger and Efstratios M. Kolibianakis

5.1 Introduction

The decapeptide gonadotropin-releasing hormone (GnRH) plays a central role in the hypothalamo-pituitary-gonadal axis which governs the menstrual cycle in adult women. GnRH is produced in the medio-basal hypothalamus in a pulsatile fashion by neurones located in the nucleus arcuatus. It acts on the pituitary gland where it orchestrates the release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Release of GnRH from the hypothalamus occurs in pulses every 70–90 minutes. Circulating GnRH is rapidly degraded by endopeptidases and therefore the half-life of the molecule is extremely short (2–5 minutes). This short half-life allows the GnRH receptors of the pituitary gland to recognize every single hypothalamic pulse of GnRH, which in turn leads to the pulsatile release of LH and FSH. FSH and LH govern follicular maturation and sex steroid production in the ovarian follicles. In women, GnRH release and thereby gonadotropin secretion are modulated – among other factors – by oestradiol (E2) and progesterone. High E2 levels elicit, by a positive feedback mechanism, the mid-cycle LH surge, which in turn induces resumption of meiosis of the oocyte, ovulation and subsequently luteinization of the dominant follicle [1].

Ovarian stimulation for in vitro fertilization (IVF) allows the growth of multiple follicles and the retrieval of a sufficient number of oocytes, required for the laboratory procedures. The growth of multiple follicles, induced by exogenous FSH administration, leads to supraphysiological E2 levels early in the follicular phase of the cycle. Untimely LH surges, evoked by the high E2 levels, may disrupt oocyte maturation and lead to a detrimental effect on oocyte quality, fertilization, endometrial receptivity and ultimately pregnancy rates [2,3]. Thus, suppression of pituitary LH release is imperative to avoid poor infertility treatment outcome [4].

Elucidation of the amino acid sequence of GnRH [5] was quickly followed by the availability of synthetic GnRH analogues with either agonistic or antagonistic action at the GnRH receptor. Continuous exposure to an agonistic GnRH analogue leads to desensitization of the pituitary gland and subsequent suppression of gonadotropin release, following an initial increase in the secretion of LH and FSH secretion, known as a flare-up effect. The flare-up effect encompasses a fivefold increase of FSH, a tenfold rise in LH and a fourfold elevation of oestradiol within approximately 12 hours of the initiation of GnRH agonist administration. It takes several days for sex steroid production to be suppressed thereafter. The advent of the GnRH analogues revolutionized infertility treatment efficacy, allowing suppression of the pituitary gland and thereby control of the LH activity, leading to the historical term ‘controlled ovarian stimulation (COS)’, e.g.

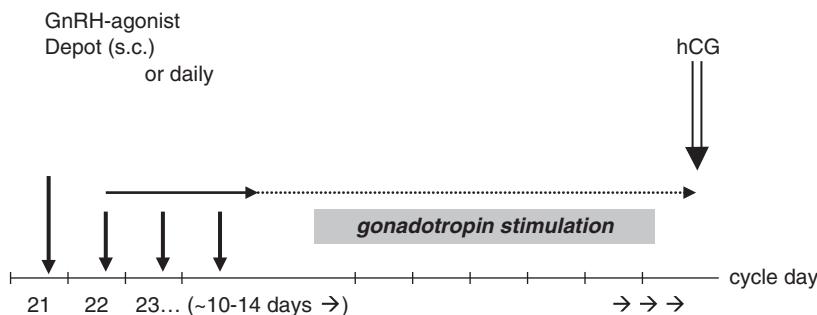


Figure 5.1
Long luteal
GnRH agonist
protocol

gonadotropin stimulation and concomitant utilization of a GnRH analogue to prevent premature LH surges.

Three main ovarian stimulation protocols utilizing GnRH analogues have been described: short agonist, long agonist protocol and antagonist, each one with a number of protocol modifications published.

5.2 GnRH Agonist Protocols

In the long agonist protocol, a GnRH agonist is started either in the luteal phase of the preceding cycle (the so-called long luteal agonist protocol) (Figure 5.1) or at initiation of menstruation (the so-called long follicular agonist protocol).

The binding of the agonistic molecule to the GnRH receptor leads to an initial flare up of gonadotropin release from the pituitary. After seven to ten days of action, the pituitary is desensitized, sex steroid production of the ovary is suppressed and ovarian stimulation with FSH is initiated. Alternatively, the GnRH agonist may be administered simultaneously with gonadotropins at the beginning of the cycle and the initial flare up is used to enhance stimulation before desensitization is achieved. This regimen is known as the flare-up (or boost) protocol or short agonist protocol.

The long follicular agonist protocols and, to a lesser degree, the long luteal agonist protocol are associated with ovarian cyst formation in a proportion of patients which may interfere with exogenous FSH stimulation [6,7].

5.3 GnRH Antagonist Protocols

Structural GnRH analogues which bind to the pituitary GnRH receptor without inducing GnRH receptor cross-linking (and thereby receptor activation) exert an antagonistic effect by competing with endogenous GnRH for the pituitary receptor [8]. Because of the lack of any intrinsic activity of these analogues, the initial 'flare-up' effect of GnRH agonist administration is not present. Moreover, antagonists lead to a rapid suppression of gonadotropin release, enabling shorter treatment regimens for COS compared to GnRH agonist protocols. The mechanism of GnRH antagonist action is dependent on the equilibrium between endogenous GnRH and GnRH antagonist administered. Modification of the dosage of GnRH antagonists administered to the patient modifies this equilibrium, and therefore the antagonistic effect of these compounds is dose-dependent [9–11]. Furthermore, triggering of final oocyte maturation with GnRH agonist instead of human chorionic gonadotrophin (hCG) is feasible in a GnRH antagonist protocol, since the

Table 5.1 The pharmacokinetic parameters of single and multiple doses of cetrorelix (daily for 14 days) and ganirelix (daily for 7 days) following subcutaneous administration. (a mean values (P5-P95), β means \pm SD, ξ median (min-max), χ AUC₀₋₄₈, δ AUC₀₋₂₄)

	Cetrorelix ^[14,15]			Ganirelix ^[10,11]	
	Single dose 3 mg	Single dose 0.25 mg	Multiple dose 0.25 mg	Single dose 0.25 mg	Multiple dose 0.25 mg
t_{max} [h]	1.5 (0.5–2) $^\xi$	1.0 (0.5–1.5) $^\xi$	1.0 (0.5–2) $^\xi$	1.1 \pm 0.3 $^\beta$	1.14 \pm 0.23 $^\beta$
$t \frac{1}{2}$ [h]	62.8 (38.2–108) $^\xi$	5.0 (2.4–48.8) $^\xi$	20.6 (4.1–179.3) $^\xi$	12.8 \pm 4.3 $^\beta$	16.23 \pm 1.64 $^\beta$
C_{max} [ng/ml]	28.5 (22.5–36.2) a	4.97 (4.17–5.92) a	6.42 (5.18–7.96) a	14.8 \pm 3.2 $^\beta$	11.16 \pm 2.41 $^\beta$
AUC [ng·h/ml]	536 (451–636) a	31.4 (23.4–42.0) a	44.5 (36.7–54.2) a	94 \pm 11 $^\beta\chi$	77.13 \pm 9.75 $^\beta\delta$
Bioavailability	85%			91%	

pituitary, following cessation of GnRH antagonist, remains sensitive and therefore responsive to a bolus dose of GnRH agonist [12].

5.4 Pharmacokinetics (PK) and Pharmacodynamics (PD) of the GnRH antagonists

Two GnRH antagonists are currently available for infertility treatment, cetrorelix and ganirelix, both at doses of 0.25 mg for daily subcutaneous injection. Cetrorelix is also available at a dose of 3 mg leading to a four-day period of pituitary suppression. Absorption of both cetrorelix and ganirelix is rapid and dose proportionality for maximum serum concentration and concentration at steady state has been shown [11,13] (Table 5.1). The decrease of LH and oestradiol secretion after antagonist administration is dose proportional, which was shown for cetrorelix at doses from 0.25 mg to 5 mg and for ganirelix at doses from 0.625 mg to 2 mg.

After a single subcutaneous (sc) administration of 0.25 mg cetrorelix, serum LH and FSH are maximally decreased by 75 percent and 23 percent respectively, at six hours after dosing, but suppression of gonadotropin and steroid activity is not complete and LH, FSH and E2 levels return to baseline within 24 hours [14]. Delay of ovulation in a natural cycle is achieved only by multiple administrations of 0.25 mg in the follicular phase. After a single sc administration of 3 mg cetrorelix, LH is suppressed for a mean of 100 hours and the LH surge is postponed for a median of 7 days.

In a study on ganirelix administration for seven days [11] it was found that following the last injection of 0.25 mg, serum LH and FSH levels had decreased by 74 percent and 32 percent respectively, compared with pretreatment values. Levels of LH and FSH reached a nadir at 4 hours and 16 hours respectively. In the 0.25 mg group, oestradiol decreased by 25 percent within 16 hours post-injection. The suppressive effect on the pituitary gland was

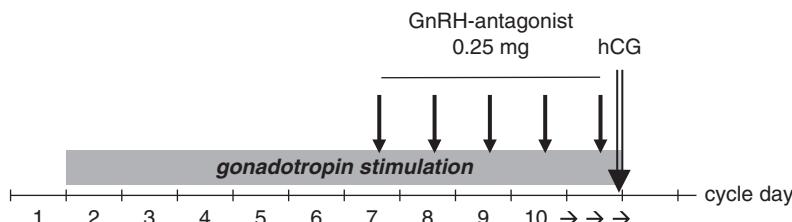


Figure 5.2
So-called fixed
GnRH antagonist
multiple-dose
protocol

rapidly reversed. After cessation of treatment, pituitary hormone and oestradiol concentrations returned to pretreatment values within 24 hours.

5.5 Are There Relevant Differences in PK/PD between the GnRH antagonists?

The PK/PD profile of the available GnRH antagonists is highly similar (Table 5.1, summarized in 16). Both cetrorelix and ganirelix appear to be safe and equipotent in the suppression of FSH and LH, which is fully effective within 4–8 hours after sc administration [17]. The level of suppression of FSH after short-term administration is comparable for cetrorelix and ganirelix but is less in absolute values as compared to LH and oestradiol. The nadir of LH suppression is reached slightly faster with ganirelix and a slightly higher bioavailability has been reported for ganirelix, indicating a higher solubility of the compound in aqueous buffers as compared with cetrorelix. Such findings may reflect slight differences in PK as well as differences in study protocols. Although they have been used to argue theoretically in favour of one drug or the other, they appear to be of limited clinical relevance, as evidenced in the phase III trial programme in which the incidence of premature LH surges in women undergoing COS was similar between the compounds tested. However, no conclusive direct comparison of ganirelix and cetrorelix for COH for IVF has been pursued.

5.6 The GnRH Antagonist Multiple Dose Protocol

Since the use of GnRH antagonist is associated with immediate pituitary suppression, administration during COS is started when a premature LH surge becomes possible. As a best estimate, this time point was chosen as stimulation day 5–6. Accordingly, the posology of both cetrorelix and ganirelix 0.25 mg stipulates that treatment with the GnRH antagonist should commence on day 5 or 6 of ovarian stimulation (Figure 5.2) (approximately 96–120 hours after start of ovarian stimulation) with urinary or recombinant gonadotropins and is to be continued throughout the gonadotropin treatment period, including the day of triggering final oocyte maturation (which is usually performed in the evening). Both morning and evening administration is feasible; however, if the GnRH antagonist is to be administered in the evening, treatment initiation is recommended on day 5 of ovarian stimulation until the evening prior to the day of hCG.

The incidence of isolated LH rises ($LH \geq 10.0$ IU/L) prior to GnRH antagonist initiation has been estimated as approximately 2–7 percent, while during GnRH antagonist treatment it is estimated as approximately 1–2 percent [18]. Premature LH surges (defined as LH rise > 10 mIU/mL with a concomitant rise in serum progesterone > 2 ng/mL) occur in less than

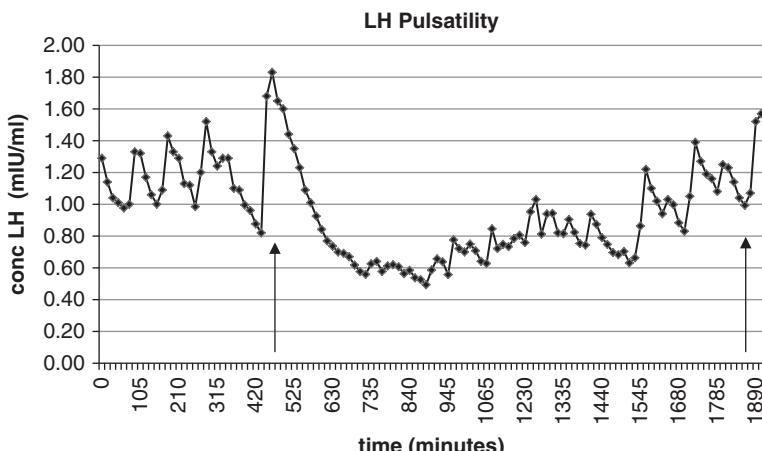


Figure 5.3 A 32-hour serum LH secretory profile on cycle day 6 in a woman undergoing ovarian stimulation with 225 IU rFSH from cycle day 2. Arrows indicate the time points of 0.25 mg sc cetrorelix administration
Source: Griesinger G, Dawson A, Schultze-Mosgau A, Finas D, Diedrich K and Felberbaum R. Assessment of luteinizing hormone levels in the gonadotropin-releasing hormone-antagonist protocol. Fertility Sterility 2006 March;85(3): 791–3.

1 percent of patients during GnRH antagonist administration with either of the two compounds available.

The suppression of the pulsatile endogenous LH release by the pituitary gland upon antagonist initiation has been well documented in the scenario of COS (Figure 5.3) [19]. In an individual patient data meta-analysis of six studies in women undergoing ovarian stimulation with rec FSH and 0.25 mg ganirelix, it was shown that endogenous LH concentration on day 8 of stimulation or on the day of triggering final oocyte maturation with hCG was not associated with the probability of ongoing pregnancy: pregnancy rates were highly similar between groups of patients with or without strong suppression of endogenous LH, both in uni-variate and multi-variate analyses accounting for age and oocyte numbers among other factors [20]. Furthermore, adding exogenous LH in a GnRH antagonist protocol does not impact on treatment outcomes in terms of pregnancy likelihood [21,22]. The suppression of FSH release by the pituitary gland upon antagonist initiation is too small to be detectable, given the comparatively large amounts of exogenous FSH utilized for stimulation [23].

5.7 Fixed versus Flexible GnRH Antagonist Multiple-Dose Protocol

As an alternative to the fixed GnRH antagonist initiation on day 5 or 6 of stimulation, a so-called flexible protocol has been developed. In a flexible GnRH antagonist protocol, the antagonist is administered based on endocrine and/or sonographic criteria indicating that LH rise is possible. These criteria have differed between studies [24].

Apparently, the fixed GnRH antagonist initiation requires less monitoring compared to a flexible approach, which translates into less burden for the patient regarding outpatient visits as well as reduced costs for sonography and/or hormone evaluations. However, the flexible GnRH antagonist protocol may avoid unnecessary administration of GnRH

antagonist in those patients in whom absence of follicular development on day 5 renders an LH rise unlikely at that point of time.

A meta-analysis of studies, comparing fixed and flexible GnRH antagonist protocols in general populations, demonstrated higher pregnancy rates with the use of the fixed protocol (odds ratio (OR) 0.7, 95% CI: 0.47–1.05, 4 RCTs, $n=484$) [24], though statistical significance was not present. It is important to note that in the comparative studies on fixed versus flexible GnRH antagonist protocol only a fraction of the patients randomized to the flexible approach indeed had a later initiation of GnRH antagonists compared to the fixed protocol study arm. This was due to the fact that the criteria used in the flexible protocol had been met by the majority of patients by day 5 of stimulation. Accordingly, the true effect of delayed GnRH antagonist initiation has not been precisely determined in these trials.

5.8 Efficacy of GnRH Antagonist Protocols

Soon after market introduction of the GnRH antagonist, a Cochrane systematic review [25] reported significantly lower clinical pregnancy rates in GnRH antagonist stimulation cycles as compared to long GnRH agonist cycles. This review has elicited a lot of concern and debate and has hampered a wider utilization of GnRH antagonists in the first years after their introduction.

Later it was shown [26] that a statistically significant difference in clinical pregnancy rate, reported by Cochrane in 2002, has never been present and that the notion of a negative impact of GnRH antagonists has been built on faulty premises. Furthermore, in 2006 another systematic review and meta-analysis showed that the probability of live birth was similar between antagonist and agonist protocols [27]. In 2011, an updated Cochrane review [28] confirmed that live birth rate was statistically indistinguishable between the protocols. Eventually, in 2016 the largest randomized trial was published in 1,050 women undergoing a first cycle of IVF or ICSI using a GnRH antagonist multiple-dose protocol or a long GnRH agonist protocol [29]. This trial included women with irregular cycles, poor ovarian reserve and polycystic ovary syndrome, and therefore the findings have broad external validity. It was found that the live birth rate was equal between the protocols. An extended analysis on cumulative live birth rates (CLBR), including embryo transfers from both fresh and frozen cycles using all oocytes collected from one initiated COC cycle within two years, also showed no benefit of the GnRH agonist protocol with a CLBR of 34.1 percent in the GnRH antagonist group versus 31.2 percent in the GnRH agonist group (OR 1.14; 95% CI: 0.88–1.48, $P = 0.32$) protocol [30].

Moreover, gonadotropin consumption is lower with GnRH antagonists [27], most likely due to the stimulation being synchronous with the natural cycle and avoiding the down-regulation preceding stimulation in long agonist protocols.

5.9 Risk of Ovarian Hyperstimulation Syndrome (OHSS)

All systematic reviews have from the beginning agreed [25,27,28] that in patients undergoing COS using hCG for triggering final oocyte maturation, the risk of developing clinically relevant OHSS is significantly decreased in GnRH antagonist protocols. The relative risk has been estimated as approximately 0.5 for OHSS requiring hospitalization [27]. Nevertheless, moderate to severe OHSS may still occur in GnRH antagonist

protocols if hCG is administered to trigger final oocyte maturation, especially in high responder patients. The incidence of severe OHSS following triggering of final oocyte maturation with hCG is approximately 1–2 percent in patients 18–36 years undergoing IVF and treated with a GnRH antagonist protocol. Therefore, an individual risk assessment for severe OHSS should be done before commencing a cycle [31], as well as at the end of the FSH stimulation phase. It has been shown in a combined analysis of three large antagonist studies encompassing 2,433 women [32] that the optimal threshold of follicles ≥ 11 mm on the day of hCG to identify those at risk of severe OHSS was 19 (sensitivity and specificity 74.3 percent and 75.3 percent, respectively). The positive and negative predictive values were 4.2 percent and 99.5 percent for severe OHSS. Accordingly, patients with follicle numbers above the threshold of 19 should be considered for secondary OHSS preventive measures, such as replacement of hCG with GnRH agonist for triggering final oocyte maturation [33,34].

5.10 Choice of Protocol in Distinct Patient Groups

For distinct patient subgroups, such as poor responders or PCOS patients, the available studies are limited and the sample sizes are generally still too small, even after pooling of several RCTs, for clinically relevant differences to be determined with confidence.

For PCOS patients, the latest Cochrane systematic review summarizes seven RCTs comparing antagonist and agonist protocol in 727 patients [35]. Ongoing pregnancy rate was highly similar between groups (OR 0.91, 95% CI: 0.67 to 1.22). Beyond efficacy, safety is of paramount importance in COS of PCOS patients. Triggering final oocyte maturation with a bolus of GnRH agonist instead of hCG in patients at high risk of OHSS is only feasible in GnRH antagonist protocols, making antagonist protocols a first choice for PCOS patients exclusively for this reason.

Several meta-analyses [28,36,37] have investigated the efficacy of antagonists in poor response patients. The available data do not indicate that the choice of GnRH analogue impacts on the likelihood of success of a patient. Secondary issues such as ease of administration and gonadotropin consumption should therefore drive the decision for selecting a GnRH analogue protocol.

5.11 Where Is Ovarian Stimulation with GnRH Analogues Heading?

GnRH antagonists have rendered the IVF treatment cycle shorter, with less side effects, less consumption of gonadotropins and less risk of OHSS while the chance of live birth is as good as in a GnRH agonist protocol. No patient group has been identified as yet that would benefit from a long or short GnRH agonist protocol. Preventing OHSS by GnRH agonist triggering and freezing of all suitable embryos is only feasible in GnRH antagonist cycles, and this has driven utilization of this protocol especially in patient groups such as oocyte donors, fertility preservation or women with PCOS. Taking all the advantages of GnRH antagonists into account, recent clinical development programmes in COS [38,39,40] have been exclusively performed in GnRH antagonist protocols, making GnRH agonist protocols a matter of the past eventually.

References

1. Knobil E. The neuroendocrine control of the menstrual cycle. *Recent Prog Horm Res.* 1980;30:1–36.
2. Stanger YD, Yovich JL. Reduced in vitro fertilization of human oocytes from patients with raised basal luteinizing hormone levels during the follicular phase. *B J Obstet Gynecol.* 1985;92:385–93.
3. Frattarelli JL, Hillensjö T, Broekmans FJ et al. Clinical impact of LH rises prior to and during ganirelix treatment started on day 5 or on day 6 of ovarian stimulation. *Reprod Biol Endocrinol.* 2013;11:90.
4. Hughes EG, Fedorkow DM, Daya S et al. The routine use of gonadotropin-releasing hormone agonists prior to in vitro fertilization and gamete intrafallopian transfer: a meta-analysis of randomized controlled trials. *Fertil Steril.* 1992;58:888–96.
5. Schally AV, Nair RMG, Redding TW et al. Isolation of the luteinizing hormone and follicle stimulating hormone-releasing hormone from porcine hypothalami. *J Biol Chem.* 1971;246:7230–6.
6. Feldberg D, Ashkenazi J, Dicker D et al. Ovarian cysts formation: a complication of gonadotropin-releasing hormone agonist therapy. *Fertil Steril.* 1989;51:42–5.
7. Biljan MM, Lapensée L, Mahutte N et al. Effects of ovarian cysts detected on the 7th day of gonadotropin-releasing hormone analog administration on the outcome of IVF treatment. *Fertil Steril.* 2000;74:941–5.
8. Reissmann T, Felberbaum R, Diedrich K et al. Development and applications of luteinizing hormone-releasing hormone antagonists in the treatment of infertility: an overview. *Hum Reprod.* 1995;10:1974–81.
9. Duijkers IJM, Klipping C, Willemse WNP et al. Single and multiple dose pharmacokinetics and pharmacodynamics of the gonadotrophin-releasing hormone antagonist Cetrorelix in healthy female volunteers. *Hum Reprod.* 1998;13:2392–8.
10. Oberye JJ, Mannaerts BM, Kleijn HJ, Timmer CJ. Pharmacokinetic and pharmacodynamic characteristics of ganirelix (Antagon/Orgalutran). Part I. Absolute bioavailability of 0.25 mg of ganirelix after a single subcutaneous injection in healthy female volunteers. *Fertil Steril.* 1999;72:1001–5.
11. Oberye JJ, Mannaerts BM, Huisman JA, Timmer CJ. Pharmacokinetic and pharmacodynamic characteristics of ganirelix (Antagon/Orgalutran). Part II. Dose-proportionality and gonadotropin suppression after multiple doses of ganirelix in healthy female volunteers. *Fertil Steril.* 1999;72:1006–12.
12. Griesinger G, Diedrich K, Devroey P, Kolibianakis EM. GnRH agonist for triggering final oocyte maturation in the GnRH antagonist ovarian hyperstimulation protocol: a systematic review and meta-analysis. *Hum Reprod Update.* 2006;12:159–68.
13. Sommer L, Zanger K, Dyong T et al. Seven-day administration of the gonadotropin-releasing hormone antagonist Cetrorelix in normal cycling women. *Eur J Endocrinol.* 1994;131:280–5.
14. Duijkers IJM, Klipping C, Willemse WNP et al. Single and multiple dose pharmacokinetics and pharmacodynamics of the gonadotrophin-releasing hormone antagonist Cetrorelix in healthy female volunteers. *Hum Reprod.* 1998;13:2392–8.
15. Erb K, Klipping C, Duijkers I et al. Pharmacodynamic effects and plasma pharmacokinetics of single doses of cetrorelix acetate in healthy premenopausal women. *Fertil Steril.* 2001;75:316–23.
16. Griesinger G, Felberbaum RE, Schultze-Mosgau A, Diedrich K. Gonadotropin-releasing hormone antagonists for assisted reproductive techniques: are there clinical differences between agents? *Drugs.* 2004;64(6):563–75.
17. Nagaraja NV, Pechstein B, Erb K et al. Pharmacokinetic and pharmacodynamic modeling of cetrorelix, an LH-RH antagonist, after subcutaneous administration in healthy premenopausal women. *Clin Pharmacol Ther.* 2000;68:617–25.

18. Frattarelli JL, Hillensjö T, Broekmans FJ et al. Clinical impact of LH rises prior to and during ganirelix treatment started on day 5 or on day 6 of ovarian stimulation. *Reprod Biol Endocrinol.* 2013 September;12:11:90.
19. Griesinger G, Dawson A, Schultze-Mosgau A, Finas D, Diedrich K and Felberbaum R. Assessment of luteinizing hormone levels in the gonadotropin-releasing hormone-antagonist protocol. *Fertility Sterility* 2006 March;85(3):791–3.
20. Griesinger G, Shapiro DB, Kolibianakis EM, Witjes H, Mannaerts BM. No association between endogenous LH and pregnancy in a GnRH antagonist protocol: part II, recombinant FSH. *Reprod Biomed Online.* 2011 October;23(4):457–65.
21. Kolibianakis EM, Kalogeropoulou L, Griesinger G et al. Among patients treated with FSH and GnRH analogues for in vitro fertilization, is the addition of recombinant LH associated with the probability of live birth? A systematic review and meta-analysis. *Hum Reprod Update.* 2007 September–October;13(5):445–52.
22. Xiong Y, Bu Z, Dai W et al. Recombinant luteinizing hormone supplementation in women undergoing in vitro fertilization/intracytoplasmic sperm injection with gonadotropin releasing hormone antagonist protocol: a systematic review and meta-analysis. *Reprod Biol Endocrinol.* 2014 November 24;12:109.
23. Griesinger Georg, Finas D, Alisch A et al. Diedrich K and Felberbaum R. FSH time-concentration profiles before and after the administration of 0.25 mg cetrorelix in the GnRH-antagonist multiple dose protocol for ovarian hyperstimulation. *Journal of Assisted Reproduction and Genetics* 2004;21:279–82.
24. Al-Inany H, Aboulghar MA, Mansour RT, Serour GI. Optimizing GnRH antagonist administration: meta-analysis of fixed versus flexible protocol. *Reprod Biomed Online.* 2005;10:567–70.
25. Al-Inany H, Aboulghar M. GnRH antagonist in assisted reproduction: a Cochrane review. *Hum Reprod.* 2002;17: 874–85.
26. Kolibianakis EM, Griesinger G. GnRH antagonists in ART In:Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice. Edited by David K. Gardner, Botros R. M. B. Rizk and Tommaso Falcone. Cambridge University Press 2011, ISBN 978-1-107-00112-1.
27. Kolibianakis EM, Collins J, Tarlatzis B et al. Among patients treated for IVF with gonadotrophins and GnRH-analogues, is the probability of live birth dependent on the type of analogue used? A systematic review and meta-analysis. *Human Reproduction Update* 2006 November–December;12:6517–1.
28. Al-Inany HG, Youssef MA, Aboulghar M et al. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Database Syst Rev.* 2011 May 11;(5):CD001750.
29. Toftager M, Bogstad J, Bryndorf T et al. Risk of severe ovarian hyperstimulation syndrome in GnRH antagonist versus GnRH agonist protocol: RCT including 1050 first IVF/ICSI cycles. *Hum Reprod.* 2016;31:1253–64.
30. Toftager M, Bogstad J, Lössl K et al. Cumulative live birth rates after one ART cycle including all subsequent frozen-thaw cycles in 1050 women: secondary outcome of an RCT comparing GnRH-antagonist and GnRH-agonist protocols. *Hum Reprod.* 2017;32: 5566–7.
31. Tarlatzis BC, Griesinger G, Leader A et al. Comparative incidence of ovarian hyperstimulation syndrome following ovarian stimulation with corifollitropin alfa or recombinant FSH. *Reprod Biomed Online* 2012;24:4101–9.
32. Griesinger G, Verweij PJM, Gates D, Devroey P, Gordon K, Stegmann BJ et al. Prediction of ovarian hyperstimulation syndrome in patients treated with corifollitropin alfa or rFSH in a GnRH antagonist protocol. *PLoS ONE* 2016;11(3):e0149615. doi:10.1371/journal.pone.0149615.

33. Griesinger G., von Otte S., Schroer A. et al. Elective cryopreservation of all pronuclear oocytes after GnRH-agonist triggering of final oocyte maturation in OHSS risk patients: a prospective, observational proof-of-concept study. *Human Reproduction* 2007;22:1348–52 Russian Excerpted Edition: *Human Reproduction Update* 2007;2:55–9.
34. Griesinger G, Berndt H, Schultz L, Depenbusch M, Schultze-Mosgau A. Cumulative live birth rates after GnRH-agonist triggering of final oocyte maturation in patients at risk of OHSS: A prospective, clinical cohort study. *Eur J Obstet Gynecol Reprod Biol.* 2010;149: 190–4.
35. Al-Inany HG, Youssef MA, Ayeleke RO et al. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Database Syst Rev.* 2016 April 29;4:CD001750.
36. Griesinger G, Diedrich K, Tarlatzis B, Kolibianakis EM. GnRH-antagonists in ovarian stimulation for IVF in patients with [1] poor response to gonadotrophins, [2] polycystic ovary syndrome, and [3] risk of ovarian hyperstimulation: a meta-analysis. *Reproductive Biomedicine Online* 2006;13:6283–8.
37. Pu D, Wu J, Liu J. Comparisons of GnRH antagonist versus GnRH agonist protocol in poor ovarian responders undergoing IVF. *Hum Reprod.* 2011;26:2742–9.
38. Fauser BC, Mannaerts BM, Devroey P et al. Advances in recombinant DNA technology: corifollitropin alfa, a hybrid molecule with sustained follicle-stimulating activity and reduced injection frequency. *Hum Reprod Update.* 2009;15:3092–1.
39. Griesinger G, Boostanfar R, Gordon K et al. Corifollitropin alfa versus recombinant follicle-stimulating hormone: an individual patient data meta-analysis. *Reprod Biomed Online.* 2016 April 20. p ii: S1472–6483(16)30068–2. doi:10.1016/j.rbmo.2016.04.005
40. Nyboe Andersen A, Nelson SM, Fauser BC et al. ESTHER-1 study group. Individualized versus conventional ovarian stimulation for in vitro fertilization: a multicenter, randomized, controlled, assessor-blinded, phase 3 noninferiority trial. *Fertil Steril.* 2017;107: 3879–6.

The Gonadotropins in IVF

r-hFSH or u-hFSH, Biosimilars, Regimens, Doses: Which Protocol

Colin M. Howles

6.1 Introduction

The early years (1980–1995) of ovarian stimulation protocols for in vitro fertilization (IVF) were shaped firstly from pioneering work in animal models (see review by Edwards [1]) and then through the use of urinary-derived gonadotropins, in particular menotropin (human menopausal gonadotropin (hMG)) and human chorionic gonadotropin (hCG), which were first scientifically described for use together in an in vitro fertilization (IVF) ovarian stimulation protocol by Steptoe and Edwards in their landmark 1970 Lancet paper [2]. Following the replacement of human embryos in 77 cases without a pregnancy and an ectopic occurring after the use of 900 IU hMG followed by hCG [3], the pioneering team decided to change track. Bob Edwards and Patrick Steptoe abandoned hMG use, and the first IVF births (two from the United Kingdom and one from Australia) were all after natural cycle IVF. A wide range of stimulation protocols were then investigated [4]; however, hMG was reintroduced in particular by the US Norfolk IVF programme [5] and then combined with clomiphene citrate (CC), which had initially been successfully used by the Melbourne groups [6]. The CC/hMG/hCG protocol was utilized at Bourn Hall Clinic in the early 1980s and proved more successful than CC alone [7].

Prior to the start of IVF, pharmaceutical preparations containing biologically active gonadotropins had been in use for about 75 years. As reviewed by Lunenfeld [8] and Howles [9], major advances in technology have brought the field of gonadotropin therapy a very long way since the era of animal-, human pituitary and urinary-derived hormones. The use of human pituitary-derived FSH for ovulation induction, from 1958 onwards, led 20 years later to a number of cases of iatrogenic Creutzfeldt–Jakob disease (CJD) in material prepared by national agencies from France, Australia and the United Kingdom. For almost 30 years, hMG had been the main gonadotropin available for clinical use. Table 6.1 lists the characteristics of FSH preparations which have been commercially available. The FSH and LH content of hMG (or menotropin) are equal in terms of biological activity (75 IU of FSH and 75 IU of LH) as measured by animal-derived bioassays. The use of the term IU's and FSH quantities in multiples of 75IU's is still used today even in the era of recombinant gonadotrophins.

The first hMGs were of low specific activity with around 370–750 ug protein per 75 IU FSH activity. In the 1980s, urinary FSH-only preparations became new therapeutic options for ovulation induction, with <1 percent ‘LH activity contamination’ but still having 95 percent protein impurity. In 1993, highly purified FSH (FSH-HP), containing <0.1 percent ‘LH contamination’, was the first highly pure biologic extract (specific

Table 6.1 Characteristics of gonadotrophin preparations commercially available

	Purity (FSH content) (%)	Mean specific FSH activity (IU/mg protein)	Injected protein per 75 IU (mcg)
hMG	< 5	~100	~750
u-FSH	< 5	~150	370–750
hMG HP	< 70	2,000–2,500	~18
u-FSH HP	> 95	~9,000 (highly variable)	6–11
r-hFSH			
Follitropin beta	–	7,000–10,000	8.1
Follitropin alfa	>99	13,645	6.1

activity ~9000 IU FSH/mg protein), and, as a result of this, could be injected subcutaneously (sc), unlike the earlier preparations which had to be administered intramuscularly (im). Today there are other ‘purified’ hMGs and u-FSH preparations available from different manufacturers. The most widely used ‘highly’ purified hMG available is still however only partially pure (specific activity of ~2500 IU mg for both FSH and LH activity) with a typical protein content of ~30 µg per 75 IU vial [10]. This compares to a total of 11 µg protein if such a 1:1 FSH:LH preparation was developed using recombinant human FSH and LH.

Following the introduction of recombinant human insulin in 1982, drugs produced through the use of recombinant DNA techniques became a welcome alternative source of complex biological proteins across medical practice. The clinical introduction in 1996 of r-hFSH, with FSH isoform distributions within the range detected for native human FSH, heralded the start of the recombinant era in reproductive medicine with the follow-on launch of both r-hLH and r-hCG. In 2010 a recombinant fusion molecule (FSH coupled to the CTP of hCG) with a longer FSH action was registered in the European Union, followed in 2014 by the first biosimilar FSH. In 2017 another FSH (follitropin delta) with different pharmacokinetic (PK) properties compared to existing FSH products entered the EU market. There is also a recent report of another modified FSH (follitropin epsilon) with different pharmacodynamic properties from follitropin alfa, which has undergone Phase I and II trials. Overall the advances in available gonadotropin preparations have brought improved product consistency and methods of injection delivery. However, urinary-derived gonadotropins are still prevalent in many countries and look likely to continue being part of the stimulation armament in years to come. As of today, the choice of which gonadotropin to use is based primarily upon the following list of factors: efficiency (oocytes retrieved), product consistency, cost per 75 IU FSH and a desire for LH activity. Other secondary factors which are more appealing to patients and nursing staff are ease of use of delivery device and formulation (liquid/freeze dried); all the former are also impacted by geographic availability.

In IVF studies comparing gonadotropins, the most feasible primary efficacy endpoint is the number of oocytes retrieved from aspirated hCG or ‘LH surge’ primed follicles. This is

the endpoint recognized by, for example, the European Medicines Agency, as pharmacologically the human FSH receptor is located only in the female, on the granulosa cell. FSH stimulation of the granulosa cell leads to repeated cell division leading to follicle growth and cell differentiation. While pregnancy and live birth are the desired outcomes for all stakeholders, implantation and pregnancy rates are influenced by a number of variables unrelated to gonadotropin stimulation of the ovaries, such as laboratory conditions and the embryo transfer technique. Pregnancy rates can vary widely between clinics within a country and also across international borders for the same patient population [11]. Over the years, the measurement of gonadotropin ‘efficacy’ has been a subject of a multitude of generally underpowered studies, which were then combined into numerous meta-analyses, yielding no major meaningful clinical difference.

6.2 FSH Glycoprotein Molecule

Natural human FSH (urinary or pituitary) is a dimeric glycoprotein, i.e. a hormone consisting of two non-identical protein components, designated the alpha and beta subunits with four carbohydrate (sugar) residues attached to specific amino acids (two on alpha and two on beta subunit). The FSH molecule exists not as a single molecular structure, but rather in a range of isoforms which change across the menstrual cycle [12]. At least 20 fractions of these have been identified [12] in human pituitary FSH from menopausal women; the pI (isoelectric point) range of FSH of various isoforms is 3.6–6.1.

Isoforms are structural variants of a protein and are differentiated by their carbohydrate moieties, which can be either acidic or basic due to the presence (or not) of sialic acid covering the terminal ends. The latter characteristic affects the isoform’s plasma half-life, and therefore also its biological activity (Howles et al. 1996). In the follicular phase of the natural menstrual cycle there is a complex interplay between the FSH isoforms secreted from the pituitary and oestradiol feedback from the growing follicle. In a series of studies Wide and colleagues [13,14] have demonstrated decreases in sialylated FSH isoforms due to oestradiol.

The more acidic isoforms have a longer half-life *in vivo* than the less acidic ones. Menopausal urine contains a majority of the more acidic isoforms [13] as these are secreted preferentially from the pituitary into the blood due to a lack of circulating oestradiol and are thus excreted in urine. The isoelectric point (pI) range for FSH isoforms in urinary-derived FSH is $\approx 3.0\text{--}5.2$. For r-hFSH (follitropin alfa and beta) the pI is between 3.5 and 6.0, but with some significant differences between the two products in the extreme pI range, alfa being slightly more acidic – 18.2 versus 9.8 percent for beta at pI 3.5–4.0 ($P = 0.03$) and 6.7 alfa versus 10.7 percent beta at pI 5.0–5.5 ($P = 0.03$) [15]. These differences were most likely due to a combination of manufacturing and production factors. Folitropin alfa and beta were produced using different mammalian cell lines as well as the fermentation and downstream purification processes. However, all the carbohydrate structures identified on follitropin alfa, for example, correspond to those found physiologically in circulating FSH (Figure 6.1).

In spite of these significant differences in the proportion of acidic versus basic isoforms, the PK characteristics of follitropin alfa and beta (as well as urinary FSH) are similar; the elimination half-life after sub-cutaneous injection is in the region of 36–40 hours. Additionally, over a 20-year period of clinical use, randomized controlled trials (RCTs) examining efficacy (oocytes retrieved) or pregnancy outcomes have not been

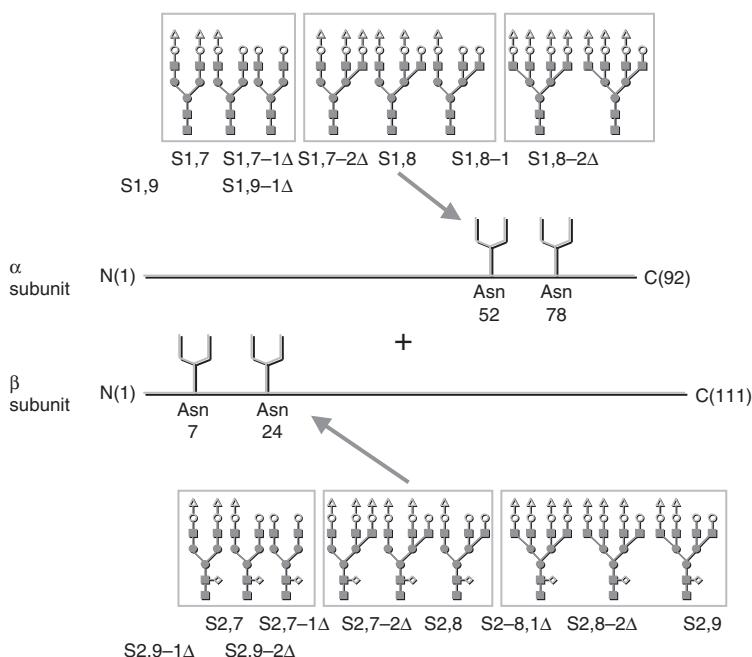


Figure 6.1 All carbohydrate structures identified on Follitropin alfa correspond to those found physiologically

demonstrated to be different between the two recombinant products. Thus, we can safely conclude that subtle differences in FSH isoform composition do not translate into differences in efficacy.

6.3 FSH Molecules Developed Using Recombinant Technology

The fact that r-hFSH preparations have a fully controlled production process from bulk to finished product, full traceability from the starting material (cell line) to the final product, unlimited supply with batch-to-batch consistency and are free from urinary protein contaminants is considered as advantageous [16,17]. The conclusion has, thus, been reached that r-hFSH, indeed, is less immunogenic than the older urinary-derived medications and, at least from this point of view and overall safety, is preferable. However, the question whether recombinant-derived FSH products are beneficial from the clinical perspective has long been debated in the literature. This is in sharp contrast with other recombinant biotech drugs used in other therapeutic areas such as diabetes or growth deficiency; the widespread use of these products was not motivated by clinical superiority over biological-/organic-derived products, but replacement was driven just on the basis of their better safety profile. This argument has never really taken hold in reproductive medicine.

Following introduction in 1996 of the first recombinant-derived h-FSH preparations (Gonal-f; follitropin alfa (Serono, now Merck) and Puregon; follitropin beta (Organon now MSD) respectively (\approx specific activity 10,000 IU FSH/ug protein)), further refinements to the production and fill process (e.g. filled by mass follitropin alfa, specific activity 13475 IU FSH/mg protein yielding 5.5 ug FSH protein per 75 IU) were developed and

commercialized. Being able to use mass units to determine the activity per dose was an important step forward as the original in vivo bioassay (Steelman Pohley) was subject to batch-to-batch variation [18].

In 2010 a long-acting formulation of r-hFSH (Elonva; corifollitropin alfa, FSH-CTP (MSD)) was commercialized first in the European Union (see review by Fauser et al. [19]). This molecule is a hybrid, having co-expression of the alfa subunit and a hybrid beta subunit of FSH with the carboxy-terminal peptide of the hCG beta subunit attached. This results in a prolonged elimination half-life after s.c. administration of around 70 hours compared with that of follitropin alfa or beta of around 36–40 hours. There are 2 dose forms marketed, 100 µg for women with body weight ≤60 kg and 150 µg FSH-CTP for women >60 kg. Corifollitropin was developed to reduce the number of FSH injections to one during the first seven days of COS and thus offer simpler ART treatment regimens. However, FSH-CTP is only registered for use in a GnRH antagonist protocol, where injections commence on day 5 or 6 of COS so there are in effect still only three to four injection-free days. Another important consideration is that an ART treatment protocol is relatively short (median duration of FSH injections 11 days) and reducing injection frequency may not be a core unmet need. With standard r-hFSH, it is feasible to reduce injection frequency (every three days, Scholtes et al. [20]). Additionally, there were also concerns regarding the incidence of OHSS associated with FSH-CTP use, and thus today it is being applied more in patients with a known poor response (low AMH, AFC) to COS.

In 2014, 20 years after the commercialization of the first r-hFSH (Gonal-f), the first biosimilar FSH (Bemfola; also designated follitropin alfa (Finox is now part of the Gedeon Richter group)) was launched in the European Union. Another biosimilar (follitropin alfa, Ovaleap; Teva) became commercially available in late 2015. A biosimilar is a medicine that has been demonstrated, through an exhaustive series of physico-chemical, in vitro, in vivo tests and confirmatory clinical trials (typically Phase I and III), to be similar in terms of quality, safety and efficacy to the reference medicinal product. This means that it has essentially the same active pharmaceutical ingredient (API), to be used at the same dose, via the same route for the same indications as the reference product. It has been postulated, incorrectly, that as a biosimilar, it may have different FSH isoforms than the originator FSH, translating into different therapeutic efficacy and safety [21]. Firstly, slight variability due to post-translational modifications can occur in any originator product batch (see article from Schiestl [22]). Secondly, it is important to point out that the goal of the biosimilar producer is to show that any difference with the reference medicinal product does not exceed the differences among batches of that product. It was noted by the European Medicines Agency in the Bemfola Assessment Report that ‘some of the observed variations are smaller than those allowed for follitropin in the European Pharmacopoeia monograph’. Overall it is therefore expected based on the reference product batches that the glycosylation pattern of a biosimilar and reference product will not be identical, but activity in vivo (as measured in the case of Bemfola by oocytes harvested after COS) is actually equivalent [23]. As discussed earlier, the significant differences in basic versus acidic FSH isoforms for follitropin alfa versus beta had no clinical implications.

The most recent r-hFSH (Rekovelle; follitropin delta (Ferring)) that has passed through clinical development and ready for commercialization in Europe has been derived using a cell line of human fetal retinal origin (PER.C6). The amino acid sequences of the a- and b-subunits are identical to that of natural human FSH, but

due to the cell line the glycosylation profile is different from follitropin alfa and beta. Follitropin delta contains both α 2,3 and α 2,6 sialylation patterns, while CHO-derived r-hFSH products exclusively carry α 2,3-linked sialic acid. A higher proportion of tetra-antennary structures and higher overall sialic acid content therefore characterizes the glycosylation profile of follitropin delta. Glycosylation is known to be associated with decreased metabolic clearance.

It is important to note that while follitropin delta has the same target (FSH receptor) as the original biological, it has been 'bio-engineered' to allow it to be in circulation for an extended period of time. This is different from follitropin alfa and beta, which were developed to have a comparable PK and pharmacodynamics (PD) profile to human urinary-derived FSH.

Studies in healthy women volunteers comparing the PK properties following a single i.v. injection of 225 IU FSH (as assessed by the Steelman Pohley bioassay) of follitropin delta or follitropin alfa showed that the former has a longer elimination half-life (30h vs 24h) and after single s.c. administration of 450 IU FSH of the two r-hFSH preparations the respective half-life was 48 versus 35 h [24]. Additionally, follitropin delta elicited a higher ovarian response when administered at equal IU doses of biological activity [25]. Based on these differences and a Phase II trial [25] an algorithm was developed for dosing based on AMH and weight (kg) of the IVF patient.

The Phase III assessor blind study [26], carried out in a GnRH antagonist protocol, employed different doses of follitropin delta administered daily according to an AMH-weight algorithm versus a 'conventional' standard dose of 150 IU per day of follitropin alfa in women aged 18–40 years of age. In the follitropin delta arm the algorithm-based dose was fixed throughout stimulation, but with follitropin alfa the starting dose was 150IU, which could be increased up to a maximum of 450 IU from day 6 of FSH stimulation. Forty percent of women recruited in both arms were 35 years or older.

In spite of a fixed starting dose of 150 IU follitropin alfa in all patients irrespective of their age (and hence AMH), the main efficacy and safety results were similar compared to an individualized approach of follitropin delta dosed according to AMH and weight. There were no significant differences in oocytes retrieved ($10.4 +/ - 6.5$ vs $10 +/ - 5.6$), percentage of clinical pregnancies (31.6 vs 30.7), percentage of incidence of moderate/severe OHSS (1.4 vs 1.4) or percentage of hospitalization due to OHSS (0.9 vs 0.3) in the follitropin alfa versus delta groups respectively. Surprisingly the individualized dosing regimen adopted in the study for follitropin delta was not associated with statistically lower cases of OHSS (total, early or late).

However, the authors reported under safety outcomes a significantly higher number of 'preventative interventions' for follitropin alfa (30 vs 15; $P = 0.005$). Preventative interventions were defined as early OHSS covered cycle cancellation, administration of GnRH agonist and administration of dopamine agonist.

In spite of the above finding, it would have been more informative for routine clinical practice if the study design had also employed individualized dosing (based on current clinical practice, e.g. using age/AMH/AFC) for follitropin alfa. This would have provided a more balanced assessment of the relative merits of follitropin delta. To date there is no clinical trial experience with follitropin delta in GnRH agonist protocols.

At the time of writing this chapter, information was published on another 'bio-engineered' recombinant FSH (follitropin epsilon (Glycotope)) that is produced in a human cell expression system with 'glycosylation optimization and adjustment'.

It was reported that the glycosylation pattern for epsilon is different from that of delta [27]. Phase I PK and PD trials comparing follitropin epsilon to follitropin alfa or urinary-derived FSH demonstrated that after a single s.c. injection of 150 IU of each product the former compound had a similar PK profile to follitropin alfa. However, multiple doses of 150 IU follitropin epsilon induced a higher follicle growth response compared to the same IU dose of follitropin alfa or urinary FSH. The results of a Phase II dose finding trial are awaited to document in more detail the PD effects of this molecule on multiple follicle growth.

6.4 Why Perform Controlled Ovarian Stimulation?

The administration of gonadotropins to promote the development of multiple ovarian follicles during controlled ovarian stimulation (COS) in assisted reproductive technology (ART) is a core foundation stone for facilitating successful treatment outcomes. By extending the time that FSH is elevated above the threshold level [28] to promote follicle development, COS, with an appropriate LH trigger, results in the harvest of a cohort typically between 5 and 20 oocytes that can be fertilized using conventional in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) techniques. A number of studies from national registries have confirmed that the number of oocytes retrieved is a robust surrogate outcome for clinical success [29]. However, there is a price to pay as far as the endocrine milieu is concerned; FSH stimulation is accompanied with supra-physiological levels of oestradiol and progesterone as each recruited follicle contributes to the overall concentrations in circulation. This has resulted in an important debate, which has recently resurfaced in view of the potential impact of elevated oestradiol and progesterone (around the last days of FSH stimulation) on implantation [30].

In the mid 1980s a revolution in stimulation practices occurred following the introduction of the GnRH agonist into the IVF clinic. The luteal start GnRH agonist protocols are still used today and based upon the early experiences [31]. The use of the GnRH agonist 'long protocol' to block gonadotrophin release from the pituitary [32,33] led to a major step forward in the clinical management of IVF patients, greatly reducing the incidence of an LH surge, allowing improved timing of hCG administration and the scheduling of oocyte retrieval, but increasing the period of drug treatment (GnRH agonist pretreatment and stimulation period), as well as increasing FSH consumption. A host of other GnRH agonist regimens appeared (short or flare for 'poor responders', ultrashort or microdose), mostly used in poor responders. The GnRH long agonist regimen still reigns worldwide (\approx 60 percent cycles) as the preferred adjunct therapy, and this is in spite of the fact that even today it is not registered in all markets (e.g. USA) for use in IVF. Its popularity is probably partly due to convenience for the clinic in terms of cycle scheduling. However, the GnRH agonist is now being seriously challenged by the GnRH antagonist protocol. The GnRH antagonist was first launched in 1999 to a tremendous fanfare (shorter treatment period, less FSH required), but it took almost ten years to find its place in the stimulation armamentaria fuelled by its ability to be coupled with GnRH agonist as the 'trigger' for timing oocyte retrieval [34,35], leading to the promise of an 'OHSS-free IVF clinic' [36].

Additionally, there has been a paradigm shift in the use of gonadotropins, from standardized to individualized COS. The ‘one size fits all’ approach is no longer considered to be the best approach. Individualized COS is designed to maximize the efficacy and safety of fertility treatment for each patient. This change in approach to treatment was facilitated by the identification of more reliable predictors of ovarian response, such as a standardized antral follicle count (AFC) using standardized ultrasound techniques [37] and anti-Müllerian hormone (AMH) [38]. Additionally, improved laboratory, culture and assessment techniques have increased the efficiency of ART treatment cycles.

However, today, due to the availability of a combination of established as well as new techniques we are beginning to witness a total re-evaluation of how ovarian stimulation is carried out. These are the use of GnRH antagonist pituitary blockade with GnRH agonist trigger, optimized COS using biomarkers such as AMH/AFC, vitrification, new generation sequencing for PGS and a move to segmented ART ('freeze only' cycles).

Thus, following careful, highly controlled laboratory culture and analysis using time-lapse technologies and/or preimplantation genetic screening (PGS), ideally one high-quality embryo can be transferred to the uterus, thus bringing closer to reality the mantra ‘one embryo one baby’.

Supernumerary oocytes and/or embryos can now be more reliably cryopreserved for future use. Vitrification is a rapid freezing technique for embryos and oocytes. Vitrified, warmed embryos have very good survival rates and the technique is becoming increasingly more widely used [39]. The success of this technique in terms of both oocyte and embryo survival rates has facilitated an increased focus on documenting the cumulative live birth rate following a combination of these strategies.

While COS is aimed at maximizing the beneficial effects of treatment, the potential risks associated with ovarian hyperstimulation syndrome (OHSS) and multiple pregnancy must be taken into account [40]. OHSS is the most serious complication of COS and is triggered or exacerbated by human chorionic gonadotropin (hCG) and results in increased capillary permeability, hemoconcentration and hypovolemia [41]. In order to minimize the incidence of OHSS, there has been an increased use of GnRH antagonist protocols coupled with a GnRH agonist [42] to induce final follicular maturation and time oocyte recovery [43]. This in itself has caused consternation with regard to the integrity of the subsequent luteal phase and its ability to support implantation, leading to a lengthy debate on how best to avoid OHSS [36] and also how to support the luteal phase [35] after fresh embryo transfer.

6.5 Summary and Concluding Remarks

The use of gonadotrophins for controlled ovarian stimulation will continue to be a cornerstone of a successful ART treatment cycle. The use of segmented ART protocols will gain ground due to the need to reduce the risk of OHSS and also because of concerns about the integrity of the stimulated endometrium to allow implantation. Additionally, vitrification as well as techniques to identify a healthy embryo are facilitating a ‘one at a time’ embryo replacement policy and a focus on cumulative live birth rates from a single course of ovarian stimulation.

References

1. Edwards RG. The history of assisted human conception with especial reference to endocrinology. *Exp Clin Endocrinol Diabetes.* 1996;104:183–204.
2. Steptoe PC, Edwards RG. Laparoscopic recovery of preovulatory human oocytes after priming of ovaries with gonadotrophins. *Lancet.* 1970; 1(7649):683–9.
3. Steptoe PC, Edwards RG. Reimplantation of a human embryo with subsequent tubal pregnancy. *Lancet.* 1976;1(7965):880–2.
4. Edwards RG, Steptoe PC, Purdy JM. Establishing full-term human pregnancies using cleaving embryos grown in vitro. *Br J Obstet Gynaecol.* 1980;87:737–56.
5. Jones HW Jr, Jones GS, Andrews MC et al. The program for in vitro fertilization at Norfolk. *Fertil Steril.* 1982 Jul;38(1):14–21.
6. Trounson AO, Leeton JF, Wood C, Webb J, Wood J. Pregnancies in humans by fertilization in vitro and embryo transfer in the controlled ovulatory cycle. *Science* 1981;212:681–2.
7. Fishel SB, Edwards RG, Purdy JM et al. Implantation, abortion, and birth after in vitro fertilization using the natural menstrual cycle or follicular stimulation with clomiphene citrate and human menopausal gonadotropin. *J In Vitro Fert Embryo Transf.* 1985;2:123–31.
8. Lunenfeld B. Historical perspectives in gonadotrophin therapy. *Human Reproduction Update* 2004;10:453–67.
9. Howles CM. Recombinant gonadotrophins in reproductive medicine: the gold standard of today. *Reprod Biomed Online.* 2006;12: 11–3.
10. Lispi M, Bassett R, Crisci C et al. Comparative assessment of the consistency and quality of a highly purified FSH extracted from human urine (urofollitropin) and a recombinant human FSH (follitropin alpha). *Reprod Biomed Online.* 2006;13: 179–93.
11. Boostanfar R, Mannaerts B, Pang S et al. A comparison of live birth rates and cumulative ongoing pregnancy rates between Europe and North America after ovarian stimulation with corifollitropin alfa or recombinant follicle-stimulating hormone. *Fertil Steril.* 2012;97:1351–8.
12. Wide L, Bakos O. More basic forms of both human follicle-stimulating hormone and luteinizing hormone in serum at midcycle compared with the follicular or luteal phase. *J Clin Endocrinol Metab.* 1993;76: 885–9.
13. Wide L, Naessén T, Sundström-Poromaa I, Eriksson K. Sulfonation and sialylation of gonadotropins in women during the menstrual cycle, after menopause, and with polycystic ovarian syndrome and in men. *J Clin Endocrinol Metab.* 2007;92:4410–7.
14. Wide L, Naessén T, Eriksson K. Effects of 17beta-oestradiol and norethisterone acetate on sulfonation and sialylation of gonadotrophins in post-menopausal women. *Ups J Med Sci.* 2010;115:97–106.
15. Horsman G, Talbot JA, McLoughlin JD, Lambert A, Robertson WR. A biological, immunological and physico-chemical comparison of the current clinical batches of the recombinant FSH preparations Gonal-F and Puregon. *Hum Reprod.* 2000;15:1898–902.
16. Howles CM. Genetic engineering of human FSH (Gonal-F). *Hum Reprod Update.* 1996;2:172–91.
17. Loumaye E, Martineau I, Piazz A et al. Clinical assessment of human gonadotrophins produced by recombinant DNA technology. *Hum Reprod.* 1996;11 Suppl 1:95–107.
18. Driebergen R, Baer G. Quantification of follicle stimulating hormone (follitropin alfa): is in vivo bioassay still relevant in the recombinant age? *Curr Med Res Opin.* 2003;19:41–6.
19. Fauser BC, Mannaerts BM, Devroey P et al. Advances in recombinant DNA technology: corifollitropin alfa, a hybrid molecule with sustained follicle-stimulating activity and reduced injection frequency. *Hum Reprod Update.* 2009;15:309–21.
20. Scholtes MC, Schnittter B, van Hoogstraten D et al. A comparison of 3-day

- and daily follicle-stimulating hormone injections on stimulation days 1–6 in women undergoing controlled ovarian hyperstimulation. *Fertil Steril.* 2004; **81**:996–1001.
21. Orvieto R, Seifer DB. Biosimilar FSH preparations: are they identical twins or just siblings? *Reprod Biol Endocrinol.* 2016; **14**:32.
 22. Schiestl M. A biosimilar industry view on the implementation of the WHO guidelines on evaluating similar biotherapeutic products. *Biologicals.* 2011; **39**:297–9.
 23. Rettenbacher M, Andersen AN, Garcia-Velasco JA et al. A multi-centre phase 3 study comparing efficacy and safety of Bemfola^(®) versus Gonal-f^(®) in women undergoing ovarian stimulation for IVF. *Reprod Biomed Online.* 2015; **30**:504–13.
 24. Olsson H, Sandstrom R, Grundemar L. Different pharmacokinetic and pharmacodynamic properties of recombinant follicle-stimulating hormone (rFSH) derived from a human cell line compared with rFSH from a non-human cell line. *J Clin Pharmacol.* 2014; **54**:1299–1307.
 25. Arce JC, Andersen AN, Fernández-Sánchez M et al. Ovarian response to recombinant human follicle-stimulating hormone: a randomized, antimüllerian hormone-stratified, dose-response trial in women undergoing in vitro fertilization/intracytoplasmic sperm injection. *Fertil Steril.* 2014; **102**:1633–40.
 26. Nyboe Andersen A, Nelson SM, Fauser BC et al. ESTHER-1 study group. Individualized versus conventional ovarian stimulation for in vitro fertilization: a multicenter, randomized, controlled, assessor-blinded, phase 3 noninferiority trial. *Fertil Steril.* 2017; **107**:387–96.
 27. Abd-Elaziz K, Duijkers I, Stöckl L et al. A new fully human recombinant FSH (follitropin epsilon): two phase I randomized placebo and comparator-controlled pharmacokinetic and pharmacodynamic trials. *Hum Reprod.* 2017; **7**:1–9.
 28. Baird DT. A model for follicular selection and ovulation: lessons from superovulation. *J Steroid Biochem.* 1987; **27**:15–23.
 29. Sunkara SK, Rittenberg V, Raine-Fenning N et al. Association between the number of eggs and live birth in IVF treatment: an analysis of 400 135 treatment cycles. *Hum Reprod.* 2011; **26**:1768–74.
 30. Bosch E, Labarta E, Crespo J et al. Circulating progesterone levels and ongoing pregnancy rates in controlled ovarian stimulation cycles for in vitro fertilization: analysis of over 4000 cycles. *Hum Reprod.* 2010; **25**:2092–100.
 31. Porter RN, Smith W, Craft IL, Abdulwahid NA, Jacobs HS. Induction of ovulation for in-vitro fertilisation using buserelin and gonadotropins. *Lancet.* 1984; **2**(8414):1284–5.
 32. Fleming R, Adam AH, Barlow DH et al. A new systematic treatment for infertile women with abnormal hormone profiles. *Br J Obstet Gynaecol.* 1982; **89**:80–3.
 33. Howles CM, Macnamee MC, Edwards RG, Goswamy R, Steptoe PC. Effect of high tonic levels of luteinising hormone on outcome of in-vitro fertilisation. *Lancet.* 1986; **2**(8505):521–2.
 34. Kol S, Muchtar M. Recombinant gonadotrophin-based, ovarian hyperstimulation syndrome-free stimulation of the high responder: suggested protocol for further research. *Reprod Biomed Online.* 2005; **10**:575–7.
 35. Humaidan P, Bredkjaer HE, Bungum L, Bungum M, Grøndahl ML, Westergaard L, Andersen CY. GnRH agonist (buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: a prospective randomized study. *Hum Reprod.* 2005; **20**:1213–20.
 36. Devroey P, Polyzos NP, Blockeel C. An OHSS-Free Clinic by segmentation of IVF treatment. *Hum Reprod.* 2011; **26**:2593–7.
 37. Broekmans FJ, de Ziegler D, Howles CM et al. The antral follicle count: practical recommendations for better

- standardization. *Fertil Steril.* 2010;94:1044–51.
38. Nelson SM, Yates RW, Lyall H et al. Anti-Müllerian hormone-based approach to controlled ovarian stimulation for assisted conception. *Hum Reprod.* 2009;24:867–75.
39. Cobo A, Garrido N, Pellicer A, Remohí J. Six years' experience in ovum donation using vitrified oocytes: report of cumulative outcomes, impact of storage time, and development of a predictive model for oocyte survival rate. *Fertil Steril.* 2015;104:1426–34.
40. Fauser BC, Diedrich K, Devroey P. Predictors of ovarian response: progress towards individualized treatment in ovulation induction and ovarian stimulation. *Hum Reprod Update.* 2008;14:1–14.
41. Aboulghar MA, Mansour RT. Ovarian hyperstimulation syndrome: classifications and critical analysis of preventive measures. *Hum Reprod Update.* 2003;9:275–89.
42. Humaidan P, Engmann L, Benadiva C. Luteal phase supplementation after gonadotropin-releasing hormone agonist trigger in fresh embryo transfer: the American versus European approaches. *Fertil Steril.* 2015;103:879–85.
43. Dosouto C, Haahr T, Humaidan P. Gonadotropin-releasing hormone agonist (GnRHa) trigger – State of the art. *Reprod Biol.* 2017;17:1–8.

Controlled Ovarian Stimulation Strategies for IVF and Programming Cycles with Steroid Pretreatment

Richard Fleming

7.1 Introduction

The need for multiple oocytes to overcome the serial inefficiencies of egg collection, human egg fertilization, embryo development and implantation was recognized in the early years of in vitro fertilization (IVF), and led rapidly to the widespread use of ovarian stimulation using exogenous follicle stimulating hormone (FSH). Today we have at our disposal many sources of FSH, and drugs controlling luteinizing hormone (LH) of both short and long duration of action. Correspondingly, we also have numerous regimes for controlled ovarian stimulation (COS) at our disposal, and they are deployed under many and diverse circumstances.

Evidence-based comparative studies are rarely useful as different methods are commonly deployed in different and limited patient categories, without universal agreements upon definitions and categorization. However, there are some essential key elements that are important, which are ignored with risk of disappointment and which indicate that much common practice is, at best, simply redundant, and worst likely to increase risks to patients. This specifically refers to the implications of the pharmacokinetics of FSH preparations, with their prolonged half-life in the circulation, which dictate that modification of stimulation doses after a week of stimulation is not the ideal way to perform COS.

One of the reasons underlying this extensive range of approaches is the broad physiological range of responses to FSH, which in turn is due to the extensive range of the functional ovarian reserve (FOR) in women of almost any age. There is also the necessity to address key events. The first is the blockade of the LH surge to avoid premature luteinization. The second is the fact that FSH-induced follicular growth from recruitment from small antral stages to large Graafian pre-ovulatory stages demands approximately 12 days. The third element, which is commonly overlooked, is the fact that follicles become more sensitive to FSH as they grow, which means that reducing doses in mid-cycle in order to reduce the degree of follicular growth does not influence continued growth and development.

7.2 Differing Circumstances of COS and Cycle Programming

In standard IVF, when embryo transfer is preferred within the cycle of treatment, it is mandatory that endometrial development is synchronous with egg maturation and subsequent embryo development. This can lead to logistical complications of clinical origin, such as when treating women with irregular ovarian activity, and also logistical sequencing issues, such as when treating egg donation and reciprocity. Even in the relatively

uncomplicated standard IVF programme, timing is often an issue as significant differences between individual responses mean flexible approaches to planning are often required.

Because of this intrinsic variability between individuals within the COS programme, some standardization may be attempted, by manipulating endogenous ovarian activity immediately prior to undertaking COS by using combined oral contraceptives (COCs) or simple progestogen (commonly norethisterone) or oestrogen pretreatment. These agents suppress pituitary FSH output and lead to suppressed ovarian activity, while manipulating endometrial growth and transformation, allowing control of menses independent of ovarian activity.

The modern IVF clinic deploys logistical programming on a considerable scale, despite sizeable evidence suggesting that as a practice it can be harmful to the outcome of the cycle. This has induced some interesting, if confusing, debate.

7.3 Ovarian Physiology and the Endocrine System

The primary function of the human ovary is folliculogenesis, which is the provision of a constant supply of small antral follicles during the reproductive years of life, which it does in excess, and independent of the endocrine system. Superimposed upon this phenomenon is the endocrine system's control of 'cyclic' recruitment of antral follicles, followed by lead follicle dominance and mono-ovulation [1].

The rate of 'initial' recruitment from the follicle pool at the start of folliculogenesis is high in young women, with a maximum at around the time of puberty (approximately 900 per month), followed by a steady decline to negligible numbers in the late 40s [2]. All the developmental stages are growth factor promoted, and it is only at the final antral stages where the intrinsic FSH receptors become functional allowing 'cyclic' recruitment by FSH. The broad range of the number of recruited pre-antral follicles seen in women of any age is the primary factor underlying the FOR and so it has profound consequences for the IVF clinic and COS (Chapter 3).

However, when follicles start to grow in response to FSH during the menstrual cycle or during COS they undergo the physiological changes that lead to follicular dominance. It is a key element of the control of mono-ovulation, which has profound implications for COS, because the follicles become very much more sensitive to FSH. As follicles grow in response to FSH, over a period of 10–14 days, they become more sensitive to FSH itself, and growth is maintained by modest and declining concentrations of FSH. Consequently, FSH dosing during COS becomes a technical challenge, addressed in detail below.

In the natural cycle, there is constant dialog between the ovary and the pituitary secreting FSH providing subtle control of stimulation, resulting in follicular selection and dominance leading to mono-ovulation. The administration of one bolus of FSH each day during COS denies us significant control over the circulating concentration of FSH (Figure 7.1), or the degree of response, where each recruited follicle becomes a 'dominant' follicle as it grows. The degree of response (number of recruited follicles) depends more on the FOR than any FSH dose manipulation.

The key points are that follicles require 10–14 days to grow from small antral stages to ovulatory stages. As they do so they all behave as dominant follicles, acquiring increased sensitivity to FSH.

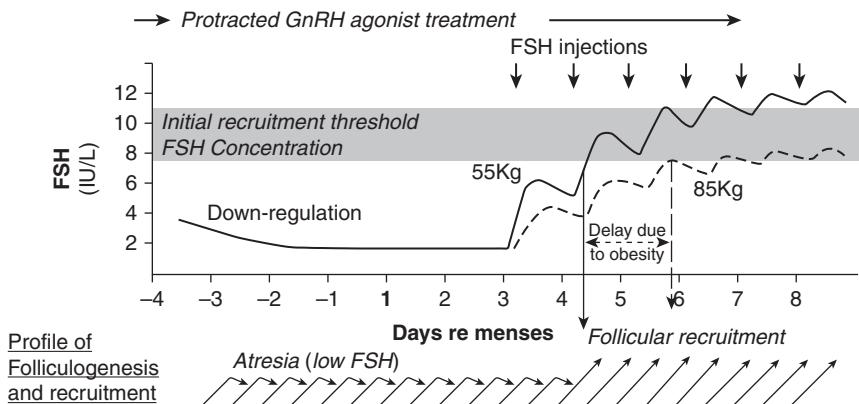
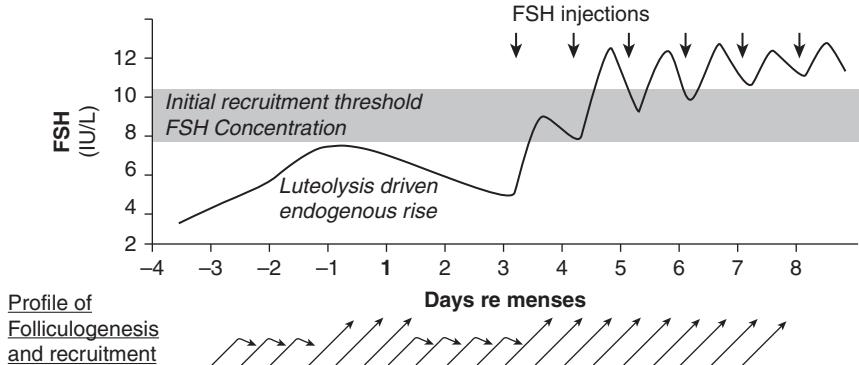
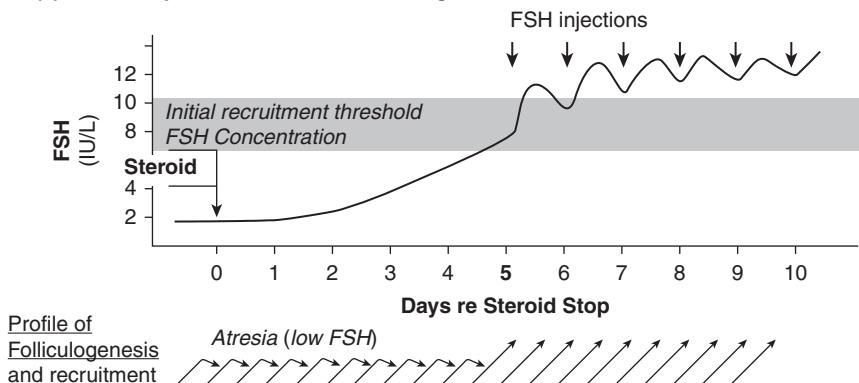
(a) Long down-regulation to COS: agonist**(b) Natural cycle to COS: antagonist****(c) Steroid pre-treatment to COS: antagonist**

Figure 7.1 Representation of FSH concentrations and follicular recruitment during (A) the basic long-course down-regulation version of COS in normal and overweight women, (B) in GnRH antagonist-controlled cycles without prior steroid suppression and (C) in GnRH antagonist-controlled cycles with steroid pretreatment

7.4 Multiple Follicular Recruitment during COS

One of the other key endocrine elements during COS is the requirement to block the spontaneous LH surge. The original solution to the problem was the suppression of LH using protracted treatment with gonadotrophin-releasing hormone (GnRH) agonists, which upon protracted administration down-regulates the pituitary gonadotrophin output. The initial pituitary response to GnRH agonists is always a ‘flare’ secretion of LH and FSH, which upon continued treatment becomes down-regulated to achieve low output levels for both hormones after a few days. When treatment is commenced in the luteal phase of the preceding cycle, menses indicates that suppression is effective, and FSH stimulation can begin whenever convenient. All follicular growth from here is induced by exogenous FSH (Figure 7.1a), by the simple administration of exogenous FSH. This elicits the growth and maturation of numerous follicles [3], and the degree of response depends upon two factors: first, the functional ovarian reserve (FOR) [4] and, second, the circulating FSH concentration, which is dictated primarily by FSH dose and body mass (Figure 7.1a).

Use of this process allows clinical control of follicular growth using exogenous FSH injections, maturation and ovulation, and the advantage is that the clinical procedures are superimposed upon a stable endocrine base of negligible follicular activity. The disadvantage is that the exogenous FSH is being administered on top of a low endogenous FSH concentration, thus requiring more time to attain the critical recruitment concentrations at a steady-state level and all stages of recruitment and growth to depend on the exogenous FSH. This generally leads to longer stimulation phases than where the exogenous FSH is superimposed upon normal pituitary activity, as in cycles controlled with GnRH antagonists without steroid pretreatment (Figure 7.1b). Different profiles of FSH and recruitment are seen when steroid pretreatment is used in antagonist controlled cycles (Figure 7.1c).

For the logistical purposes of programming within a clinical programme, the start of FSH injections may be delayed for a considerable period of time at this ‘down-regulation’ stage, without affecting the outcome of the cycle. Indeed, in cases of endometriosis, a protracted period of pituitary suppression induced by GnRH agonist and exogenous steroids (add back levels of activity) may even improve outcomes [5].

The availability of GnRH antagonists in 2000 allowed shorter COS methods wherein treatment for the LH surge blockade is performed only when the patient is at risk of such an event. In the initial controlled studies comparing antagonists with agonists, lower egg yields were universally recorded in the antagonist arm. Of course, this can be deemed an advantage in women with a high FOR.

However, because ‘cyclic’ recruitment starts in the late luteal phase/early follicular phase, the timing of FSH injections is somewhat restricted. This restriction can be overcome by suppressing endogenous cyclic recruitment, usually by administration of exogenous steroids to suppress endogenous pituitary activity and follicular recruitment (Figure 7.1c). Consequently, steroid programming of cycles controlled by GnRH antagonists has been widely adopted, despite evidence suggesting harmful effects of such practice.

7.5 FSH Starting Doses and Responses

Figure 7.1a shows FSH concentrations and profiles of follicular recruitment in women with a healthy FOR treated with FSH injections after treatment with GnRH agonist starting in the preceding luteal phase. The FSH concentration values take two or three days to achieve levels eliciting recruitment (at the crossing of the critical value) and four or five days to

achieve steady-state values [6]. Multiple follicular recruitment and growth are induced when the FSH concentration consistently attains the noted critical values in the circulation. The actual concentration attained after standard dose administration is influenced by body mass, adiposity and factors associated with clearance rates [7]. Of course, the number of follicles responding to the FSH is mostly dictated by the FOR.

In most cycles of long-course GnRH agonist-controlled ovarian stimulation the FSH concentrations exceed the threshold values required for maximal follicular recruitment. However, on a worldwide basis large variations in drug starting doses and dose escalations or reductions are deployed, and scientific examination has rarely accommodated the critical element of the patient's FOR and/or her body mass.

It should be noted that the relatively slow evolution of both circulating FSH concentrations and complex control mechanisms for follicular growth renders clinical control by manipulation of the FSH dose to be imprecise at best.

Historically, the dosing used most widely with GnRH agonist control was 225 IU/day, which generally results in maximal or near-maximal follicular recruitment during the standard long-course COS. A comparison of responses to higher doses (300 IU/day) in women categorized by their antral follicle count (AFC) as a measure of their FOR [8] showed negligible effect of the higher doses on any category of ovarian reserve. This indicates that 225 IU daily recombinant FSH (recFSH) achieves maximal follicular response in the majority of cases. As in most such studies, body mass was not addressed directly, but it was clear that the extra dosing provided in the 300 IU group achieved no tangible benefit over the 225IU schedule, even in women with a low FOR. Furthermore, comparative approaches in women diagnosed as 'poor responders' (low FOR) have shown that doses up to 450 IU/day fail to achieve increased oocyte yields [9].

Women with a high AFC all showed high egg yields with both doses [8], putting them at increased risk of ovarian hyperstimulation syndrome (OHSS), and perhaps the main lesson from their experience is that lower doses should be adopted from the start in women with a high AMH or AFC (FOR). Randomized comparison of starting doses of 150 IU versus 225 IU in unselected patients did show lower egg yields with the lower dose [10], indicating that responses can be attenuated with differential starting (and maintained) dose.

The reduced risks of excessive responses (and OHSS) when lower FSH doses are used in these women, and also when using antagonist protocols, are in line with modern practice [11]. The recent introduction of recombinant human FSH using a graduated dosing schedule based upon AMH category and body mass was able to demonstrate that COS can be made more efficient (more cases with ideal egg yields) and safer (fewer excessive responses) compared with starting stimulation with a modest dose and changing the dose based upon initial responses [12].

The key element is that dose changes after a week of stimulation fail to achieve the desired effect of reducing the incidences of both excess and poor responses. This is due to the two factors of FSH pharmacokinetics and follicular physiology.

7.6 Pharmacokinetics of Exogenous FSH and Dose Modification

The simplest biology of COS is seen in the GnRH-agonist down-regulated cycle, as all follicular 'cyclic' recruitment and development are driven by the exogenous FSH, administered daily. Figure 7.1a shows a schematic representation of classic long-course GnRH agonist treatment cycle with its sequence of FSH suppression and subsequent replacement,

leading from antral follicle atresia to recruitment and growth. Because the half-life of FSH in the circulation is around 30 hours, daily sub-cutaneous administration of FSH leads to increasing circulating concentrations. The net effect is an accumulation of circulating FSH concentrations over the first four or five days at which point they achieve ‘steady-state’ levels [6]. Although FSH concentrations do increase for a few hours after each injection, they then decline to the steady-state value approximately 24 hours later. This steady-state level is usually sufficient to elicit the recruitment and growth of all FSH-sensitive follicles. The first recognizable consequence of this recruitment is an increase in circulating oestradiol, and after about a week of injections, multiple follicles are detected by ovarian ultrasound. In such conventional COS, where endogenous FSH contributes very little to the circulating FSH levels during stimulation, most cases would see maintained FSH administration until the development of a sizeable cohort of follicles is achieved. This usually takes between 10 and 13 days of FSH treatment, and dose manipulation is often undertaken during the second week of treatment, with the aim of modifying the degree of response.

Once the threshold concentration for recruitment is breached full follicular recruitment is effected by the elevated, though fluctuating, FSH concentrations. It also shows the delaying effect of high body mass upon circulating FSH concentrations ('85 kg'), which may be a component profoundly influencing follicular recruitment, and which is often given insufficient attention.

7.7 Discontinuation of FSH Injections: Implications from Follicular Dynamics

The two most important elements of the recruitment phenomenon in COS are the pharmacokinetics of FSH itself and the ability of follicles to respond to FSH, which increases as they mature ('dominance'). The former was explored in the 1990s with the introduction of recFSH preparations [6], and there were numerous lessons to be drawn from this, although perhaps not taken to heart by many of us.

It should be noted that attempts to modify the ‘steady-state’ concentration will require a further two to four days to become re-established – and of course some further days after that before a clinical effect related to that change of dose can be observed – thus dose changes require at least a week to determine effect.

The clearest evidence from studies of dose changes during the follicular phase derives from dose discontinuation, from which we may draw indications regarding dose reduction.

Figure 7.2 (adapted from [13]) shows the profiles of FSH concentrations during and after seven days of injections of rFSH (Folitropin α; 150 IU sc daily). Following discontinuation on day 7, the circulating concentration of FSH takes a full three days to decline significantly to sub-maximal levels, and a further day to achieve values where we might expect to see a reduction in ‘cyclic’ recruitment potential. However, because the follicles are now sensitized to FSH, growth continues apace. Table 7.1 shows the data starting after seven days of injections (150 IU daily), and the first column (day ‘0’) representing the last day of injections. The oestradiol (E2) is seen to continue its rise two days following discontinuation, and then declines by approximately 40 percent on the third day – representing the first observed effect of FSH discontinuation. As the enzyme responsible for E2 biosynthesis (the aromatase enzyme in the granulosa cells) requires direct FSH stimulation, this probably does not reflect any changes in follicular dynamics taking place at this stage.

Table 7.1 Data from the study by Porchet et al. (1998) [6], with interpretation of recruitment and follicular growth phenomena

Days since FSH injection	0	1	2	3	4	5
Days since FSH start	7	8	9	10	11	12
FSH [IU/L]	8.0	8.4	5.6	3.0	1.5	1.1
E2 [pmol/L]	1500	2800	3300	2000	900	300
Total FD volume ($\text{mm}^3 \times 1000$)	18	25	26	27	32	38
Number of large follicles (FD>10 mm)	5					14

Interpretation						
E2 biosynthesis	+++	+++	++	+	-	-
Cyclic recruitment	+++	+++	++	++	-	-
Recruited follicle growth	+++	+++	+++	+++	+++	++

Note: Day '0' represents the last stimulation day and subsequent days are those following discontinuation of FSH injections. '+++' represents maximal effect, '++' represents sub-maximal effect and '-' represents negligible effect. E2 = oestradiol

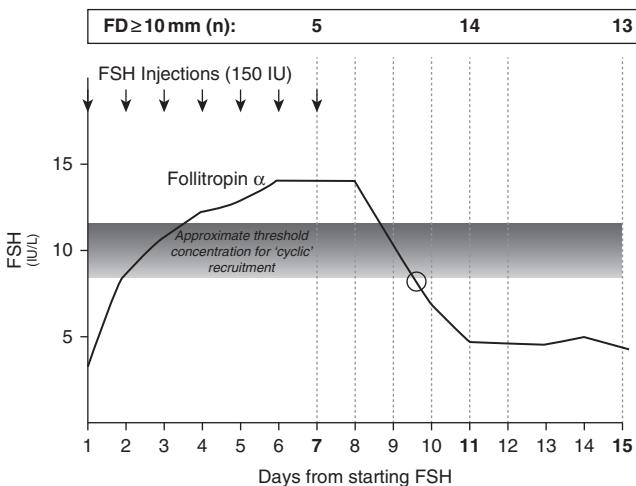


Figure 7.2 The effects of seven days of FSH injections upon circulating FSH and Graafian follicle recruitment over a 15-day observation period. Data adapted from Olsson et al. 2014 [13].

The most important finding [13] is that the observed follicular mass continued to develop, uninterrupted, for the full five days of observation. Table 7.1 shows the details of the FSH doses and the likely interpretation of its consequences. We may be fairly confident that the FSH concentrations declined sufficiently to inhibit new 'cyclic' recruitment from day 3 after discontinuation (day 10 of 'stimulation'), but recruited follicular growth was maintained for at least two days after this. This refers back to the increased follicular sensitivity of 'dominant' follicles of the normal cycle and that the sensitivity of follicles to

FSH once in the growing phase is sufficient to support continued development for a significant period of time with relatively low degrees of FSH stimulation. Correspondingly, dose reduction after a week of stimulation is unlikely to either achieve a reduction in the total follicular mass or increase safety (reduced OHSS risk).

In clinical terms, the decline in circulating E2 during the later stages of this experiment may be a hazard for endometrial receptivity, but the maintained follicular growth out to day 11 or 12 from the start of stimulation, despite the declining FSH from day 9, is critically important, and frequently overlooked when planning treatment dosing regimes.

This has important implications for safety when concerned about potentially excessive responses.

We can conclude that simply reducing the dose after a week or so of stimulation will not influence follicular recruitment and growth and therefore have no impact upon risks. Correspondingly, other steps should be undertaken when considering risk mitigation in excess responders.

The key points from these studies are as follows:

1. It takes more than two days without FSH to see a significant decline in the circulating FSH concentration.
2. A decline to below the recruitment threshold concentration takes further time.
3. It takes a further delay before a decline in oestradiol is observed.
4. The recruited follicles continue to grow for at least five days after discontinuation of FSH treatment, due to their increased sensitivity to FSH.
5. This growth is seen in both numbers and sizes of follicles [13].

The data regarding FSH discontinuation ([6] and [13]) reveal how formidable the system of follicular survival and FSH sensitivity can be, and how daily dose modification is unlikely to achieve significant influence upon responses, and why different approaches from the start of stimulation are more likely to achieve desired outcomes compared with modifications during treatment.

7.8 Suboptimal Initial Responses and Dose Increases in Standard Regimes

When the initial response is a sub-optimal, it is tempting to increase the FSH dose. However, when considering the pharmacokinetics and follicular growth dynamics, this may not be an appropriate action.

As described earlier, because of the pharmacokinetics of FSH, resetting the ‘steady-state’ circulating concentration of FSH will take a further two to three days from the change and a further week or more to identify follicles recruited by the re-established ‘steady-state’ concentration. That is it would take a total of nine days to see effect and a further few days for these follicles to achieve pre-ovulatory dimensions. That would demand a total stimulation period close to three weeks – something rarely undertaken.

Consequently, if the patient has a reasonable ovarian reserve, and her response appears to be inappropriately low after a week or so of stimulation, it is likely that a better course of action would be to discontinue stimulation at that stage, with the aim of starting stimulation again, with a higher starting dose.

The overall indications from this section are that any dose modification after a week of stimulation is unlikely to achieve either desired endpoint. The aim should be to start with the correct dose of FSH.

7.9 Steroid Pretreatment and 'Cyclic' Recruitment

The aim of steroid pretreatment in a COS programme is to suppress gonadotrophin output from the pituitary resulting in down-graded ovarian activity, allowing practical issues to be resolved in a timely manner. If FSH stimulation is initiated immediately after discontinuation of the steroid treatment, then we would expect follicular recruitment to be similar to that shown in Figure 7.1a – as with GnRH agonist down-regulation, because there would be no endogenous activity present. However, if the cycle is intended to be controlled by a GnRH antagonist, we would expect the endogenous FSH to rise soon after discontinuation of steroid administration, leading to 'cyclic' recruitment starting when the higher values are achieved, as in the normal cycle, so we would (see the recruitment profile shown in Figure 7.1c).

The timing of follicular recruitment does not stand alone, as endometrial development has also to be considered in cycles of fresh IVF, and steroid pretreatment can be used to satisfy this role too.

7.10 The Short Agonist 'Flare' Protocol: A Requirement for Steroid Pretreatment

The 'flare' regimen is commonly deployed in women with expected poor response and where dose increases are usually obviated by starting with high daily doses on top of stimulated levels of endogenous FSH. The immediate effect of GnRH agonist administration is a large output of pituitary FSH and LH. The rise of FSH can be seen for a variable amount of time generally lasting three to five days, and this can be deployed as the initial recruitment stimulus. The method has a number of variables, but the numerous published studies lack both clear endocrine evaluation (efficiency of LH surge blockade) and also clear consensus about its potential value, or in what category of patients it may have advantages.

The protocol does appear to perform well in some circumstances [9], but can appear to achieve poor results in other circumstances [14]. Few studies have explored the endocrine effects of the critical stages of the method.

The simultaneous initial flare rise of FSH and LH at treatment initiation can be a source of concern, as the elevated LH is potentially capable of rescuing the corpus luteum from the previous cycle, and thereby eliciting elevations in progesterone in the early follicular phase of the stimulation cycle.

Figure 7.3 shows a schematic representation of the protocol and how the use of norethisterone pretreatment can be used to obviate this rise in progesterone. In this case, therefore, a steroid programming phase prior to treatment is essential for biological/clinical reasons, rather than clinic convenience [15].

This demonstrates again that there may be clinical benefit from steroid as a programming agent prior to treatment, suppressing ovarian function prior to stimulation as in the classic long-course GnRH agonist control.

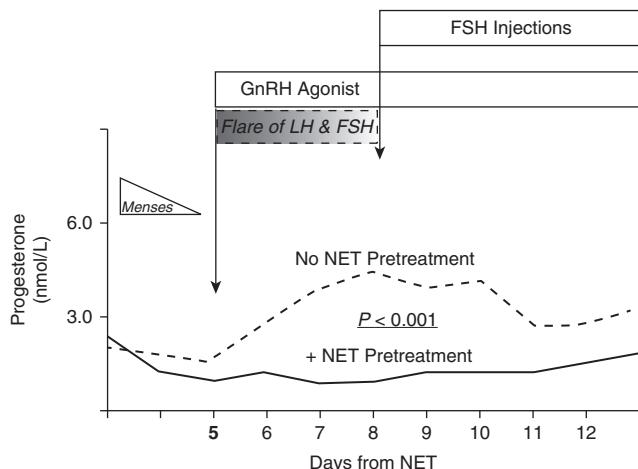


Figure 7.3 Progesterone profiles in the follicular phase after initiation of GnRH agonist during cycles managed with a 'flare' protocol with or without norethisterone (NET) pretreatment. The use of NET effectively eliminated the rise in progesterone elicited by the flare effect of GnRH agonist initiation

Single-centre studies examining results from 'flare' protocol cases rarely have suitable control cases which can provide absolute validation, because they are performed in patients who have had numerous previous cycles and perhaps other confounding elements. The results from such prospective studies show mixed outcomes. Despite this doubt, many programmes deploy the method frequently, mainly in women with a reduced FOR and previous poor response.

Table 7.2 shows an analysis [16] of embryo implantation rates (fetal hearts per 100 embryos transferred) categorized by the number of available embryos in a young (<37 years) cohort from the programme at the GCRM (UK). The restricted available embryo numbers were compared with cases treated with GnRH antagonist and GnRH agonist control. It is clear that embryos derived from 'flare' cycles with NET pretreatment, despite deriving from women with a low AMH (FOR), showed implantation rates equivalent to those of the other two methods performed in women with higher AMH values. This is further circumstantial evidence indicating that the steroid pretreatment, in this case a technical requirement, did not undermine embryo potential and had no impact upon clinical outcome.

7.11 Starting FSH Stimulation and Programming of COS with Exogenous Steroids

Cycles managed with the long-course GnRH agonist control, with luteal phase initiation, have intrinsic flexibility with regard to starting stimulation, as the patients can be 'held' for many days following menses because the down-regulation blocks endogenous ovarian activity.

In contrast, Figure 7.1b shows that cycles using GnRH antagonists are less flexible. They are limited by the fact that endogenous cyclic follicular recruitment starts when the endogenous FSH rises at the demise of the corpus luteum and into the beginning of the treatment cycle. Delaying FSH injections after the first few days of the cycle risks having two discontinuous cohorts of follicles – the few follicles recruited endogenously (perhaps a single lead follicle) and those recruited by the subsequent FSH injections [17]. Figure 7.1c shows that steroid treatment during the preceding luteal phase obviates any endogenous rise

Table 7.2 Implantation rates for embryos transferred in the fresh cycle which derived from the three main protocol types according to the number of available embryos in women <37 years. The antagonist protocol was used in women with a high AMH, the long agonist method was used in women with a moderate AMH and the flare protocol was deployed in women with reduced AMH

	Cases	Age	AMH	Implantation rate by embryos available (FHs/100 embryos transferred)		
	n	(y)	pmol/L	1 or 2	3 or 4	5 or 6
Antagonist	507	31.9	49.3	25.1	28.2	37.3
Agonist	461	32.8	19.5	22.7	29.5	37.5
Flare	358	33.5	9.4	22.4	29.0	39.4
Anova (p)			<0.001	NS	NS	NS

in FSH until discontinuation of such treatment. This allows clinical control over the changes in FSH exposure of the ovary; correspondingly, programming with steroid treatments in the ‘cycle’ prior to the stimulation cycle is widely practised.

Complications of any programme of ovarian stimulation can include untimely menses, cyst formation (either spontaneous or due to GnRH agonist initiation) and a failure to achieve a thin endometrium prior to the start of stimulation. It can therefore be deemed appropriate to manifest some control over the starting processes by suppressing current ovarian function using oral contraception steroid treatment prior to COS. This can be in the form of a full sequence of oestrogen and progestogen (combined oral contraception, COC), or simple application of a progestogen, such as norethisterone, if the patient is already oestrogenized.

7.12 GnRH Antagonist Control and Programming with Steroid Pretreatment

Figure 7.1b shows that the dynamic of follicular recruitment in cycles controlled by GnRH antagonists is different from long-course GnRH agonist control, as initial follicular recruitment is undertaken by endogenous FSH activity at the end of the previous luteal phase and the start of the new cycle. As discussed earlier, it can be expedient to control the start of follicular cyclic recruitment, and this requires an understanding of the dynamics or responses upon discontinuation of steroid pretreatment.

Examination of the endocrine and physiological effects of these steroid agents [18] has helpfully catalogued the effects of discontinuation of the steroid treatment (COC, norethisterone alone or oestrogen alone) associated with a subsequent cycle controlled with GnRH antagonist. Effective suppression of FSH and LH concentrations was achieved during and at the end of the steroid treatment, and following discontinuation of treatment, FSH rose steadily to a peak at five days, when it is presumed that endogenous recruitment was beginning (Figure 7.1c). This five-day window should be exploited, therefore, for timing the start of exogenous FSH administration. A similar dynamic is shown in Figure 7.3 for the ‘flare’ program. Menses occurred prior to the five-day peak of FSH, so there was no logistical interference related to endometrial function. Interestingly, the suppressive effect and

recovery were weakest for oestradiol alone, and the follicular responses to stimulation were less homogenous in this case [18].

In GnRH antagonist-controlled cycles with steroid pretreatment (Figure 7.1c) recruitment will start simultaneously to the FSH injections; correspondingly, a requirement for more days of stimulation, compared with the natural cycle approach (Figure 7.1b), may be expected and these have been recorded in some studies, as have higher egg yields, presumably because recruitment is more of the nature of that seen in GnRH agonist-treated cycles (Figure 7.1a). However, the larger meta-analyses do not fully substantiate these individual findings [19, 20]. This may be due to the selective approach to patient entry criteria in many prospective studies.

7.13 Steroid Treatment Prior to GnRH Antagonist-Controlled Cycles and Systematic Review

Clinical benefits of steroid pretreatment have been systematically reviewed using reasonably large numbers of randomized cases [20], and the concept that steroid pretreatment may achieve greater homogeneity of responses has not been fully supported. However, there is broad agreement that they do offer protection against spontaneous ovarian cysts – which occur during down-regulation with GnRH agonists – and their use may be associated with a higher demand for FSH (longer follicular phases) and with achieving higher egg yields depending on the cohort under examination. These differences are modest if they exist at all.

Disconcertingly, some randomized studies have concluded that the practice of steroid pretreatment resulted in lower clinical pregnancy rates during the fresh cycle. There has been an evolutionary series of conclusions regarding these issues. The initial examination (847 patient cycles in randomized controlled trials) showed that pre-treatment resulted in a greater consumption of FSH, as predicted earlier, with no change in egg yields, and no difference in clinical pregnancy rates. However, a subsequent review [19] by the same group, involving 1343 patient cases, showed a lower pregnancy rate associated with the pretreatment practice.

In 2015, the conclusion was thoughtfully challenged [20] by authors, suggesting that the choice of studies included in the later meta-analysis was technically flawed because some of the included studies did not comply with current practice, particularly the absence of a five-day treatment-free interval prior to stimulation. The response [21] from the original authors was a helpful reanalysis, excluding some errant studies, but which indicated that the finding remained true after exclusion of studies with these failings.

No hypothesis potentially explaining the effect has been proposed, which perhaps by now should have been forthcoming.

We have already established that exposure to steroid treatment prior to stimulation in cycles controlled with GnRH agonists has potentially beneficial effects in some specific circumstances, and we have concluded that they are unlikely to have direct negative effects in the flare protocol, where direct benefit can be determined. Correspondingly, this implies that any apparent negative effect upon clinical pregnancy outcome is not due to any direct influence of the steroids themselves, and a concept of how the apparent negative effect is manifested would be useful.

The more recent and larger review [20] ($n = 3069$ randomized cases) concluded that pretreatment with COC pills or oestradiol valerate gave no advantage concerning numbers of oocytes or an impact upon pregnancy rates. Consequently, it is likely that the practice of

using steroid pretreatment in routine IVF programmes of cycles controlled with GnRH antagonists will remain commonplace because of the significant element of convenience it provides for a busy clinic and its patients.

7.14 Conclusion

While there are large numbers of variations of protocols for using FSH as the stimulant for COS, and a large variation in the cohort of patients in which they are applied, there is little compelling evidence to support one method compared with another in particular categories of patients. On the other hand, the dosing schedules used in different programmes often fail to address the reality of FSH pharmacokinetics and follicular dynamics. These two phenomena dictate that most of the dose manipulations undertaken are redundant, and the key for the future is getting the starting dose right. The approaches using AMH and body mass so far have achieved the best results.

Steroid pretreatment for programming of cycles, particularly in GnRH antagonist-controlled cycles, can achieve practical benefit, and despite a debate over a possible negative impact upon pregnancy rates, it will continue to be widely used in practice. The most effective means of doing this are COC preparations or simply norethisterone alone.

References

1. Baird DT, Factors regulating the growth of the preovulatory follicle in the sheep and human, *J Reprod Fertil.* 1983, **69**, 343–52.
2. Wallace WH, Kelsey TW, Human ovarian reserve from conception to the menopause, *PLoS One*, 2010, 8772.
3. Fauser BC, Van Heusden AM, Manipulation of human ovarian function: physiological concepts and clinical consequences, *Endocr Rev.* 1997, **18**, 71–106.
4. Nelson SM, Yates RW, Fleming R, Serum anti-Müllerian hormone and FSH: prediction of live birth and extremes of response in stimulated cycles—implications for individualization of therapy, *Hum Reprod.* 2007, **22**, 2414–21.
5. de Ziegler D, Gayet V, Aubriot FX et al., Use of oral contraceptives in women with endometriosis before assisted reproduction treatment improves outcomes, *Fertil Steril.* 2010, **94**, 2796–9.
6. Porchet HC, Le Cotonnec JY, Loumaye E, Clinical pharmacology of recombinant human follicle stimulating hormone (FSH). III. Pharmacokinetic-pharmacodynamic modeling after repeated subcutaneous administration, *Fertil Steril.* 1994, **61**, 687–95.
7. Olivennes F, Trew G, Borini A et al., Randomized, controlled, open-label, non-inferiority study of the CONSORT algorithm for individualized dosing of follitropin alfa, *Reprod Biomed Online.* 2015, **30**, 248–57.
8. Jayaprakasan K, Hopkisson J, Campbell B et al., A randomised controlled trial of 300 versus 225 IU recombinant FSH for ovarian stimulation in predicted normal responders by antral follicle count, *BJOG.* 2010, **117**, 853–62.
9. Schimberni M, Ciardo F, Schimberni M et al., Short gonadotropin-releasing hormone agonist versus flexible antagonist versus clomiphene citrate regimens in poor responders undergoing in vitro fertilization: a randomized controlled trial, *Eur Rev Med Pharmacol Sci.* 2016, **20**, 4354–61.
10. Wikland M, Bergh C, Borg K et al., A prospective, randomized comparison of two starting doses of recombinant FSH in combination with cetrorelix in women undergoing ovarian stimulation for IVF/ICSI, *Hum Reprod.* 2001, **16**, 1676–81.
11. La Marca A, Grisendi V, Giulini S et al., Live birth rates in the different combinations of the Bologna criteria poor ovarian responders: a validation study, *J Assist Reprod Genet.* 2015, **32**, 931–7.

12. Nyboe Andersen A, Nelson SM, Fauser BC et al., ESTHER-1 study group., Individualized versus conventional ovarian stimulation for in vitro fertilization: a multicenter, randomized, controlled, assessor-blinded, phase 3 noninferiority trial, *Fertil Steril.* 2016, [Epub ahead of print], PMID:27912901.
13. Olsson H, Sandström R, Grundemar L, Different pharmacokinetic and pharmacodynamic properties of recombinant follicle-stimulating hormone (rFSH) derived from a human cell line compared with rFSH from a non-human cell line, *J Clin Pharmacol.* 2014, **54**, 1299–307.
14. Sunkara SK, Coomarasamy A, Faris R, Braude P, Khalaf Y, Long gonadotropin-releasing hormone agonist versus short agonist versus antagonist regimens in poor responders undergoing in vitro fertilization: a randomized controlled trial, *Fertil Steril.* 2014, **101**, 147–53.
15. Fleming R, Yates R, Coutts JRT, Presented to the Endocrine Society annual meeting, 1994.
16. Fleming R, Ambrose P, Mitchell P, Adam C, Gaudoin M, Manuscript in preparation.
17. Blockeel C, Sterrenburg MD, Broekmans FJ et al., Follicular phase endocrine characteristics during ovarian stimulation and GnRH antagonist cotreatment for IVF: RCT comparing recFSH initiated on cycle day 2 or 5, *J Clin Endocrinol Metab.* 2011, **96**, 1122–8.
18. Cédrin-Durnerin I, Bständig B, Parneix I et al., Effects of oral contraceptive, synthetic progestogen or natural estrogen pretreatments on the hormonal profile and the antral follicle cohort before GnRH antagonist protocol, *Hum Reprod.* 2007, **22**, 109–16.
19. Griesinger G, Kolibianakis EM, Venetis C, Diedrich K, Tarlatzis B, Oral contraceptive pretreatment significantly reduces ongoing pregnancy likelihood in gonadotropin-releasing hormone antagonist cycles: an updated meta-analysis, *Fertil Steril.* 2010, **94**, 2382–4.
20. Sobotka V, Streda R, Mardesic T, Tosner J, Heracek J, Steroids pretreatment in assisted reproduction cycles, *J Steroid Biochem Mol Biol.* 2014, **139**, 114–21.
21. Griesinger G, Venetis CA, Tarlatzis B, Kolibianakis EM, To pill or not to pill in GnRH-antagonist cycles: the answer is in the data already!, *Reprod Biomed Online.* 2015, **31**, 42588.

Monitoring the Stimulated IVF Cycle

John L. Yovich

8.1 Introduction

Historically, the first successful three births from in vitro Fertilization (IVF) cycles were conducted by tracking the natural cycle (Louise Brown in 1978, Alastair Macdonald in 1979 and Candice Reed in 1980). This followed that the two centres involved (in Oldham and Melbourne) had reached a point of frustration from nearly a decade of unsuccessful use of stimulated cycles. The first pioneers Steptoe and Edwards, working in Oldham, found that ovarian stimulation generated a disturbed luteal phase which they could not correct by progestogen supplements, mainly norethisterone. The Australian team working in Melbourne under Professor Carl Wood became divided into two programmes: one under gynaecologist Ian Johnston and scientist Alex Lopata pursuing natural cycles, and the other under gynaecologist John Leeton and veterinary scientist Alan Trounson pursuing Clomid (clomiphene citrate) stimulation with urine-derived human chorionic gonadotrophin (hCG) trigger. The former succeeded first, but the result was not readily reproduced; the latter began generating live births in 1981 and continued with a substantial ongoing pregnancy rate.

In those early years, tracking the natural cycle utilized the Higonavis multiwell test – sensitized sheep red cells would agglutinate in the presence of luteinizing hormone (LH) or hCG in a test urine sample which took around two hours to perform. It had to be performed 6–8 hourly and the subsequent arrangement for the laparoscopic follicle aspiration was an estimate, usually performed around 30–32 hours after the first positive Higonavis result. Louise Brown resulted from 4 pregnancies arising from 68 laparoscopies performed in Oldham, where 32 cases achieved an embryo transfer (ET) procedure (i.e. 5.9 percent of ovum pick-up (OPU) attempts or 12.5 percent of ETs). Of those four pregnancies, Louise was born on 25 July 1978; the next miscarried at 11 weeks and was shown to be a XXX triploidy; the third was a male delivered pre-term on 26 November (male, Courtney Cross died at birth and categorized as a post-amniocentesis loss at 21 weeks); and finally healthy boy Alistair Macdonald was delivered at term on 14 January 1979 [1].

The Monash programme applying stimulated cycles (Clomid/hCG) in Melbourne started delivering babies in 1981 from a series of 14 pregnancies arising from 115 initiated cycles (12.2 percent pregnancy rate), resulting in 9 births during 1981–1982 (7.8 percent live birth rate). Soon after, the group from Norfolk Virginia headed by Howard Jones delivered their first infant Elizabeth Carr in December 1981 following ovarian stimulation using urine-derived human menopausal gonadotrophins (uHMG; Pergonal), with hCG trigger

and Proluton injections providing progesterone for luteal support. In 1982, several other teams around the world delivered IVF infants, including my small team from Perth, Western Australia; Wilfred Feichtinger from Vienna, Austria; Seigfried Trotnow from Erlangen, Germany; Rene Frydman from Paris, France; and Paul Devroey from Brussels, Belgium – we all used some form of ovarian stimulation and hCG triggers. In fact my unit PIVET is so named as an acronym for ‘Programmed’ IVF & ET, representing the most efficient system from all perspectives – the patient, the clinic and the hospital services. HMG was also used by Subhas Mukherjee, who has been belatedly and posthumously acknowledged as generating the world’s second IVF infant [2]: a girl born in Calcutta, India, on 3rd October 1978 (details published in 1997 by TC Anand Kumar [3], architect with Indira Hinduja of India’s official first IVF infant, born in 1986).

All these early pregnancies were achieved with minimal monitoring, other than menstrual cycle timing and detection of the LH surge. There were two key advances in monitoring pre-ovulatory follicular development. The first was the development of ELISA – enzyme-linked immunosorbent assays on serum (to replace the tedious radio-immunoassay processes on 24-hour urine samples). These were developed in the 1970s and introduced into IVF monitoring by the early 1980s. The second was the progression to transvaginal ultrasound (U/S) for the accurate detection and measurement of follicles developing in the ovaries. It also was utilized in replacing laparoscopy by transvaginal ultrasound-guided needle aspiration for oocyte retrieval [4]. In fact, there were several European groups presenting the transvaginal ultrasound story at the 1984 meeting held at Finlandia Hall in Helsinki chaired by Markku Seppala [5] and which laid the foundations for ESHRE, the European Society of Human Reproduction and Embryology [6]. These included Susan Lenz, Matts Wickland, Lars Hamberger, Wilfried Feichtinger and Peter Kemeter.

The structure of gonadotrophin-releasing hormone (GnRH) was identified by Andrew Schally [7] and Roger Guillemin [8] independently in Baylor College Houston, for which they shared the Nobel Prize, 1977. This soon led to modifications of the decapeptide in the development of analogues, both agonist (GnRH-a) and antagonist (GnRH-ant). By the end of the 1980s, agonists were introduced to prevent premature LH elevations. By 2000, safer variants of the antagonists were introduced for immediate down-regulation, improving stimulation cycle control (using rFSH) and enabling agonist triggers, in such cycles, decreasing the chance of the life-threatening complication of ovarian hyperstimulation syndrome; OHSS.

8.2 Initial Screening Tests

Details of the screening tests for new patients, both male and female, are shown in Figure 8.1. All abnormal results require specific considerations, including referrals for specialist management – e.g. abnormal Pap smears, breast lump and testicular lump. Medical conditions must be stabilized prior to fertility treatment – e.g. abnormal liver, renal or thyroid function tests and evidence of genital tract infection. Specific management considerations will be highlighted for other detections – e.g. abnormal thrombophilia profile, varicocele detection and hyperprolactinaemia.

Some of these tests can ideally be incorporated in the recommended Assessment Cycle for logistic benefit. If more than one year advances since original screening, all tests require updating, including the recording of weight and height for body mass index (BMI) calculation, excepting those which do not change, e.g. blood group, karyotype and Rubella titre, when adequate.

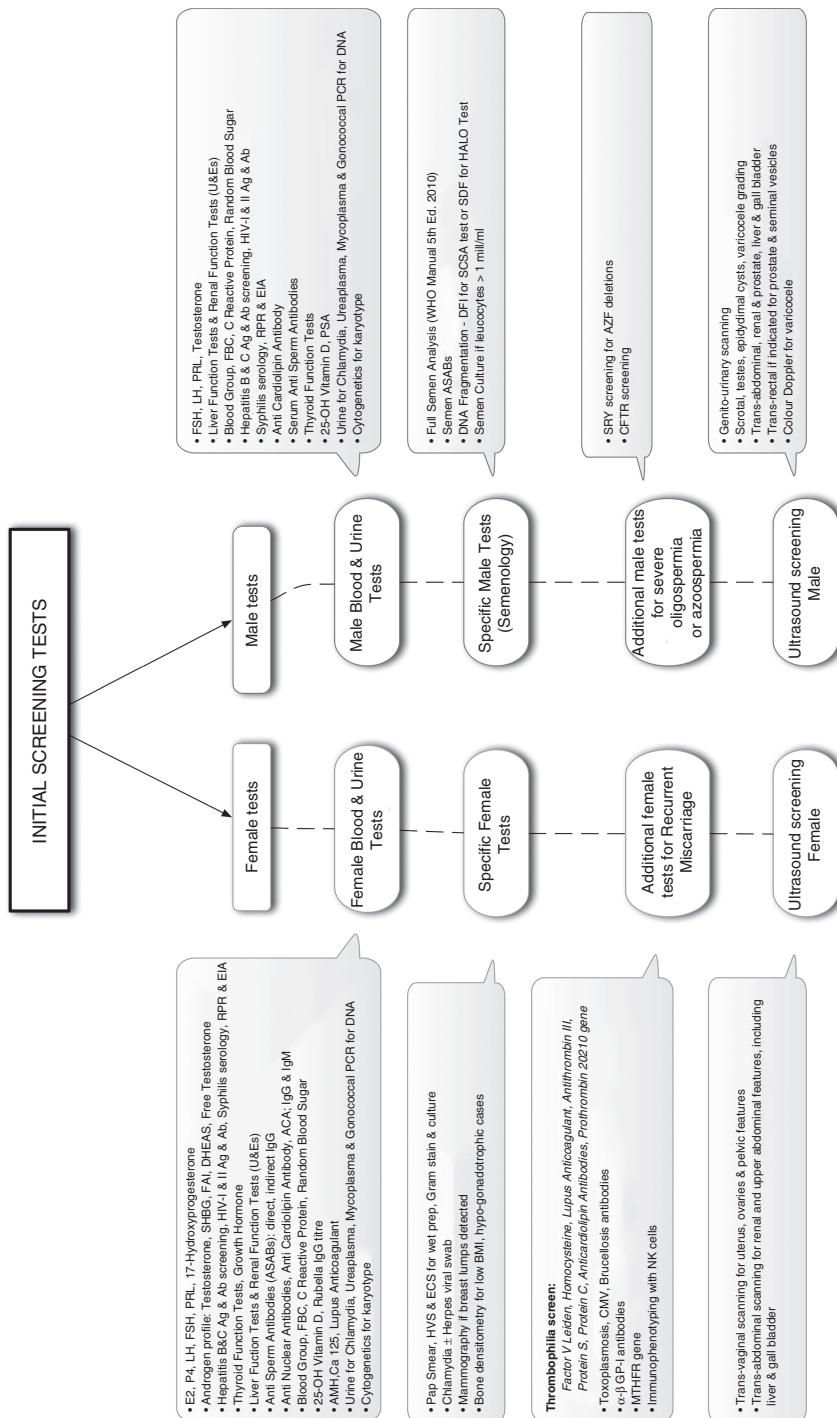


Figure 8.1 Initial screening tests for new couples attending for infertility and/or recurrent miscarriage

8.3 Assessment Cycle

Ideally, all new patients should conduct an Assessment Cycle which includes the described tests and a mid-luteal clinical review around day 21 to plan the specific Fertility Programme, including IVF cycle management. (Patients attending repeat cycles can attend day 21 without repeating the Assessment Cycle to arrange IVF or frozen embryo transfer (FET cycle.) The following describes a full fertility assessment, which can be modified for clear andrology cases or where restricting tests for ‘bare essentials’. The Assessment Cycle is displayed in algorithmic form in Figure 8.2.

Day 2 Blood test to determine if hormones are baseline (see Box 8.1 for conversions):

Box 8.1 Conversion formulae from SI units to conventional units and weight vs IU

E2 oestradiol 17 β , conversion pmol/L to pg/mL

250 pmol/L = 68 pg/mL

1,000 pmol/L = 272 pg/mL

12,000 pmol/L = 3269 pg/mL

P4 progesterone, conversion nmol/L to ng/mL

5.0 pmol/L = 1.6 ng/mL

60 pmol/L = 18.9 ng/mL

600 pmol/L = 189 ng/mL

PRL Prolactin, conversion mIU/L to ng/mL

750 mIU/L = 35.25 ng/mL

Testosterone, conversion nmol/L to ng/mL

2.0 nmol/L = 0.58 ng/mL

10 nmol/L = 2.9 ng/mL

20 nmol/L = 5.8 ng/mL

FSH & LH Gonadotrophins IU/L to mIU/ml

2 IU/L = 2 mIU/mL

12 IU/L = 12 mIU/mL

AMH Anti-Müllerian hormone

10 pmol/L = 1400 pg/mL

HCG Human Chorionic Gonadotrophin

25 IU = 25 mIU/mL

Ovidrel = rHCG Pregnyl = uHCG

Ovidrel 250 mcg ~ 6,500 IU uHCG

Ovidrel 250 mcg X2 ~ 13,000 IU uHCG

Ovidrel 250 mcg X3 ~ 19,500 IU uHCG

rHCG – recombinant Human Chorionic Gonadotrophin

uHCG – urinary Human Chorionic Gonadotrophin

Serum 17 β Oestradiol	E2 <250 pmol/L
Serum progesterone	P4 <5.0 nmol/L
Serum Luteinizing hormone	LH < 12 IU/L
Serum Follicle Stimulating hormone	FSH < 12 IU/L
Serum prolactin	PRL < 750 mIU/L

Day 5 AFC (antral follicle count)

categorized A to E in PIVET algorithms

female androgen profile – Testosterone, Sex hormone binding globulin (SHBG), Free androgen index (FAI), androstenedione, Dehydroepiandrosterone sulphate (DHEAS)

Insulin growth factor IGF1, IGF binding protein (IGFBP3) for growth hormone

- | | |
|------------|--|
| Days 7–10 | HyCoSy (Hystero-Contrast-Sonography) evaluation of uterine cavity and patency of fallopian tubes along with check of pelvic features. Hysterosalpingogram (HSG) is a less preferred option. |
| Days 10–12 | Blood test & Transvaginal Ultrasound; U/S ovarian Tracking Scan |
| Days 12–14 | Post-coital test (PCT) 6–14 hrs post-coitus in the immediate pre-ovulatory phase. This includes Insler cervical scoring requiring score $\geq 6/12$ and detection of motile spermatozoa within the mucus at HPF X400. Any sperm detected can be considered ‘positive’, but ≥ 5 motile spermatozoa constitutes a strongly positive test. |
| Day 21 | Mid-luteal with blood (E2, P4 & resting cortisol) and Transvaginal U/S scan review to plan specific fertility programme. |

After the review of the investigations, the ongoing management can include specific treatment of conditions (e.g. male referrals for varicocele and testicular lesions or female referral for auto-immune conditions or pituitary evaluation for hyper-prolactinaemia). Preliminary surgeries for the female include laparoscopic procedures for endometriosis, adenomyosis, fibroids, hydrosalpinges and ovarian cysts, as well as hysteroscopic procedures for endometrial polyps and septate uterus. Other configuration anomalies require strategic discussions [9]. At this stage consideration of gamete storage for sperm or oocytes should be considered. These may include considerations of malignancy or pre-chemotherapy or when there are logistic considerations, such as husbands working as FiFo (fly-in-fly-out) workers in remote locations. Otherwise cases will be considered for

1. a series (maximum $\times 3$) of natural cycle tracking with timed intercourse ovulation induction \pm timed intercourse (maximum $\times 3^*$)
2. intra-uterine insemination (IUI) (maximum $\times 3$)
3. IVF-related protocols

Although each of these programmes would require up to ten treatments to fully explore their potential to generate a pregnancy, *we discourage more than three attempts, as those who fail experience physical, mental, emotional and financial

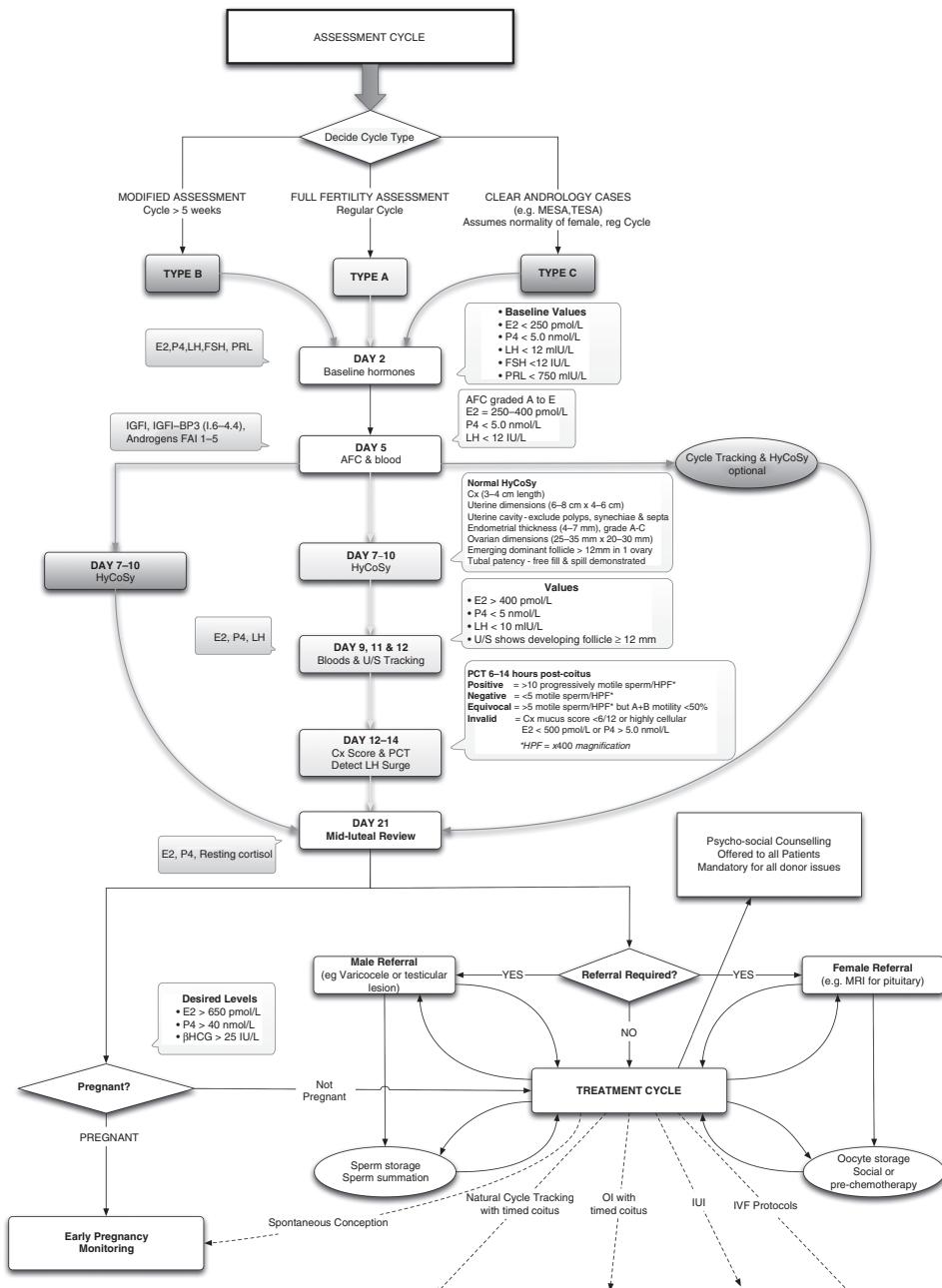


Figure 8.2 Algorithmic display of the Assessment Cycle which includes a mid-luteal clinical review at which the various clinical management programmes are considered along with specific medical and surgical management issues for each partner

exhaustion. This subsequently works against their opportunities within the more favourable IVF-related programmes. Furthermore, the time delay for females beyond age 35 years works unfavourably and causes them to avoid or restrict to one or two such 'low-prognosis' treatment cycles, in order to progress to the better-prognosis IVF-related opportunities.

The relevance of undertaking an 'Assessment Cycle' can be seen from many perspectives, including as a comparable therapeutic benefit. PIVET has recorded about a 5 percent persistent annual spontaneous pregnancy rate, resulting in over 36 years of conducting these cycles. Most of the pregnancies result from cycles with defined tubal patency and a strongly positive PCT, but a small number of pregnancies have also arisen following negative or equivocal findings at the PCT as well as the HyCoSy evaluation and sometimes both. We believe there are therapeutic benefits from the 'tubal flushing' process as well as 'arranging coitus' at the optimal time. HSG using lipiodol is also reported favourably in this regard.

8.4 Counselling

At this stage psycho-social counselling is offered to all patients but is mandatory where donor gametes or embryos are being considered, or where stress, depression or anxiety is a clinical feature. The number of such counselling sessions is predicated upon the circumstances of the couple, psychological needs identified by the counsellor and desires of individual patients, both males and females. Some patients benefit from psychiatric referral; others require special considerations, e.g. where histories include backgrounds of violence, criminal history or marital disharmony. It is unwise to proceed with IVF-related treatments unless both partners are comfortable with the processes, have provided fully informed consent and have resolved any outstanding domestic issues. Unresolved issues such as previous domestic conflict may require consideration by a clinical panel which includes the Counsellor, the Medical Director, a member of the Institutional Ethics Committee and a member of the Patient Support Group. The rights of patients, the need to avoid discrimination at any level, including age or disabilities, and the responsibilities of the clinic are all important considerations which are considered in relevant Codes of Practice along with State legislations [10].

It is against the above background that monitoring of the stimulated IVF cycle is described as practiced at PIVET.

8.5 Flare Agonist (GnRH-a) Protocol

Agonist Flare is preferred for women who do not have any significant risk for OHSS. This applies to older women ≥ 40 years old and those with lower AFC categories; C, D and E in the PIVET algorithms [11] and lower AMH ratings [12] (see Figures 8.3 and 8.4).

Day 21 Clinical Review

Transvaginal U/S and high vaginal swab (HVS); endo-cervical swab (ECS) (if not performed earlier within the Assessment Cycle).

Ensure appropriate control of medical conditions along with counselling and discussions about costs, prior to starting.

If an ovarian cyst is detected, this may be an obvious corpus luteum (tracked during an Assessment Cycle; otherwise of an uncertain nature). Ultrasound can be reviewed on day 2 or 3 of the treatment cycle, deferring stimulation if the cyst persists. In such a situation, combined oral contraceptive (COC) pill to suppress one or two cycles is administered and an ultrasound is performed on day 21 to recheck. (Routine use of COC prior to IVF is not favoured.)

Day 2 blood test to determine if baseline

E2 <250 pmol/L

P4 <5.0 nmol/L

LH <12 IU/L

FSH <12 IU/L

PRL <750 mIU/L

If elevated, review blood test in 2–3 days for baseline. If persistently elevated, cancel and consider Pill suppression prior to next cycle Day-2.

Commence GnRH-a

Decapeptyl® (triptorelin acetate) 100®mcg sc daily (mane); or

Lucrin® (leuprolide acetate) 500 mcg start, can reduce to 200 mcg sc daily (mane); or

Synarel® (nafarelin acetate) 1 puff (200 mcgm bd) each nostril – mane x1 & nocte x1

Day 3

Commence rFSH according to PIVET algorithm (Figure 8.3 for Gonal-f; Figure 8.4 for Puregon®). The rFSH dosage is selected depending on age and AFC category, choosing the box which accords with BMI. The dosage can then be adjusted downwards if AMH level is discordantly high, indicating that a lower rFSH dosage is needed. Other variables requiring adjustment include baseline (days 1 to 3) serum FSH level, smoking history and whether the programme is planned as a freeze-only of oocytes or embryos. As defined in the algorithm, these aspects translate to a higher rFSH dosage.

Some cases can be considered for the long-acting rFSH – corifollitropin (Elonva®) – as shown in the green sector (see following and Figures 8.3 and 8.4). As with Gonal-f® and Puregon®, Elonva® is given on day 3 and is followed by the calculated rFSH dosage from day 9 if not yet ready for the hCG trigger [13]. This applies to most cycles.

Day 9

Blood (E2, P4, LH)

Transvaginal U/S scan for ovarian follicles and endometrium (features and thickness). Endometrium should be advancing from hyperechoic to iso-echoic pattern at this stage with thickness > 6 mm.

Track follicles >10 mm

Note follicle number ≤10 mm

Gonal-F, Puregon & Elonva Desk Chart

卷之三

Assessed FSH and smokers

Where FSH is less than 8 IU/L, with no history of smoking, use values as shown

- Smokers move two columns to the right

Where FSH is between 8 & 12 IU/L, with no history of smoking, move one column to the right

— Smokers move two columns to the right

Where F_{SH} is greater than 12 U/L , move two columns to the right

- Smokers and non-smokers read same colonics!

104

Erythrocyte Donors

25 |U increments also suit Pyrecon Pen

• 11

$$\text{Elonva} = \begin{cases} 1 & \text{if } 100\mu\text{g for } Wt \leq 60\text{kg} \\ 2 & \text{if } 150\mu\text{g for } Wt > 60\text{kg} \end{cases}$$

፩፻፲፭ የዕለታዊ ወንድ

Figure 8.3 PIVET rFSH dosing algorithm designed for 125 IU increments which suits the Gonal-f pen. The green area covers dosages which can be replaced by Elionva (long-action co-cyclitropine). Adapted from Davis, D., et al., 2011 and reprinted in Deardorff, Diamond, O'Neil, 2011.

Puregon, Gonal-F & Elionva Desk Chart

AMH AFC*	≤30 pM/L		25-29 pM/L		20-24.9 pM/L		A (20-29 follicles)		B (13-19 follicles)		15-19.9 pM/L		16-17 (19 follicles)		16-17 (18-19 follicles)		D (5-8 follicles)		5-9.9 pM/L		< 5.0 pM/L								
	16-17	18-19	20-21	22-23	20-21	22-23	30-35	16-17	18-19	20-21	22-23	30-35	16-17	18-19	20-21	22-23	30-35	16-17	18-19	20-21	22-23	30-35	16-17	18-19	20-21	22-23	30-35		
AMH	A++ (≤ 40 follicles)																												
BMI	16-17	18-19	20-21	22-23	20-21	22-23	30-35	16-17	18-19	20-21	22-23	30-35	16-17	18-19	20-21	22-23	30-35	16-17	18-19	20-21	22-23	30-35	16-17	18-19	20-21	22-23	30-35		
20	41.7	41.7	50.0	58.3	58.3	58.3	66.7	66.7	75.0	75.0	75.0	75.0	83.3	91.7	91.7	100.0	108.3	116.7	116.7	125.0	135.3	141.7	141.7	150.0	150.0	166.7	166.7	175.0	
21	41.7	41.7	41.7	50.0	58.3	58.3	58.3	58.3	66.7	75.0	75.0	75.0	83.3	91.6	91.6	100.0	108.3	116.6	116.6	125.0	132.0	133.3	141.6	150.0	150.0	168.3	168.3	183.3	
22	41.7	41.7	41.7	50.0	58.3	58.3	58.3	58.3	66.7	75.0	75.0	75.0	83.3	91.6	91.6	100.0	108.3	116.7	116.7	125.0	133.3	141.7	141.7	150.0	150.0	168.3	168.3	183.3	
23	50.0	50.0	50.0	58.3	66.6	66.6	66.7	66.7	75.0	83.3	83.3	83.3	91.7	91.7	91.7	100.0	108.3	116.7	116.7	125.0	133.3	141.7	141.7	150.0	150.0	166.7	166.7	183.3	
24	50.0	50.0	50.0	58.3	66.6	66.6	66.7	66.7	75.0	83.3	83.3	83.3	91.7	91.7	91.7	100.0	108.3	116.7	116.7	125.0	133.3	141.7	141.7	150.0	150.0	166.7	166.7	183.3	
25	50.0	50.0	50.0	58.3	66.6	66.6	66.7	66.7	75.0	83.3	83.3	83.3	91.7	91.7	91.7	100.0	108.3	116.7	116.7	125.0	133.3	141.7	141.7	150.0	150.0	166.7	166.7	183.3	
26	58.3	58.3	58.3	66.7	75.0	75.0	75.0	75.0	83.3	91.6	91.7	91.7	100.0	108.3	116.7	116.7	125.0	132.0	141.6	150.0	150.0	158.3	166.7	175.0	175.0	183.3	183.3		
27	58.3	58.3	58.3	66.7	75.0	75.0	75.0	75.0	83.3	91.6	91.6	91.6	100.0	100.0	100.0	108.3	116.7	125.0	132.0	141.6	150.0	150.0	158.3	166.7	175.0	175.0	183.3	183.3	
28	58.3	58.3	58.3	66.7	75.0	75.0	75.0	75.0	83.3	91.7	91.7	91.7	100.0	108.3	116.7	125.0	133.3	141.7	150.0	158.3	166.7	175.0	183.3	183.3	183.3	183.3	183.3		
29	66.7	66.7	66.7	66.7	75.0	75.0	75.0	75.0	83.3	91.7	91.7	91.7	100.0	108.3	116.7	125.0	133.3	141.7	141.7	150.0	158.3	166.7	175.0	183.3	183.3	183.3	183.3	183.3	
30	66.7	66.7	66.7	66.7	75.0	75.0	75.0	75.0	83.3	91.7	91.7	91.7	100.0	108.3	116.7	125.0	133.3	141.7	141.7	150.0	158.3	166.7	175.0	183.3	183.3	183.3	183.3	183.3	
31	75.0	75.0	75.0	83.3	91.6	91.7	91.7	91.7	100.0	108.3	116.7	125.0	125.0	125.0	125.0	125.0	133.3	141.6	141.6	141.7	141.7	150.0	158.3	166.7	175.0	183.3	183.3		
32	75.0	75.0	83.3	83.3	83.3	83.3	83.3	83.3	91.6	100.0	100.0	108.3	116.6	125.0	125.0	125.0	133.3	141.6	150.0	150.0	150.0	158.3	166.6	175.0	175.0	183.3	183.3		
33	83.3	83.3	83.3	83.3	91.7	91.7	91.7	91.7	100.0	108.3	116.7	125.0	133.3	133.3	141.7	150.0	158.3	166.7	175.0	183.3	183.3	183.3	183.3	183.3	183.3	183.3	183.3		
34	83.3	83.3	83.3	83.3	91.7	91.7	91.7	91.7	100.0	108.3	116.7	125.0	133.3	141.7	141.7	141.7	150.0	158.3	166.7	175.0	183.3	183.3	183.3	183.3	183.3	183.3	183.3		
35	83.3	83.3	83.3	83.3	91.7	91.7	91.7	91.7	100.0	108.3	116.7	125.0	133.3	141.7	141.7	141.7	150.0	158.3	166.7	175.0	183.3	183.3	183.3	183.3	183.3	183.3	183.3		
36	100.0	100.0	100.0	108.3	116.6	116.6	116.6	116.6	100.0	108.3	116.6	125.0	125.0	125.0	125.0	125.0	133.3	141.6	150.0	150.0	150.0	158.3	166.6	175.0	175.0	183.3	183.3		
37	100.0	100.0	100.0	108.3	116.6	116.6	116.6	116.6	100.0	108.3	116.6	125.0	125.0	125.0	125.0	125.0	133.3	141.6	150.0	150.0	150.0	158.3	166.6	175.0	175.0	183.3	183.3		
38	108.3	108.3	108.3	108.3	116.7	116.7	116.7	116.7	125.0	133.3	133.3	133.3	141.6	150.0	150.0	150.0	150.0	158.3	166.7	175.0	183.3	183.3	183.3	183.3	183.3	183.3	183.3		
39	108.3	108.3	108.3	108.3	116.7	116.7	116.7	116.7	125.0	133.3	133.3	133.3	141.6	150.0	150.0	150.0	150.0	158.3	166.7	175.0	183.3	183.3	183.3	183.3	183.3	183.3	183.3		
40	116.7	116.7	116.7	116.7	116.7	116.7	116.7	116.7	125.0	133.3	133.3	133.3	141.6	150.0	150.0	150.0	150.0	158.3	166.7	175.0	183.3	183.3	183.3	183.3	183.3	183.3	183.3		
41	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	
42	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	
43	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3
44	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3
45	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3

Increased FSH and Smokers

Where FSH is less than 8 IU/L, with no history of smoking, use values as shown

— Smokers move two columns to the right

Where FSH is between 8 & 12 IU/L, with no history of smoking, move one column to the right

— Smokers move two columns to the right

Where FSH is greater than 12 IU/L, move two columns to the right

— Smokers and non-smokers read same column

*Antral Follicle Count based on number of antral follicles < 10 cm

Oocyte Donors

Aiming for 10-12 oocytes, move four columns to the right

Consider GnRH Agonist trigger if > 10 follicles

e.g., Triptorelin 100 mcg x 2

Piveteau Algorithm

Where FSH is less than 8 IU/L, with no history of smoking, use values as shown

— Smokers move two columns to the right

Where FSH is between 8 & 12 IU/L, with no history of smoking, move one column to the right

— Smokers move two columns to the right

Where FSH is greater than 12 IU/L, move two columns to the right

— Smokers and non-smokers read same column

Puregon, Gonal-F & Elionva Desk Chart

Where FSH is less than 8 IU/L, with no history of smoking, use values as shown

— Smokers move two columns to the right

Where FSH is between 8 & 12 IU/L, with no history of smoking, move one column to the right

— Smokers move two columns to the right

Where FSH is greater than 12 IU/L, move two columns to the right

— Smokers and non-smokers read same column

Piveteau Algorithm

Where FSH is less than 8 IU/L, with no history of smoking, use values as shown

— Smokers move two columns to the right

Where FSH is between 8 & 12 IU/L, with no history of smoking, move one column to the right

— Smokers move two columns to the right

Where FSH is greater than 12 IU/L, move two columns to the right

— Smokers and non-smokers read same column

Piveteau Algorithm

Where FSH is less than 8 IU/L, with no history of smoking, use values as shown

— Smokers move two columns to the right

Where FSH is between 8 & 12 IU/L, with no history of smoking, move one column to the right

— Smokers move two columns to the right

Where FSH is greater than 12 IU/L, move two columns to the right

— Smokers and non-smokers read same column

Piveteau Algorithm

Where FSH is less than 8 IU/L, with no history of smoking, use values as shown

— Smokers move two columns to the right

Where FSH is between 8 & 12 IU/L, with no history of smoking, move one column to the right

— Smokers move two columns to the right

Where FSH is greater than 12 IU/L, move two columns to the right

— Smokers and non-smokers read same column

Piveteau Algorithm

Where FSH is less than 8 IU/L, with no history of smoking, use values as shown

— Smokers move two columns to the right

Where FSH is between 8 & 12 IU/L, with no history of smoking, move one column to the right

— Smokers move two columns to the right

Where FSH is greater than 12 IU/L, move two columns to the right

— Smokers and non-smokers read same column

Piveteau Algorithm

Where FSH is less than 8 IU/L, with no history of smoking, use values as shown

— Smokers move two columns to the right

Where FSH is between 8 & 12 IU/L, with no history of smoking, move one column to the right

— Smokers move two columns to the right

Where FSH is greater than 12 IU/L, move two columns to the right

— Smokers and non-smokers read same column

Piveteau Algorithm

Where FSH is less than 8 IU/L, with no history of smoking, use values as shown

E2 > 1000 pm/L (can increase rFSH if required)

P4 < 5.0 pm/L (consider early trigger if elevated; and cryopreserve best embryos).

If LH elevated (≥ 5 IU) or P4 elevated (≥ 5 nm/L) consider early trigger (if several pre-ovulatory follicles present) or switch to GnRH-ant 0.5 mg daily (this higher dose is required as the LH or P4 elevation indicates that down-regulation is inadequate).

If an excess of 15 or more follicles are developing, switch to GnRH-ant, enabling agonist trigger. This conversion requires at least two, preferably three, days to ensure that the agonist down-regulation effect has worn off enabling the agonist trigger to be effective, as a new flare effect.

GnRh-ant options

Cetrotide® (cetrorelix acetate)	Standard dose	0.25 mg
	High dose	0.5 mg
Ganirelix® (ganirelix acetate)	Standard dose	0.25 mg
	High dose	0.5 mg

Days 11–12

Blood (E2, P4, LH) and transvaginal U/S scan for endometrium (features and thickness) and ovarian follicles.

Endometrium should be advancing from iso-echoic to hypo-echoic pattern (compared with myometrium) and thickness >7.5 mm.

Trigger when 2 follicles ≥ 18 mm.

Otherwise continue stimulation and re-assess in two days.

If endometrial features or thickness is unfavourable, add Trental® (Pentoxifylline)/Vitamin E regimen along with E2 or E2/Viagra® (sildenafil) pessary regimen daily until embryo transfer.

Trigger Options and Luteal Phase Management

Urinary hCG (uHCG; Pregnyl® 5000 IU, 10,000 IU & 20,000 IU) is nowadays replaced by recombinant rHCG (Ovidrel®). See Table 8.1 for conversion of units.

Ovidrel®	250 mcg x2 sc is standard
Ovidrel ®	250 mcg x1 sc if ≥ 15 follicles
(or agonist trigger if converted to antagonist)	
Ovidrel®	250 mcg x3 if ≤ 4 follicles despite high dosage rFSH ≥ 300 IU

OPU interval

Standard 36–37 hours

If leading follicle ≥22 mm, or previous history of premature egg release, add Nurofen® (ibuprofen) cover 200 mg tds after trigger.

Shorter interval may be considered if P4 and LH are elevated (indicating threatened premature surge); however, PIVET policy is to double GnRh-ant dosage to 0.5 mg in the morning and give standard trigger in the evening (Table 8.1). In such cases consider undertaking transvaginal U/S just prior to OPU, to determine if ovulated. If ovulation has taken place, pregnancies can occasionally be achieved following intercourse or IUI in such cases where fallopian tubes demonstrated patent on HyCoSy evaluation undertaken in the Assessment Cycle, but consider multiple pregnancy risk.

Table 8.1 Details of ovulation trigger strategies (dependent upon follicle numbers) and luteal management protocols (dependent upon number of oocytes retrieved) Trigger and Luteal Support Schedule for Standard IVF Cycles. All trigger cycles, the trigger day is 2. The presumed ‘ovulation’ day/TVOA is day 0. Ovidrel support when ordered is given on days 4, 7,10 & 13 post-TVOA ‘ovulation’ which is the same as days 6, 9, 12, & 15 post-trigger

Follicle number (≥12 mm) & E2 at trigger	Trigger	Oocyte number at TVOA	Luteal support
1 ≤4 follicles at high rFSH AACEP protocol Shanghai protocol All patients ≥40 yrs ≤2 MII oocytes previous E2 <4000 pm/L	Ovidrel x 3 pens	≤4 oocytes	Ovidrel 5 clicks, days 4,7,10,13 + P4 400 mg bd E2/P4 combo nocte (commence day after TVOA)
2 5–12 follicles Or E2 <12,000 pm/L	Ovidrel x 2 pens	5–12 oocytes 13–15 oocytes >15 oocytes	Ovidrel 5 clicks, days 4,7,10,13 + P4 400 mg bd (commence day after TVOA) IMP Cabergoline regimen post-TVOA Reduce Ovidrel to 3 clicks, days 4,7,10,13 + P4 400 mg bd IMP Cabergoline regimen post-TVOA Replace Ovidrel with full HRT

Table 8.1 (cont.)

	Follicle number (≥12 mm) & E2 at trigger	Trigger	Oocyte number at TVOA	Luteal support
3	13–19 follicles or E2 >12,000 pm/ L but <20,000 pm/L	Decapeptyl 100 mcg/mL x 2 or Synarel 0.2 mg x 2 (1 puff each nostril)	<20 oocytes	IMP Cabergoline regimen post-TVOA Commence full HRT day after trigger
4	≥20 follicles or E2 >20,000 pm/L Donors & cases with excess follicles 12–14 mm	Decapeptyl 100 mcg/mL x 2 or Synarel 0.2 mg x 2 (1 puff each nostril)	≥20 oocytes	Cabergoline post-trigger Freeze All Provera 10 mg x 12 days starting from day after TVOA Arimidex 1 mg x 10 days from TVOA for cases ≥20 oocytes
5	Replace Ovidrel with E2/P4 pessaries tds or full HRT- if blood test results during luteal phase: E2 >6000 pm/L P4 >600 nm/L			

Note:

This new schedule removes the option of Ovidrel trigger for >12 follicles and assumes all high responses result from antagonist (or antagonist conversion) cycles.

If not an antagonist cycle, a high response cycle (≥13 oocytes or E2 >12,000 pm/L) should be cancelled for re-cycling to antagonist regimen.

Luteal phase day 9 post-trigger

(day 7 post-OPU)

Blood test for E2 and P4

Ideal E2 range 1000 pm/L to 6000 pm/L

Ideal P4 range 60 nmol/L to 600 nm/L

If levels below range, add appropriate pessary, e.g. P4 400 mg or E2/P4 (2mg/400mg) bd or tds.

If levels above range, indicates OHSS risk – hence cancel Ovidrel® injections; replace with P4 pessaries and monitor patient according to increased monitoring protocol (IMP) regimen (Box 8.2).

Box 8.2 Increased monitoring protocol for women at OHSS risk along with management strategy for cases developing severe OHSS

1 IMP

for women at risk for OHSS:

Do not use HCG in luteal phase; replace with HRT regimen
Introduce Cabergoline, ideally at time of trigger (see below)
Daily telephone or Skype contact with clinic
Report daily weight; notify if >1 kg increase per day
Maintain boosted fluid input 2–3 litres per day – sports drinks ideal
Report persistent dark urine (should be light lemon colour)
Report daily girth; notify if >4 cm increase
Return to clinic if any concern; e.g. abdominal distention, breathlessness
Check SG of urine:
SG ≥ 1015 , for IV fluids; 2 litres over 2 hours
SG >1020 , 3 litres iv fluids over 3 hours
SG >1030 ; 4 litres iv fluids over 4 hours saline/ Hartman's regimen
Anti-emetic and mild analgesics as required
Trans-abdominal ultrasound if distended or breathless

2. OHSS management:

Admit to hospital if major ascites or pleural effusion, or persistent nausea, pain or respiratory distress.

Closer attention to iv. fluid balance management \pm urinary catheter
Consider paracentesis or pleural drain
Clexane cover (enoxaparin 40 mg daily) if high PCV, hypovolaemic or hypercoagulable state.
Cabergoline 1 mg daily (nocte) for 10 days, preferably beginning with trigger.
Aromatase inhibitor, e.g. Anastrozole 1 mg or letrozole 2.5 mg daily also useful to limit severity and duration of OHSS
Hyponatraemia and hypo-albuminaemia will self-correct slowly on above schedule (albumin infusion usually avoidable).

The final blood test for pregnancy detection is performed on day 17 post-OPU (day 19 post trigger) to measure E2, P4 and β hCG (see early pregnancy monitoring).

8.6 Antagonist (GnRH-ant) Protocol

Antagonist is the preferred protocol for all patients with higher AFC and AMH ratings [12], especially A groups and the first cycle with B groups (15–19 antral follicles according to PIVET algorithms). This protocol is mandatory for all donors and those undertaking oocyte banking where higher egg numbers are deliberately intended (with rFSH dosage adjusted upwards, i.e. four boxes to the right). GnRH agonist trigger should be used in such cases to counter any risk for OHSS.

Day 21 Clinical Review

Update any relevant tests if not part of the Assessment Cycle. Perform HVS and ECS to exclude pathogens, bearing in mind that transvaginal oocyte aspiration (TVOA) is

undertaken with minimized vulvo-vaginal preparation and no sterilizing fluids. Perform transvaginal U/S to exclude ovarian cysts.

Perform hormone test for FSH, LH, E2 and P4. In women with an irregular cycle or those who are amenorrhoeic, consider for start if baseline values are low (denote as day 2 as no cycle for dating).

Ensure appropriate control of medical conditions along with counselling and costing discussions prior to start.

Day 2 Blood test to determine if baseline

E2 <250 pmol/L

P4 <5.0 nmol/L

LH < 12 IU/L

FSH < 12 IU/L

PRL < 750 mIU/L

If elevated, review blood test in two to three days for baseline; otherwise cancel if FSH or LH levels show persisting elevation. Suppress levels with oral contraceptive for one to two months (oestrogenic pill, e.g. Microgynon® 50 preferred).

Day 2 (actual or adjusted). Commence antagonist, e.g. Decapeptyl® 100 mcg daily (mane preferred).

Day 3

Commence rFSH or uFSH according to PIVET Algorithm rFSH Gonal-f® or Puregon® Pergoveris® (rFSH+rLH) can be used x1 amp if 150–250 IU; x2 amps if >250 IU to 450 IU Long-acting corifollitropin (Elonva®) can be used if AFC gradings B to E (see green sector in Figure 8.5.)

Day 7

Blood test for response:

E2 >500 pm/L and <2000 pm/L

Adjust dosage rFSH accordingly by 1 increment (box to right if too low; rarely box to left if too high)

P4 <5.0 nm/L

LH <12 IU/L

Commence antagonist when E2>500 pm/L

250 ug daily; Cetrotide® or Orgalutran®

If P4 ≥5.0 nm/L or LH ≥12 IU/L, increase antagonist to 500 ug daily

If P4 ≥5.0 nm/L and LH <2.0 IU, commence Luveris® (rLH) 75 IU daily

Day 9 blood test + Transvaginal U/S scan for follicles and endometrium (features and thickness). Ideally iso-echoic and >6.5 mm at this stage.

E2: expect >100 percent rise from day 7

P4: expect <5.0 nm/L; otherwise ensure Luveris® 75 IU and increase antagonist to 500 mcg.

Gonal-F & Elonva Lite Desk Chart

ANH AF/C*	25-29.9 pm/L		20-24.9 pm/L		16-17 (30-39 follicles)		A+ (30-39 follicles)		B (20-29 follicles)		16-17 (13-19 follicles)		C (13-19 follicles)		15-19.9 pm/L		10-14.9 pm/L	
	>30 pm/L A++ (≥ 40 follicles)		16-17 (8-19 20-21 22-29 30-35)															
BMI	20	25.0	25.0	37.5	37.5	37.5	37.5	37.5	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
21	25.0	25.0	37.5	37.5	37.5	37.5	37.5	37.5	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
22	25.0	25.0	37.5	37.5	37.5	37.5	37.5	37.5	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
23	37.5	37.5	50.0	50.0	50.0	50.0	50.0	50.0	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
24	37.5	37.5	50.0	50.0	50.0	50.0	50.0	50.0	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
25	37.5	37.5	50.0	50.0	50.0	50.0	50.0	50.0	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
26	50.0	50.0	62.5	62.5	62.5	62.5	62.5	62.5	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0
27	50.0	50.0	62.5	62.5	62.5	62.5	62.5	62.5	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0
28	50.0	50.0	62.5	62.5	62.5	62.5	62.5	62.5	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0
29	62.5	62.5	75.0	75.0	75.0	75.0	75.0	75.0	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5
30	62.5	62.5	75.0	75.0	75.0	75.0	75.0	75.0	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5
31	62.5	62.5	75.0	75.0	75.0	75.0	75.0	75.0	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5	87.5
32	75.0	75.0	87.5	87.5	87.5	87.5	87.5	87.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
33	75.0	75.0	87.5	87.5	87.5	87.5	87.5	87.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
34	75.0	75.0	87.5	87.5	87.5	87.5	87.5	87.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
35	87.5	87.5	100.0	100.0	100.0	100.0	100.0	100.0	112.5	112.5	112.5	112.5	112.5	112.5	112.5	112.5	112.5	112.5
36	87.5	87.5	100.0	100.0	100.0	100.0	100.0	100.0	112.5	112.5	112.5	112.5	112.5	112.5	112.5	112.5	112.5	112.5
37	87.5	87.5	100.0	100.0	100.0	100.0	100.0	100.0	112.5	112.5	112.5	112.5	112.5	112.5	112.5	112.5	112.5	112.5
38	100.0	100.0	112.5	112.5	112.5	112.5	112.5	112.5	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0
39	100.0	100.0	112.5	112.5	112.5	112.5	112.5	112.5	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0
40	100.0	100.0	112.5	112.5	112.5	112.5	112.5	112.5	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0	125.0
41	112.5	112.5	125.0	125.0	125.0	125.0	125.0	125.0	137.5	137.5	137.5	137.5	137.5	137.5	137.5	137.5	137.5	137.5
42	112.5	112.5	125.0	125.0	125.0	125.0	125.0	125.0	137.5	137.5	137.5	137.5	137.5	137.5	137.5	137.5	137.5	137.5
43	112.5	112.5	125.0	125.0	125.0	125.0	125.0	125.0	137.5	137.5	137.5	137.5	137.5	137.5	137.5	137.5	137.5	137.5
44	125.0	125.0	137.5	137.5	137.5	137.5	137.5	137.5	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0
45	137.5	137.5	150.0	150.0	150.0	150.0	150.0	150.0	162.5	162.5	162.5	162.5	162.5	162.5	162.5	162.5	162.5	162.5

Algorithm dosages	
Aiming for 4-8 oocytes Antagonist cycle only	
Use GnRH Antagonist trigger e.g., Triptorelin 100 mcg 2x	
Smokers move two columns to the right	
Where FSH is less than 8 IU/L, with no history of smoking, use values as shown	
Smokers move two columns to the right	
Where FSH is between 8 & 12 IU/L, with no history of smoking, move one column to the right	
Smokers move two columns to the right	
Where FSH is greater than 12 IU/L, move two columns to the right	
Smokers and non-smokers read same column	
*Antral Follicle Count based on number of antral follicles <1.0 cm	

Increased FSH and Smokers

Where FSH is less than 8 IU/L, with no history of smoking, use values as shown

— Smokers move two columns to the right

Where FSH is between 8 & 12 IU/L, with no history of smoking, move one column to the right

— Smokers move two columns to the right

Where FSH is greater than 12 IU/L, move two columns to the right

— Smokers and non-smokers read same column

Algorithm dosages

Aiming for 4-8 oocytes

Antagonist cycle only

Use GnRH Antagonist trigger

e.g., Triptorelin 100 mcg 2x

Smokers move two columns to the right

Where FSH is less than 8 IU/L, with no history of smoking, use values as shown

Smokers move two columns to the right

Smokers move two columns to the right

Smokers move two columns to the right

Smokers and non-smokers read same column

Smokers move two columns to the right

Smokers and non-smokers read same column

Smokers move two columns to the right

Smokers and non-smokers read same column

Smokers move two columns to the right

Smokers and non-smokers read same column

Smokers move two columns to the right

Smokers and non-smokers read same column

Smokers move two columns to the right

Smokers and non-smokers read same column

Smokers move two columns to the right

Smokers and non-smokers read same column

Smokers move two columns to the right

Smokers and non-smokers read same column

Smokers move two columns to the right

Smokers and non-smokers read same column

Smokers move two columns to the right

Smokers and non-smokers read same column

Version 1.1 6 December 2016

Figure 8.5 PIVET rFSH dosing algorithm designed for the LITE protocol. It aims to collect fewer eggs with minimized monitoring and out-of-pocket costs. It is modified from Figures 8.3 and 8.4 to select cases with a favourable prognosis for a single blastocyst embryo transfer

Transvaginal U/S scan – expect cohort of follicles 12–14 mm appearing (do not adjust rFSH dosage on basis of U/S; rely on E2 rise for guidance. Note that PIVET algorithm ensures stable dosage but elevation may be required for low dosages ≤ 100 IU).

If thin endometrium < 6 mm add Vitamin E 1000 IU bd/Trental® 400 mg bd oral regimen. Ideally the endometrium should be iso-echoic or hypo-echoic (compared to the myometrium) at this stage.

Add oestradiol pessaries 10–20 mg nocte until embryo transfer, or if historical thin endometrium use E2/Viagra® pessaries 25 mg:25 mg up to twice daily (mane and nocte).

Days 11–12

Blood and Transvaginal U/S; expect

E2 6,000–12,000 pm/L

P4 < 5.0 nm/L

LH < 2.5 IU

If P4 or LH is elevated, increase antagonist dosage from 250 mcg to 500 mcg; may also consider early trigger if follicles are of sufficient pre-ovulatory size follicles.

Generally, trigger when 2 follicles ≥ 18 mm; otherwise continue stimulation and reconsider in two days.

The majority of triggers are given on day 11 or 12.

See Table 8.2 for triggers and Luteal phase management.

Trigger–OPU interval:

Standard 36–37 hours

If lead follicle ≥ 22 mm, or previous history of premature ovulation, 36 hours plus Nurofen® cover 200 mg tds after trigger.

May consider shorter interval if P4 and LH elevated (indicating threatened premature surge); however, PIVET policy is double antagonist in the morning and standard Ovidrel® or agonist trigger in the evening according to Table 8.1. In such cycles consider U/S just prior to OPU, to determine if ovulated. Pregnancies can occasionally be achieved following intercourse or IUI in such cases if fallopian tubes are patent, but consider multiple pregnancy risk.

Luteal phase day 9 post-trigger (= day 7 post-OPU)

Blood test for E2 and P4

Ideal E2 range 1000 pm/L to 6000 pm/L

Ideal P4 range 60 nmol/L to 600 nm/L

If levels below range, add appropriate pessary, e.g. P4 400 mg or E2/P4 (2 mg/400 mg bd or tds).

If levels above range, it indicates OHSS risk – hence cancel hCG injections; replace with P4 pessaries and monitor patient according to the IMP regimen.

Final blood test for pregnancy detection performed day 17 post-OPU (day 19 post-trigger) measuring E2, P4 and β hCG (see early pregnancy monitoring).

8.7 Long Down-Regulation (LDR) Protocol

The former gold standard, LDR is nowadays reserved for poor prognosis patients and specialized management cases.

Day 21 Clinical Review

Update any relevant tests, e.g. Pap smear or HPV screen.

Transvaginal U/S to exclude ovarian cysts.

Hormone test if irregular or amenorrhoeic cycle; may consider for start if certain of anovulatory or mid-luteal status.

Anovulatory amenorrhoeic

E2 <250 pm/L

P4 <5 nm/L

LH <12 IU/L

Mid-luteal

E2 250–650 pm/L

P4 20–60 nm/L

LH <12 IU/L

FSH <12 IU/L

PRL <750 mIU/L

Ensure appropriate control of medical conditions along with counselling and costing discussions prior to start.

Commence down-regulation, e.g. Lucrin® 500 mcg daily or Decapeptyl® 100 mcg daily.

Day 2 blood test to determine if baseline (down-regulated). Where amenorrhoeic, perform this test ten days after commencing down-regulation, classify as 'day 2'.

E2 <250 pmol/L

P4 <5.0 nmol/L

LH <12 IU/L

If not down-regulated, perform transvaginal U/S to check for ovarian cyst.

Repeat blood test and U/S in two to three days for baseline; otherwise cancel or adjust rFSH schedule if FSH or LH levels are elevated. May suppress cycle with COC; then resume schedule day 21 for day 2; otherwise revert to agonist regimen. Elevated E2 usually indicates persistent ovarian follicular cyst, elevated P4 indicates persistent luteal cyst. Such cycles best managed by cancellation and COC suppression.

Can reduce agonist dosage if clearly down-regulated;

E2 < 100 pm/L

P4 < 1.0 nm/L

LH < 2.0 IU

E.g. Lucrin® 200 mcg/day. No benefit to reduce Decapeptyl®

Commence rFSH on day 3 (actual or adjusted for amenorrhoeic cycles – 11 days after commencing down-regulation) as described for agonist cycle (above). Elonva® can be used as described (above and below).

Day 9

Blood (E2, P4, LH) & Transvaginal U/S scan

Track follicles >10 mm

Note follicle number ≤10 mm

E2 > 1000 pm/L (can increase rFSH if required)

P4 < 5.0 pm/L (consider early trigger if elevated; cryopreserve best embryos)

If LH elevated consider early trigger or switch to antagonist 0.5 mg daily (down-regulation inadequate).

If excess follicles, ≥15 emerging, switch to antagonist, as previously described, enabling agonist trigger.

Days 11–12

Blood (E2, P4, LH) & T/V scan

Trigger when 2 follicles ≥ 18 mm

Otherwise continue stimulation and re-assess in two days

Trigger and Luteal support according to Table 8.2

Luteal phase day 9 post-trigger (= day 7 post OPU)

Blood test for E2 and P4

Ideal E2 range 1000 pm/L to 6000 pm/L

Ideal P4 range 60 nmol/L to 600 nm/L

If levels below range; add appropriate pessary, e.g. P4 400 mg or E2/P4 (2 mg/400 mg bd or tds).

If levels above range, indicates OHSS risk hence cancel hCG injections; replace with P4 pessaries and monitor patient according to IMP regimen.

Final blood test for pregnancy detection performed day 17 post-OPU (=day 19 post-trigger) measuring E2, P4 and βhCG (see early pregnancy monitoring).

8.7.1 Long Down-regulation Variant: AACEP

This regimen was described by the Nevada group [13] when LDR was the ‘gold standard’. It was intended for poor-prognosis cases (i.e. repetitive failures) and the acronym describes:

Agonist – antagonist conversion with oestrogen priming.

Day 21 commences as described above for LDR.

Day 2 blood test to confirm baseline levels. Day 2 is defined as day of menstrual cycle; alternatively for amenorrhoeic women, this is 10th day following commencement of agonist. If not adequately suppressed continue for three days and retest, until down-regulated; ideally:

E2 < 100 pm/L

P4 < 1.0 nm/L

LH < 2.0 IU

Can accept:

E2 <250 pm/L

P4 <5 nm/L

LH < 12 IU/L

At this stage, usually day 2, convert to antagonist 250 mcg on alternate days. (For amenorrhoeic cycles ‘day 2’ is ten days after commencing down-regulation – see above.) Also commence daily oestrogen. PIVET policy utilizes a vaginal pessary of micronized 17 β -oestradiol, 5 mg applied daily.

Commence rFSH day 3, usually maximal dosage 450 IU.

Thereafter monitor as LDR with day 9 blood and U/S scan. However, the E2 level only reflects the effectiveness of the pessary; the follicle scanning becoming all important. P4 and LH are also monitored and managed as previously described for LDR.

The E2 effect is usually excellent for endometrial thickness and most cases develop 4±2 follicles. Our standard is to apply Ovidrel® x3 as the trigger, regarding the ovaries as ‘under-vascularized’; however, these cases can certainly suit the idea of a Combined trigger (e.g. Ovidrel® + Decapeptyl®).

Depending upon the endometrial thickness, the E2 pessaries can be ceased at trigger, or continued to TVOA. Thereafter Luteal Support is according to the Table 8.2 schedule. This can include E2/P4 ‘combo’ pessaries tds to maintain a thicker endometrium.

Mid-luteal and final 19 blood test is performed as standard.

8.8 Occasional Schedules

Several stimulation regimens are either entrenched historically or involve the introduction of new stimulatory agents. This chapter does not embrace the use of adjuvant therapies such as DHEA or Growth Hormone but includes those which most IVF units will apply for a small subset of patients, although occasionally as a preferred schedule. The use of biosimilar FSH preparations are embraced within the PIVET rFSH dosing schedules, having similar dosages.

8.8.1 Clomiphene or Tamoxifen

In Australia, these agents generated most of the first pregnancies and were continued through the 1980s until the introduction of GnRH analogues became more readily associated with the use of gonadotrophins [14]. The Clomiphene dosage was 50 mg–100 mg for five days; higher dosages were associated with scotomata and sometimes a permanent loss of parts of the visual fields. At the lower dosages there was a large safety factor as most cases generated only 1–4 follicles. In fact as ovulation induction, without any monitoring, the generation of twins was uncommon and triplets rather rare. In fact, the prevailing view was that eggs were failing to release, even with the use of hCG triggers [15]. Another view was that Clomiphene had adverse effects on the endometrium, inhibiting implantation. For this reason it was applied for only five days in the early follicular phase and the earlier schedule of days 5–9 was changed to 2–6 when applied for IVF. We still have a place for these oral agents, but the use of tamoxifen appears less detrimental to the endometrium and enables a schedule of days 2–10. OHSS was absolutely rare with these agents and caused some IVF facilities to revert to this ‘Minimal Stimulation’ schedule to avoid the problem [16].

Monitoring such cases could be done with minimal testing although our policy applied the same schedule as the flare agonist cycle. This lent itself well as increasingly programmes added (urinary) uFSH and later rFSH in order to maintain reasonable pregnancy rates [14]. This, of course, became associated with the problem of premature LH surges (which was uncommon with clomiphene or tamoxifen alone), hence the urgent uptake of GnRH analogues by the end of the 1980s, as soon as effectiveness was demonstrated.

8.8.2 Elonva® Schedule

The long-acting rFSH corifollitropin (Elonva®) is popular with those patients wishing to minimize injections. It has been advised for women classified as normo-responsive but avoided in over-responders [17]. Our experience with this preparation indicates:

It acts in a step-down fashion (boosting the first two to three days)

– this explains why many cases show rapid follicular growth

It carries its effect across six to seven days (fading over days 6–7)

It can be used with GnRH-agonist or GnRH-antagonist cycles

It can be used successfully across a broad range of rFSH dosage 200 IU – 375 IU in Categories B to E inclusive

It must not be used with AFC categories A, A+ or A+++ i.e. ≥ 20 antral follicles as the OHSS risk is inordinately high, hence Elonva is confined to the Green Sector in PIVET Algorithms.

On day 7 (cycle day 9) a decision is required to convert to standard rFSH daily dosage if not yet ready for trigger. Our published data [13] showed overall the median trigger day for Elonva across AFC categories B, C and D was cycle day 11 (ranging from day 9 to day 12), whereas for standard rFSH, the median trigger day was cycle day 13 (ranging day 10 to day 16).

Elonva dosage 100 mcg or 150 mcg is selected according to the woman's weight (≤ 60 kg or > 60 kg respectively).

8.8.3 Shanghai Protocol

Designed for women with extremely poor prognosis whose main feature is POR (poor ovarian response). It involves immediate stimulation in the luteal phase after the first cycle OPU. Hence it is also known as the Dual Stimulation (Duo-Stim) protocol or Double Stimulation Protocol [19].

In Stage 1, it applies letrozole 2.5 mg days 2–5 along with clomiphene 50 mg for day 2 until trigger with Gonal-f® 150 IU from day 6 until trigger.

Monitoring is not necessary until day 12, for consideration of trigger: E2, P4 and Transvaginal U/S only for ovarian follicle.

If follicle ≥ 16 mm trigger and collect – fertilize and cryopreserve at cleavage stage.

If follicle < 16 mm, continue clomiphene and Gonal-f®; rescan and trigger when ≥ 16 mm, proceeding to TVOA.

If follicle fails to advance to ≥ 16 mm, give Decapeptyl® trigger but do not undertake OPU (ovum pick-up) procedure.

For advanced follicle ≥ 16 mm trigger with agonist, e.g.. Decapeptyl® 200 mcg and consider Nurofen® 200 mg tds if follicle ≥ 22 mm to avoid premature egg release. TVOA is undertaken at 36 hours.

Stage 2 commences day after TVOA or 3rd day after trigger without TVOA.

Letrozole 2.5 mg for five days from day after TVOA.

Gonal-f® 225 IU from 2nd day after TVOA until 2nd trigger

Provera® (medroxyprogesterone acetate) 10 mg b.d. from 3rd day after TVOA until 2nd trigger.

Monitoring unnecessary until 12th day after TVOA as no changes to the schedule will be undertaken.

Trigger: E2, P4 and Transvaginal U/S only for ovarian follicle.

if follicle ≥ 16 mms trigger and collect – fertilize and cryopreserve at cleavage stage.

If follicle <16 mm, continue clomiphene and Gonal-f®, trigger and TVOA if ≥ 16 mms

If failing to develop trigger but do not undertake OPU.

Trigger is agonist, e.g. Decapeptyl® 200 mcg and Nurofen® 200 mg tds are given to avoid premature egg release. TVOA is undertaken at 36 hours.

All resulting embryos are cryopreserved and no fresh embryo transfer procedures are undertaken. The woman is subsequently reviewed to plan for an FET cycle, ideally with an HRT schedule [20] as most women in this category have disordered menstrual cycles, not suited to natural cycle transfers.

Usually 1–2 oocytes are recovered at Stage 1 and 3–4 at Stage 2. However, occasionally nil are collected at either stage, indicating all options for autologous egg recovery are exhausted. Such cases have invariably progressed through various schedules including AACEP and minimal stimulation. Consideration for donor oocytes is recommended.

8.8.4 LITE Protocol

This schedule has been designed to reduce out-of-pocket costs to enable patients with a favourable prognosis to access IVF where financial considerations are an imperative. The term LITE, ‘light’, refers to an adjusted regimen designed to collect fewer (4–6) oocytes such that the likelihood will be the transfer of a single blastocyst and no embryos remaining for cryopreservation. Furthermore, the case selection and treatment schedule exclude the likelihood of OHSS, hence monitoring can be minimized.

Case selection requires BMI <32 Kg/m², AMH >10 pm/L, AFC >8 antral follicles, baseline FSH <10 IU/L. Patients undertaking this regimen have standard consultations, examinations and Assessment Cycles. Complex treatment regimens are not suitable for LITE as full effort needs to be applied in such regimens. Similarly, those with underlying disorders, including medical, mental or gynaecological, e.g. endometriosis, are not eligible. Those seeking donor gametes or embryos, surrogacy or preimplantation diagnosis or screening, PGD/PGS, are also not eligible for this low-cost programme as the administrative costs are higher. The aim is to apply a minimized stimulation schedule without adjuncts, but includes IVF and ICSI with fresh or frozen sperm, aiming for blastocyst culture and a strict single embryo transfer (SET) policy. If more than one good quality blastocyst arises, patients have the option of cryopreservation at conventional costing. The cases are managed with minimized monitoring as follows: An antagonist regimen is applied using a modified

PIVET Algorithm with rFSH dosages reduced 4 boxes to the left from standard (Figure 8.5). This enables the Gonal-f® pen as well as Elonva® (in the green sector).

Day 21

Same as a standard antagonist cycle.

Day 2

Blood test to determine if baseline same as standard antagonist cycle.

Day 3

Commence rFSH (Gonal-f(R) or Elonva®) according to PIVET Algorithm. uHMG (Menopur®, Ferring) may be preferred as it contains LH activity; perhaps precluding need for LH add-back, although the latter appears more effective [21], especially in women with severely down-regulated or absent LH, such as those with hypothalamic hypogonadism.

Day 7

Blood test for response:

E2 >350 pm/L and <2000 pm/L

Adjust dosage rFSH accordingly by 1 increment (box to right if too low; rarely box to left if too high)

P4 <5.0 nm/L

LH <12 IU/L

Commence antagonist when E2>350 pm/L

250ug daily; Cetrotide® or Orgalutran®

If P4 ≥5.0 nm/L or LH ≥12 IU/L, increase antagonist to 500ug daily

If P4 <5.0 nm/L and LH <2.0 IU, commence Luveris® (rLH) 75 IU daily

Day 9 (not monitored in LITE)

Days 11–12

Blood and Transvaginal U/S; expect

E2 2,000–6,000 pm/L

P4 <5.0 nm/L

sLH <2.5 IU

If P4 or LH elevated, increase antagonist dosage from 250 mcg to 500 mcg immediately and consider early trigger.

Generally trigger when 2 follicles ≥18 mm; otherwise continue stimulation and reassess in two days.

The vast majority of triggers are given on day 11 or 12.

LITE cases for cancellation if follicles >12 or E2 ≥12,000 pm/l.

Patients may elect to convert to conventional programme with standard costings at this stage.

Trigger dosage is Ovidrel® 250 mcg x1.

Luteal Support is Crinone bd®

Mid-luteal bloods – not monitored

Final blood measure E2, P4 and β hCG for pregnancy detection performed day 17 post-OPU (day 19 post-trigger). Pregnancy is monitored to week 8 (see below) but without adjunctive hormonal supports, although such can be offered with attendant extra costs.

8.8.5 Pergoveris

The conversion from uHMG to rFSH around 1990 was driven by a safety concern to avoid any infective element derived from human tissues. Certainly, the revelation was that prions were behind the cases of Creutzfeldt–Jakob Disorder (CJD); this condition occurring in a small number of those given pituitary extracts such as HPG and HGH drove the recombinant technology. However, this led to concern from many long established IVF units that the ultra-refined rFSH product lacked some quality which may have been provided by the multiple isoforms of FSH or the LH/HCG activity present in the urinary products. The introduction of GnRH-ant regimens propelled this idea, hence Merck Serono has released Pergoveris®-150 IU rFSH +75 IU rLH in each ampoule. There is data suggesting that the rFSH/rLH combination may be superior to uHMG, especially in severely LH-suppressed women or those women with hypothalamic hypogonadism [21].

Nonetheless we propose to explore this gonadotrophin combination in an adapted PIVET Algorithm across the range of rFSH 150–350 IU using one ampoule (150 IU FSH/75 IU LH) in the range 150–225 IU rFSH or two ampoules (300 IU FSH/150 IU LH) for the range 250–300 IU rFSH. However, we have no useful data generated at this stage and we have some concern that those women requiring higher dosages of rFSH will concurrently be exposed to higher amounts of LH which may be seen as adverse in those women who have higher basal levels [21].

8.9 Early Pregnancy Monitoring

The blood test performed at the end of the IVF cycle on luteal phase day 19 post-trigger (day 17 post-OPU) is tested for β hCG, E2 and P4. Pregnancy is diagnosed with β hCG \geq 25 IU/L applying the Siemens Centaur platform. Although the assay detects levels \geq 2 IU with coefficient of variability <7 percent, it is our policy not to declare pregnancy at levels <25 IU as there may sometimes be a prolonged effect from the Ovidrel® injections when applied for luteal support despite the last being four days prior. Furthermore, pursuing pregnancy support treatments for levels <25 IU has proven fruitless in our experience but causes much anxiety from the patient's perspective. We have also found it relevant to undertake to also measure E2 (with ideal range 1000 pm/L to 6000 pm/L) and P4 (with ideal range 60 nmol/L to 600 nm/L).

PIVET policy undertakes hormonal monitoring each week thereafter to seven or eight weeks [22] until a clear diagnosis is determined by the finding of a viable fetus within an intra-uterine gestational sac. Again, although ultrasound may detect viable pregnancy as early as day 35 (five weeks), the failure to detect such can be flawed for many other causes, especially slower-developing or delayed-implantation pregnancies, where an accurate diagnosis cannot be certain until day 49 (seven weeks). It is therefore our policy to continue pregnancy supports in all cases where β hCG levels are rising until week 7.

Any support hormones given during the luteal phase of the IVF cycle is continued through to the seven-week diagnostic scan (other than Ovidrel® injections; except when indicated for recurrent miscarriage). If a viable intra-uterine pregnancy is detected, the

luteal supports are continued until nine weeks when a reducing schedule is introduced, ceasing all supports by week 12, excepting progestagens in high risk pregnancies.

For those with β hCG ≥ 25 IU at nominated 'week four', a blood sample is checked each week to week seven and hormonal supports are adjusted to maintain E2 > 1000 pm/l and P4 above 60 nm/l. This means applying pessaries containing micronized progesterone 400 mg or P4/E2 500 mg/2 mg. This hormonal support regimen was developed following close tracking of many pregnancies from the earliest years of our programme [23]. However, despite this active support policy, not all pregnancies progress in a normal fashion and the following may also be identified: Biochemical pregnancy: these show a fading β HCG by week 7, sometimes falling below 25 IU. At these points, the support hormones are withdrawn and the patient usually has a typical 'delayed menses'. However, we have learnt to monitor these cases for at least one more week to ensure the β hCG level reduces below 10 IU when further monitoring is unnecessary. Ectopic pregnancy is diagnosed classically by a sub-optimally rising β HCG level, along with falling P4 levels and definitive ultrasound scan at day 49 showing an empty uterine cavity (or a pseudosac without fetal evidence). At this stage most cases are quite amenable to methotrexate (MTX) treatment. Such cases are monitored with blood and T/V scan over the next two weeks to determine an appropriate fall in β hCG levels. Sometimes a second MTX dose is required and occasionally a laparoscopy is required for definitive treatment of the ectopic pregnancy – usual tubal but ovarian and secondarily implanted cases have been found over our almost 40 years of experience.

We have also published on heterotopic pregnancies [24], sometimes with a surviving uterine pregnancy and associated ruptured tubal ectopic gestation. Suffice it to say that each of those cases has set its own challenge, but the best result has been achieved by applying hormonal supports to day 49, thereafter responding to the ultrasound evidence and proceeding to laparoscopy if the woman has pelvic pain and associated fluid (blood) in the Pouch of Douglas. Occasionally the diagnosis is actually ovarian torsion combined with a viable uterine pregnancy. Most cases have been amenable to laparoscopic unravelling of the ovarian ligament following which a plication suture is applied to prevent future episodes.

Pregnancy of unknown location (PUL) is increasingly diagnosed as we find the early application of methotrexate (MTX) resolves many uncertain cases. Sometimes this occurs at a combined hysteroscopic and laparoscopic search where ectopic gestation is suspected but no clear tubal or abdomino-pelvic pregnancy can be detected. The main possibility is of an early interstitial or cornual gestation, sometimes intramural in a former caesarean scar or occasionally as a cervical gestation. MTX 50 mg injected into each cornual site has resolved all cases we have experienced. A consensus statement on PULs covers nomenclature, definitions and outcomes [25].

The more common scenario of course is a failed intrauterine pregnancy described as a blighted ovum (no fetal detection) or a delayed miscarriage where viability of a detected fetus is not able to be confirmed. Our policy is to monitor to week 7 in such cases, maintaining the hormonal supports. A failed pregnancy will show a marked reduction in crown-rump length, often with associated sub-chorionic haematoma and continuing absence of fetal heart, whereas an occasional viable pregnancy will show enlargement and fetal heart detection; much to the surprise of the ultrasonographer. In our experience, approximately 50 percent of such cases have continued as normal pregnancies to term.

Progressing pregnancies are referred for obstetric management and includes first-trimester screening increasingly with non-invasive fetal DNA testing. This is beyond the

remit of this chapter, but suffice it to state that pregnancies arising from a subfertile background have a higher risk for fetal anomalies, pre-term births, intra-uterine growth restriction and complex obstetric outcomes. There are many advances in management of such issues in recent times; hence, the issue of careful monitoring is an essential continuum. The IVF clinic should hand over its pregnancies with clear management instruction such as the need for longer-term progesterone support in many higher-risk scenarios.

8.10 Conclusion

The earliest IVF cycles – both unstimulated and stimulated – were conducted with minimal monitoring. They were conducted on the principles of the natural menstrual cycle which indicated that the ovulatory LH surge occurred around day 12 and the egg was released sometime around 38 hours after its onset. Those early patients were young, certainly the women were <35 years, and to this day, that group has the best prognosis. However, the expectations arising from the assisted reproductive technologies around IVF have created a need to find solutions to treat the full array of infertility disorders, including male factor, across the entire reproductive age range. So too is ART expected to resolve associated challenges such as the problems of recurrent miscarriages and genetic disorders – both genetic mutations and chromosomal aneuploidies, particularly trisomies. These expectations have created a high demand for ART procedures, hence regulatory processes, both legislative and self-regulatory, have evolved. These increasingly expect absolute safety (e.g. avoiding OHSS) and demand sensitivity to community concerns (e.g. avoiding multiple pregnancies and adverse obstetric outcomes). To achieve these ideals explains the reasons for an increasingly sophisticated monitoring system behind each IVF treatment cycle and the ensuing pregnancy.

References

- Yovich JL. A clinician's personal view of assisted reproductive technology over 35 years. *Reprod Biol* 2011; **11**: 31–42.
- Malhotra J. (Ed). Pathfinders; Extraordinary doctors, extraordinary achievements; An Aspire initiative. Pugmarks Mediaa Allahabad, Uttar Pradesh, India 2016; pp84.
- Anand Kumar TC. Architect of India's first test tube baby: Dr. Subhas Mukherjee (16 Jan 1931 to 19 Jul 1981). *Current Science* 1997; **72**: 526–531.
- Wikland MD, Nilsson L, Hanson R, Hamberger L, Jenson PO. Collection of human oocytes by the use of sonography. *Fertil Steril* 1983; **39**: 603–608.
- Seppälä M, Edwards RG (eds). *In vitro fertilization and Embryo Transfer*. Annals of the New York Academy of Sciences 1985; **442**: p 619. New York.
- Brown S. ESHRE –the first 21 years. ESHRE publication 2005: p 273.
- Schally AV, Kastin AJ, Arimura A. Hypothalamic follicle-stimulating hormone (FSH) and luteinizing hormone (LH)-regulating hormone: structure, physiology, and clinical studies. *Fertil Steril* 1971; **22**: 703–721.
- Burgus R, Butcher M, Ling N et al. Molecular structure of the hypothalamic factor (LRF) of ovine origin monitoring the secretion of pituitary gonadotropin hormone of luteinization hormone (LH). *C R Acad Sci Hebd Seances Acad Sci D* 1971; **273**: 1611–1613. [Article in French]
- Tomažević T, Ban-Frankež H, Virant-Klun I et al. Septate, subseptate and arcuate uterus decrease pregnancy and live birth rates in IVF/ICSI. *Reprod Biomed Online* 2010; **21**: 700–705.
- Van Keppel M, Yovich JL. Nurturing the best interests of the future child. Annual Meeting of Fert Soc Aust, Sydney, September 2013 (For submission).

11. Yovich JL, Alsbjerg B, Hinchliffe P, Keane KN. PIVET rFSH dosing algorithms for individualised controlled ovarian stimulation enables optimised pregnancy productivity rates and avoidance of ovarian hyperstimulation syndrome. *Drug Design, Development & Therapy* 2016; **10**: 2561–2573.
12. Nelson SM. Biomarkers of ovarian response: current and future applications. *Fertil Steril* 2013; **99**: 963–969.
13. Fisch JD, Keskintepe L, Sher G. Gonadotropin-releasing hormone agonist/antagonist conversion with estrogen priming in low responders with prior in vitro fertilization failure. *Fertil Steril* 2008; **89**: 342–347.
14. Yovich J, Grudzinskas G. The management of infertility; a manual of gamete handling procedures. Heinemann Medical Books, Oxford UK 1990; see pp 21–37.
15. Craft I, Shelton K, Yovich JL, Smith D. Ovum retention in the human. *Fertil Steril* 1980; **34**: 537–541.
16. Bodri D, Kawachiya S, Brucker MD et al. Cumulative success rates following mild IVF in unselected infertile patients: a 3-year, single-centre cohort study. *Reprod Biomed Online* 2014; **28**: 572–581.
17. Pouwer AW, Farquar C, Kremer JA. Long-acting FSH versus daily FSH for women undergoing assisted reproduction. 2012 Cochrane Database Syst Rev Jun 13;(6): CD009577. doi:10.1002/14651858.CD009577.pub2
18. Yovich JL, Keane KN, Borude G, Dhaliwal SS, Hinchliffe PM. Finding a place for Corifollitropin within the PIVET FSH Dosing Algorithms. 2017 *Reprod Biomed Online*; *in press*.
19. Kuang Y, Chen Q, Hong Q et al. Double stimulations during the follicular and luteal phases of poor responders in IVF/ICSI programmes (Shanghai protocol). *Reprod Biomed Online* 2014; **6**: 684–691.
20. Yovich JL, Conceicao JL, Stanger JD, Hinchliffe PM, Keane KN. Mid-luteal serum progesterone concentrations govern implantation rates for frozen embryo transfers conducted under hormone replacement. *Reprod BioMed Online* 2015; **31**, 180–191.
21. Büehler KF, Fischer R. Recombinant human LH supplementation versus supplementation with urinary hCG-based LH activity during controlled ovarian stimulation in the long GnRH-agonist protocol: a matched case-control study. *Gynecol Endocrinol* 2012; **28**: 345–350.
22. Lower A, Shenton P, O'Shea F, Yovich JL. The value of serum levels of oestradiol, progesterone and beta-human chorionic gonadotrophin in the prediction of early pregnancy losses. *Hum Reprod* 1992; **7**: 711–717.
23. Yovich JL, Stanger JD, Yovich JM, Tuvik AI. Hormonal profiles in the follicular phase, luteal phase and first trimester of pregnancies arising from in-vitro fertilization. *Br J Obstet Gynaecol* 1985; **92**: 374–384.
24. Yovich JL, McColm SC, Turner SR, Matson PL. Heterotopic pregnancy from in-vitro fertilisation. *J Vitro Fert Embryo Transfer* 1985; **2**: 146–150.
25. Barnhart K, van Mello NM, Bourne T et al. Pregnancy of unknown location: A consensus statement of nomenclature, definitions and outcome. *Fertil Steril* 2010; **95**: 857–866.

Preventing Ovarian Hyperstimulation Syndrome

Michele Kwik

9.1 Introduction

Ovarian hyperstimulation syndrome (OHSS) is an iatrogenic condition that is associated with significant morbidity. It can be classified into mild, moderate or severe (Table 9.1). Severe OHSS affects 1 percent of women who undergo treatment with assisted reproductive technology (ART). It is characterized by abdominal bloating and discomfort, primarily associated with vascular endothelial growth factor (VEGF) mediated fluid shifts from the intravascular compartment into the third spaces. In severe cases, fluid shifts occur into the pleural cavity, resulting in pleural effusions and shortness of breath. Abdominal pain, nausea and vomiting may exacerbate the intravascular volume depletion, and women are therefore at higher risk of venous thromboembolic events and morbidity and mortality associated with this.

This phenomenon is exacerbated by the presence of human chorionic gonadotrophin (HCG). In controlled ovarian stimulation (COS), multiple ovarian follicles are stimulated by the administration of follicle-stimulating hormone (FSH). HCG is frequently used to trigger oocyte maturation prior to transvaginal oocyte retrieval. OHSS may therefore be divided into early onset and late onset. Early OHSS typically occurs in the first eight days following HCG trigger injection. Late OHSS is thought to be related to

Table 9.1 Classification of severity of OHSS

Grade	Symptoms
Mild OHSS	Enlarged ovaries, abdominal discomfort, abdominal bloating
Moderate OHSS	Moderate abdominal pain Nausea and vomiting Ascites
Severe OHSS	Severe abdominal pain and ascites Pleural effusion Haematocrit >0.45 Electrolyte abnormalities (hyponatraemia, hyperkalaemia) Oliguria

pregnancy-induced HCG production, and therefore symptoms are prolonged and more severe in the setting of an ART cycle with consequent pregnancy.

9.2 Diagnosis

The diagnosis of OHSS is mainly made on clinical grounds. Women with mild OHSS will complain of abdominal discomfort and bloating. Mild to moderate OHSS is often managed in an outpatient setting, with regular patient monitoring. Women who have significant ascites may require paracentesis to ease discomfort from abdominal distension, and this may be performed as an outpatient procedure.

Women may experience nausea, vomiting and shortness of breath with increasing severity of OHSS. These symptoms are a good guide for when hospital admission should be considered – in particular the inability to speak in full sentences, and vomiting with poor urine output.

Serology may show an increased haematocrit and electrolyte imbalances (hyponatraemia, hyperkalaemia) with severe OHSS, and hospital admission should be considered if serum markers are significantly deranged.

9.3 Treatment of OHSS

Several international bodies have developed their own guidelines for management of OHSS based on review of the evidence and expert opinion. These include the American Society for Reproductive Medicine [1], the Royal College of Obstetricians and Gynaecologists [2], the Institute of Obstetricians and Gynaecologists and the Royal College of Physicians of Ireland [3], the Joint Society of Obstetricians and Gynaecologists of Canada–Canadian Fertility and Canadian Fertility and Andrology Society [4], and the Royal Australian and New Zealand College of Obstetricians and Gynaecologists [5, 6].

Women with OHSS frequently complain of abdominal distension and pain, and simple analgesics are employed to keep them comfortable. Paracetamol is most commonly used, while non-steroidal anti-inflammatory agents should be avoided as they may interfere with implantation and may also compromise renal function in women with severe OHSS. Paracentesis may be performed to ease discomfort from abdominal distension. Women should be advised to drink to thirst, and the ART clinician should monitor symptoms and the woman's urine output.

Women with OHSS who are admitted to hospital are often unable to tolerate oral intake, and anti-emetics should be used to encourage drinking and eating. A strict fluid balance should be recorded, with judicious use of intravenous hydration in the early stages of OHSS, as intravenous fluids may exacerbate third-space fluid retention. Routine observations should include peripheral oxygen saturation levels, as women can also get pleural effusions.

Due to third-space fluid shifts from the intravascular compartment, women are intra-vascularly volume depleted with raised haematocrit levels. Women should therefore also be prophylactically anticoagulated, particularly in the in-patient setting.

The treatment of OHSS is mainly supportive, and given time, the condition will resolve spontaneously. OHSS symptoms often improve with the onset of menses. However, in the setting of pregnancy, symptoms may take weeks to resolve.

Therefore, the prevention of OHSS in the first instance is an important consideration for all clinicians offering ART services.

9.4 Prevention of OHSS

There are two critical arms to the prevention of OHSS.

The initial preventative strategies involve identification of women who are at risk of OHSS in the first instance and modification of the ovarian stimulation protocol to reduce this risk.

The second level of prevention occurs once women have commenced COS. All women who commence COS are monitored with serial serum oestradiol levels and follicular growth on ultrasound scans. During monitoring, women may then be identified as being at risk of developing OHSS should the cycle proceed.

(1) Identification of women at risk prior to ovarian stimulation (Box 9.1) [7]

Women with underlying polycystic ovarian syndrome (PCOS) and polycystic ovaries (PCO) are at higher risk of developing OHSS. Several retrospective studies have found increased association of OHSS in women with PCOS, and a systematic review in 2005 found an odds ratio of 6.8 for the development of OHSS in the setting of PCO.

Elevated anti-Müllerian hormone (AMH) levels are predictive of OHSS [8]. AMH levels measure ovarian reserve, as AMH is produced by the antral follicles in the ovary. It is routinely used as a marker for ovarian reserve prior to commencing ART. Low AMH levels are associated with a lower risk of OHSS [9], and indeed may be used to counsel and manage patients' expectations on the potential oocyte yield following COS.

Younger women and those with a low BMI are at higher risk of developing OHSS.

It is important to recognize that all these groups of women are at higher risk of OHSS, and a *lower dose of FSH* for ovarian stimulation should be considered. Consideration should also be given as to the stimulation protocol, as studies have clearly found a lower association of OHSS with *antagonist protocols* as opposed to long down-regulation protocols (OR 0.43), with no difference in pregnancy rates [10]. The antagonist protocol is well tolerated, with start of ovarian stimulation on day 2 or 3 of the menstrual cycle. A gonadotrophin-releasing hormone (GnRH) antagonist is then added to inhibit the luteinizing hormone (LH) surge associated with rising oestradiol levels, and oocytes are ready for retrieval after approximately 11 days of stimulation.

In women with PCOS, a systematic review found that the incidence of OHSS was significantly lower in women given *metformin during IVF cycles*, compared to placebo (OR 0.27) [11].

(2) Identification of women at risk of OHSS during controlled ovarian stimulation [12]

The progress of women undergoing ART cycles is routinely monitored by serum oestradiol levels and serial ultrasound scans measuring follicular growth. Women with many moderate-sized follicles are at risk of OHSS, as are women with high levels of oestradiol on the day of HCG trigger administration.

Box 9.1 Women at risk of OHSS

- Young age
- Low body mass index (BMI)
- Polycystic ovarian syndrome
- Polycystic ovaries on pelvic ultrasound scan
- Elevated anti-Müllerian hormone levels

The rate of OHSS has been found to be higher after retrieval of more than 15 oocytes, and therefore recognition of women at risk of OHSS should trigger some changes in the ART protocol.

As the symptoms of OHSS are exacerbated by the presence of HCG, one option would involve *cancellation of the cycle* altogether, before administration of HCG trigger injection.

However, many couples who commence an ART cycle have the expectation that they will have an embryo transfer and potentially conceive in that cycle. Therefore, once a woman has been identified as being at risk of developing OHSS during the stimulation phase of the cycle, there are certain measures that may be instigated to salvage the cycle.

(a) Coasting

Coasting involves the reduction or omission of gonadotrophins, in an attempt to slow follicular growth. There appears to be no detrimental effect on live birth rates, although studies are inconclusive with regard to whether this strategy actually decreases the incidence of OHSS.

(b) Changes to the trigger injection

The HCG trigger injection is important, as it induces maturation of the oocytes, so that meiosis is initiated and suspended at metaphase II. The trigger injection also causes the cumulus-oocyte complex to detach from the side of the follicle wall, so that the oocyte is freely floating in the follicular fluid for aspiration transvaginally.

As the symptoms of OHSS are exacerbated by HCG, a frequently used option is to reduce the dose of HCG trigger, with evidence that a dose of 2500 IU of HCG is sufficient to induce oocyte maturation. Alternatively, the trigger used may not involve HCG at all.

(c) GnRH agonist trigger

In women undergoing an IVF cycle using a GnRH antagonist protocol, a GnRH agonist may be used to trigger oocyte maturation, and this has been associated with a reduced rate of OHSS (OR 0.1) [13]. Administration of a GnRH agonist causes an initial flare of endogenous LH levels, to induce oocyte maturation via direct action on follicular LH receptors.

However, GnRH agonist triggering is ineffective in women who are already on a GnRH long down-regulation IVF protocol, as these women are already on a GnRH agonist and their pituitary has been down-regulated. It is also ineffective in women with hypothalamic hypogonadism, as the hypothalamic-pituitary axis is incapable of responding with a flare of endogenous gonadotrophins.

The use of a GnRH trigger has also been associated with a reduced pregnancy rate [13], as the resulting luteal phase is shortened, due to premature involution of corpora lutea. Various groups have looked at adding a small dose of HCG in the luteal phase, or using intensive oestrogen and progestogen luteal phase support in IVF cycles where a GnRH agonist has been used as a trigger, in an attempt to maintain pregnancy rates.

However, currently most clinicians who have administered a GnRH agonist trigger would freeze all embryos and will transfer in a subsequent cycle. Not only is there a concern about the endometrium, but the GnRH agonist trigger was given in the first place due to a risk of OHSS and therefore attempts to create a pregnancy in that cycle would be inappropriate.

Once eggs are retrieved, freezing of all the resulting embryos is an option, to prevent exposure of the woman to HCG associated with pregnancy. Studies show that this may prevent the occurrence of late-onset OHSS, but not early onset OHSS, if an HCG trigger was administered. However, the symptoms of early OHSS will resolve more quickly should the IVF cycle not result in a pregnancy.

Should embryo transfer proceed, it would be prudent to only replace a single embryo. Multiple pregnancies are another risk factor for OHSS.

Again, to prevent exposure to HCG, progesterone support rather than pregnyl (HCG) injections may be used for luteal phase support. Both are equally efficacious for luteal phase support.

(d) Other strategies

Various treatments have been investigated to prevent the development of OHSS once oocytes have been retrieved. These include dopamine agonists, and a recent systematic review of eight RCTs found that *cabergoline* 500 mcg once a week was associated with a reduced occurrence of early OHSS. Cabergoline is reasonably well tolerated, although women should be warned of postural hypotension as the main side effect.

Recently a small study ($n = 200$) showed that *intravenous calcium infusions* in women at risk of OHSS in the days following oocyte retrieval were associated with a reduction in the incidence of OHSS compared to placebo [14].

Currently, there is insufficient evidence to recommend the use of *intravenous albumin* and *hydroxy-ethyl starch* around the time of oocyte retrieval in the prevention of OHSS.

9.5 Summary

OHSS is an important iatrogenic complication of ovarian stimulation, and the prevention of OHSS is a priority for the clinician. So much so that some clinicians are working toward a 0 percent OHSS rate, by only prescribing antagonist protocols with GnRH agonist trigger and freezing of all embryos generated [15].

References

1. The Practice Committee of the American Society for Reproductive Medicine. Ovarian hyperstimulation syndrome. Fertility and Sterility 2008;9(3); S188–S193.
2. Royal College of Obstetricians and Gynaecologists (RCOG) The Management of Ovarian Hyperstimulation Syndrome Green-top Guideline No. 5 September 2006.
3. Institute of Obstetricians and Gynaecologists, Royal College of Physicians of Ireland, Directorate of Strategy and Clinical Programmes and Health Service Executive. Clinical Practice Guideline, Ovarian Hyperstimulation Diagnosis and Management 2012.
4. Joint Society of Obstetricians and Gynaecologists of Canada-Canadian Fertility and Andrology Society Clinical Practice Guidelines Committee. Shmorgun D, Claman P, Gysler M et al. The diagnosis and management of ovarian hyperstimulation syndrome: No. 268, November 2011. Int J Gynaecol Obstet. 2012;116:268–273.
5. Boothroyd C, Karia S, Andreadis N et al. and The Australasian CREI Consensus Expert Panel on Trial evidence (ACCEPT) group, Consensus statement on prevention and detection of ovarian hyperstimulation syndrome. Australian and New Zealand Journal of Obstetrics and Gynaecology. 2015; 5:523–534. doi:10.1111/ajo.12406

6. Kwik M, Karia S, Boothroyd C, RANZCOG CREI consensus statement on treatment of ovarian hyperstimulation syndrome. *Australian and New Zealand Journal of Obstetrics and Gynaecology*, 2015;55:413–419. doi:10.1111/ajo.12389
7. Mathur RS, Tan BK. British fertility society policy and practice committee: Prevention of ovarian hyperstimulation syndrome. *Human Fertility* 2014;17(4):257–268.
8. Lee TS, Liu CH, Huang CC et al. Serum AMH and estradiol levels as predictors of OHSS in ART cycles. *Hum Reprod* 2008;23(1):160–167.
9. Luke B, Brown MB, Morbeck DE et al. Factors associated with OHSS and its effect on ART treatment and outcome. *Fertil Steril* 2010;94(4):1399–1404.
10. Al-Inany HG, Youssef MA, Aboulghar M et al. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Database of Systematic Reviews*, 5, Art. No.: CD001750. Doi:10.1002/14651858. CD001750.pub3.
11. Tang T, Glanville J, Orsi N et al. The use of metformin for women with PCOS undergoing IVF treatment. *Human Reprod* 2006;21(26):1416–1425.
12. Mathur R, Kailasam C, Jenkins J. Review of the evidence base strategies to prevent ovarian hyperstimulation syndrome. *Hum Fertil* 2007;10:75–85.
13. Youssef MA, Van der Veen F, Al-Inany HG et al. Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist-assisted reproductive technology. *Cochrane Database Syst Rev* 2014 October 31;(10): CD008046. doi:10.1002/14651858. CD008046.pub4.
14. El-Khayat W, Elsadek M. Calcium infusion for the prevention of ovarian hyperstimulation syndrome: a double-blind randomized controlled trial. *Fertil Steril* 2015;103(1):101–105.
15. Banker M, Garcia-Velasco JA. Revisiting ovarian hyper stimulation syndrome: towards OHSS free clinic. *J Hum Reprod Sci*. 2015 January–March;8(1):13–17.

Oocyte Recovery

Anthony J. Rutherford

10.1 Introduction

It is easy to forget that it is 1978 was 40 years ago, since the birth of Louise Brown [1]. Surrounded by technology today, it is incredible to imagine the difficulties faced by those early pioneers Steptoe and Edwards in collecting oocytes at the precise time needed for fertilization in vitro. It was the prowess of Steptoe in laparoscopy, then a relatively new surgical procedure in gynaecology, that drew Edwards to Oldham, where Louise was conceived. Thus began the most famous partnership in in vitro fertilization (IVF) history, providing the first step in the ladder to develop assisted conception, giving millions of couples the opportunity of family life. Retrieval of oocytes by operative laparoscopy was widely practiced until the mid-1980s [2]. Notwithstanding the need for a general relaxant anaesthesia, the major disadvantage of laparoscopy was the availability of an operating theatre, and the need in up to one-third of cases to schedule the procedure to a time dependent on an early LH surge. Two major advances changed the face of IVF. The first was the use of ultrasound-guided oocyte collection which did not require general anaesthesia, and the second was the use of GnRH analogues to programme IVF cycles, allowing all egg collections to be safely performed at the surgeon's discretion without compromising oocyte quality. Susan Lenz in 1982 was the first to employ ultrasound, using the echolucent urinary bladder to give access to the pelvis, allowing oocytes to be collected by passing a needle safely through the abdominal wall [3]. Some of these egg collections were performed under local anaesthesia. Up until this point in time, the need for laparoscopy limited IVF to a hospital environment, where operating theatres were available, but the advent of a variety of techniques using local anaesthesia allowed assisted conception units to be developed in outpatient or clinic facilities. Initially, the use of ultrasound-guided techniques was not met with uniform approval. Professor Stuart Campbell, and his research team in King's in 1984, who introduced ultrasound-guided oocyte procedures into the United Kingdom, was heavily criticized for using the trans-vesical approach. At a meeting at the Royal Society of Medicine, this approach was described by Patrick Steptoe as 'barbaric' and by Robert Winston as 'dangerous', with concerns expressed by Robert Edwards about the integrity of the oocyte spindle. Campbell's group further refined their technique using a transurethral approach – TUDOR [5]. Dellenbach [6] described using a transabdominal ultrasound-guided transvaginal approach, while Feichtinger [7] was the first to describe using a modified ultrasound sector probe and a transvaginal approach, taking advantage of the juxtaposition of the ovaries to the top of the vagina. Early transvaginal ultrasound probes were mechanical sector scanners, some of quite alarming sizes, being quickly replaced by probes which were smoother, smaller using linear array technology. The remainder of this

chapter describes the current use of transvaginal oocyte retrieval, with the occasional use of an abdominal approach where required. Each section concludes with a small 'practice point summary', indicating our current practice for practical guidance.

10.2 Principles

The guiding principle behind any successful oocyte collection is to provide the embryologist with healthy oocytes. The aim is to retrieve the oocyte from each and every follicle aspirated, with the minimum risk of damage to the delicate cumulus-oocyte complex, and transfer to the laboratory without a significant drop in temperature. The procedure itself needs to be performed in a stable, comfortable and safe environment, with a minimal risk of complications. To achieve this goal, a suitable facility with a procedure room attached to a laboratory, equipped not only with the necessary instruments for vaginal oocyte retrieval but also with the appropriate safety apparatus to allow delivery of sedation anaesthesia with adequate resuscitation if required in an emergency is needed. In addition, as in any medical scenario appropriately trained and competent staff must be available.

10.3 Equipment

10.3.1 Ultrasound

Ultrasound imagery and technology has advanced enormously over the past 30 years. Modern equipment using three-dimensional (3D) technology and a variety of transducers of varying frequencies can achieve high-resolution images, which can, if required, be used in combination with colour flow Doppler to identify vascular anatomy and function. Advances in data assessment using more sophisticated computers and software can allow sonographic-based automated volume follicle volume assessment. The first attempts at a transvaginal ultrasound-guided transvaginal approach to egg collection were described by Feichtinger in 1986 [7], using a newly developed transvaginal transducer (Kretztechnik-Zipf, Austria), with a needle guide attached to the probe. The probe was a sector scanner with an acoustic frequency of 5 MHz, a maximum penetration depth of 10 cm, a 240° field of view and an image frequency of 8 images a minute. Their technique involved using a spring-guided puncture device. Since these early days both ultrasound technology and needle design have improved significantly. Although 3D ultrasound has been tried as a means to collect oocytes, with 3D puncture procedures performed in close to real-time ultrasound, there is a delay of approximately 5 seconds per follicle from coasting to resuming real-time ultrasound to allow volume calculation and precise location of the needle tip in the follicle [8]. Therefore, in most clinical situations where the ovaries are accessible transvaginally, conventional 2D transvaginal ultrasound probes are employed. Occasionally, where the ovaries are highly mobile, abdominal pressure may be required to push the ovaries closer to the vagina. In rare circumstances where this is not possible, a transabdominal, transvesical approach may still be used.

Assessing the volume of follicular fluid that is expected at aspiration can be calculated using the formula $V = \frac{4}{3} \pi r^3$, where V = volume and r = radius. The calculated volumes for different follicle diameters (extrapolated from radius) are shown in Table 10.1.

Table 10.1 The diameter-to-volume ratio for follicles

Follicle diameter (mm)	Follicle volume (ml)
6	0.1
7	0.2
8	0.3
9	0.4
10	0.5
11	0.7
12	0.9
13	1.1
14	1.4
15	1.8
16	2.1
17	2.6
18	3.0
19	3.6
20	4.2

Practice Point

We employ a Voluson P8, with an E8C-RS curved array multi-frequency transvaginal transducer, which has an angle of around 20°, to ease the collection procedure using a disposable plastic needle guide, E8385M. The probe is dressed with a non-latex sterile probe cover, with the handle and cord covered with a plastic sheath, commonly used in the general theatre to cover the laparoscope light lead. This maintains cleanliness and aids cleaning of the probe after each collection.

10.3.2 Needle Design

There are a number of properties to take into consideration when designing a needle for transvaginal oocyte recovery. The aim is to collect the oocyte from the follicle into a suitable container to be passed to the scientist as quickly as possible without physical damage to the cumulus–oocyte mass and a significant loss of temperature. The needle needs to be long enough to safely pass through the vaginal wall into the ovary, but the dead space, representing the volume of the cylinder created by the cross-sectional interior area of the needle and its length [9], needs to be minimized, so collected oocytes pass into the collecting tube and do not remain in the tube. Historically the diameter of the needle employed varies between 14 and 16 gauge, giving internal diameters of between 1.6 and 1.194 mm respectively [3,10], with relatively short bevels [11]. Today, the most commonly used needles are 16 to 17 gauge which have a dead space of 1.0–1.2 cc respectively. The needle needs to be as rigid as possible to ensure that it stays in the course of the guideline visible on screen, and it is vital that the tip

of the needle is visualized at all times. Most needles are scored close to the tip to aid visualization. The rigidity of the oocyte needle decreases as the gauge increases, even though the actual width of the wall of the needle is a little different [12]. The end of the needle needs to be sharp so that it allows easy entry through the tunica ovarica, and more importantly when entering the follicle, to keep the increase in pressure within the follicle to a minimum. Most needles have a bevelled tip to aid entry. The diameter of the needle needs to be great enough such that the inner channel is of a width to allow passage of the cumulus–oocyte complex intact, but small enough to minimize tissue trauma, diminishing the risk of serious bleeding if blood vessels are entered, and reducing pelvic discomfort [11,13]. A study comparing 15-, 17- and 18-gauge single-lumen needles showed no difference in recovery rates, but a significant reduction in perceived pain when the smaller diameter needle was employed [11]. As the needle length increases, the velocity and flow rate decrease [14]. Likewise, the flow rate decreases as the needle diameter falls. The needle is attached to a tubing system made of materials that are not toxic to gametes and that retain temperature well.

10.3.3 Physics of Suction Aspiration

Before the onset of monitored accurate electrical pumps, the high aspiration pressures associated with handheld syringes were shown to detrimentally impact the zona pellucida [15]. A suction source with a graduated controlled aspiration pressure is required to drain the follicular fluid in a controlled manner, without exerting too much pressure. The aspiration pressure is determined to some extent by the Hagen–Poiseuille law, which determines the steady flow through pipes [15]. Poiseuille's law says that increasing the length of the tube decreases the pressure at the end in proportion to the percentage that the length was increased. The aspiration pressure is also determined by the diameter of the operating needle channel, with even relatively small decreases in diameter decreasing the pressure dramatically; a 10 percent fall in diameter results in decreasing the pressure by 65.5 percent. Pressure and flow rates using a Wallace 17-gauge needle and a rocket suction pump at 100 mm of mercury (Hg) will give a flow rate of 0.42 ml/second, which equates to aspirating a 20 mm follicle, with a volume of 4 ml, in 9.6 seconds. The maximum flow rate achieved through a 17-gauge needle with 150 mm Hg is 0.486 ml/second. The diameter of the needle has a significant impact on the velocity and vacuum pressure required to maintain flow rate. The velocity in a 17-gauge needle will be 20 percent higher than a 16-gauge needle. The longer the needle and the smaller the internal diameter, the greater the vacuum required to maintain velocity. To maintain the same flow rate in a smaller diameter needle the velocity will be increased. However, increasing the aspiration pressure and flow rate too much has the potential to cause damage to the cumulus oocyte mass, with the pressure at the tip of the needle critical.

The pressure within an individual follicle is dependent on size, shape and position within the ovary with increased pressure in bigger follicles [15]. Pressure within the follicle is further increased by approximately 60 mm Hg as the needle presses on the follicular wall, such that there is a risk that when the follicle is entered fluid escapes past the needle, unless the seal is tight. Potentially this follicular fluid escape could be avoided if the vacuum is applied before the follicle is entered, as the build-up in aspiration pressure has occurred outside the follicle, but this needs to be balanced by the fact that in these circumstances as the needle enters the follicle the flow rate dramatically increases, causing turbulence within the tube, which may damage the oocyte. If suction is applied once the follicle is entered, flow rates normally take one to two seconds to stabilize, with a rapid rise in flow rate followed by

a more stable gradual rise to maximum flow. Assuming a good seal around the needle tip, flow remains constant until the follicle starts to collapse, and then the flow rate falls. If the vacuum is stopped too soon before the needle is removed from the follicle, there is risk of back flow, which could leave the oocyte in the follicle. Again the flow rate increases significantly as the needle is withdrawn from the ovary completely.

Practical Point

We use a 16-gauge dual-channel needle (Cook), with a suction pressure of 138 mm Hg. Suction is applied once the needle is inserted into the first follicle, and as the fluid drains the needle tip is gently rotated as required to keep the flow rate relatively constant until the follicle is emptied. Each follicle is drained in turn using the next follicle aspirated to clear the needle dead space. The follicular fluid is then given to the embryologist to identify the oocyte. If possible, all follicles are drained through one vaginal puncture on each side.

10.3.4 Damage to the Cumulus–Oocyte Complex

Damage to the oocyte cumulus–complex can occur from turbulence, vacuum pressure or velocity, and it can occur in the needle, vacuum lines or the follicle. There is a vacuum gradient down the collection system. The vacuum at the needle tip is only approximately 5 percent of that at the vacuum pump. The oocyte is exposed to ever increasing vacuum pressures along the collection system. This may cause the oocyte to swell or the zona to crack. High velocities may cause the cumulus cells to become detached. There will be significant difference in the flow rate within the needle, with higher flow rates centrally (laminar flow). This may exert a drag pressure on the cumulus cells. In a 17-gauge needle the cumulus–oocyte complex (COC) will occupy 25 percent of the diameter of the needle. Turbulence may throw the oocyte around in the tube and cause stripping of cumulus cells or the zona pellucida to crack. The transition from motionless to the velocity of the flow rate as the oocyte enters the needle may cause the cumulus cells to be stripped. This is more likely to cause damage in smaller follicles. Increasing flow rate or increasing the diameter of the needle may result in increased shear stress pressure on the COC. The larger the diameter of the needle, the greater the pressure gradient from the centre of the needle to the outside wall. Most flow rates used during the oocyte recovery are likely to keep the flow laminar, but if velocity increases too much then turbulent flow may result which can cause further stress. Turbulent flow is more likely to occur on entry of the COC into the needle tip. Vigorous flushing and sharply moving the bevel may also increase pressure.

Practical Point

Concentrate on keeping the pressure within the collection system constant, with the tip of the needle in the ovary throughout the procedure. At the end of the procedure, or if the needle is withdrawn, stop suction to avoid sucking in air, which then causes frothing (cappuccino effect) with turbulence and possible damage to the oocyte. Flush the needle using warmed culture media.

10.3.5 Follicular Flushing

Pregnancy rates are thought to be directly related to the numbers of oocytes retrieved [16]. In most studies, oocytes are recovered from approximately 80 percent of follicles aspirated.

Originally most oocyte collection needles used to be single channel; if an oocyte was not retrieved in the initial aspirate, flushing was required. This could result in the oocyte moving back and forwards up in the operating channel of the needle. Dual-channel needles were designed to allow follicular flushing, by providing a second channel down which flush medium could be dispensed. Initial studies did seem to show that this was of some benefit [17]. Waterstone and Parson [18] found that using a double-lumen needle 27.7 percent oocytes were collected in the initial aspirate and 55.2 percent from the dead space of the needle. Flushing a further three times provided 13.9 percent more oocytes, and a further three flushes yielded an additional 3.2 percent, concluding that flushing an additional six times would increase the oocyte recovery rate by 20 percent, a figure similar to other research [19]. Rose [9] argues that in women with more than 12 recovered oocytes, statistical calculations suggest that flushing to gain an extra 20 percent of oocytes is unlikely to change the pregnancy rate by more than 2 percent. Indeed, in randomized controlled trials where flushing was compared to no flushing, the number of eggs collected, the fertilization rates, the quality of embryos formed and the subsequent pregnancy rates in either arm were not found to be significantly different. However, an argument was put forward to flush in women having mild or minimal stimulation with low numbers of follicles, and in those women with a low ovarian response to stimulation, as the loss of an oocyte in these patients would have a greater impact. Levens and colleagues [21] addressed whether flushing was of any benefit in poor responders, defined as women with fewer than 8 follicles > 12 mm on day of hCG trigger, and concluded that there was no difference in recovery rates with 6.5 ± 2.2 with the single-lumen needle compared to 7.2 ± 2.3 with the double-lumen needle, while flushing resulted in a twofold increase in the procedure time. Mendez Lozano et al. [22] performed flushing in 271 women with a single follicle recovering 84.5 percent of oocytes with 55.5 percent of recovered oocytes in the initial aspirate, 35.8 percent in the first flush, 10.8 percent in the second, 5.9 percent in the third and 2.9 percent in the fourth. There have been a plethora of small studies looking at the benefit of flushing in low responders, with some showing a small benefit by flushing and others showing a potentially lower pregnancy rate [23,24]. Unfortunately, most of the studies comparing flushing and no flushing have involved relatively small numbers of patients, making it difficult to draw firm conclusions. However, there are two meta-analyses, using the most appropriate of these investigations, both of which reached the same conclusion, namely that flushing does not increase the oocyte yield, improve the pregnancy or live birth rate, but does substantially lengthen the oocyte collection procedure with the consequent increase in the need for analgesia [25,26]. Wongtra-ngan et al. showed that without flushing the operative time was significantly shorter, by between 3 and 15 minutes ($P < 0.001$), and subsequently the dose of analgesia significantly decreased ($P < 0.00001$). Despite this seeming lack of evidence of benefit, many clinics still use a flushing technique.

Practical Point

In our experience in Leeds, we use a standard 16-gauge double-lumen needle in all patients (Cook), but tend not to use the flush unless recovery rates are poor, or where the patient has less than five follicles on the preoperative ultrasound assessment.

10.4 Temperature Control

The meiotic spindles of the human oocyte consist of microtubules and like most mammals are extremely sensitive to temperature fluctuation, both up and down [27,28]. The spindle structure can dissemble relatively quickly on cooling, but takes longer to recover on rewarming. The temperature drop and the length of time the oocyte is exposed to cooling will determine whether the spindle will recover. Relatively minor drops of temperature from 37° to 33° can be tolerated for less than 10 minutes, before rewarming, but greater drops in temperature for longer periods of time will result in permanent damage [29,30]. This is very pertinent in assisted conception, as the oocyte is subject to temperature change at the time of the oocyte recovery and during manipulation in the laboratory. The meiotic spindles are responsible for the chromosome alignment and separation of the maternal chromosomes during fertilization. Therefore, any disruption to the spindle may lead to abnormal chromosome distribution, leading subsequently to failed fertilization or aneuploidy [28]. At the time of the oocyte harvest, the follicular fluid temperature can drop up to $7.7 \pm 1.3^\circ\text{C}$, in addition to the dissolved oxygen levels and the increasing pH [31]. Where used, the temperature of the media used to flush follicles can drop by up to 5° if handheld in preloaded syringes. Closed workstations where temperature, oxygen concentration and pH can be more reliably controlled have been introduced to reduce these potential stresses. Working with a closed isolator-based workstation may be technically more challenging but protects against ambient conditions, promoting the development of better quality blastocysts and higher pregnancy rates [32].

Practice Point

In Leeds, all oocyte procedures are performed in a room with an ambient temperature of 26°C. There is a small connecting hatch through to the laboratory, where the embryologist is working in a Grade A temperature-controlled workstation. All equipment used in the oocyte collection that comes into contact with the oocyte is pre-heated to 37°C in an incubator based in the procedure room. All the collecting tubes are kept in a tube heater at the same temperature. The last equipment to be assembled is the oocyte collecting system. This system is flushed with flushing media at 37°C immediately before the onset of the procedure. If flushing is employed we use small preloaded syringes of flush media which are kept in the incubator until used. Each follicle is aspirated in turn and the collecting tube immediately given to the embryologist to identify the oocyte.

10.4.1 Analgesia

The primary role of analgesia is to provide a safe comfortable environment in which the egg collection can be performed without difficulty, yet is able to facilitate a speedy recovery by the minimal use of anaesthetic and analgesic agents. The obvious advantage of an ultrasound-based approach is that in most circumstances the oocyte collection procedure does not require a full general anaesthetic. That said, since inception a variety of methods of pain relief have been employed for a transvaginal oocyte recovery [33], including general anaesthetic, conscious sedation, plus or minus local anaesthesia, local anaesthesia alone, either para cervical or pre-ovarian block [34] (local anaesthetic inserted in the vaginal wall in the lateral fornix close to the ovarian surface), regional approach, including both epidural and spinal

anaesthesia, and finally, perhaps the most intriguing, electro-acupuncture [35]. The facility used for oocyte recovery, whether it is on an isolated site or part of a large hospital complex, and the presence or absence of an anaesthetist, and the monitoring equipment available will determine what analgesic options are feasible [36]. The other consideration is the medical health of the woman. Most women having oocyte collection will be ASA (American Society of Anaesthesiologists) grade I-II, but there will be women who do have life-affecting disorders (ASA III), who will need an anaesthetist, and some require full hospital facilities. This highlights the importance of pre-assessment to identify high-risk patients (ASA III+), and if found, the need to have a protocol in place for the oocyte collection to be performed on a central hospital site. Examples would include patients with a history of major cardiac disease, or those with an established risk of a clotting diathesis.

The majority of oocyte collections in the United Kingdom and North America are performed under 'conscious sedation' [37,38]. Conscious sedation is defined as depression of the central nervous system such that procedures can be performed, but throughout the operation verbal contact is maintained with the patient. This level of sedation must be such that the patient remains conscious, retains protective reflexes and is able to respond to verbal commands. However, it is important to remember that sedation is a continuum, from the awake state to general anaesthesia, and is classified by the ASA into four stages through minimal sedation, moderate 'conscious' sedation, deep sedation and finally general anaesthesia [39]. The personnel qualified to give conscious sedation are now generally competency based, and a sedation practitioner may have medical or nursing qualifications. Generally, from a safety perspective, the sedation practitioner should not be the operator performing the procedure. A separate individual should be responsible for supervising the sedation. If this is not possible, it is permissible to have an operating practitioner prescribing medication, which is administered by a nurse practitioner, as long as the nurse has intermediate life-support skills and training.

It is also important to recognize that the type of medication used will depend on the level of training of the sedation practitioner. There remains the potential for over-sedating patients, which is relevant because deep sedation is more likely to incur airway obstruction, hypoventilation and cardiovascular depression. All patients should have venous access and supplementary oxygen, and be monitored during the procedure with intermittent non-invasive blood pressure, pulse oximetry, continuous ECG, respiratory rate and preferably capnography to observe carbon dioxide levels. The latter is used as pulse oximetry alone is a poor guide to alveolar function in a patient on supplementary oxygen. The two most commonly employed medications are the anxiolytic midazolam (Versed®), along with the narcotic analgesic fentanyl (Sublimaze®). Another frequently used drug is propofol (Diprivan®), an agent more commonly used to induce general anaesthesia. It has a much narrower therapeutic index when used in conscious sedation, and is given in either small boluses or by an infusion pump by a trained anaesthetist. Initially, there was concern from studies in mice that propofol may have adverse impact on oocyte development, encouraging parthenogenetic division [40]. In human research, while propofol increases in the follicular fluid during the course of the oocyte collection procedure, there is no evidence that this has a harmful impact on the rate of oocyte immaturity, fertilization, cleavage and early embryo development [41].

Unlike many procedures performed under conscious sedation, it is important to remember that oocyte retrieval has certain trigger points where pain may be more intense and expected. This includes the initial insertion of the needle and the potential movement of the ovary during the procedure itself. For this reason, a combination of both a sedative and

a powerful analgesic is required. A paracervical block (PCB) alone results in pain scores 2.5 times higher than when used in conjunction with intravenous sedation [42,43]. Lidocaine (Xylocaine®) gel, while potentially avoiding any risk of toxicity from absorbed lidocaine, is unfortunately less effective than PCB in reducing pain associated with insertion of the needle ($P<0.001$) or the pain associated with the egg collection (<0.01) [44]. A preoperative sedative will reduce anxiety, and peri-operative analgesic requirements, yet recovery will be longer, delaying discharge home. [45] Finally, patient-controlled analgesia has been tried for oocyte retrieval but shown to be less effective than physician controlled analgesia [46,47]. In conclusion, there is no hard evidence that one method of delivery or drug is superior to another [48]. As with most medical interventions there are pros and cons of each modality.

Practical Point

Our clinic is based on an isolated hospital site with day case procedure rooms without recourse to general anaesthesia. We have two lists of oocyte retrievals running side by side, supervised by one consultant anaesthetist, with a trained nurse practitioner providing conscious sedation for the second list. All patients have preoperative assessment using a standardized questionnaire. Those with ASA scores above II are assigned to the consultant list. If there are serious health concerns, patients have their oocyte procedure on the main hospital site. This occurs rarely. Patients are administered a 50 mcg bolus dose of fentanyl initially, followed two or three minutes later by midazolam 2.5 mg. A local paracervical block is performed with 12–20 mls of lidocaine 1 percent, dependent on their body mass. Further boluses of fentanyl and midazolam are given dependent on response up to a maximum of 100 mcg fentanyl and 5 mg of midazolam. Most oocyte procedures are scheduled 40 minutes apart, with most taking less than 30 minutes, of which operating time is 10–15 minutes. Everyone is given supplementary oxygen and is monitored with pulse oximetry and capnography, as well as intermittent non-invasive blood pressure assessment. Rarely, anxious patients or those with multiple follicles which have been preferentially placed on the consultant anaesthetist list may need a small top bolus of propofol.

10.4.2 Cleaning the Vagina before Oocyte Retrieval

Theoretically inserting a needle through the vaginal wall from essentially an unsterile environment, into the abdomen-a sterile field, increases the risk that intraperitoneal infection may occur. However, pelvic infection after transvaginal oocyte recovery is thankfully rare, irrespective of whether prophylactic antibiotics are used with an incidence of 0.6 percent in a large series of 2670 cases [49]. Others have found a serious infection rate of 1.3 percent in a large series of 674 women, where antibiotics were administered [50]. Therefore, the use of prophylactic antibiotics remains controversial [51]. Bacterial vaginosis (BV) is a common infection found in 19 percent of women undergoing IVF treatment [52], and is associated with a significantly increased risk of preclinical pregnancy loss (OR 2.36, 95% CI; 1.24–4.51). In an attempt to reduce the risk of an abdominal infection, povidone iodine 1 percent (Betadine®) has been used to cleanse the vagina prior to the oocyte collection procedure. In a randomized controlled trial comparing Betadine® with a simple normal saline douche in 334 women undergoing oocyte retrieval, no difference was found in the fertilization rates (45.5 versus 47.8 percent) or cleavage rates (49.8 versus 52.1 percent) respectively. However, the pregnancy rate was significantly lower in the Betadine® group (17.2 compared to

30.3 percent), indicating that Betadine® appeared to have an adverse impact on outcome [53]. Likewise, even where the Betadine® is removed by douching with normal saline, there was a higher biochemical pregnancy rate where Betadine was employed [54].

Practice Point

Clindamycin cream, known to be effective against BV, is administered in the five days leading up to the oocyte recovery. At the start of the procedure a simple douche with normal saline removes any excess vaginal discharge before the local anaesthetic is inserted. Antibiotics are administered prophylactically to those women with a known endometrioma, or those in whom the procedure was difficult or prolonged.

10.4.3 Complications of Transvaginal Oocyte Recovery

Transvaginal ultrasound-guided oocyte retrieval is a safe well-tolerated procedure, with a low risk of serious complications. Blood loss of the oocyte procedure is calculated using pre- and post-operative haemoglobin measurements and estimated to be approximately 230 ml [55]. Those most at risk are those with multiple follicles to aspirate, such as women with PCOS [56]. Occasionally, patients may bleed from the vaginal puncture sites, but in most cases this bleeding will settle with the application of pressure. Rarely a haemostatic suture may be required if bleeding persists. Rarely, more significant bleeding can occur, with an incidence of 1–2 per 1000 cases [49]. This is most likely to be ovarian, but could arise from a punctured iliac vessel. It is important to be vigilant as most women having oocyte retrieval are young and healthy, and tolerate a significant blood loss, compensating later than expected. Patients should be warned of this possibility, and a 24-hour emergency contact number should be provided on discharge. If admission is required, this should be to a hospital facility versed in dealing with hyperstimulated patients to avoid over aggressive surgery, which may compromise future fertility. Often the bleeding site is difficult to identify, and simple oversowing of capsular puncture sites will be sufficient to control haemorrhage [49]. Infection is the other significant complication that can present in the first week after the oocyte collection procedure, but may be delayed up to six weeks [57]. Pelvic infection with pyrexia and pelvic tenderness requiring antibiotic therapy occurred in 9 cases in a large series of 2677 oocyte collection procedures, with 9 more significant infections with abscess formation giving an overall incidence of 0.6 percent [49], which is similar to the rate of pelvic tubo-ovarian abscess found in other large series [57,58]. Those most at risk are those with endometrioma [59], although the risk is too small to justify the routine removal of endometrioma before treatment [60]. There are other more unusual but extremely rare complications reported, such as ureteric injury [61], damage to the appendix or vertebral osteomyelitis [62].

10.4.4 How Much Discomfort Does Oocyte Recovery Cause from a Patient's Perspective – What Should We Tell Our Patients?

Esposito and colleagues [57] surveyed women undergoing oocyte collection in stimulated cycles. Women were asked to rate the inconvenience and pain associated with oocyte collection procedure using a numeric rating scale of zero to ten. Data on analgesic usage

and the time taken to return to work and normal activity were also recorded. The mean pain score immediately post operation was 4.6, and at 24 hours 3.9, on a scale of 1 to 10. Most women returned to normal activity and work within two days (mean 1.7, SD 1.5 days and mean 1.8, SD 1.5 respectively). Immediate post-operative discomfort was not found to be correlated with the number of oocytes collected. However, the number of oocytes collected positively correlated with the quantity and duration of analgesics consumed and the time taken to resume work and normal activity.

10.4.5 The Oocyte Collection Checklist

As described in the Checklist Manifesto (Gawande 2010) [58], having a surgical checklist decreases the risk of steps being missed. It is recommended that units devise a checklist that they use prior to oocyte collection, including equipment and patient checks.

References

1. Steptoe PC, Edwards RG. Birth after re-implantation of a human embryo. *Lancet* 1978; **2**: 366.
2. Steptoe PC, Webster J. Laparoscopy for oocyte recovery. *Ann NY Acad Sci* 1985; **442**: 178–81.
3. Lenz S, Lauritsen JK. Ultrasonically guided percutaneous aspiration of human follicles under local anaesthesia: a new method for collecting oocytes for in vitro fertilization. *Fertil Steril*. 1982; **38**: 673–7.
4. Royal Society of Medicine Symposium, 1985. Mrs Vinay Sharma – personal communication.
5. Parsons J, Booker M, Goswamy R et al. Campbell S: Oocyte retrieval for in-vitro fertilisation by ultrasonically guided needle aspiration via the urethra. *Lancet* 1:1076, 1985.
6. Dellenbach P, Nisand I, Moreau L et al. Transvaginal sonographically controlled follicle puncture for oocyte retrieval. *Fertil Steril*. 1985; **44**: 656–662.
7. Feichtinger, W. and Kemeter, P. Transvaginal sector scan sonography for needle guided transvaginal follicle aspiration and other applications in gynecologic routine and research. *Fertil. Steril.* 1986; **45**, 722–725.
8. Feichtinger W. Follicle aspiration with interactive three-dimensional digital imaging (Voluson): a step toward real-time puncturing under three-dimensional ultrasound control. *Fertil Steril*. 1998; **70** (2): 374–377.
9. Rose B I. Follicle flushing for oocyte retrieval: Targeted analysis for patients with few follicles. *IVF Lite* 2014; **1**: 75–80.
10. Lewin A, Laufer N, Rabinowitz R, Schenker J. Ultrasound guided oocyte recovery for in vitro fertilization: an improved method. *J In Vitro Fert Embryo Transfer*. 1986; **3**: 370–3.
11. Aziz N, Biljan MM, Taylor CT, Manasse PR, Kingsland CR. Effect of aspirating needle caliber on outcome of in-vitro fertilization. *Hum Reprod*. 1993; **8**: 1098–100.
12. Wikipedia. Needle gauge comparison chart. http://en.wikipedia.org/wiki/Needle_gauge_comparison_chart, accessed April 25, 2014.
13. Wiklund M, Blad S, Bungum L et al. A randomized controlled study comparing pain experience between a newly designed needle with a thin tip and a standard needle for oocyte aspiration. *Hum Reprod*. 2011; **26**: 1377–83.
14. Horne R, Bishop CJ, Reeves G, Wood C, Kovacs GT. Aspiration of oocytes for in-vitro fertilization. *Hum Reprod Update*. 1996; **2**: 77–85.
15. Lowe B, Osborn JC, Fothergill DJ, Lieberman BA. Factors associated with accidental fractures of the zona pellucida and multipronuclear human oocytes following in-vitro fertilization. *Hum Reprod*. 1988; **3**: 901–4.

16. Sunkara SK, Rittenberg V, Raine-Fenning N et al. Association between the number of eggs and live birth in IVF treatment: An analysis of 400 135 treatment cycles. *Hum Reprod* 2011; **26**: 1768–74.
17. Elhussein E, Balen AH, Tan SL. A prospective-study comparing the outcome of oocytes retrieved in the aspirate with those retrieved in the flush during transvaginal ultrasound directed oocyte recovery for in vitro fertilization. *Br J Obstet Gynaecol* 1992; **99**: 841–844.
18. Waterstone JJ, Parsons JH. A prospective-study to investigate the value of flushing follicles during transvaginal ultrasound-directed follicle aspiration. *Fertil Steril* 1992; **57**: 221–223.
19. Bagtharia S, Haloob ARK. Is there a benefit from routine follicular flushing for oocyte retrieval? *J Obstetrics & Gynaecology*, 2005; **25** (4): 374–376.
20. Kingsland CR, Taylor CT, Aziz N, Bickerton N. Is follicular flushing necessary for oocyte retrieval? A randomized trial. *Human Reproduction* 1991; **6** (3): 382–383.
21. Levens ED, Whitcomb BW, Payson MD, Larsen FW. Ovarian follicular flushing among low-responding patients undergoing assisted reproductive technology. *Fertil Steril*. 2009; **91**: 1381–1384.
22. Mendez Lozano DH, Brum Scheffer J, Frydman N et al. Optimal reproductive competence of oocytes retrieved through follicular flushing in minimal stimulation IVF. *Reprod Biomed Online* 2008; **16**: 119–23.
23. Mok-Lin E, Brauer AA, Schattman G, Zaninovic N, Rosenwaks Z, Spandorfer S. Follicular flushing and in vitro fertilization outcomes in the poorest responders: a randomized controlled trial. *Human Reproduction* 2013; **28** (11): 2990–2995.
24. Malhotra N, Dolkar D, Mahey R, Singh N. To flush or not to flush: a randomized controlled trial comparing follicular flushing and direct aspiration at oocyte retrieval in poor responders undergoing IVF. *Fertil & Steril*. 2017; **108** (3): 237–238.
25. Levy G, Hill MJ, Ramirez CI et al. The use of follicle flushing during oocyte retrieval in assisted reproductive technologies: a systematic review and meta-analysis. *Human Reproduction* 2012; **27** (8): 2373–2379.
26. Wongtra-ngan S, Vutyavanich T, Brown J. Follicular flushing during oocyte retrieval in assisted reproductive techniques. *Cochrane Database of Systematic Reviews* 2010, Issue 9. Art. No.: CD004634
27. Sun XF, Wang WH, Keefe DL. Overheating is detrimental to meiotic spindles within in vitro matured human oocytes. *Zygote* 2004; **12**(1): 65–70.
28. Wang WH, Meng L, Hackett R, Odenbourg R, Keefe DL. Limited recovery of meiotic spindles in living human oocytes after cooling-rewarming observed using polarized light microscopy. *Human Reproduction* 2001; **16** (11), 2374–2378.
29. Almeida, P., & Bolton, V. The effect of temperature fluctuations on the cytoskeletal organisation and chromosomal constitution of the human oocyte. *Zygote*, 1995; **3**(4), 357–365.
30. Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J, Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil and Steril*. 1990; **54** (1): 102–108.
31. Redding GP, Bronlund JE, Hart AL. The effects of IVF aspiration on the temperature, dissolved oxygen levels, and pH of follicular fluid. *J Assist Reprod Genet* 2006; **23**: 37–40.
32. Hyslop L, Prathalingam N, Nowak L, Fenwick J, Harbottle S et al. (2012) A Novel Isolator-Based System Promotes Viability of Human Embryos during Laboratory Processing. *PLOS ONE* 7(2): e 31010.
33. Kwan I, Bhattacharya S, Knox F, McNeil A. Pain relief for women undergoing oocyte retrieval for assisted reproduction. *Cochrane Database Syst Rev* 2013; **1**: CD004829.
34. Cerne A, Bergh C, Borg K et al. Pre-ovarian block versus paracervical block for oocyte

- retrieval. *Human Reproduction* 2006; **21** (11): 2916–2921.
35. Gejervall AL, Stener-Victorin E, Möller A et al. Electro-acupuncture versus conventional analgesia: a comparison of pain levels during oocyte aspiration and patients' experiences of well-being after surgery. *Hum Reprod* 2005; **20**: 728–35.
 36. Royal College of Anaesthetists. Guidelines on the Provision of Anaesthetic Services (GPAS), Chapter 19, Guidance on the Provision of Sedation Services 2016.
 37. Yasmin E, Dresner M, Balen A. Sedation and anaesthesia for transvaginal oocyte collection: An evaluation of practice in the UK. *Hum Reprod* 2004; **19**: 2942–5.
 38. Ditkoff EC, Plumb J, Selick A, Sauer MV. Anesthesia practices in the United States common to in vitro fertilisation (IVF) centers. *J Assist Reprod Genet*. 1997; **14** (3): 145–147.
 39. Sheahan CG, Mathews DM. Monitoring and delivery of sedation. *British Journal of Anaesthesia* 2014; **113** (2): 37–47.
 40. Janssenswillen C, Christiaens F, Camu F and Steirteghem A (1997) The effect of propofol on parthenogenetic activation, in vitro fertilisation and early development of mouse oocytes. *Fertil Steril* **67**, 769–774.
 41. Ben-Shlomo I, Moskovich R, Golan J et al. The effect of propofol anaesthesia on oocyte fertilisation and early embryo quality. *Human Reproduction* 2000; **15** (10): 2197–2199.
 42. Ng EH, Tang OS, Chui DK, Ho PC. A prospective, randomized, double-blind and placebo-controlled study to assess the efficacy of paracervical block in the pain relief during egg collection in IVF. *Hum Reprod*. 1999; **14**: 2783–2787.
 43. Corson SL, Batzer FR, Gocial B, Kelly M, Gutmann JN, Go KJ, English ME. Is paracervical block anesthesia for oocyte retrieval effective? *Fertil Steril*. 1994; **62**: 133–136.
 44. Tummon I, Newton C, Lee C, Martin J. Lidocaine vaginal gel versus lidocaine paracervical block for analgesia during oocyte retrieval. *Human Reproduction* 2004; **19** (5): 1116–1120.
 45. Ng EHY, Miao B, Ho PC. Anxiolytic premedication reduces preoperative anxiety and pain during oocyte retrieval. A randomised double-blinded placebo-controlled trial. *Human Reproduction* 2002; **17** (5): 1233–1238.
 46. Bhattacharya S, MacLennan F, Hamilton MPR, Templeton A. How effective is patient-controlled analgesia? A randomised comparison of two protocols for pain relief during oocyte recovery. *Human Reproduction*. 1997; **12** (7): 1440–1442.
 47. Lok IH, Chan MTV, Chan DLW et al. A prospective randomized trial comparing patient-controlled sedation using propofol and alfentanil and physician-administered sedation using diazepam and pethidine during transvaginal ultrasound-guided oocyte retrieval. *Human Reproduction* 2002; **17** (8): 2101–2106.
 48. Kwan I, Bhattacharya S, Know F, McNeil A. Conscious sedation and analgesia for oocyte retrieval during IVF procedures: a Cochrane review. *Human Reproduction* 2006; **21** (7): 1672–1679.
 49. Bennett SJ, Waterstone JJ, Cheng WC, Parsons J. Complications of transvaginal Ultrasound-Directed Follicle Aspiration: A review of 2670 Consecutive Procedures. *J Assisted Reproduction & Genetics* 1993; **10** (1): 72–77.
 50. Tureck RW, Garcia CR, Blasco L, Mastrianna L Jr. Perioperative complications arising after transvaginal oocyte retrieval. *Obstet Gynecol* 1993; **81**: 590–3.
 51. Sowerby E, Parsons J. Prevention of iatrogenic pelvic infection during in vitro fertilization – current practice in the UK. *Hum Fertil (Camb)* 2004; **7**: 135–40.
 52. Van Oostrom N, De Sutter P, Meys J, Verstraeten H. Risks associated with Bacterial Vaginosis in infertility patients: a systematic review and meta-analysis. *Human Reproduction* 2013; **28** (7): 1809–1815.

53. Van Os HC, Roozenburg BJ, Janssen-Caspers HA et al. Vaginal disinfection with povidone iodine and the outcome of in-vitro fertilisation. *Human Reproduction* 1992; **7** (3): 349–350.
54. Hannoun A, Awwad J, Zreik T, Ghaziri G, Abu-Musa A. Effect of betadine vaginal preparation during oocyte aspiration in in-vitro fertilisation cycles on pregnancy outcome. *Gynaecol Obstet Invest.* 2008; **66** (4): 274–278.
55. Dessole S, Rubattu G, Ambrosini G et al. Blood loss following non-complicated transvaginal oocyte retrieval for in vitro fertilization. *Fertil Steril.* 2001; **76**: 205–6.
56. Liberty G, Hyman JH, Eldar-Geva T et al. Ovarian hemorrhage after transvaginal ultrasonographically guided oocyte aspiration: a potentially catastrophic and not so rare complication among lean patients with polycystic ovary syndrome. *Fertil Steril.* 2010; **93**: 874–9.
57. Dicker D, Ashkenazi J, Feldberg D et al. Severe abdominal complications after transvaginal ultrasonographically guided retrieval of oocytes for *in vitro* fertilization and embryo transfer. *Fertil Steril* 1993; **59**: 1313–5.
58. Tureck RW, García CR, Blasco L, Mastroianni L, Jr. Perioperative complications arising after transvaginal oocyte retrieval. *Obstet Gynecol* 1993; **81**: 590–3.
59. Moini A, Malekzadeh F, Amirkaghmaghi E et al. Risk factors associated with endometriosis among infertile Iranian women. *Arch Med Sci* 2013; **9**: 506–14.
60. Jayaprakasan K, Becker C, Mittal M on behalf of the Royal College of Obstetricians and Gynaecologists. The Effect of Surgery for Endometriomas. Scientific Impact paper number 55. BJOG 2017; DOI: 10.1111/1471–0528.14834
61. Miller PB, Price T, Nichols JE, Jr., Hill L. Acute ureteral obstruction following transvaginal oocyte retrieval for IVF. *Hum Reprod* 2002; **17**: 137–8.
62. Almog B, Rimon E, Yovel I et al. Vertebral osteomyelitis: a rare complication of transvaginal ultrasound-guided oocyte retrieval. *Fertil Steril* 2000; **73**: 1250–2.
63. Esposito J, Qin S, Kovacs G. How much inconvenience and discomfort does modern IVF treatment cause? *Glob J Reprod Med* 2017; **1**(2):MS.ID.555557
64. Gawande A. *The Checklist Manifesto*. 2010 Profile Books London.

The Use of DHEA to Improve Oocyte Quality

Ben Kroon

11.1 Introduction

The holy grail of assisted conception is to improve the longevity of the female reproductive lifespan. In women, the lifetime supply of oocytes undergoes an inexorable decline until menopause is reached, signalling the end of natural reproductive opportunities. Unfortunately, long before oocyte numbers are exhausted there is a decline in oocyte quality, clinically signified by reduced fecundability, increased miscarriage and increased chromosomal abnormalities in offspring. These characteristics are the result of deterioration in oocyte cytoplasmic and nuclear competence. Spindle formation, chromosomal segregation, mitochondrial function and cytoskeleton integrity are globally impaired in the ageing oocyte. In the case of women undergoing assisted reproduction, the clinical consequence of oocyte ageing is poor fertilization, slow (or abnormal) cell division, reduced blastocyst formation and resulting poor pregnancy rates.

11.2 Oocyte Competence

Women of advanced reproductive age, a term which arguably includes all women ≥ 35 years of age, but which without doubt includes all women ≥ 40 years of age, have globally poorer reproductive outcomes. Overlapping with this group of women, in a less well-defined manner, are the outcomes of those women with premature ovarian ageing (POA), demonstrated clinically by raised age-specific FSH, poor antral follicle counts and reduced AMH for age [1]. These two groups of women are likely to fit the Bologna criteria for ‘poor responders’, defined clinically by at least two of the following three features being present: (i) advanced maternal age or any other risk factor for poor ovarian response; (ii) a previous poor ovarian response; and (iii) an abnormal ovarian reserve test [2].

While not clearly represented in the literature, all clinicians experienced in reproductive medicine will also care for a cohort of women who may not be either reproductively ‘old’ or have POA, yet have persistently poor embryo quality, reduced blastocyst formation and prolonged time to conception, often without a sufficient biological explanation. Frustratingly for the clinician, while age and ovarian reserve are easily identified, there are no accepted markers for oocyte quality. Biomarkers from follicular fluid have been investigated, and some units use polarized light microscopy to assist with oocyte selection; however, this use is far from mainstream. The currently accepted surrogate measure for oocyte quality is to observe its function in vitro, when fertilized with spermatozoa.

11.3 Factors Affecting Oocyte Competence

Predominantly age, but also a variety of environmental factors, is likely to impact on oocyte competence. The most frequently encountered environmental factors thought to impact on oocyte competence are obesity and smoking. Overweight and obese women ($\text{BMI} \geq 25 \text{ kg/m}^2$) have been demonstrated in a meta-analysis of 33 studies to have a statistically reduced clinical pregnancy rate ($\text{RR} = 0.90, P < 0.0001$) and live birth rate ($\text{RR} = 0.84, P = 0.0002$) and a significantly higher miscarriage rate ($\text{RR} = 1.31, P < 0.0001$) than normal weight women [3]. Cigarette smoking in women similarly reduces the odds of pregnancy in vitro fertilization (IVF) cycles compared to non-smokers ($\text{OR } 0.66, 95\% \text{ CI } 0.49\text{--}0.88$) [4]. The relative impact of these exposures on endometrium and oocyte is however unclear. Additional adverse environmental factors have been proposed to impact on oocyte competence, including psychological stress, androgen deficiency and oxidative stress induced by advanced glycation end products [5].

11.4 The Role of Adjuvant Therapies for Poor Oocyte Quality

In cases of IVF failure where poor embryo development is felt to be related to poor oocyte quality, whether this is related to age, seen in the setting of POA or observed with no sufficient biological explanation, an opportunity may exist for improving outcomes by improving oocyte quality. Adjuvant therapies proposed to improve oocyte quality include melatonin (reviewed elsewhere in this publication), coenzyme Q10 (CoQ10) and DHEA (dehydroepiandrosterone), while differing stimulation regimens involving the use of growth hormone or luteinizing hormone have also been proposed. Coenzyme Q10, a critical component of cellular energy generation, has been investigated in older women undergoing IVF. In a randomized double-blind study performed in a small number of women, CoQ10 administration had no significant effect on oocyte aneuploidy, although the frequency of oocyte aneuploidy was 46.5 percent with CoQ10 treatment compared with 62.8 percent in the placebo-treated group, suggesting that its further investigation as a pretreatment for IVF may be worthwhile [6].

11.5 DHEA

DHEA and DHEAS (dehydroepiandrosterone-sulphate) are androgenic steroid prohormones produced from cholesterol predominantly in the adrenal cortex, but also in the ovaries and brain. In women, adrenal production of DHEA and DHEAS contributes substantially to overall androgen levels. Most circulating DHEA exists as DHEAS, the sulphate ester of DHEA. DHEAS is a stable compound with minimal episodic variation, making it an excellent serum target to provide a direct measure of adrenal androgen activity. Modest elevations of DHEAS may be found in hyperprolactinaemic women and those with anovulatory polycystic ovarian syndrome (PCOS), while dramatically elevated levels are suggestive of an adrenal tumour.

DHEA is a metabolic precursor for both androgen and oestrogen production. Neither DHEA nor DHEAS itself activates steroidogenic receptors, but instead it acts as a reservoir for androgen (and oestrogen) production, which in turn activates their relevant receptors. The pathway for conversion of steroids to DHEA is shown in Figure 11.1.

When DHEA is administered, there is a dose-dependent increase in circulating testosterone, dihydrotestosterone and oestrogens [7]. DHEA is generally administered as an oral

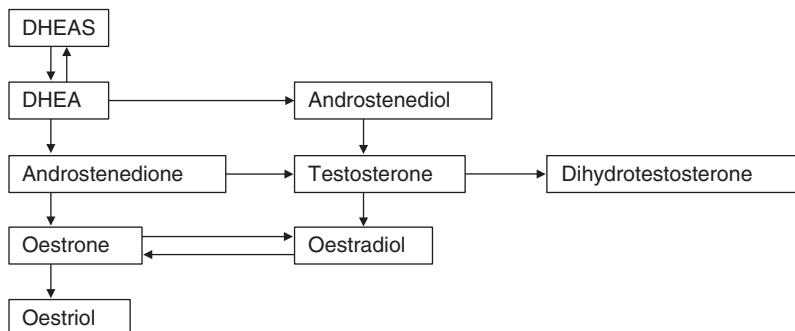


Figure 11.1
The DHEA steroidogenic pathway

preparation, with most orally ingested DHEA being converted to DHEAS in the intestines and liver. It is worth noting that when DHEA is administered prior to IVF, DHEAS may interfere with progesterone immunoassays used in clinical ART programmes, resulting in serum progesterone levels that may not reflect truly bioactive progesterone [8].

11.5.1 Proposed Uses for DHEA

Over a woman's reproductive lifespan, androgen levels, including DHEA and DHEAS, undergo a natural decline [9]. For this reason, androgens, including DHEA, have been much studied for their potential benefits in rejuvenating the ageing body. DHEA has been proposed to have many therapeutic properties, including neuroprotection, antidepressant activity and to play a role in improved bone health, libido, cognition and body composition to name just a few. It is heavily marketed in many countries worldwide and may be available in varying jurisdictions either with or without a script, pre-prepared as a commercial product or compounded as required by chemists.

Not all of the claimed benefits of DHEA have in fact been borne out in studies. The Endocrine Society clinical guidelines recommend against the general use of DHEA for sexual, cognitive and metabolic dysfunction, bone health and general well-being, as there is no clear evidence of benefit and evidence of safety is lacking [10]. In post-menopausal women there is also insufficient data to recommend its use [11].

The side effect profile of DHEA is consistent with its androgenic action. Oily skin and acne are frequently encountered, while prolonged use may be associated with metabolic changes, hirsutism and voice changes.

11.5.2 Follicular Role of Androgens

At the follicular level androgens have an obligatory role as oestrogen precursors. In the theca cell, luteinizing hormone stimulates the steroidogenic pathway to produce androgens, which then diffuse into the granulosa layer where aromatase activity facilitates the conversion to oestrogens (the two-cell, two-gonadotrophin theory of steroidogenesis) (Figure 11.2).

Thus, androgens in low concentrations are critical to follicular growth and development. Androgens (of any origin) may however have detrimental effects, as excessive follicular concentrations of androgens result in the preferential conversion of androgens to more potent 5 α -reduced androgens and may also result in the inhibition of aromatase activity and

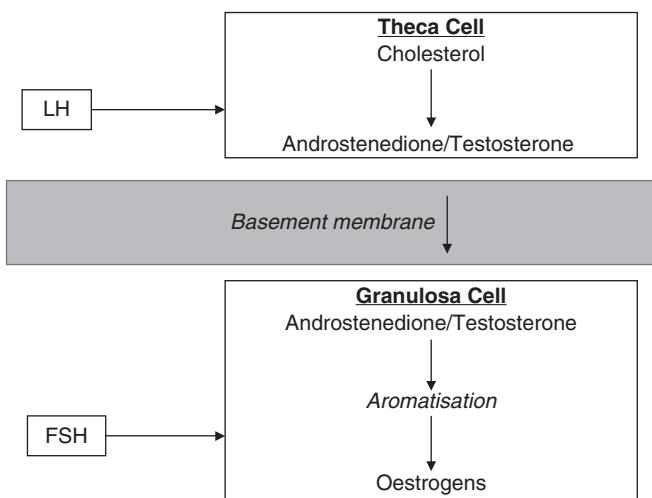


Figure 11.2 Follicular conversion of androgens to oestrogens

anovulation. The follicle-packed ovaries of women with PCOS are an example of this phenomenon.

11.5.3 Mechanisms for DHEA Action in Infertility

The first reported use of DHEA in fertility management documented an improved response to ovarian stimulation in a small group of patients with previous poor ovarian response [12]. Since then, numerous studies have investigated both the mechanism for its proposed reproductive benefits and the true impact of supplementation on clinical outcomes. There are many hypotheses to explain how DHEA may exert a positive influence on reproductive outcomes. The clinical observation that a hyper-androgenic environment leads to increased antral follicles is consistent with androgens having a trophic effect upon follicular growth and survival. Androgen treatment for as little as three to ten days has been shown to result in a significant increase in primary follicles in the animal model. Androgen treatment increases IGF-1 and IGF-1 receptor mRNAs, suggesting that an androgen-induced activation of IGF-1 signalling may play a role in triggering primordial follicle entry into the follicular pool [13]. Androgen treatment is also associated with a significant increase in granulosa cells and reduced granulosa cell death, with increased growth in secondary and small antral follicles [14]. Androgens induce and upregulate FSH receptors in granulosa cells, preventing atresia and amplifying FSH stimulatory effects [15]. Finally, administration of DHEA provides increased androgen substrate and may enhance ovarian follicular steroidogenesis [16].

In the setting of reproductive medicine, where clinicians are frequently challenged by poor ovarian response, a treatment that may positively influence the number of antral follicles available for recruitment, and the action of these gonadotrophins on those follicles, is worth further investigation.

11.5.4 Evidence for the Role of DHEA in Infertility Management

Early positive findings that DHEA use in women with poor ovarian reserve resulted in improved oocyte and embryo numbers, embryo quality and decreased cycle cancellation rates stimulated further interest in the true effect of DHEA [17].

A Cochrane review published in late 2015 summarized the impact of androgen treatment (DHEA and testosterone) for women undergoing assisted reproduction [18]. Twelve randomized controlled trials were identified that compared DHEA to either placebo (six studies) or no treatment (six studies). Of these, all but two included women who had been identified (by a variety of criteria) as ‘poor responders’. The treatment protocol varied between studies, but most used a daily oral dose of 75 mg DHEA for 12 weeks as a pre- and then co-treatment with a long gonadotropin-releasing hormone agonist (GnRHa) protocol.

The review found that DHEA pre-treatment was associated with an improvement in rates of live birth or ongoing pregnancy (OR 1.88, 95% CI 1.30–2.71; 8 RCTs, N = 878, moderate quality evidence). However, when trials at high risk of bias were removed, the effect size was reduced and no longer reached significance (OR 1.50, 95% CI 0.88–2.56; 5 RCTs, N = 306). This review found similar results for the use of testosterone, with pretreatment being associated with higher live birth rates (OR 2.60, 95% CI 1.30–5.20; 4 RCTs, N = 345, moderate evidence). However, when studies at high risk of bias were removed, only one study remained, demonstrating no difference in live birth rate when testosterone was administered (OR 2.00, 95% CI 0.17–23.49; 1 RCT, N = 53) (Figure 11.3).

The use of DHEA prior to IVF did not reduce miscarriage rates (OR 0.58, 95% CI 0.29–1.17; eight RCTs, N = 950, moderate quality evidence) (Figure 11.4).

The authors concluded that DHEA pretreatment may be associated with improved live birth rates; however, there was insufficient evidence to draw any conclusions about the safety of either treatment, and further research is required.

Since the publication of the Cochrane review in late 2015, several additional studies have added to the worldwide literature. In 2016, a double-blind RCT investigating the effect of DHEA on antral follicle count and ovarian response to gonadotrophin stimulation in ‘normal responders’ demonstrated no significant difference in this population [19]. The proposed mechanism of action of DHEA would anticipate this finding.

A further RCT of 140 women with poor ovarian response (according to the Bologna criteria) compared the use of DHEA 25 mg three times daily for 12 weeks prior to IVF/ICSI with no treatment. In this study of women undergoing cycles of controlled ovarian hyper-stimulation using antagonist pituitary suppression, those randomized to DHEA were found to have a significantly higher clinical pregnancy rate (32.8 vs 15.7 percent, $p=0.029$), ongoing pregnancy rate (28.5 vs 12.8 percent), number of retrieved oocytes (6.9 ± 3 vs 5.8 ± 3.1 , $p=0.03$), fertilization rate (62.3 ± 27.4 vs 52.2 ± 29.8 , $p=0.039$), reduced gonadotropins use (3383 ± 717.5 IU vs 3653.5 ± 856 IU, $p=0.045$) and days of stimulation (11.6 ± 1.8 vs 12.6 ± 1.06 , $p=0.001$) [20].

Recent systematic reviews not just of RCTs have found variably in favour of [21], against [22] and non-committal [23] about the use of DHEA supplementation.

The advice from governing bodies regarding use of DHEA as an adjuvant therapy is consistently negative. The British Fertility Society recommends that ‘the available evidence does not support the routine use of DHEA as an adjuvant in IVF cycles. The use of DHEA in this setting cannot therefore be supported’ [24]. The Endocrine Society clinical (2014) guidelines recommend against the general use of DHEA for infertility [11]. Similarly, the

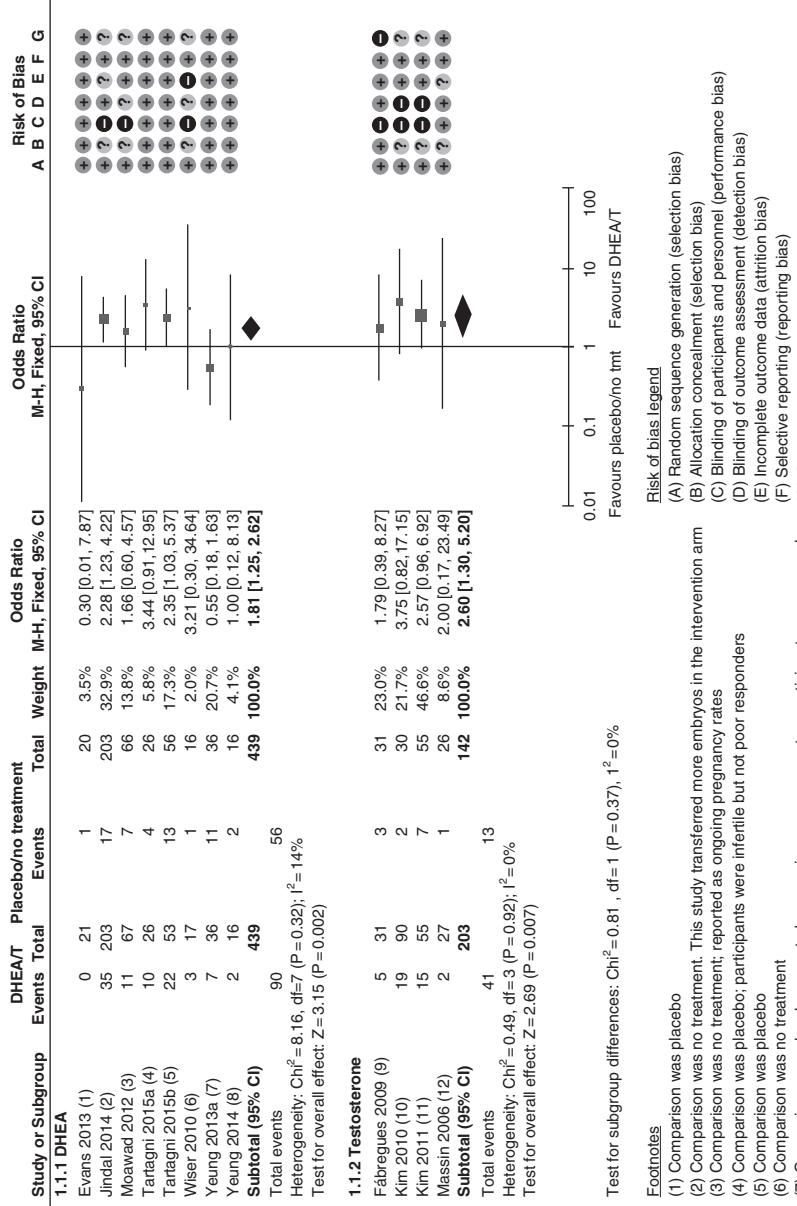


Figure 11.3 DHEA or testosterone versus placebo/no treatment, live birth/ongoing pregnancy rates. From [18] Reproduced with permission from John Wiley and Sons

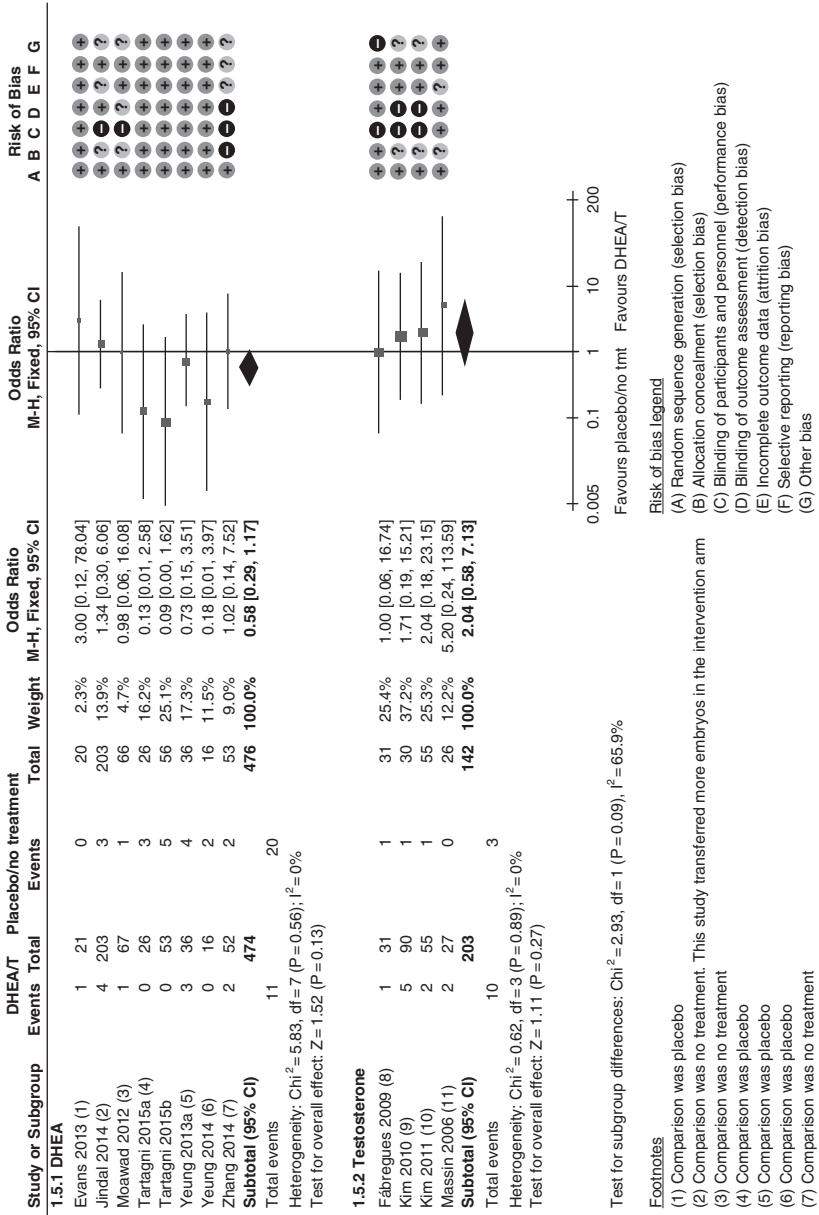


Figure 11.4 DHEA or testosterone versus placebo/no treatment, miscarriage rate. From [18] Reproduced with permission from John Wiley and Sons

National Institute for Health and Care Excellence (NICE) guidelines advise not to use DHEA as adjuvant treatment in IVF protocols [25].

11.6 Conclusion

Clinicians worldwide are faced daily with the clinical conundrum of how to improve IVF success rates in women of advanced reproductive age, premature ovarian ageing and those with persistently poor embryo quality. DHEA pretreatment is an adjuvant therapy proposed to improve IVF outcomes in women with poor ovarian response. Despite over a decade of investigation into the mode of action and true clinical efficacy of this androgen pro-hormone, there remains a lack of good quality evidence to support its routine introduction into clinical practice. While the side effect profile of DHEA makes it generally well tolerated, the potential effect of long-term androgen supplementation has not been sufficiently addressed. The best evidence from a meta-analysis of available RCTs indicates that DHEA supplementation may be associated with an improvement in live birth rates; however, there remains clinical uncertainty and caution should be exercised until further well-designed trials are available. In the meantime, many clinicians and patients are likely to continue to turn to DHEA when faced with repeated IVF failure.

References

1. Gleicher N, Kushnir VA, Albertini DF, Barad DH. (2016). Improvements in IVF in women of advanced age. *J Endocrinol.*, 230 (1), F1–6. Epub May 6.
2. Ferraretti E, La Marca A, Fauser B et al. on behalf of the ESHRE working group on Poor Ovarian Response Definition. (2011). ESHRE consensus on the definition of ‘poor response’ to ovarian stimulation for in vitro fertilization: the Bologna criteria. *Human Reproduction*, 26(7), 1616–1624.
3. Rittenberg V, Seshadri S, Sunkara SK et al. (2011). Effect of body mass index on IVF treatment outcome: an updated systematic review and meta-analysis. *Reprod Biomed Online*, 23(4), 421.
4. Augood C, Duckitt K, Templeton AA. (1998). Smoking and female infertility: a systematic review and meta-analysis. *Hum Reprod.*, 13(6), 1532.
5. Meldrum DR, Casper RF, Diez-Juan A et al. (2016). Aging and the environment affect gamete and embryo potential: can we intervene? *Fertil Steril.*, 105(3), 548–559.
6. Bentov Y, Hannam T, Jurisicova A, Esfandiari N, Casper RF. (2014). Coenzyme Q10 supplementation and oocyte aneuploidy in women undergoing IVF-ICSI treatment. *Clin Med Insights Reprod Health*. 8, 31–36.
7. Arlt W, Justl HG, Callies F et al. (1998). Oral dehydroepiandrosterone for adrenal androgen replacement: pharmacokinetics and peripheral conversion to androgens and estrogens in young healthy females after dexamethasone suppression. *J Clin Endocrinol Metab.*, 83, 1928–1934.
8. Franasiak JM, Thomas S, Ng S et al. (2016). Dehydroepiandrosterone (DHEA) supplementation results in supraphysiologic DHEA-S serum levels and progesterone assay interference that may impact clinical management in IVF. *Journal of Assisted Reproductive Genetics*, 33(3), 387–391.
9. Davison SL, Bell R, Donath S, Montalto JG, Davis SR. (2005). Androgen levels in adult females: changes with age, menopause, and oophorectomy. *J Clin Endocrinol Metab.*, 90, 3847–3853.
10. Wierman ME, Arlt W, Basson R et al. (2014). Androgen therapy in women: a reappraisal: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab.* 99(10), 3489–3510.
11. Elraiyah T, Sonbol MB, Wang Z et al. (2014). The benefits and harms of systemic dehydroepiandrosterone (DHEA) in

- postmenopausal women with normal adrenal function: a systematic review and meta-analysis. *J Clin Endocrinol Metab.* 99, 3536–3542.
12. Casson PR, Lindsay MS, Pisarska MD, Carson SA, Buster JE. (2000). Dehydroepiandrosterone supplementation augments ovarian stimulation in poor responders: a case series. *Hum Reprod.* 15, 2129–2132.
13. Vendola K, Zhou J, Wang J et al. (1999). Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary. *Biol Reprod.*, 61, 353–357.
14. Vendola KA, Zhou J, Adesanya OO, Weil SJ, Bondy CA. (1998) Androgens stimulate early stages of follicular growth in the primate ovary. *J Clin Invest.*, 101, 2622–2629.
15. Nielsen ME, Rasmussen IA, Kristensen SG et al. (2011). In human granulosa cells from small antral follicles, androgen receptor mRNA and androgen levels in follicular fluid correlate with FSH receptor mRNA. *Molecular Human Reproduction*, 17(1), 63–70.
16. Haning RV Jr, Hackett RJ, Flood CA et al. (1993). Plasma dehydroepiandrosterone sulfate serves as a prehormone for 48% of follicular fluid testosterone during treatment with menotropins. *Clin Endocrinol Metab.*, 76, 1301–1307.
17. Barad D, Gleicher N. (2006). Effect of dehydroepiandrosterone on oocyte and embryo yields, embryo grade and cell number in IVF. *Human Reproduction*, 21, 2845–2849.
18. Nagels HE, Rishworth JR, Siristatidis CS, Kroon B. Androgens (dehydroepiandrosterone or testosterone) for women undergoing assisted reproduction. *Cochrane Database of Systematic Reviews*, 2015, Issue 11. Art. No.: CD009749. DOI: 10.1002/14651858.CD009749.pub2.
19. Yeung T, Chai J, Li R et al. (2016). A double-blind randomised controlled trial on the effect of dehydroepiandrosterone on ovarian reserve markers, ovarian response and number of oocytes in anticipated normal ovarian responders. *British Journal of Obstetrics and Gynaecology*. 123(7), 1097–1105.
20. Kotb MM, Hassan AM, AwadAllah AM. (2016). Does dehydroepiandrosterone improve pregnancy rate in women undergoing IVF/ICSI with expected poor ovarian response according to the Bologna criteria? A randomized controlled trial. *European Journal of Obstetrics, Gynecology and Reproductive Biology*, 200, 11–15.
21. Zhang M, Niu W, Wang Y et al. (2016). Dehydroepiandrosterone treatment in women with poor ovarian response undergoing IVF or ICSI: a systematic review and meta-analysis. *J Assist Reprod Genet.*, 33(8), 981–991.
22. Triantafyllidou O, Sigalos G, Vlahos N. (2016). Dehydroepiandrosterone (DHEA) supplementation and IVF outcome in poor responders. *Hum Fertil (Camb)*. 7, 1–8. [Epub ahead of print]
23. Qin JC, Fan L, Qin AP. (2016). The effect of dehydroepiandrosterone (DHEA) supplementation on women with diminished ovarian reserve (DOR) in IVF cycle: Evidence from a meta-analysis. *J Gynecol Obstet Biol Reprod (Paris)*. 2016 May 19. p ii: S0368-2315(16)00003-X. doi: 10.
24. Nardo L, El-Toukhy T, Stewart J, Balen AH, Potdar N. (2015). British fertility society policy and practice committee: adjuvants in IVF: evidence for good clinical practice. *Human Fertility*, 18 (1), 2–15, DOI: 10.3109/14647273.2015.985454
25. National Institute for Health and Care Excellence 2016.

Implications of Polycystic Ovary Syndrome on Oocyte Quality

Zi-Jiang Chen and Linlin Cui

12.1 Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age. It is characterized by a cluster of clinical manifestations, including ovulation dysfunction, hirsutism, acne, and polycystic ovarian morphology on ultrasonography. A number of endocrine and metabolic alterations, such as hypersecretion of luteinizing hormone (LH) and androgen, hyperinsulinemia, obesity, and dyslipidemia, are evident in a majority of affected individuals [1]. Although the infertility associated with PCOS is primarily due to chronic anovulation, the outcomes of fertility treatment were also compromised by potential poor oocyte quality with lowered fertilization rate and increased miscarriage risk [2]. In this chapter, we review PCOS-specific changes during oogenesis, nuclear and cytoplasmic maturation, and epigenetic regulation in humans.

12.2 Oogenesis in PCOS

Normal oogenesis and oocyte maturation are requisite for pregnancy. Oogenesis starts at fetal phase and forms a so-called “resting pool” of oocytes which is actually invisible and hard to evaluate. As the follicles enter the “growing pool”, the primordial follicles develop into primary follicles and become quantitative. with the beginning of anti-müllerian hormone (AMH) secretion. PCOS is characterized by increased pre-antral and small antral follicles developing. It is reasonable to suppose an alteration in this initial transition process. Three hypotheses have been proposed to be responsible for the multi-follicles recruitment and arrest: i) a larger resting pool formed at fetal stage, ii) accelerated transition to growing pool, and iii) decreased loss of oocytes. Good evidence comes from the histologic sections of cortical biopsies from the ovaries of women with PCOS. They showed a six-fold increased density of follicles at both primordial and primary stage. An elevated percentage of primordial-to-primary follicles was also found [3]. Alterations in secretion and action of androgen, gonadotropin, and anti-Müllerian hormone (AMH) are well demonstrated pathogenic mechanisms [12,13]. Hyper-secretion of androgen (HA) was supposed to accelerate early growth of primordial follicle into pre-antral follicles through impairing hypothalamic – pituitary – ovarian (HPO) axis regulation, inducing persistent increases in gonadotropin-releasing hormone (GnRH) pulse frequency, as well as stimulating hypersecretion of LH over FSH and related increased LH/FSH ratio. Increased number of pre-antral follicles and enhanced secretion ability of individual follicles thus leads to over-production of AMH, which was supported by the 2-3 times increase of serum AMH level and higher follicular fluid (FF)-derived AMH per follicle being detected in PCOS patients, compared with normal women [4] [5]. AMH is an inhibitor of follicle stimulating hormone (FSH) and

cAMP-stimulated aromatase activity. Over-secretion of AMH will exacerbate follicular FSH resistance in the growing FSH-dependent follicles, attenuates the FSH stimulating effect, and inhibits aromatase activity in granulosa cells that contributes to chronic HA. There is evidence reported on the association of neuroendocrine deregulation with impaired oocyte competence from the beginning of foliculogenesis in PCOS, but the molecular mechanism is unknown [6]. Better in vitro fertilization (IVF) outcomes, such as higher cumulative pregnancy and live birth rates, in polycystic ovarian morphology (PCOM)-only women compared with PCOS patients suggested furthermore that changes of intra and extra ovarian environment played an dominant role rather than multi-follicle recruitment [7].

12.3 Oocyte Maturation in PCOS

Oocyte maturation encompasses three essential developmental programs: (i) nuclear maturation, (ii) epigenetic maturation, and (iii) cytoplasmic maturation. They do not occur one after another. Actually, epigenetic modification and cytoplasmic maturation occur throughout oocyte growth, and in parallel with nuclear maturation [8]. Alterations in any of these three programs may affect oocyte quality and therefore lead to a diminished fertility potential, including fertilization rate and embryo competence. Although contradictory results have been reported, majority of studies suggests adverse changes happened during oocyte maturation and consequently conferred to impaired oocyte competence in PCOS.

12.3.1 Nuclear Maturation

Nuclear maturation refers to the reinitiation of the first meiotic division and progression to metaphase II with a stable arrest. By the induction of preovulatory LH surge, the arrested oocyte reinitiates meiosis, and complete homologous chromosomes separation and assembly. PCOS patients have typically hyper-secretion of LH even in follicular phase. It is reasonable to suspect that tonic elevated LH and premature LH receptor expression in somatic follicular cell would activate reinitiation of meiosis before ovulation, result in impaired extrusion of the first polar body, and consequently lead to chromosomal abnormality of the oocyte. However, in PCOS women undergoing IVF or intracytoplasmic sperm injection (ICSI), a similar or even higher MII oocyte rate has been observed compared with normal women [9–11]. Besides, according to available studies, embryonic aneuploidy rate in women with PCOS was comparable to normal controls, which also supported a normal chromosomal assembly during oocyte nuclear maturation [12].

12.3.2 Epigenetic Maturation

Epigenetic maturation occurs with oocyte growth. It sets gamete-specific imprints on the genome and regulates gene expression through oogenesis and maturation process. Genomic imprint includes changes in DNA methylation, acetylation, and methylation of different lysine residues in histone proteins. Both the epigenetic reprogramming and the timing of the process are essential for normal meiosis and embryonic development. Omics data from oocytes and supporting somatic cells from PCOS women and normal counterparts highlighted differential expression of genes involved in spindle dynamics, homologous recombination, as well as chromosome alignment, such as *Mater/NALP5*, *BNC1*, and *FMN2*, which was hypothesized to natively affect oocyte quality [13]. Transcriptional profiles of

cumulus-oocyte complexes (COC) in PCOS also revealed differing expression involving a subset of key genes of oocyte nuclear maturation, including *CTNNB1*, *SFRP4*, and *TNIK* within Wnt signaling, *GABRA5*, *GRIN2A*, and *LHCGR* associated with receptor interactions, as well as *ADAMTS-9* and *ANK2* involved in cytoskeleton and extracellular matrix [14]. Noncoding RNAs caused RNA silencing is another way to regulate genetic expression. Differential expression of miRNAs indicated altered regulation of genes of Wnt, MAPK and Notch signaling pathways, as well as oocyte meiosis and maturation, while lncRNAs expression in PCOS granulosa cells suggested impacted folliculogenesis, oocyte maturation, and ovarian steroidogenesis [15]. Nevertheless, it should be emphasized that the current results cannot prove the etiological effect of epigenetic changes on oocyte competence in PCOS due to a lack of direct evidence on oocyte maturation or fertilization.

12.3.3 Cytoplasmic Maturation

Cytoplasmic maturation defines the process in the cytoplasm beyond meiosis, preparing the oocyte for fertilization and early embryonic development. It is under close regulation of subtle intrafollicular microenvironment. Estradiol was found to play an essential role in oocyte cytoplasmic maturation which was antagonized by androgen. It was demonstrated that the follicle fluid hyperandrogenic environment together with the elevated 5 α -reductase activity in women with PCOS interferes with estradiol action in oocyte cytoplasmic maturation [16, 17].

12.4 Metabolic Disturbance as a Rationale for Impaired Oocyte Quality

Several studies suggested metabolism-induced changes in the oocyte through the intrafollicular microenvironment in PCOS. It was found that women with PCOS and obesity had smaller oocytes compared with non-PCOS women [18], though solid evidence is still lacking about the effect of oocyte size on developmental competence and pregnancy outcome. But lower fertilization rate and repeated implantation failure were confirmed in obese PCOS women with insulin resistance [19]. Besides, PCOS women have increased levels of free fatty acid (FFA) profiles in both plasma and follicular fluid, which were positively correlated with poor COC morphology and embryo fragmentation score [20]. FFA-induced lipotoxicity, protein secretion alteration, and endoplasmic reticulum stress are the potential negative factors on oocyte quality. Enhanced positive feedback loop between advanced glycation end products (AGEs) and AGE receptors (RAGEs) in PCOS is another suggested explanation of how metabolic disturbance affected oocyte competence. It was reported that obesity, insulin resistance, and hyperglycemia can enhance AGE-RAGE actions which were negatively correlated with the number of MII oocytes and fertilization rate [21].

12.5 Potential Effect of Heterogeneous Phenotypes

As stated in the beginning, several studies with negative or even opposite results also argued for a comparable oocyte quality in PCOS. It was reported that pregnancy rate of IVF was similar between women with PCOS and tube factor based on data from the Society for Assisted Reproductive Technologies registry. Moreover, the live birth rate was even higher in advanced aged women with a previous diagnosis of PCOS [22]. The most possible reason may be the heterogeneity in etiology and in vivo microenvironment between different

phenotypes. It was found that clinical pregnancy rate was increased in patients with all three phenotypes compared with those with only ovulatory dysfunction and hyperandrogenism [23]. The fertilization rate in PCOS was found to be lower in conventional IVF/ICSI cycles, but comparable in IVM cycles. That also indicated the adverse effect of PCOS-specific in vivo microenvironment on oocyte competence [11, 24, 25]. Another reason may be due to the possibly slower age-dependent decrease of competent antral follicles in PCOS compared with normal ovulated women.

12.6 Conclusions

Deficiency of oocyte developmental competency exists in PCOS. Although many indications and much evidence have been obtained, the story is still far from completed. Many key questions remain unresolved. Is the nuclear maturation affected in PCOS? Which pathway was the target of the endocrinological and metabolic microenvironment dysfunctional? What are the PCOS-specific changes of maternal effect factors? What are the functional changes caused by the differential gene expression? When and how does the detrimental effect from granulosa cells work on the oocyte? How to reverse these pathophysiological changes? The answers are essential to understand the fundamental mechanisms of PCOS on oocyte quality, and thus critical for novel therapeutic targets to improve reproductive outcome.

References

1. Dumesic, D.A., S.E. Oberfield, E. Stener-Victorin et al., Scientific statement on the diagnostic criteria, epidemiology, pathophysiology, and molecular genetics of polycystic ovary syndrome. *Endocr Rev* 2015, **36**(5): pp. 487–525.
2. Qiao, J. and H.L. Feng, Extra- and intra-ovarian factors in polycystic ovary syndrome: impact on oocyte maturation and embryo developmental competence. *Hum Reprod Update* 2011, **17**(1): pp. 17–33.
3. Webber, L.J., S. Stubbs, J. Stark et al., Formation and early development of follicles in the polycystic ovary. *Lancet* 2003, **362**(9389): pp. 1017–21.
4. Homburg, R. and G. Crawford, The role of AMH in anovulation associated with PCOS: a hypothesis. *Hum Reprod* 2014, **29**(6): pp. 1117–21.
5. Stubbs, S.A., J. Stark, S.M. Dilworth, S. Franks, and K. Hardy, Abnormal preantral folliculogenesis in polycystic ovaries is associated with increased granulosa cell division. *J Clin Endocrinol Metab* 2007, **92**(11): pp. 4418–26.
6. Dewailly, D., G. Robin, M. Peigne et al., Interactions between androgens, FSH, anti-Müllerian hormone and estradiol during folliculogenesis in the human normal and polycystic ovary. *Hum Reprod Update* 2016, **22**(6): pp. 709–24.
7. Li, H.W., V.C. Lee, E.Y. Lau et al., Cumulative live-birth rate in women with polycystic ovary syndrome or isolated polycystic ovaries undergoing in-vitro fertilisation treatment. *J Assist Reprod Genet* 2014, **31**(2): pp. 205–11.
8. Eppig, John J., M.M.V., CarrieMarin Bivens, and Rabindranath de La Fuente, Regulation of Mammalian Oocyte Maturation, in E.Y.A. Peter and C.D. Leung (eds.) *The Ovary*. 2004 Elsevier Academic Press, Oxford UK. pp. 118–23.
9. Sigala, J., C. Sifer, D. Dewailly et al., Is polycystic ovarian morphology related to a poor oocyte quality after controlled ovarian hyperstimulation for intracytoplasmic sperm injection? Results from a prospective, comparative study. *Fertil Steril* 2015, **103**(1): pp. 112–18.
10. Esinler, I., U. Bayar, G. Bozdag, and H. Yarali, Outcome of intracytoplasmic sperm injection in patients with polycystic ovary syndrome or isolated polycystic ovaries. *Fertil Steril* 2005, **84**(4): pp. 932–7.

11. Heijnen, E.M., M.J. Eijkemans, E.G. Hughes et al., A meta-analysis of outcomes of conventional IVF in women with polycystic ovary syndrome. *Hum Reprod Update* 2006, **12**(1): pp. 13–21.
12. Weghofer, A., S. Munne, S. Chen, D. Barad, and N. Gleicher, Lack of association between polycystic ovary syndrome and embryonic aneuploidy. *Fertil Steril* 2007, **88**(4): pp. 900–5.
13. Wood, J.R., D.A. Dumesic, D.H. Abbott, and J.F. Strauss, 3rd, Molecular abnormalities in oocytes from women with polycystic ovary syndrome revealed by microarray analysis. *J Clin Endocrinol Metab* 2007, **92**(2): pp. 705–13.
14. Huang, X., C. Hao, X. Shen et al., Differences in the transcriptional profiles of human cumulus cells isolated from MI and MII oocytes of patients with polycystic ovary syndrome. *Reproduction* 2013, **145**(6): pp. 597–608.
15. Liu, S., X. Zhang, C. Shi et al., Altered microRNAs expression profiling in cumulus cells from patients with polycystic ovary syndrome. *J Transl Med* 2015, **13**: p. 238.
16. Tesarik, J. and C. Mendoza, Direct non-genomic effects of follicular steroids on maturing human oocytes: oestrogen versus androgen antagonism. *Hum Reprod Update* 1997, **3**(2): pp. 95–100.
17. Vassiliadi, D.A., T.M. Barber, B.A. Hughes et al., Increased 5 alpha-reductase activity and adrenocortical drive in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* 2009, **94**(9): pp. 3558–66.
18. Marquard, K.L., S.M. Stephens, E.S. Jungheim et al., Polycystic ovary syndrome and maternal obesity affect oocyte size in in vitro fertilization/intracytoplasmic sperm injection cycles. *Fertil Steril* 2011, **95**(6): pp. 2146–9, 2149 e1.
19. Cano, F., J.A. Garcia-Velasco, A. Millet et al., Oocyte quality in polycystic ovaries revisited: identification of a particular subgroup of women. *J Assist Reprod Genet* 1997, **14**(5): pp. 254–61.
20. Niu, Z., N. Lin, R. Gu, Y. Sun, and Y. Feng, Associations between insulin resistance, free fatty acids, and oocyte quality in polycystic ovary syndrome during in vitro fertilization. *J Clin Endocrinol Metab* 2014, **99**(11): pp. E2269–76.
21. Merhi, Z., Advanced glycation end products and their relevance in female reproduction. *Hum Reprod* 2014, **29**(1): pp. 135–45.
22. Kalra, S.K., S.J. Ratcliffe, and A. Dokras, Is the fertile window extended in women with polycystic ovary syndrome? Utilizing the Society for Assisted Reproductive Technology registry to assess the impact of reproductive aging on live-birth rate. *Fertil Steril* 2013, **100**(1): pp. 208–13.
23. Ramezanali, F., M. Ashrafi, M. Hemat et al., Assisted reproductive outcomes in women with different polycystic ovary syndrome phenotypes: the predictive value of anti-Mullerian hormone. *Reprod Biomed Online* 2016, **32**(5): pp. 503–12.
24. Sahu, B., O. Ozturk, M. Ranieri, and P. Serhal, Comparison of oocyte quality and intracytoplasmic sperm injection outcome in women with isolated polycystic ovaries or polycystic ovarian syndrome. *Arch Gynecol Obstet* 2008, **277**(3): pp. 239–44.
25. Siristatidis, C., T.N. Sergentanis, P. Vogiatzi et al., In vitro maturation in women with vs. without polycystic ovarian syndrome: A systematic review and meta-analysis. *PLoS One* 2015, **10**(8): p. e0134696.

The Use of Melatonin to Improve Oocyte Development

Shavi Fernando and Luk Rombauts

13.1 Melatonin

13.1.1 Secretion

Melatonin (*N*-acetyl-5-methoxytryptamine) is a neuro-hormone mainly synthesized in the pineal gland. It is produced in response to ambient darkness, with plasma levels increasing between 1800 and 2000 hours and peaking between midnight and 0500 hours. The diurnal variation is significant, with levels before 0900 hours being five times higher than those after 1100 hours.

13.1.2 Actions and Safety of Melatonin

13.1.2.1 Classical Actions

In humans, melatonin is associated with sleep disturbances, including insomnia, and much of the literature is focused on this area. It has been identified as a key factor in the regulation of circadian rhythms and there is modern concern that prolonged exposure to artificial lighting beyond those that are natural might reduce endogenous melatonin exposure. Interestingly, while melatonin regulates seasonal differences in reproductive activity in many species, this does not apply to primates, suggesting that its role extends further than simply the control of sleep patterns in these species.

13.1.3 Oxygen Scavengers and 'Oxidative Stress'

Normal physiological functions result in the production of reactive oxygen species (ROS). While these can be necessary for certain biological processes, an excess of ROS can damage cells and is referred to as 'oxidative stress'. ROS can be neutralized and rendered inert by the use of oxygen scavengers, or 'antioxidants' such as melatonin.

By the nature of the physiology of oxygen species, when an antioxidant is utilized, it has the potential to become an ROS itself. Melatonin is unique. Not only is it a potent antioxidant that has the capability of augmenting the action of naturally occurring oxygen scavengers, but it is also a suicidal terminal antioxidant, meaning that it does not promote oxidation under any circumstance. In addition, its secondary and tertiary metabolites are capable of acting as antioxidants (Figure 13.1) [1].

It is therefore not surprising that melatonin has garnered such interest in several disciplines of medicine in which oxidative stress has been implicated, including endometriosis, glaucoma, diabetes and even in adjuvant therapy to prevent side effects of chemotherapy and radiotherapy.

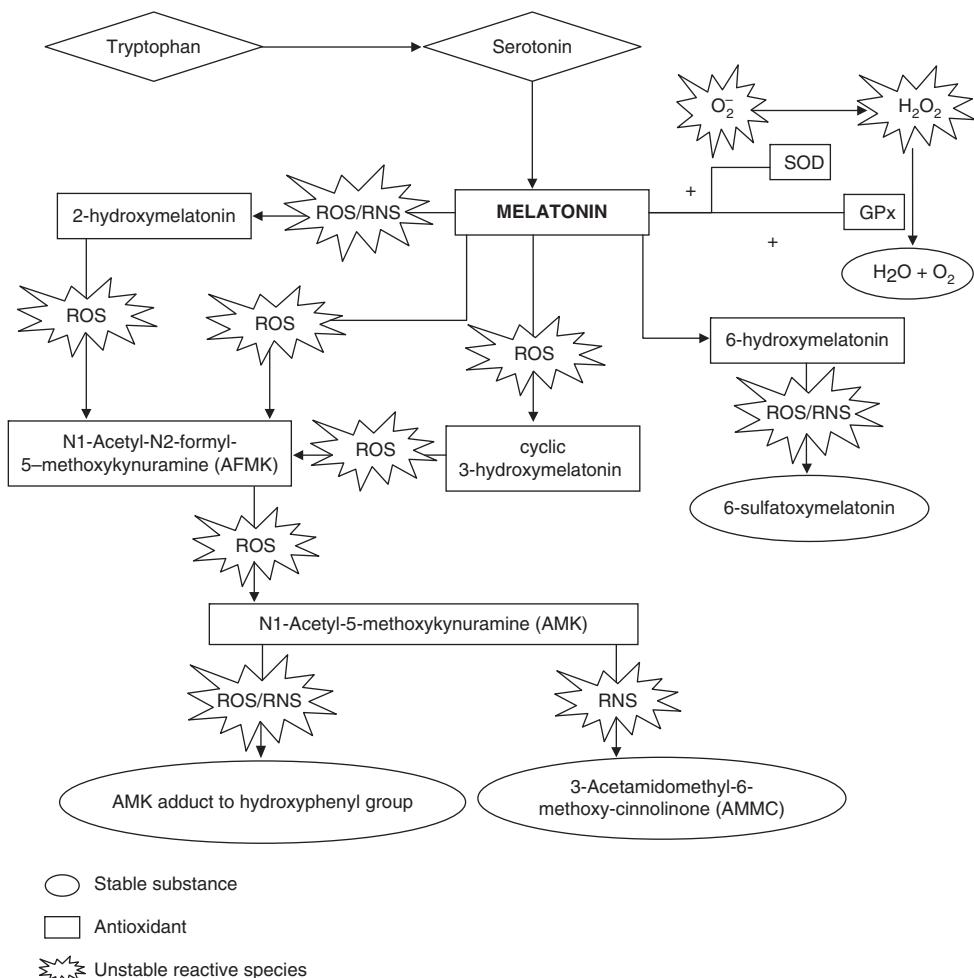


Figure 13.1 Actions of melatonin and its metabolites. Reproduced with permission from Fernando et al. [1], extrapolated from Hardeland [26], Reiter et al. [27] and Watson [28]

GPx: glutathione peroxidase; SOD: superoxide dismutase; ROS: reactive oxygen species; RNS: reactive nitrogen species

13.1.4 The Role of Melatonin in Reproduction

As discussed earlier, melatonin is a circadian hormone, meaning that its daily variation is significant. Few human studies have been performed to assess the levels of melatonin in relation to the menstrual cycle; however, those that have suggest that serum levels are minimal just prior to ovulation, with a peak occurring during the luteal phase [2]. This variation, which may be in response to menstrual hormonal changes, implies that the roles of melatonin may change dependent on the stage of the menstrual cycle, also suggesting a role in reproductive activity.

It is well known that shift-workers experience greater menstrual disturbance and circadian irregularity than daytime workers. Investigators have shown that shift-workers have altered menstrual patterns [3]. While having significantly different serum melatonin levels, these women do not experience concomitant alteration in FSH and LH levels [4]. This might suggest that it is the melatonin that is responsible for the menstrual disturbance that occurs in these women.

The variation of melatonin levels during the menstrual cycle and its apparent ability to modify the release of gonadotrophic hormones are illustrated in the observation that in high doses and when combined with progesterone, melatonin has the ability to inhibit ovulation [5]. However, it is impossible to determine whether this effect is due to the progesterone or the melatonin, and no study has so far been done to clarify this issue.

Receptors for melatonin have also been identified on granulosa cells, which therefore represents another potential site of melatonin action. This also explains the finding that melatonin preferentially concentrates in the ovaries (with follicular fluid concentrations being much higher than those found in the serum), and in particular, in larger and more mature ovarian follicles [6]. This indicates that the total antioxidant capacity within large follicles is higher than that within small follicles, suggesting a role of melatonin in the maturation and development of follicles and perhaps even oocytes. However, whether it is the melatonin that results in follicular maturity or maturity that results in higher levels of melatonin is still not clear.

In addition to its apparent influence on the menstrual cycle and fertility, the role of melatonin has also been investigated during pregnancy itself, with levels increasing after 24 weeks [7]. Interest is gathering around its influence on preventing preeclampsia, intrauterine growth restriction and neonatal neurological morbidity.

Therefore, the importance of melatonin in the human reproductive cycle has been illustrated consistently and its effect seems to be primarily related to its ability to reduce oxidative stress.

13.1.5 Safety

The treatment of infertile and pregnant populations is a sensitive subject, particularly with regard to drug safety. Before melatonin can be recommended for use in this population, as well as determining efficacy, it is imperative to assess its safety profile.

In both animal and human studies, melatonin has proven to have no significant teratogenic effects, and even in extremely high doses (5–20 mg/day for up to 12 weeks) it has not been associated with either significant sedative effects or hepatotoxicity.

There have been case reports of melatonin negatively affecting autoimmune conditions through its immune-stimulatory capabilities. This includes the potential to exacerbate rheumatoid arthritis and autoimmune hepatitis. While causality has not yet been proven, until further research is performed, it is recommended that melatonin be avoided in those with autoimmune conditions.

13.2 Infertility Treatment

13.2.1 Oxidative Stress in ART

Oxidative stress impairs the in vitro fertilization (IVF) process from start to finish, and has the potential to reduce the quality of oocytes and embryos and therefore limit the

successful pregnancy outcome after IVF treatment. The hormonal influence of ovarian stimulation results in a disturbance to the antioxidant milieu present in the ovarian follicles, resulting in a higher risk of *in vivo* oxidative stress to the developing oocyte. Once removed from the antioxidant-rich follicular fluid, oocytes are left vulnerable to free radical attack. They may also be exposed to high levels of oxygen while being transported and while in incubators, with higher ambient oxygen tensions being associated with higher levels of oxidative stress.

A retrospective analysis of 138 intracytoplasmic sperm injection (ICSI) patients found that the follicular fluid in patients who achieved a successful pregnancy had a significantly higher level of antioxidants and significantly lower level of ROS [8]. While the study was small, this supports the hypothesis that intra-follicular oxidative stress impairs IVF success rates.

Oxidative stress has also been implicated in the pathogenesis of male factor infertility, particularly through its influence on the endogenous oxygen scavenger, superoxide dismutase, despite the necessity of ROS for normal sperm capacitation events.

With growing evidence suggesting the negative effects that oxidative stress can have on the treatment of infertility, investigators have begun to focus on the potential benefits of 'low-risk' oxygen scavenger therapies such as melatonin.

13.2.2 The Role of Melatonin in Assisted Reproductive Technology

Oxidative stress-related cellular damage can occur at every stage of the IVF process, and it is suggested that oxygen scavengers such as melatonin might be able to protect oocytes and embryos from such damage. There have been a number of interventional studies focusing on the use of oral melatonin treatment as an adjuvant therapy during the stimulation protocol of an IVF cycle. Currently available human studies assessing the use of melatonin in IVF are discussed in the following and summarized in Table 13.1.

13.2.3 Effects of Melatonin on Oocyte Quality

Mitochondria contain their own antioxidants to help balance the amount of ROS produced during cellular respiration and energy production. Melatonin can improve the efficiency of electron transport in mitochondria, which results in a drop in the production of excess ROS and subsequently protects DNA from damage, sometimes even more effectively than specific mitochondrial antioxidants.

Following the process of normal ovulation, the production of inflammatory mediators is associated with an increase in ROS. These species result in an inhibition of oocyte maturation, meaning that from the time of ovulation, oocyte quality begins to diminish. A recent murine study showed that this may occur as early as eight hours after ovulation and progresses rapidly. By showing that the addition of melatonin to oocyte culture media prevented these effects, resulting in a higher proportion of oocytes reaching the blastocyst stage, this study demonstrated that an imbalance of ROS can impair oocyte quality and that melatonin can inhibit these effects [9].

Tamura and colleagues showed that melatonin concentrations within the follicular fluid are directly proportional to follicular growth and development [10]. They are also inversely proportional to the levels of oxidative stress markers in follicular fluid. This finding demonstrates that the follicular fluid from healthy follicles contains a balance of oxidative and antioxidative species that is protective of the oocyte.

Table 13.1 Summary of human studies assessing the use of melatonin in IVF

Study	Design	NICE Level of evidence	Sample size	Intervention	Control	Outcomes
Melatonin alone						
Tamura et al. 2008 [11]	Prospective cohort	2 ⁺	115	3 mg melatonin po from day 5 to oocyte collection (n=56)	No melatonin (n=59)	No difference in fertilization or clinical pregnancy rate
Tamura et al. 2008 [11]	Uncontrolled before-after study	2 ⁻	112	3 mg melatonin po from day 5 to oocyte collection (n=56)	Previous cycle without melatonin (n=56)	Higher fertilization rate in melatonin cycle (50 vs 20.2%)*
Nishihara et al. 2014 [29]	Uncontrolled before-after study	2 ⁻	97	3 mg melatonin po for at least 2 weeks leading up to HCG trigger in second cycle (n=97)	No melatonin in first cycle (n=97)	No difference in pregnancy rate
Batioglu et al. 2012 [30]	Single-blinded randomized controlled trial (only embryologists were blinded)	1 ⁻	85	3 mg melatonin po (n=40)	No melatonin (n=45)	Higher percentage of MII oocytes in melatonin group (81.9 vs 75.8%)*
						Higher number of G1 embryos (3.2 vs 2.5)*
						No difference in number of oocytes, fertilization rate or clinical pregnancy rate

Table 13.1 (cont.)

Study	Design	NICE Level of evidence	Sample size	Intervention	Control	Outcomes
Eryilmaz et al. 2011 [18]	Unblinded randomized controlled trial	1-	60	3 mg melatonin po from day 3–5 until HCG injection (<i>n</i> =30)	No melatonin (<i>n</i> =30)	Higher number of oocytes in melatonin group (11.5 vs 6.9)* Higher MII oocyte counts (9.4%)*
Tamura et al. 2012 [6]	Uncontrolled before – after study	2-	9	3 mg melatonin po from day 5 of menstrual cycle to oocyte collection (<i>n</i> =9)	Previous cycle without melatonin (<i>n</i> =9)	Higher rate of good embryos in melatonin cycle (65 vs 27%)*
Combinations with melatonin						
Pacchiarotti et al. 2016 [22]	Double-blinded randomized controlled trial	1+	526	PCOS patients only 400mcg folate (<i>n</i> =195)		Higher percentage of mature oocytes in melatonin group compared to controls (48.2 vs 38.0%)* Higher percentage of G1 embryos (45.7 vs 25.6%)*

Rizzo et al. 2010 [19]	Unblinded randomized controlled trial	1-	65	3 mg melatonin daily + 2 g myoinositol po bd + 200 mcg folic acid po bd from day of GnRH administration (<i>n</i> =32)
				2 g myoinositol po bd + 200 mcg folic acid po bd from day of GnRH administration (<i>n</i> =33)
Unfer et al. 2011 [21]	Uncontrolled before – after study	2-	46	2 g myoinositol po + 200 mcg folic acid po in the morning and 3 mg melatonin po + 2 g myoinositol po + 200 mcg folic acid po in the evening for 3 months leading to second cycle of IVF

IVF: in vitro fertilization; NICE: National Institute for Health and Care Excellence; *statistically significant; G1: grade 1; G2: grade 2; M1: meiosis I; MII: meiosis II; lCSL: intracytoplasmic sperm injection; HCG: human chorionic gonadotrophin; GnRH: gonadotrophin-releasing hormone; po: per oral; bd: twice per day

The supplementation of oocyte culture media with melatonin is more controversial, with conflicting animal results in the literature. A murine study found that melatonin supplementation of culture media in vitro appears to have a dose-response relationship between the amount of melatonin used and the number of mature oocytes [11]. These findings are in agreement with a bovine study assessing the same outcome [12]. Unfortunately, other animal studies have found that, rather than higher doses always being more protective, there may be an optimal level of melatonin concentration in culture media, ranging from 10^{-6} to 10^{-9} M, with both lower and higher doses being associated with negative effects [13, 14]. Human studies are in agreement with the latter hypothesis, finding a media melatonin concentration of 10^{-5} M to 10^{-9} M to be optimal for improving nuclear maturation [15], implantation rate and maybe even pregnancy rates (not statistically significant) [16], and agreeing that higher doses appeared to worsen outcomes.

While it appears that melatonin does have a beneficial effect on oocyte culture media, before supplementation is universally recommended, further human studies are required to determine the optimal dose.

Much of the literature surrounding melatonin in infertility treatments has been positive, with a recent review concluding that oral administration of melatonin reduces intra-follicular oxidative stress and increases fertilization rates [6]. However, the answer does not seem to be as simple, with most studies being hampered by a ‘within patients’ study design, where patients are used as their own controls. Drawing conclusions based on these studies is, therefore, fraught with hazard as any effect demonstrated must be assumed to be explained by a regression towards the mean [17].

Clinical human studies have largely been promising; however, all have been limited by study design limitations.

In a small open-label randomized controlled trial assessing melatonin supplementation in women with sleep disturbances undergoing IVF [18], women were randomized to receive 3 mg nocte of oral melatonin during their ovarian stimulation or no additional treatment. Despite the finding of an increase in the number of oocytes, number of mature oocytes and improvement in the quality of embryos, the authors could not control for the number of previous failed IVF attempts (mean duration of infertility was six to seven years), the use of other concurrent adjuvants or the aetiology of infertility.

Despite these significant limitations, these findings were in agreement with another open-label randomized trial in which 80 women were randomized to receive melatonin or no treatment during their ovarian stimulation. The investigators found an increase in clinical pregnancy rate which did not reach statistical significance (likely because of the relatively small sample size). While this study also found an increase in the proportion of mature oocytes and high-quality embryos, patients with cancelled cycles were excluded from the analysis, making these results subject to bias and difficult to interpret with accuracy.

A common limitation of the clinical human studies already discussed is that they lack a placebo control. Perhaps understandably, randomizing IVF patients to a placebo control when there may be even a slight possibility that the intervention has a beneficial effect can be logistically challenging. Other investigators have attempted to circumvent this challenge by using adjuvant combinations as a control group. This also does not sufficiently provide a solution to the question of the usefulness of melatonin in IVF. For example, a prospective controlled trial that compared myo-inositol (insulin sensitizing agent) and folate

supplementation (control group) to myo-inositol, folate and melatonin (intervention group) found that the intervention group experienced improvements in mature oocyte number and embryo quality [19]. Melatonin is known to synergize with other antioxidants, and whether or not the observed positive effect is related to melatonin or its interaction with myo-inositol is still not adequately addressed, especially as myo-inositol itself is associated with improved IVF outcomes.

13.2.4 Melatonin in Embryo Culture Media

Oocyte culture media environment is very important when considering ROS, and the same can be said of embryo culture media. While animal studies have shown an overall beneficial effect of melatonin supplementation of embryo culture media, there is a paucity of human data. Similarly to supplementation of oocyte culture media, supplementation of embryo media appears to have an ‘optimal range’ of melatonin concentration (10^{-5} and 10^{-11}M) [20], with higher concentrations being harmful.

Once again, further human studies would be required before suggesting inclusion of embryo culture supplementation in standard IVF treatment protocols.

13.2.5 Effects of Melatonin on Pregnancy Rates – Human Studies

Irrespective of the potentially positive effects of melatonin on oocyte and embryo number and quality, the outcome of importance for an infertile couple is live birth rate, or ‘take home baby’ rate. Thus far, no study has successfully achieved an estimate of this outcome in human patients treated with melatonin. Indeed, even those studies that have aimed to determine the efficacy of melatonin in improving clinical pregnancy rates have considered it in combination with folic acid and myo-inositol. On average, these studies have suggested positive effects of melatonin in these populations.

Rizzo et al. [19] in a prospective trial of 65 women compared myo-inositol and folate supplementation to myo-inositol, folate and melatonin. They found a trend towards a higher clinical pregnancy rate in the melatonin group, but this did not reach statistical significance.

Another prospective longitudinal cohort study addressed the effects on myo-inositol and melatonin supplementation in 46 women who failed to conceive in previous IVF cycles because of poor oocyte quality [21]. Participants were treated with myo-inositol 4 g/day and melatonin 3 mg/day for three months and then underwent another IVF cycle. After this treatment, there were statistically significant improvements in the number of mature oocytes and fertilization rate. As discussed previously, because this was a before-after study and patients were only included if they failed to conceive in their first cycle, it is difficult to comment on the significance of this clinical pregnancy rate as an appropriate control group was not used.

In the largest double-blind randomized-controlled trial addressing these agents, which looked specifically at patients with PCOS undergoing their first cycle of ICSI, Pacchiarotti et al. [22] allocated 165 patients to triple therapy (myo-inositol 4 g, folic acid 400 mcg and melatonin 3 mg per day) and 166 patients to myo-inositol and folic acid alone and 195 patients to receive only folic acid. With this larger sample size, they found higher numbers of mature oocytes and grade 1 embryos in patients treated with triple therapy, supporting the role of melatonin in the treatment of infertility caused by PCOS. This is arguably the largest and best-designed study published to date addressing the question of the efficacy of melatonin in IVF treatment. However, this does not necessarily demonstrate an

independent effect of melatonin on embryo quality or oocyte maturity, and as discussed previously, may represent a synergistic effect with myo-inositol and folic acid, although this has not been proven.

Tamura et al. [11] assessed 115 patients who failed to become pregnant in a previous cycle of IVF/ET, with a fertilization rate of less than or equal to 50 percent. They used a dose of 3 mg/day of melatonin in the subsequent IVF cycle during ovarian stimulation. Compared with their first cycle, the fertilization rate was significantly higher after taking melatonin in their second cycle (50.0 ± 38.0 vs 22.8 ± 19.0 percent, $p < 0.01$). Again, while this is supportive of a beneficial effect of melatonin, the within-subjects' design limits definitive conclusions.

Overall, only a limited number of clinical studies have investigated the use of melatonin to improve pregnancy outcomes in infertile women. These studies have generally been poorly designed, have often compared combination regimens, have investigated a narrow range of melatonin doses and have been unable to conclusively identify an independent positive role for melatonin on clinical pregnancy rates and live birth rates after IVF. There clearly is a need for a large randomized double-blind placebo-controlled trial to investigate whether oral melatonin increases clinical pregnancy rates in IVF patients and which dose provides maximal benefit, and such a study, the MIART trial, is currently being conducted which will address many of the limitations of previous studies [23].

13.2.6 Systematic Reviews and Meta-Analyses

Only one meta-analysis has been performed specifically assessing the use of melatonin in IVF [24]. This systematic review and meta-analysis of five randomized controlled trials found a pooled risk ratio of 1.21 (95% CI 0.98–1.50) in favour of melatonin for the outcome of clinical pregnancy rate. This meta-analysis did not include the recent large randomized trial by Pacchiarotti and colleagues [22]. The authors suggested that the adequacy of the data evaluating the usefulness of melatonin is poor, and that it should not yet be recommended for routine use. While they did not find any worsening of the outcomes of IVF, the authors commented on the lack of live birth rate as an outcome measure as well as the imprecision encountered in all studies considered.

On the other hand, melatonin is also known to be remarkably safe, with the Cochrane systematic review and meta-analysis finding no association between antioxidant supplementation and adverse effects for women involved in treatment [25]. This meta-analysis, which considered studies of melatonin as well as other antioxidants, found a similar non-statistically significant improvement in clinical pregnancy rate when using any antioxidant (OR 1.30, 95% CI 0.92–1.85) with a total sample size of over 2000 patients [25].

13.3 Conclusion and Future Directions

While the beneficial nature of melatonin, an endogenous antioxidant, has been known for decades, the investigation into the role of melatonin in the treatment of infertility is still in its infancy. Good quality evidence has emerged from other disciplines indicating the utility of melatonin in the treatment of a variety of medical conditions. There is also evidence to support the use of melatonin as analgesic in temporomandibular disorders and as a method of reducing oxidative stress and improving dyspnoea in patients with chronic obstructive pulmonary disease. Despite this, melatonin use in infertility treatment still lacks adequate evidence to recommend routine use.

Infertility treatments are associated with significant levels of ROS, which have the potential to negatively affect the quality of oocytes and embryos. Melatonin shows promise as an adjunctive therapy in the treatment of infertility. Its unique antioxidative characteristics and safety profile make it an ideal potential adjuvant therapy to be further investigated in well-designed randomized trials.

References

1. Fernando, S. and L. Rombauts, *Melatonin: shedding light on infertility?—A review of the recent literature*. J Ovarian Res, 2014. 7: p. 98.
2. Tang, P. et al., *Plasma melatonin profile and hormonal interactions in the menstrual cycles of anovulatory infertile women treated with gonadotropins*. Gynecol Obstet Invest, 1998. 45(4): pp. 247–252.
3. Lawson, C.C. et al., *Rotating shift work and menstrual cycle characteristics*. Epidemiology, 2011. 22(3): pp. 305–312.
4. Miyauchi, F., K. Nanjo, and K. Otsuka, *Effects of night shift on plasma concentrations of melatonin, LH, FSH and prolactin, and menstrual irregularity*. Sangyo Igaku, 1992. 34(6): pp. 545–550.
5. Voordouw, B. et al., *Melatonin and melatonin-progestin combinations alter pituitary-ovarian function in women and can inhibit ovulation*. J Clin Endocrinol Metab, 1992. 74(1): pp. 108–117.
6. Tamura, H. et al., *The role of melatonin as an antioxidant in the follicle*. J Ovarian Res, 2012. 5(1).
7. Nakamura, Y. et al., *Changes of serum melatonin level and its relationship to feto-placental unit during pregnancy*. J Pineal Res, 2001. 30(1): pp. 29–33.
8. Bedaiwy, M. et al., *Effect of follicular fluid oxidative stress parameters on intracytoplasmic sperm injection outcome*. Gynecol Endocrinol 2012. 28(1): pp. 51–55.
9. Lord, T. et al., *Melatonin prevents postovulatory oocyte aging in the mouse and extends the window for optimal fertilization in vitro*. Biol Reprod, 2013.
10. Tamura, H. et al., *Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate*. J Pineal Res, 2008. 44.
11. Tamura, H. et al., *Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate*. J Pineal Res, 2008. 44(3): pp. 280–287.
12. Kang, J. et al., *Effects of melatonin on in vitro maturation of porcine oocyte and expression of melatonin receptor RNA in cumulus and granulosa cells*. J Pineal Res, 2009. 46(1): pp. 22–28.
13. Salimi, M. et al., *The effect of melatonin on maturation, glutathione level and expression of HMGB1 gene in Brilliant Cresyl Blue (BCB) stained immature oocyte*. Cell J, 2014. 15(4): pp. 294–301.
14. Bahadori, M.H. et al., *Melatonin effect during different maturation stages of oocyte and subsequent embryo development in mice*. Iran J Reprod Med, 2013. 11(1): pp. 11–18.
15. Wei, D. et al., *Supplementation with low concentrations of melatonin improves nuclear maturation of human oocytes in vitro*. J Assist Reprod Genet, 2013. 30: pp. 933–938.
16. Kim, M. et al., *Does supplementation of in-vitro culture medium with melatonin improve IVF outcome in PCOS?* Reprod Biomed Online, 2013. 26(1): pp. 22–29.
17. Rombauts, L., *Is there a recommended maximum starting dose of FSH in IVF?* J Assist Reprod Genet, 2007. 24(8): pp. 343–349.
18. Eryilmaz, O. et al., *Melatonin improves the oocyte and the embryo in IVF patients with sleep disturbances, but does not improve the sleeping problems*. J Assist Reprod Genet, 2011. 28(9): pp. 815–820.
19. Rizzo, P., E. Raffone, and V. Benedetto, *Effect of the treatment with myo-inositol plus folic acid plus melatonin in comparison with a treatment with myo-inositol plus folic acid on oocyte quality and pregnancy*

- outcome in IVF cycles. A prospective, clinical trial. Embase European Review for Medical and Pharmacological Sciences, 2010. 14(6): pp. 555–561.
20. Wang, F. et al., *Melatonin improves the quality of in vitro produced (IVP) bovine embryos: implications for blastocyst development, cryotolerance, and modifications of relevant gene expression*. PLoS One, 2014. 9(4): p. e93641.
 21. Unfer, V. et al., *Effect of a supplementation with myo-inositol plus melatonin on oocyte quality in women who failed to conceive in previous in vitro fertilization cycles for poor oocyte quality: a prospective, longitudinal, cohort study*. Gynaecol Endocrinol, 2011. 27(11): pp. 857–861.
 22. Pacchiarotti, A. et al., *Effect of myo-inositol and melatonin versus myo-inositol, in a randomized controlled trial, for improving in vitro fertilization of patients with polycystic ovarian syndrome*. Gynecol Endocrinol, 2016. 32(1): pp. 69–73.
 23. Fernando, S. et al., *A pilot double-blind randomised placebo-controlled dose-response trial assessing the effects of melatonin on infertility treatment (MIART): study protocol*. BMJ Open, 2014. 4(8): p. e005986.
 24. Seko, L. et al., *Melatonin supplementation during controlled ovarian stimulation for women undergoing assisted reproductive technology: systematic review and meta-analysis of randomized controlled trials*. Fertil Steril, 2014. 101(1): pp. 154–161.
 25. Showell, M. et al., *Antioxidants for female subfertility*. Cochrane Database of Systematic Reviews, 2013(8).
 26. Hardeland, R., *Melatonin metabolism in the central nervous system*. Curr Neuropharmacol, 2010. 8(3): pp. 168–181.
 27. Reiter, R. et al., *Peripheral reproductive organ health and melatonin: ready for prime time*. Int J Mol Sci, 2013. 14(4): pp. 7231–7272.
 28. Watson, R., *Melatonin in the Promotion of Health* 2nd edition. 2011, United States: CRC Press.
 29. Nishihara, T. et al., *Oral melatonin supplementation improves oocyte and embryo quality in women undergoing in vitro fertilization-embryo transfer*. Gynecol Endocrinol, 2014. 30(5): pp. 359–362.
 30. Batioğlu, A. et al., *The efficacy of melatonin administration on oocyte quality*. Gynecological Endocrinology, 2012. 28(2): pp. 91–93.

The Use of LH Supplements to Improve the Response to Ovarian Stimulation

Rachael Rodgers and William Ledger

14.1 Structure of LH

Luteinizing hormone (LH) is a heterodimeric glycoprotein comprised of two non-covalently linked subunits, designated α and β . The α subunit consists of 92 amino acids, the gene for which is located on chromosome 6, at 6q12-q21. The α subunit in LH is identical to the α subunit of human chorionic gonadotropin (hCG), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH). The β subunit of LH consists of 121 amino acids (with a 24 amino acid leader sequence that is cleaved post-transcription) and is encoded by the *LHB* gene located on chromosome 19 at 19q13.32 [1]. It is the β subunit that conveys the biological specificity of the hormone [2].

Post-translational modifications such as the addition of carbohydrate side chains including sialic and sulfonic acid moieties create upwards of 30 LH isoforms. These isoforms differ in their half-life, bioactivity and signalling properties. Naturally occurring variants of LH that differ in their biological effect have been identified. One such variant is associated with unexplained infertility and subfertility due to ovulatory dysfunction, and this genetic variant may be more prevalent among women who demonstrate resistance to ovarian stimulation with FSH. Another variant of LH involving a single amino acid substitution of serine for glycine at amino acid 102 in the β subunit has been linked to menstrual disorders and male infertility [1].

LH binds to the LH receptor, a G-protein-coupled receptor with seven transmembrane domains. It is a member of the rhodopsin subfamily of glycoprotein receptors. Receptor activation requires the binding of LH (or a hormone structurally similar to LH) to the large NH₂-terminal extracellular region of the receptor. LH receptors are expressed by both granulosa and theca cells of the ovaries as well as in some extra gonadal sites. Inactivating mutations of the LH receptor have been associated with cases of menstrual disturbance, infertility and the empty follicle syndrome [3].

14.2 Function of LH

LH is synthesized and secreted by the gonadotropic cells of the anterior pituitary in response to pulsatile gonadotropin-releasing hormone (GnRH) from the hypothalamus. LH is believed to have different functions at different times during the ovarian cycle. During the follicular phase, basal concentrations of LH are required to support oestrogen production (as per the two-cell, two-gonadotropin hypothesis) and for optimal follicular development. The importance of the presence of LH during the follicular phase of the menstrual cycle is demonstrated in women with hypogonadotropic hypogonadism (discussed in the

following). The LH surge that occurs mid-cycle initiates final oocyte maturation and triggers ovulation, with ovulation occurring 38–40 hours after the LH surge. During the luteal phase, LH maintains corpus luteal function – importantly the secretion of progesterone – until hCG production by trophoblast cells assumes this function. If pregnancy does not occur, progesterone concentrations fall and menstruation occurs.

14.3 The Two-Cell, Two-Gonadotropin Hypothesis

Fevold [4] used hypophysectomized rats to determine that the presence of both LH and FSH is required for adequate production of oestrogen. They demonstrated that the administration of FSH plus LH resulted in a marked increase in oestrogen secretion compared with administration of FSH alone. This work led to the development of two-cell, two-gonadotropin hypothesis that states that both LH and FSH are required for oestrogen production and proper follicular development. LH binds to LH receptors on theca cells and stimulates androgen production (androgens are precursors to oestrogens in the steroid synthesis pathway). The androstenedione and testosterone produced by the theca cells then diffuse across the basement membrane into the nearby granulosa cells where these hormones are converted by cytochrome p450 aromatase into oestrone and 17β oestradiol respectively. Oestrone is then converted into 17β oestradiol by hydroxysteroid dehydrogenase 1 (17β HSD1) [5,6]. Aromatase activity is stimulated by the binding of FSH to FSH receptors on the granulosa cell membrane. Only the granulosa cells possess aromatase, but granulosa cells do not possess the steroidogenic enzymes needed for synthesis of androgens. Thus, two cells (theca and granulosa cells) and two hormones (LH and FSH) must act in concert to allow oestrogen production (see Figure 14.1).

14.4 Pharmacology of Superovulation for IVF

Although the first pregnancies derived from in vitro fertilization (IVF) followed collection of a single oocyte following the natural LH surge in a spontaneous ovarian cycle, the development of technologies to extract gonadotropins from human pituitary and later human post-menopausal urine allowed production of pharmaceutical-grade gonadotropin preparations that could be administered to women to induce a state of superovulation. This allowed for stimulation of growth of multiple follicles and collection of large numbers of oocytes from a single stimulated IVF cycle, greatly improving chances of pregnancy following IVF. These early gonadotropin preparations contained both FSH and LH activity. FSH was clearly the active molecule in the induction of multi-follicular development and the presence of LH activity was largely disregarded. The picture changed in the 1990s when recombinant technology was first used to derive “pure” FSH, LH and hCG using Chinese hamster ovarian cells transfected with the human genes for each of these three hormones. Large-scale bioreactor production provided reproductive medicine specialists with the ability to undertake stimulation with FSH alone or with added LH. This allowed separation of the pharmacological activities of the two gonadotropins for the first time. Many studies followed, most sponsored by the pharmaceutical companies that produced rival products. This chapter will review the extensive literature concerning the question of whether adding LH to FSH during superovulation can improve the ovarian response and produce more or better quality oocytes and higher pregnancy and live birth rates.

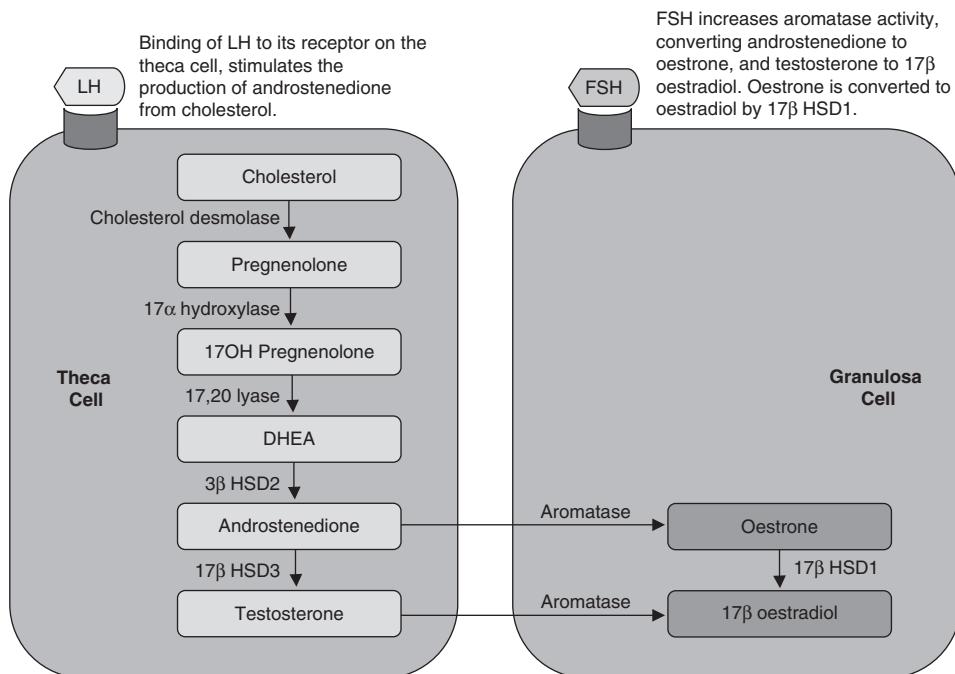


Figure 14.1 The two-cell, two-gonadotropin hypothesis – LH binds to receptors on theca cells and stimulates the conversion of cholesterol into rostenedione and testosterone. These hormones enter granulosa cells where they are converted into 17 β oestradiol.

14.5 Ovarian Stimulation Involving Addback LH, hCG or hMG

The literature that describes possible benefits of LH supplementation during ovarian stimulation is made more complex by the use of different types of LH-containing molecules in different studies. The most straightforward use concerned recombinant LH added to recombinant FSH for superovulation. However, others use hCG, which exhibits LH-like activity, or human menopausal gonadotropin (hMG), which has LH activity primarily conveyed by pituitary hCG contained in the urine collected from post-menopausal women.

HCG and LH are likely to have developed from a common ancestral gene and they continue to share an 82 percent amino acid sequence homology. The β subunit of hCG contains an additional 24 amino acid carboxy-terminal extension, which is thought to have arisen as the consequence of two evolutionary changes to the DNA – a single base deletion at position 1,540 that puts the hCG sequence out of synchronicity with the terminal seven codons of the β subunit of LH, and two base pair insertions at positions 1,612/3. The net effect of these DNA changes is that a previously untranslated region of the gene has become a coding region [2]. The 24 amino acid extension to hCG conveys biological stability (hCG is ten times more stable than LH). LH has a considerably shorter half-life of 30–60 minutes than hCG, which has a half-life of approximately 37 hours [7].

Despite the structural and functional similarities of LH and hCG, it is important to note that they are not bio-identical, and the two hormones differ in their physiological purposes. LH is produced by the gonadotropes of the anterior pituitary. Four different forms of hCG with differing functions may be present in a female – hCG produced by villous syncytiotrophoblast cells of the embryonic placenta; hyperglycosylated hCG produced by cytotrophoblast cells; hCG-free β subunit produced by some malignancies; and a sulphated variant of hCG produced by the pituitary in low levels. The primary function of hCG relates to the maintenance of pregnancy, with hCG having many important roles such as supporting corpus luteal production of progesterone, promoting angiogenesis within the uterine vasculature and modulating the immune system to prevent immune rejection of the implanting embryo. Hyperglycosylated hCG produced by the pituitary is thought to have a similar action to pituitary LH during the menstrual cycle [7].

While both LH and hCG bind to the LH receptor, the two hormones are not functionally identical. Casarini et al. [8] demonstrated that hCG was five times more potent than LH in terms of intracellular cAMP production, although the maximal effect of LH was significantly faster (10 minutes) than hCG (1 hour). In human granulosa cells, continuous exposure for 36 hours to hCG resulted in significantly higher cAMP production than seen after continuous exposure to LH. Activation of the cAMP/PKA pathway stimulates progesterone production by granulosa cells. Conversely, exposure of cells to LH resulted in more ERK and AKT activation than when exposed to hCG. AKT and ERK1/2 pathways are involved in cell proliferation, differentiations and survival. Aromatase expression by granulosa cells involves cAMP/PKA, ERK1/2 and AKT pathways, with these pathways playing an important role in oocyte maturation and follicular growth. The authors concluded that binding of LH or hCG to the LH receptor resulted in the activation of different pathways, and indicated that the receptor was able to discriminate between the binding of the two hormones.

14.6 Rationale for LH Supplementation during Ovarian Stimulation

The value of adding LH to FSH during ovarian stimulation is still debated. GnRH analogues, either agonist or antagonist, are routinely used during superovulation to prevent premature ovulation and loss of oocytes. GnRH analogues cause a profound suppression of serum LH concentrations in a proportion of normogonadotropic women, with LH concentrations of <0.5UI/L in 12 percent of women and <1.0IU/L in 64 percent of women reported in a long GnRH agonist cycle by Humaidan et al. [9]. Another study [10] reported 49 percent of women had a serum LH concentration of <0.5IU/L on day 8 of a long GnRH agonist cycle. Some authors have suggested that low LH concentrations during a stimulation cycle may result in poorer cycle outcomes [9,10,11]. Additionally, FSH induces expression of LH receptors on the granulosa cells of the developing follicles in the mid-follicular phase of the cycle. Binding of LH to these LH receptors activates the cAMP/PKA pathway, and may then become an additional stimulant of follicular growth. As the follicle develops, it decreases its dependence on FSH and becomes increasingly responsive to LH, which may also stimulate production of other growth factors [12]. Hence, there is a theoretical rationale to support the hypothesis that FSH alone is not sufficient to produce optimal ovarian response to superovulation.

The concept of an 'LH window' has been proposed based on studies on induction of ovulation, such as that by Shoham et al. [13], who conducted a double-blind, randomized, placebo-controlled trial in which women with LH <1.2 IU/L and FSH <5.0 IU/L who desired pregnancy received either 75 IU lutropin alfa (recombinant human LH, rLH) or placebo in conjunction with 150 IU follitropin alpha (recombinant human FSH, rFSH). Significantly more women in the group that received rLH reached the primary end point of at least one follicle ≥ 17 mm and serum oestradiol level ≥ 400 pmol/L on the day of hCG administration and a mid-luteal phase progesterone level of ≥ 25 nmol/L. The 'LH Window' theory proposed that without the presence of a threshold concentration of LH during the follicular phase, there would be insufficient oestradiol production for adequate follicle development, endometrial proliferation and formation of the corpus luteum [12].

The counterargument is that the low LH concentrations that persist during pituitary suppression with GnRH analogues are sufficient to maintain follicular and oocyte development. In a study of superovulation using the GnRH antagonist ganirelix to prevent premature ovulation, the Ganirelix dose-finding study group [14] reported a median LH concentration of 1.7 IU/L on the day of hCG trigger with the administration of 0.250 mg GnRH antagonist ganirelix from day 6 of the stimulation cycle. In women receiving reduced doses of ganirelix (0.0625 mg and 0.125 mg), median LH concentrations on the day of hCG trigger were higher (3.6 and 2.5 IU/L respectively). However, despite the lower LH concentrations in the 0.250 mg ganrelax group, there was no decrease in the number of oocytes collected, in the total or good quality number of embryos collected, or the implantation, miscarriage, vital pregnancy or ongoing pregnancy rate. Chappel et al. [15] suggested that LH needs to bind to <1 percent of LH receptors in order to elicit a maximal steroidogenic response.

Adding weight to the case that supplementation with LH is not beneficial during FSH superovulation is the finding from large studies that LH concentrations do not correlate with pregnancy in cycles in which a GnRH antagonist is administered during rFSH-only stimulation. A study by Doody et al. [16] reported ongoing pregnancy rates in women undergoing GnRH antagonist cycles with rFSH-only stimulation. Women were stratified into LH concentrations centiles of <P25, P25–P75 and >P75 on day 1, 5 and 8. There was no difference in pregnancy rates between the groups, despite LH concentrations of <0.91 IU/L on day 8 in the women in the <P25 group.

Several meta-analyses support the claim that LH supplementation is not required during ovarian stimulation, with no increase in pregnancy or live birth rates [17,18,19,20]. Some of these studies reported benefits in terms of higher oestrogen levels on the day of hCG trigger administration [17] and improved oocyte yield [17]. All of these meta-analyses have limitations, including broad patient inclusion criteria and differing treatment protocols, as outlined earlier.

A meta-analysis [18] that included seven randomized controlled trials (RCTs) (701 women) reported no difference in the live birth rate (OR 0.92, 95% CI 0.65–1.31, $p=0.65$) or clinical pregnancy rate (OR 0.85, 95% CI 0.57–1.26) with the addition of rLH to the FSH stimulation regime. There was also no difference in the secondary outcomes, including the amount of FSH required for ovarian stimulation, the duration of FSH stimulation, oestradiol concentration on the day of trigger, the total or mature number of oocytes collected and the fertilization rate. The inclusion criteria, stimulation protocol, method of LH surge suppression, method of fertilization (IVF or ICSI), day of embryo

transfer (conducted on days 2–5) and type of luteal support (progesterone and/or hCG) differed between the studies. Women differed in their baseline demographics such as age and the indication for artificial reproductive technology. Six of the seven studies used rFSH for ovarian stimulation; one study [21] used highly purified urinary FSH. In this latter study of 31 women, there was a trend towards a worse clinical pregnancy per initiated cycle in the group of women receiving rLH (64.7 versus 35.7 percent in the FSH-only versus the FSH-plus-rLH groups, OR 0.21, 95% CI 0.04–1.05), although this did not reach statistical significance. In five of the seven studies, GnRH agonists (leuprorelin, triptorelin or buserelin) were used to prevent premature LH surges; in the remaining studies GnRH antagonists (cetrorelix daily or single dose cetrorelix) were used. The criteria for administering the hCG trigger injection, fertilization methods (IVF or ICSI), day of embryo transfers (2–5 days after oocyte collection) and luteal support differed between the studies.

A meta-analysis by Baruffi et al. [17] included five RCTs in which LH addback was studied (434 women) and reported significantly higher oestradiol concentrations on the day of hCG trigger ($p<0.0001$, WMD=514, 95% CI 368–660) and a greater number of mature oocytes collected ($p=0.0098$, WMD=0.88, 95% CI 0.21–1.54), but no difference in the clinical pregnancy rate per oocyte retrieval, the total number of oocytes retrieved, the implantation rate, miscarriage rate, the total amount of rFSH required for stimulation or the number of days of stimulation. Only RCTs containing normogonadotropic, ‘good prognosis’, patients were included.

A meta-analysis by Oliveira et al. [20], which included only RCTs of studies that used rFSH stimulation protocols in conjunction with the use of a GnRH agonist to prevent a premature LH surge, included four studies and a total of 505 women. This meta-analysis concluded that the addition of rLH to the stimulation protocol did not improve the total number of oocytes collected, the number of mature oocyte collected, the clinical pregnancy rate per oocyte retrieved, implantation rate or miscarriage rate. The study did report that when rLH was added to the stimulation regime, fewer days of stimulation were required ($p<0.0001$, WMD=−0.198, 95% CI −0.24 to −0.16), the total amount of rFSH administered was reduced ($p<0.0001$, WMD=−192, 95% CI −220 to −164) and serum oestradiol levels of the day of hCG administration were increased ($p<0.0001$, WMD 49.4, 95% CI 38.4–60.4). Women aged 18–37 years were included in the studies. One study [22] specifically excluded women if they had a poor response (≤ 2 oocytes recovered) in a previous cycle.

A Cochrane review (Mochtar et al., 2007) of 14 trials including 2612 women concluded that there was no statistical difference in pregnancy outcomes with the addition of rLH, although the data suggested there may be a beneficial effect with rLH supplementation in respect to pregnancy loss and in women who were poor responders to FSH-only stimulation. Of the 11 trials that used a GnRH agonist protocol, there was no difference in live birth rates (two trials, 222 women, OR 1.51, 95% CI 0.79–2.87), clinical pregnancy rates (seven trials, 1256 women, OR 1.15, 95% CI 0.91–1.45) or ongoing pregnancy (seven trials, 1409 women, OR 1.22, 95% CI 0.95–1.56). Of the three trials that used a GnRH antagonist, there were no trials that reported the outcome of live birth. There was no difference in clinical pregnancy rates (one trial, 49 women, OR 0.79, 95% CI 0.26–2.43) or in ongoing pregnancy rates (two trials, 166 women, OR 0.83, 95% CI 0.39–1.80). Regarding pregnancy loss, in the eight GnRH agonist studies that reported miscarriage rate, there was a non-statistically significant trend towards reduced pregnancy loss with the use of rLH supplementation (506 women, OR 0.59, 95% CI 0.35–1.02). In the two GnRH antagonist

cycles, this trend was not replicated (176 women, OR 2.37, 95% CI 0.77–7.33). In the three trials of poor responders that included 310 women, there was a significant increase in the pregnancy rate with the administration of rLH (OR 1.85, 95% CI 1.10–3.11).

The conclusion that can be drawn from these meta-analyses is that the addition of rLH to rFSH stimulation regimes is not necessary in the majority of superovulation cycles in which women respond normally to FSH stimulation. However, co-administration of LH or LH-like molecules (hCG or hMG) may be helpful to specific sub-groups of women. One group that clearly requires such support is the one with hypogonadotropic hypogonadism. This group will now be discussed, followed by analysis of other less clear-cut indications for LH supplementation.

14.7 The Use of LH Supplementation in Women with Hypogonadotropic Hypogonadism

Balasch and colleagues, in [23], described approaches to superovulation in a patient with isolated hypogonadotropic hypogonadism. In her first cycle, she was administered 150 IU recombinant FSH; in her second cycle she was administered 150 IU human urinary menopausal gonadotropin (hMG); and in her third cycle she received 150 IU highly purified human urinary FSH plus oestradiol valerate. In these cycles, her oestradiol concentrations on the day of hCG trigger were 228, 5073 and 257 pg/mL respectively, demonstrating the marked effect of a medication with LH-like activity on oestradiol production from the follicle. Use of hMG also increased the number of follicles ≥ 14 mm on the day of trigger (10, 22 and 15 respectively). While the total number of oocytes retrieved did not markedly differ between the courses (7, 14 and 11 respectively), the fertilization rate was significantly improved in the cycle in which the women received HMG, with fertilization rates of 28, 93 and 27 percent occurring, resulting in a total of 2, 13 and 3 embryos created in each of the cycles.

A randomized crossover study of ten women with hypogonadotropic hypogonadism was performed by Couzinet et al. (1988). The women included in the study did not want to conceive, but participated in order to determine their ovulatory potential. Women received either highly purified FSH or urinary hMG at a dose of 225 IU per day for ten days. Circulating oestradiol became significantly higher in cycles in which hMG was administered (2474 \pm 675 compared with 760 \pm 253 pmol/L in FSH-only cycles, $p < 0.05$) after ten days of gonadotropin treatment. HCG was administered to eight of the ten women receiving hMG (hCG was withheld in two women due to either an oestradiol level of $>11,000$ pmol/L or >3 follicles at ≥ 16 mm), with all eight women indicating evidence of ovulation. HCG was administered to nine of ten women receiving FSH (one woman was excluded due to >3 follicles at ≥ 16 mm), with six women showing evidence of ovulation, suggesting an improved ovulation rate after triggering with hCG in women with hypogonadotropic hypogonadism who received urinary hMG compared to highly purified FSH.

A subsequent randomized trial used recombinant FSH (rFSH) with different doses of recombinant human LH (rLH) for ovulation induction in women with hypogonadotropic hypogonadism [24]. Women were randomized to receive rLH in doses of 0, 25, 75 or 225 IU per day in addition to a fixed dose of 150 IU rFSH. Both androstenedione and oestradiol concentrations were increased in a manner that correlated with the dose of rLH. Androstenedione concentrations ranged from 3.6 \pm 0.9 nmol/L in women receiving no

rLH to $6.7+/-1.3$ nmol/L in women receiving 225 IU rLH. Oestradiol concentrations ranged from $65+/-4$ pmol/L for women receiving no rLH to $2441+/-904$ for women receiving 225 IU rLH. Follicular development was also improved in women receiving 75IU or 225 IU rLH daily. The end point of this study was follicular development (at least one follicle with a diameter of $>=17$ mm), a preovulatory oestradiol of $>=400$ pmol/L and a mid-luteal progesterone level of $>=25$ nmol/L. The proportion of patients who achieved this end point increased with the dose of rLH ($p=0.0001$). Pregnancy data were not reported. However, this study clearly demonstrated the need for LH during FSH-induced folliculogenesis in women with hypogonadotropic hypogonadism, although only small amounts of rFSH were needed to produce adequate secretion of oestradiol and follicle growth, and serum concentrations of LH were low in all the groups throughout the study period.

14.8 Is There a Role for LH Co-Administration in Superovulation for Poor Responders?

Studies that attempt to address this question are many in number but generally poor in quality. Most 'poor responder' patients are older, or have premature ovarian insufficiency, with low numbers of primordial and small antral follicles and hence will always respond sub-optimally to superovulation whatever stimulation regime or dose is used. One problem that has bedevilled attempts to meta-analyse studies in this area has been the lack of an agreed definition of a 'poor responder'. A despairing systematic review identified over 40 different definitions of poor ovarian response that have been used in published trials [25]. Criteria used to define 'poor responders' include the age of the patient, previous cycle cancellations, antral follicle count, basal FSH concentrations, number of follicles visualized on the final day of ovarian stimulation, the total number of oocytes retrieved, the number of mature oocytes retrieved, the number of good quality embryos created, oestrogen concentration on the final day of stimulation and the dose of gonadotropin chosen by the clinician for ovarian stimulation. This lack of consensus concerning the definition of poor ovarian response has led to considerable heterogeneity between studies, making it impossible to perform a good quality and meaningful meta-analysis of these studies.

More recently, attempts have been made to produce a standardized classification of the 'poor responder' patient in order to improve agreement between studies. The Bologna criteria are the basis of a consensus statement produced by the ESHRE working group on Poor Ovarian Response Definition [26]. The consensus document states that the minimum criteria required to define a poor ovarian response are the presence of at least two of the following features:

- i. Advanced maternal age (≥ 40 years) or any other risk factor for poor ovarian response
- ii. Previous poor ovarian response (≤ 3 oocytes) with a conventional stimulation protocol
- iii. An abnormal ovarian reserve test, defined as AFC $<5-7$ follicles or AMH $<0.5-1.1$ ng/mL

The consensus document further states that two episodes of a poor ovarian response after maximal stimulation are sufficient to define a patient as a poor responder, even in the absence of advanced maternal age or abnormal test of ovarian reserve.

The Bologna criteria have been criticized as several sub-populations of women with different biological characteristics fall under the one classification. Bias may be introduced

Group 1 <35 years with sufficient prestimulation ovarian reserve parameters (AFC≥5, AMH≥1.2 ng/mL) with an unexpectedly poor or suboptimal response after standard ovarian stimulation a. Poor response: <4 oocytes retrieved b. Suboptimal response: 4–9 oocytes retrieved	Group 2 ≥35 years with sufficient prestimulation ovarian reserve parameters (AFC≥5, AMH≥1.2 ng/mL) with an unexpectedly poor or suboptimal response after standard ovarian stimulation a. Poor response: <4 oocytes retrieved b. Suboptimal response: 4–9 oocytes retrieved
Group 3 <35 years with poor prestimulation ovarian reserve parameters (AFC<5, AMH<1.2 ng/mL)	Group 4 ≥35 years with poor prestimulation ovarian reserve parameters (AFC<5, AMH<1.2 ng/mL)

Figure 14.2 Poseidon classification of poor ovarian responders

into a study that compares different methods of treating poor responders if the randomized groups differ in their baseline characteristics despite being correctly classified by the Bologna criteria. For example, one group may be much younger than the other. The breadth of the Bologna criteria also leads to persistent heterogeneity between different groups [27].

The Poseidon categorization of poor ovarian responders was subsequently developed, with subcategories of poor responders to try to improve homogeneity (see Figure 14.2). Different clinical treatment strategies may be required for each of these groups in order to optimize their fertility outcome.

14.9 LH Supplementation in Poor Responders

The extreme heterogeneity of the literature concerning efficacy of LH supplementation for ‘poor responders’ makes it pointless to attempt to use standard meta-analytical methods to derive meaningful conclusions. Even within a single trial, the poor responders included may not have the same biological characteristics and consequently may not respond in the same manner to rLH supplementation. For example, one RCT included women who were either expected to respond poorly to stimulation due to their age (35–41 years) or because they were young (<35 years) but had pre-stimulation investigations suggesting a reduced ovarian reserve (FSH>12 IU/mL and AFC≤5) [28]. It is conceivable that these two groups of women may respond differently to rLH supplementation, and indeed the ability of women in different age groups to respond differently to rLH has been shown by Bosch et al. [29].

Most RCTs investigating rLH supplementation during ovarian stimulation either show a non-significant trend towards a beneficial effect in terms of clinical pregnancy [29,30] or no effect at all [28,31,32,33]. Other isolated RCTs have shown a statistically significant beneficial effect (Ferraretti et al., 2004; Ruvolo et al., 2007). Two meta-analyses have suggested an advantage of rLH supplementation in poor responders [34,19], although

another found no benefit (Fan et al., 2013). All of these meta-analyses are problematic, due to the heterogeneity of the groups of women defined as ‘poor responders’, the disparate treatment protocols in the individual RCTs or the choice of studies included in the meta-analysis. For example, the Fan et al. [35] meta-analysis included only three studies (total 458 women). One study (Ferraretti et al., 2004) that met the stated inclusion criteria was excluded by the authors of the meta-analysis as they disagreed with the definition of poor responder used in the RCT and stated that ‘common sense’ told them that the large effect size was unlikely to be replicable in studies with a larger sample size. Thus, the authors of the meta-analysis introduced negative bias into their analysis and unsurprisingly reported no benefit.

A further demonstration of the overall poor quality of the literature in this area is that very few studies of rLH supplementation of poor responders report live birth rates as an outcome. One small study (Ferraretti et al., 2004) of women who ‘stagnated’ in their follicular development from days seven to ten reported a beneficial effect of rLH supplementation (details of this study are outlined in the following). An RCT by Vuong et al. [36] studied the effect of rLH supplementation on 240 women aged ≥ 35 years (not specifically poor responders) and failed to detect an improvement in live birth rate with rLH supplementation (16.7 versus 17.5 percent, rFSH-only versus rFSH plus rLH, 95% CI -9.5–11.2). One meta-analysis (Lehert et al., 2014) reported a non-significant trend towards improvement in live birth rate with rLH supplementation (RR 1.30, 95% CI 1.01–1.21).

A phase III, single-blind, randomized parallel-group clinical trial funded by Merck used an interactive voice response system to assign 477 women to receive rFSH plus rLH and 462 women to receive rFSH alone in a long GnRH agonist protocol [33]. Women were included if they met the Bologna criteria for poor ovarian responders. An intention-to-treat analysis was conducted and reported no statistical difference between women receiving rFSH plus rLH and women receiving rFSH alone in terms of the number of oocytes retrieved (3.3 versus 2.6, $p=0.182$), clinical pregnancy rate (14.1 versus 16.8 percent, $p=0.320$), ongoing pregnancy rate (11.0 versus 12.4 percent, $p=0.599$) or live birth rate (10.6 versus 11.7 percent, $p=0.663$). A beneficial effect of rFSH-only treatment was demonstrated in regard to the biochemical pregnancy rate (17.3 versus 23.9 percent, $p=0.020$). While the authors report a beneficial effect of the addition of rLH on the live birth rate of women with moderate or severe poor ovarian response, and a beneficial effect of the use of rFSH-only in women with mild poor ovarian response, these results should be interpreted with caution as they arise from a post hoc analysis.

14.10 Is LH Supplementation Beneficial in Older Women Undergoing Superovulation?

A prospective RCT of 84 patients with a basal FSH ≥ 10 IU/mL and who were ≥ 40 years of age undergoing IVF assessed the effects of adding rLH to the stimulation regime [32]. A flare protocol in which ovarian stimulation commenced on day 2 with administration of a GnRH agonist (leuprolide acetate) and 375 IU rFSH was used. In the supplementation group, 150 IU rLH was commenced on day 7. Ongoing pregnancy rate per retrieval and the implantation rate per embryo transferred were the primary outcome measures. The secondary outcomes were the number of days of gonadotropin treatment, oestradiol levels on the day of hCG administration, number of developed follicles, number of retrieved oocytes, fertilization rate, cumulative embryo score and the number of

transferred embryos. The authors found no significant differences in any of the primary or secondary measures between the two groups of women. The pregnancy rate in the rFSH-plus-rLH group was 10/42 (23.81 percent) versus 9/42 (21.43 percent) in the FSH-only group ($p=0.441$). The miscarriage rate was 3/10 (30 percent) versus 2/9 (22.22 percent) in the rFSH-plus-rLH versus rFSH groups respectively ($p=0.498$). Live birth rate was not reported.

Bosch and colleagues [29] reported on an RCT of rFSH alone versus rFSH plus rLH that was conducted in two groups of patients – women up to 35 years of age (380 women) and women aged 36–39 years (340 women). In the younger patients, there was no benefit observed with the addition of rLH. In the older group of women the results were mixed. The implantation rate was significantly higher in the rFSH-plus-rLH group (26.7 versus 18.6 percent, OR 1.15, 95% CI 1.04–2.33), but the difference in ongoing pregnancy rate did not reach statistical significance (rFSH plus rLH versus rFSH only, 33.5 versus 25.3 percent, OR 1.49, 95% CI 0.93–2.38). Interestingly, the total number of oocytes was higher in the rFSH-only group (10.1 versus 8.4, $p=0.008$), but the number of mature oocytes was not (7.0 versus 6.6, $p=0.303$). The fertilization rate of the oocytes obtained from the rFSH-plus-rLH group was slightly higher (61.2 versus 68.0 percent, $p=0.027$), suggesting that the oocytes obtained from the rFSH-plus-rLH group may have been of better quality. However, it is also conceivable that the improved implantation rate seen in the rFSH-plus-rLH group was due to an endometrial effect of rLH, as fresh embryo transfers were conducted in this study. Finally, the number of cryopreserved oocytes was higher in the rFSH-only group (1.2 versus 0.7, $p=0.008$), so the eventual cumulative live birth rate may be equivalent between the two groups.

The possible effect of variation in the timing of commencement of rLH was investigated by Behre et al. [37]. In this open-label RCT women aged 36–40 years were assigned to receive early rLH supplementation during ovarian stimulation with FSH (rLH commenced on day 1) compared with delayed rLH supplementation (rLH commenced on day 6). The study reported no significant differences in their primary outcome of the number of oocytes retrieved (9.7+/-6.9 versus 10.9+/-6.5 in the rLH from day 1 group compared to the rLH from day 6 group); however, there was a significant increase in the secondary outcomes of implantation rate and clinical pregnancy rate (both per started cycle and per embryo transfer) in the group of women receiving rLH from day 1, again suggesting a possible endometrial effect of rLH addback.

14.11 Is LH Supplementation Beneficial in Women with Reduced Ovarian Reserve Undergoing Superovulation?

A study by Musters et al. [28] of 244 women who were expected to respond poorly to rFSH superovulation according to pre-stimulation assessment of ovarian reserve (age 35–41 years or age < 35 years with $\text{FSH} > 12 \text{ IU/mL}$ and $\text{AFC} \leq 5$) assessed the possible benefit of the addition of rLH to the stimulation regime. The participants underwent a long GnRH agonist protocol, with a starting dose of gonadotropin dependent on the AFC on day 5. The group reported no significant difference in the ongoing pregnancy rate (rFSH plus rLH versus rFSH-only, 13 versus 12 percent, RR 1.1, 95% CI 0.57–2.16) or in the proportion of top-quality embryos (rFSH plus rLH versus rFSH-only, 17 versus 11 versus, mean difference 0.06, 95% CI -0.01–0.14).

Conversely, one study that showed a beneficial effect of rLH supplementation was the prospective RCT of 108 women by Ferraretti et al. (2004). Women <30 years were commenced on 150 IU rFSH and women 30–37 years were commenced on 225 IU rFSH. Women >37 years were excluded from the study. Women who initially had a normal follicular response (defined as >10 antral follicles of ≥8 mm and oestradiol of ≥100 pg/mL) and who then showed a plateau in follicular growth (no increase in oestradiol level or follicular size on days 7–10 of stimulation) were eligible for inclusion. The women were randomized into treatment groups of rFSH only (increased dose), rFSH-plus-rLH supplementation and rFSH-plus-hMG supplementation. This study reported an improved implantation and live birth rate in women supplemented with rLH compared with women receiving an increased dose of rFSH or rFSH plus hMG (implantation rates: rFSH-only versus rFSH plus rLH versus rFSH plus hMG, 14.1 versus 36.8 versus 7.4 percent; Live birth rates: 22 versus 40.7 versus 18 percent; $p<0.05$ for rFSH plus rLH versus rFSH-only and rFSH plus hMG). Of note, the women included in this study do not meet generally accepted criteria of poor responders; rather, these women were normal responders who ‘stagnated’ in their follicular growth during ovarian stimulation.

Another study that reported a beneficial effect of rLH supplementation was conducted by Ruvolo et al. (2007). Women who had previously undergone ovarian stimulation with rFSH and had required a total of >3000 IU FSH were selected as potential poor responders for this RCT. The identified women were all commenced on 225 IU rFSH. On day 8, if testing indicated an oestradiol concentration of <180 pg/mL and ultrasound indicated at least six follicles with a diameter of 7–10 mm and no follicles with a diameter of >12 mm, the women became eligible for entry into the study. The 42 eligible women were randomized to either continue on 225 IU rFSH only (18 women, mean age 36.33 \pm 2.1 years), or to continue at the same dose of rFSH plus 75–150 IU rLH (24 women, mean age 33.00 \pm 3.1 years). The primary outcome was the difference in the DNA fragmentation rate in the cumulus cells, indicating apoptosis. The authors reported a higher apoptosis rate in the cumulus cells of the women receiving rFSH only. There was no difference in the total number of oocytes retrieved between the groups; however, there was a lower number of immature oocytes in the rFSH-plus-rLH group (0.58 versus 2.33, $p<0.01$). The number of transferred embryos (2.91 versus 1.56, $p<0.01$) and the pregnancy (15.6 versus 12.5 percent, $p<0.01$) and implantation (45.4 versus 25.0 percent, $p<0.01$) rates were higher in the rFSH-plus-rLH group compared to the rFSH-only group. The authors conclude that rLH supplementation may improve the chromatin quality of cumulus cells, resulting in an improved mature oocyte yield.

14.12 Other Studies of Poor Responders

A prospective RCT of 145 women compared rFSH-only ovarian stimulation with rFSH-plus-rLH stimulation and rFSH plus hCG [31]. High doses of rFSH (600 IU) were used for all groups with the addition of 75 IU rLH or 75 IU rhCG daily in the two experimental groups. Poor responders were classified as having ≤12 antral follicles (a definition not consistent with either the Bologna or the Poseidon criteria). Women were excluded if they were over 42 years of age or if they had only one ovary or if their basal FSH concentration was >12 IU/L. There was no statistically significant difference in the outcome measures of pregnancy rate (positive β-hCG), clinical pregnancy rate (fetal cardiac activity), mature oocytes retrieved, peak

oestradiol levels, days of stimulation, total FSH administered, number of embryos transferred or cycle cancellation rate.

14.13 Conclusions

Gonadotropins have been used for superovulation for almost 50 years. During that time there have been many advances in the quality, efficacy and ease of use of the pharmaceutical products that are available to patients. This in turn has contributed to the improvement in pregnancy and live birth rates seen after IVF over the decades. Commercial competition has inevitably led to over-inflation of claims of superiority of one product over another based on small differences observed in underpowered studies. Any analysis of an area of research that is so heavily supported by the pharmaceutical industry must be tempered with scepticism. Accepting this, we suggest that the current evidence base shows the following:

- LH supplementation during controlled ovarian stimulation is **not beneficial** in normal responders, whether in agonist- or antagonist-controlled superovulation cycles.
- LH supplementation is **necessary** in ovulation induction and superovulation in women with **hypogonadotropic hypogonadism**.
- LH supplementation **may be beneficial for older patients or predicted poor responders**, but the literature is difficult to interpret and any benefit is likely to be at best modest. Most poor responders have **diminished ovarian reserve which cannot be repaired** with gonadotropins.

References

1. Choi J, Smitz J. *Luteinizing hormone and human chorionic gonadotropin: origins of difference*. Molecular and Cellular Endocrinology 2014; **383**: 203–213.
2. Talmadge K, Vamvakopoulos NC, Fiddes JC. *Evolution of the genes for the b subunits of human chorionic gonadotropin and luteinizing hormone*. Nature 1984; **307**: 37–40.
3. Menon KMJ, Menon B. *Structure, function and regulation of gonadotropin receptors – a perspective*. Molecular and Cellular Endocrinology 2012; **356**: 88–97.
4. Fevold HL. *Synergism of the follicle stimulating and luteinizing hormones in producing estrogen secretion*. Endocrinology 1941; **28**: 33–36.
5. Garzo VG, Dorrington JH. *Aromatase activity in human granulosa cells during follicular development and the modulation by follicle-stimulating hormone and insulin*. American Journal of Obstetrics & Gynecology 1984; **148**: 657–662.
6. Steinkampf MP, Mendelson CR, Simpson ER. *Regulation by follicle-stimulating hormone of aromatase cytochrome P-450 in human granulosa cells*. Molecular Endocrinology 1987; **1**: 465–471.
7. Cole LA. *Biological functions of hCG and hCG-related molecules*. Reproductive Biology and Endocrinology 2010; **8**: 102.
8. Casarini L, Lispi M, Longobardi S et al. *LH and hCG action on the same receptor results in quantitatively and qualitatively different intracellular signalling*. PLoS One 2012; **7** (10): e46682, 1–15.
9. Humaidan P, Bungum L, Bungum M, Andersen CY. *Ovarian response and pregnancy outcome related mid-follicular LH levels in women undergoing assisted reproduction with GnRH agonist down-regulation and recombinant FSH stimulation*. Human Reproduction 2002; **17**: 2016–2021.
10. Westergaard LG, Laursen SB, Andersen CY. *Increased risk of early pregnancy loss by profound suppression of luteinizing hormone during ovarian stimulation in normogonadotropic women undergoing assisted reproduction*. Human Reproduction 2000; **15**: 1003–1008.

11. Fleming R, Rehka P, Deshpande N et al. *Suppression of LH during ovarian stimulation: effects differ in cycles stimulated with purified urinary FSH and recombinant FSH.* Human Reproduction 2000; **15**: 1440–1445.
12. Esteves SC, Alvgi C. *The role of LH in controlled ovarian stimulation.* In Ghuman S (ed), Principles and Practice of Controlled Ovarian Stimulation in ART, 2015; Springer.
13. Shoham Z, Smith H, Teko T et al. *Recombinant LH (lutropin alfa) for the treatment of hypogonadotropic women with profound LH deficiency: a randomized, double-blind, placebo-controlled, proof-of-efficacy study.* Clinical Endocrinology 2008; **69**: 471–478.
14. Ganirelix Dose-finding Study Group. *A double-blind, randomized, dose-finding study to assess the efficacy of the gonadotropin-releasing hormone antagonist ganirelix (Org 37462) to prevent premature luteinizing hormone surges in women undergoing ovarian stimulation with recombinant follicle stimulating hormone (Puregon).* Human Reproduction 1998; **13**: 3023–3031.
15. Chappel SC, Howles C. *Reevaluation of the roles of luteinizing hormone and follicle-stimulating hormone in the ovulatory process.* Human Reproduction 1991; **6**:1206–1212.
16. Doody K, Devroey P, Gordon K, Witjes H, Mannaerts B. *LH concentrations do not correlate with pregnancy in rFSH/GnRH antagonist cycles.* Reproductive BioMedicine Online 2010; **20**:565–567.
17. Baruffi RLR, Mauri AL, Petersen CG et al. *Recombinant LH supplementation to recombinant FSH during induced ovarian stimulation in the GnRH-antagonist protocol: a meta-analysis.* RBM Online 2007; **14**: 14–25.
18. Kolibianakis EM, Kalogeropoulou L, Griesinger G et al. *Among patients treated with FSH and GnRH analogues for in vitro fertilization, is the addition of recombinant LH associated with the probability of live birth? A systematic review and meta-analysis.* Human Reproduction Update 2007; **13**: 445–452.
19. Mochtar MH, Van der Veen, Ziech M, van Wely M. *Recombinant luteinizing hormone (rLH) for controlled ovarian hyperstimulation in assisted reproductive cycles.* Cochrane Database of Systematic Reviews 2007; **18**(2): CD005070.
20. Oliveira JBA, Mauri AL, Petersen CG et al. *Recombinant luteinizing hormone supplementation to recombinant follicle-stimulating hormone during induced ovarian stimulation in the GnRH-agonist protocol: a meta-analysis.* Journal of Assisted Reproduction and Genetics 2007; **24**: 67–75.
21. Sills ES, Levy DP, Moomjy M, McGee M, Rosenwaks Z. *A prospective, randomized comparison of ovulation induction using highly purified follicle stimulating hormone alone and with recombinant human luteinizing hormone in in-vitro fertilization.* Human Reproduction 1999; **14**: 2230–2235.
22. Tarlatzis B, Tavmergen E, Szamatowicz M et al. *The use of recombinant human LH (lutropin alfa) in the late stimulation phase of assisted reproduction cycles: a double-blind, randomized, prospective study.* Human Reproduction 2006; **21**: 90–94.
23. Balasch J, Miro F, Burzaco I et al. *The role of luteinizing hormone in human follicle development and oocyte fertility: evidence from in-vitro fertilization in a woman with long-standing hypogonadotropic hypogonadism and using recombinant human follicle stimulating hormone.* Human Reproduction 1995; **10**: 1678–1683.
24. European Recombinant Human LH Study Group. *Recombinant human luteinizing hormone (LH) to support recombinant human follicle-stimulating hormone (FSH)-induced follicular development in LH- and FSH-deficient anovulatory women: a dose finding study.* Journal of Clinical

- Endocrinology and Metabolism 1998; **83**: 1507–1514.
25. Polyzos NP, Devroey P. A systematic review of randomized trials for the treatment of poor ovarian responders: is there any light at the end of the tunnel? Fertility and Sterility 2011; **96**: 1058–1061.
 26. Ferraretti AP, La Marca A, Fauser BCJM et al. ESHRE consensus on the definition of “poor response” to ovarian stimulation for in vitro fertilization: the Bologna criteria. Human Reproduction 2011; **26**(7): 1616–1624.
 27. Papathanasiou A. Implementing the ESHRE “poor responder” criteria in research studies: methodological implications. Human Reproduction 2014; **29**: 1835–1838.
 28. Musters AM, van Wely M, Mastenbroek S et al. The effect of recombinant LH on embryo quality: a randomized controlled trial in women with poor ovarian reserve. Human Reproduction 2012; **27**: 244–250.
 29. Bosch E, Labarta E, Crespo J et al. Impact of luteinizing hormone administration on gonadotropin-releasing hormone antagonist cycles: an age-adjusted analysis. Fertility and Sterility 2011; **95**: 1031–1036.
 30. De Placido G, Alviggi C, Perino A et al. Recombinant human LH supplementation versus recombinant human FSH (rFSH) step-up protocol during controlled ovarian stimulation in normogonadotropic women with initial inadequate ovarian response to rFSH. A multicentre, prospective, randomized controlled trial. Human Reproduction 2005; **20**: 390–396.
 31. Berkkanoglu M, Isikoglu M, Aydin D, Ozgur K. Clinical effects of ovulation induction with recombinant follicle-stimulating hormone supplemented with recombinant luteinizing hormone or low-dose recombinant human chorionic gonadotropin in the midfollicular phase in microdose cycles in poor responders. Fertility and Sterility 2007; **88**: 665–669.
 32. Barrenetxea G, Agirregoitko JA, Jimenez MR et al. Ovarian response and pregnancy outcome in poor-responder women: a randomized controlled trial on the effect of luteinizing hormone supplementation on in vitro fertilization cycles. Fertility and Sterility 2008; **89**: 546–553.
 33. Humaidan P, Chin W, Rogoff D et al. Efficacy and safety of follitropin alfa/ lutropin alfa in ART: a randomized controlled trial in poor ovarian responders. Human Reproduction 2017; **32**: 544–555.
 34. Lehert P, Kolibianakis EM, Venetis C et al. Recombinant human follicle-stimulating hormone (rhFSH) plus recombinant luteinizing hormone versus r-hFSH alone for ovarian stimulation during assisted reproductive technology: systematic review and meta-analysis. Reproductive Biology and Endocrinology 2014; **12**: 17.
 35. Fan W, Li S, Chen Q et al. Recombinant luteinizing hormone supplementation in poor responders undergoing IVF: a systematic review and meta-analysis. Gynecological Endocrinology 2013; **29**(4): 278–284.
 36. Vuong TNL, Phung HT, Ho MT. Recombinant follicle-stimulating hormone and recombinant luteinizing hormone versus recombinant follicle-stimulating hormone alone during GnRH antagonist ovarian stimulation in patients aged ≥ 35 years: a randomized controlled trial. Human Reproduction 2015; **30**: 1188–1195.
 37. Behre HM, Howles CM, Longobardi S. Randomized trial comparing luteinizing hormone supplementation timing strategies in older women undergoing ovarian stimulation. Reproductive BioMedicine Online 2015; **31**: 339–346.

Growth Hormone as an Adjunct to Controlled Ovarian Hyperstimulation for IVF

Rui Wang, Roger Hart, and Robert J. Norman

15.1 Physiology

Growth hormone is a protein synthesized, stored and secreted by the anterior pituitary gland. Apart from its effects on growth and metabolism, it plays important roles in pubertal development and growth. It is thought to be important in menstruation and reproduction by affecting gametogenesis and steroidogenesis.

It is known that deficiency or insufficiency of growth hormone causes delay of puberty unless treated with synthetic growth hormone [1]. Growth hormone may activate cells directly by binding to the growth hormone receptor or indirectly by inducing the production of insulin-like growth factor-I (IGF-1) [2]. The IGF-1 acts as a systemic growth factor and also a negative feedback regulator of growth hormone secretion [3]. The growth hormone receptor is also expressed in granulosa, cumulus cells and the oocytes but does not appear to control the local expression of IGF-1 in the ovary. Most of the effects of growth hormone appear to be mediated by systemic indirect effects of growth hormone rather than local actions of growth hormone on the follicle. Both systemic and local ovarian IGF-1 appear to be involved in controlling follicular development [3]. Growth hormone may increase the intra-ovarian production of IGF-1, sensitizing the ovary to gonadotrophin stimulation, stimulating follicular development, oestrogen production and oocyte maturation [2]. Therefore growth hormone could potentially play an important role in controlled ovarian stimulation protocol in IVF.

15.2 Evidence on the Use of Growth Hormone in IVF

The first pilot study on the use of growth hormone as an adjuvant to ovarian stimulation was published in 1988 [4]. This study reported seven ovulatory infertile women with hypogonadotropic hypogonadism treated with a combination of human menopausal gonadotrophins (hMG) and growth hormone, including four undergoing in vivo fertilization and three in vitro fertilization (IVF). It showed that the addition of growth hormone to gonadotropin therapy significantly augmented the ovarian response. Since then, randomized controlled trials (RCTs) have been conducted to examine the role of growth hormone as an adjuvant to ovarian stimulation for IVF [4].

The first RCT reporting the use of growth hormone solely in IVF was published in 1991 [5]. In this study, Owen and colleagues included 25 suboptimal responders who had less than six oocytes collected with less than four embryos developed in a previous IVF cycle. They randomized the participants into two groups: 13 with growth hormone co-treatment (24 IU, alternate days) starting on the first day of hMG till the use of human chorionic gonadotropin (hCG) administration and 12 in the placebo group. They found that the use of

growth hormone significantly reduced the total dosage of hMG but showed no differences in other outcomes [5].

15.2.1 Use of Growth Hormone in Women with Poor Ovarian Response

Since the first RCT in the United Kingdom, several RCTs on patients with a documented poor response to ovarian stimulation – ‘poor responders’ – have been published in other countries. In 1994, a Swedish group published a placebo-controlled, double-blinded, four-leg parallel pilot study including 40 poor responders (defined as at least two previously performed and failed IVF attempts) [6]. They found that the number of oocytes retrieved and the amount of hMG required did not differ between the groups, while the oocyte fertilization rate and the serum and follicular concentration of IGF-1 were higher in the growth hormone group. The authors concluded that the overall results did not support growth hormone as a clinically useful adjuvant treatment. In the same year, a Chinese group published an RCT of 27 poor responders and found that the use of growth hormone increased the levels of serum IGF-I and the pregnancy rate, but did not improve ovarian response or lead to a reduction in the amount of hMG required for ovarian stimulation [7].

In 1995, Dor and colleagues randomized 14 women who previously failed to conceive and demonstrated a poor response to ovarian stimulation during treatment in a double-blind, placebo-controlled study [8]. The authors found no differences in the ovarian stimulation outcomes, including the number of hMG ampoules used, the number of follicles (>14 mm), serum oestradiol concentration on the day of administration of the oocyte trigger injection, the number of oocytes retrieved and fertilized and the number of embryos transferred. None of the participants in this study conceived. A year later, Suikkari and colleagues investigated 22 poor responders in a placebo-controlled, dose-comparison study [9]. Participants in this study were randomized into placebo, growth hormone 4 IU or 12 IU group. The results showed that there was no improvement in cycle outcomes with daily use of either 4 or 12 IU of growth hormone, while serum IGF-I levels were highest in the group administered 12 IU of growth hormone.

The first Cochrane systematic review on the use of growth hormone in IVF was published in 2000 [10]. The pooled analysis of six studies of poor responders showed no statistically significant difference in terms of pregnancy per cycle (odds ratio (OR) 2.55, 95% CI 0.64–10.12). The authors called for further well-powered studies to address this question.

In 2007, another Chinese group compared 40 women with poor ovarian responses co-administrated with growth hormone plus aspirin and without co-treatments [11]. They found an increased rate of oocytes collected, oocyte maturation and fertilization in those women administered growth hormone plus aspirin, while no significant differences in the number of replaced embryos or pregnancy rate were detected. A subsequent Turkish RCT of 61 poor responders demonstrated no difference in clinical pregnancy rate between the control group and the group administered growth hormone (12/31 vs 6/30) [12].

Since 2009, there have been several well-conducted systematic reviews and meta-analyses of RCTs on this topic. In 2009, a systematic review and meta-analysis including six RCTs was published in the *Human Reproduction Update* [13]. This meta-analysis included 169 women with poor ovarian response undergoing ovarian stimulation for IVF. It showed that the addition of growth hormone significantly increased the proportion of women reaching embryo transfer (rate difference +22%, 95% CI +7 to +36), clinical

pregnancy (rate difference +16%, 95% CI +4% to +28%) and the live birth rate (rate difference +17%, 95% CI +5% to +30%). Another systematic review of RCTs evaluated different strategies to improve the pregnancy rate in poor responders undergoing IVF [14]. Five of these RCTs compared ovarian stimulation with and without the use of growth hormone, and pooled analysis suggested that addition of growth hormone significantly increased live birth rate in poor responders (OR 5.22, 95% CI 1.09–24.99).

The most recent Cochrane systematic review was updated in 2010 [15]. This review included ten RCTs of women undergoing IVF. Subgroup analysis of women with poor ovarian response showed a statistically significant increase in both live birth rates (OR 5.39, 95% CI 1.89–15.35) and pregnancy rates (OR 3.28, 95% CI 1.74–6.20) without increasing adverse events. The authors addressed that interpretation of results should be with a degree of caution, as the number of studies and the sample size were both small.

While in all the previous studies, growth hormone was used as a co-treatment in a GnRH-a down-regulation IVF protocol, in 2013, an Iranian RCT studied the use of growth hormone in a GnRH-antagonist protocol. This study included 82 poor responders and found that women with growth hormone had more retrieved oocytes (6.10 ± 2.90 vs 4.80 ± 2.40 , $p = 0.035$) and embryos generated in an IVF cycle (3.7 ± 2.89 vs 2.7 ± 1.29 ($p = 0.018$), but no significant differences in implantation, chemical and clinical pregnancy rates were detected [16]. In 2015, an Egyptian group published an RCT of 145 poor responders in a microflare protocol [17]. They found that growth hormone increased the number of oocytes retrieved (7.2 ± 1.5 vs 4.7 ± 1.2 , $P < 0.001$), metaphase II oocytes observed (5.2 ± 1.2 vs 2.8 ± 1.0 , $P < 0.001$) and fertilized oocytes generated (4.2 ± 1.1 vs 2.5 ± 0.7 ; $P < 0.001$). Again, there were no differences in terms of the implantation and clinical pregnancy rates. More recently, another Egyptian group published an RCT of 141 poor responders in a GnRH-antagonist protocol [18]. Similarly, the authors found that the addition of growth hormone increased the number of collected oocytes (7.58 ± 1.40 vs 4.90 ± 1.78), metaphase II oocytes (4.53 ± 1.29 vs 2.53 ± 1.18), fertilized oocytes (4.04 ± 0.96 vs 2.42 ± 1.03) and transferred embryos (2.89 ± 0.45 vs 2.03 ± 0.81), while no significant difference in clinical pregnancy rate per cycle (22.1 vs 15.1 percent) or live birth rate per cycle was evident (14.7 percent vs 10.9 percent).

All previous studies have relatively small sample sizes and these studies were not powered for fertility outcomes. Therefore, the conclusions with respect to pregnancy outcomes, especially live birth, were underpowered. An Australian group conducted by far the largest RCT on growth hormone – the LIGHT study. This study was intended to recruit 390 women with poor ovarian response while it enrolled 136 participants and was presented at the European Society of Human Reproduction and Embryology 2016 annual meeting [19]. It showed improvement in the ovarian response in women administered growth hormone, but not live birth.

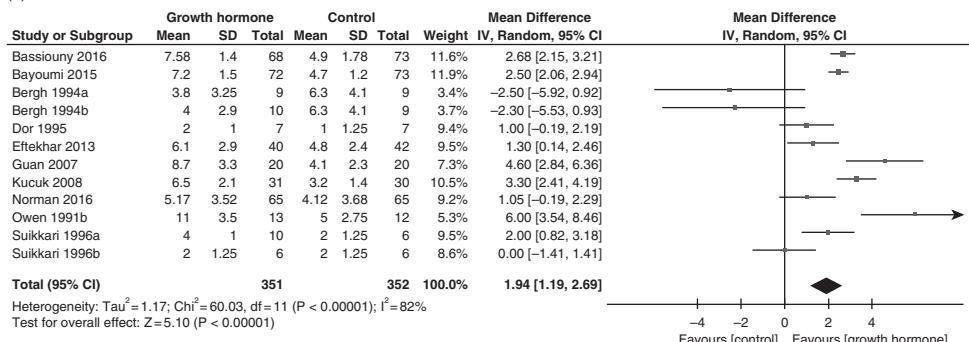
An updated meta-analysis was performed by Hart et al. (2017) with all the available studies analysed (Figure 15.1) [20]. It showed that in poor responders undergoing ovarian stimulation, the use of adjunct growth hormone increased the number of retrieved oocytes (mean difference (MD) 1.94, 95% CI 1.19–2.69) and fertilized oocytes (MD 1.72, 95% CI 1.13–2.31). There was a benefit noted in terms of positive pregnancy test (OR 1.79, 95% CI 1.26–2.55) and clinical pregnancy (OR 1.51, 95% CI 1.00–2.88) while not in live birth (OR 1.54, 95% CI 0.86–2.74). The authors noted the heterogeneous definitions of poor responders and use of ovarian stimulation protocols. Therefore, the results should be interpreted with caution.

15.2.2 Use of Growth Hormone in Women with Normal or High Ovarian Response

The evidence of use of growth hormone in women other than poor responders is limited. Although the first published RCT on growth hormone in IVF included 25 suboptimal responders, 18 out of the 25 had ultrasound-diagnosed polycystic ovaries (PCO) [5]. In this subset of women with PCO, the use of growth hormone increased the number of follicles, oocytes, fertilization and cleavage embryos [5].

In 1992, a Finnish research group reported an RCT of 54 women with normal menstrual cycle co-treated with growth hormone or placebo in IVF [21]. They found that the number of hMG ampoules, follicles and oocytes were similar in both groups. An Israeli group published a similar study in the same year [22]. They randomized 42 normal ovulatory women with mechanical factor infertility into two groups, a growth hormone and a placebo group. Similarly, there were no significant differences between

(a)



(b)

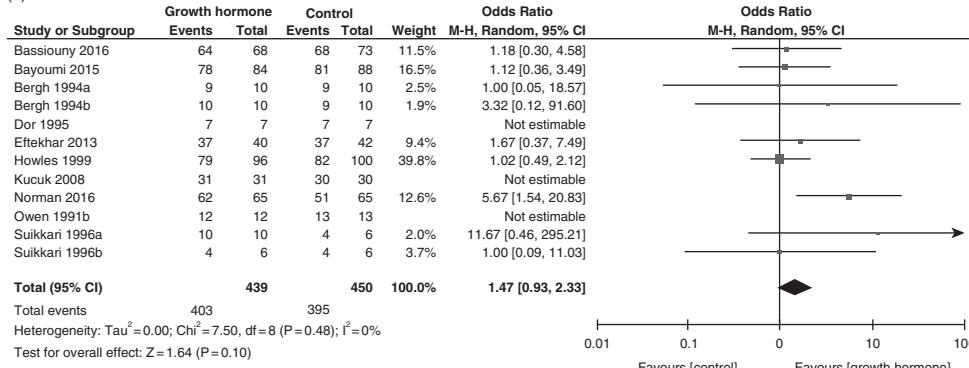
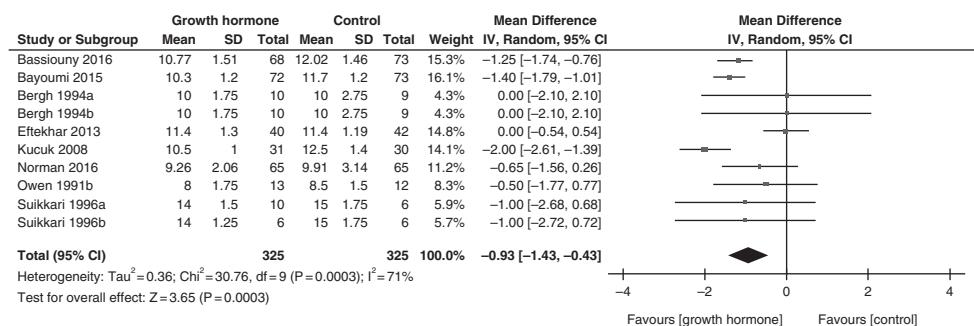


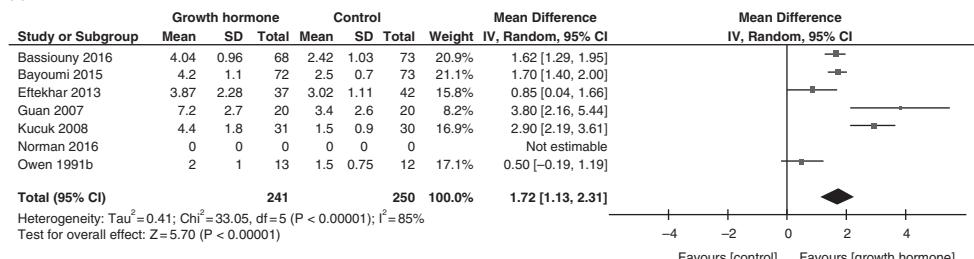
Figure 15.1 (a) 'Forest plot of comparison: oocytes collected per cycle started. Not all patients reached oocyte retrieval'. (b) 'Patients reached oocyte retrieval and had at least one oocyte retrieved'. (c) 'The duration of stimulation'. (d) 'Number of fertilized oocytes for women per cycle started (some data are presented by patients who had oocytes retrieved)'. (e) 'Patients with an embryo available for transfer per cycle started'. (f) 'Positive pregnancy test per cycle started'. (g) 'Clinical pregnancy per cycle started'. (h) 'Live birth per cycle started'. (From Hart et al. *Curr Opin Obstet Gynecol* 2017, 29(3):119–125.) [20]

Figure 15.1 (Cont.)

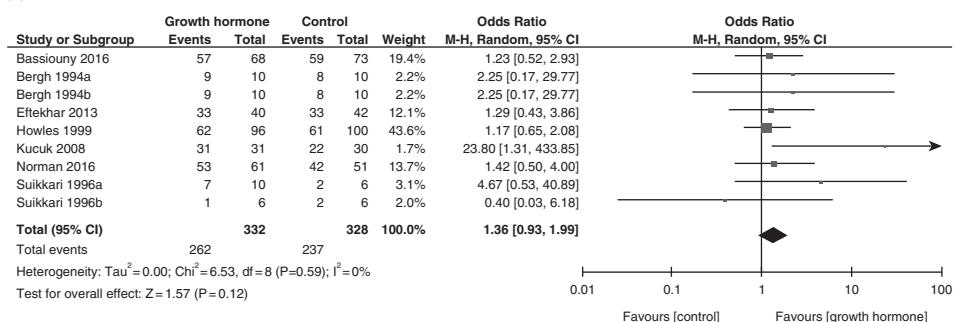
(c)



(d)



(e)



(f)

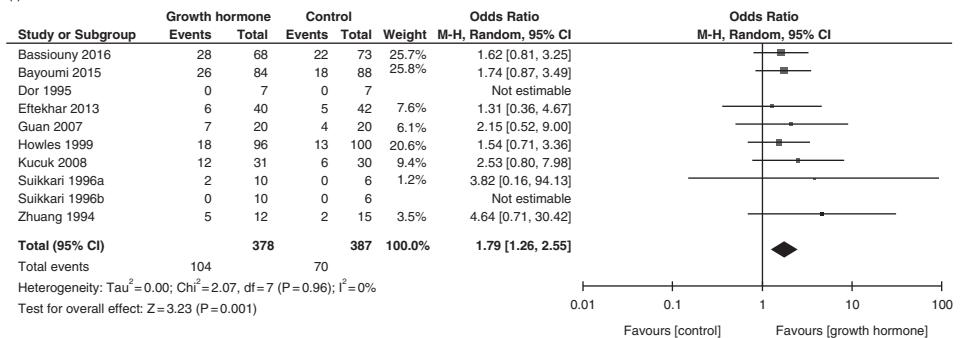
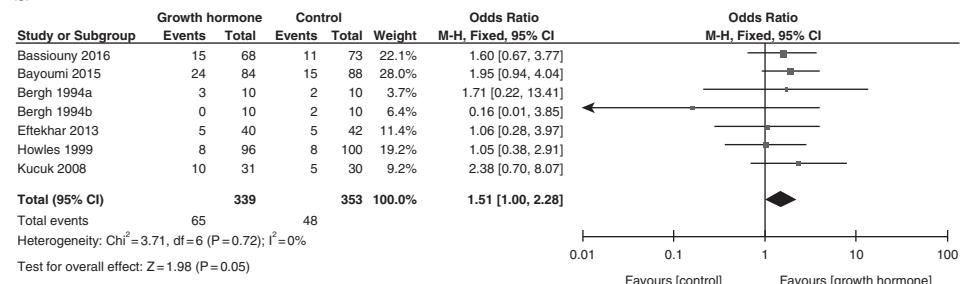
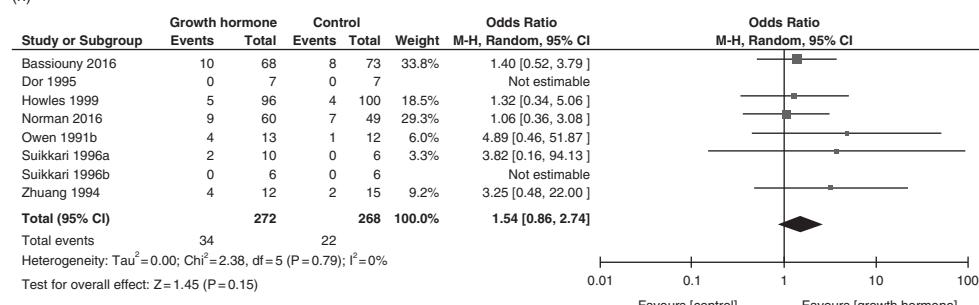


Figure 15.1 (Cont.)

(g)



(h)



two groups with respect to the number of hMG ampoules used, serum oestradiol and the number of follicles, oocytes and embryos generated. The embryo morphology and rate of cleavage were also similar. Moreover, there was no difference in the implantation or clinical pregnancy rates per embryo transfer. The Cochrane review in 2010 included these two studies in a pooled analysis and showed no difference in outcome measures in the routine use of adjuvant growth hormone in IVF protocols [15].

15.2.3 The Dosage and Duration of Growth Hormone

Currently there is no standard dosage or duration of growth hormone in an ovarian stimulation protocol. In most studies growth hormone was co-administrated together with gonadotrophins or even before ovarian stimulation. The dosage and duration varied from study to study, as summarized in Table 15.1.

15.2.4 Adverse Events of Growth Hormone

The evidence of adverse events from the published RCTs is limited. One study [6] reported two participants with slight oedema during treatment that resolved spontaneously after 1 to 2 days, while three studies [9, 12, 18] reported no adverse events. Given the relative small sample sizes of these RCTs, adverse events of growth hormone need to be confirmed in future large studies.

Table 15.1 The dosage and duration of growth hormone

Study	Dosage	Duration
Owen 1991 [5]	24 IU, i.m. per day	Alternate days for a maximum period of 2 weeks until the administration of hCG
Tapanainen 1992 [21]	24 IU i.m. per day	On alternate days starting on cycle day 4 until the day of last hMG injection
Younis 1992 [22]	12 IU s.c. per day	On days 1, 3, 5 and 7 of hMG treatment
Bergh 1994 [6]	0.1 IU/kg body weight per day, s.c.	Started after GnRH down-regulation, continued until sufficient follicular maturation was achieved, for a maximum of 25 days from the start of stimulation
Zhuang 1994 [7]	2 IU i.m. per day	On alternate days for 6 times
Dor 1995 [8]	18 IU s.c. per day	On days 2, 4, 6 and 8
Suikkari 1996 [9]	12 or 4 IU s.c. per day	From day 3 of spontaneous menstrual cycle
Guan 2007 [11]	4 IU i.m. per day	On alternate days starting from mid-luteal phase of preceding cycle along with GnRH-a
Kucuk 2008 [12]	4mg (12 IU) s.c. per day	From day 21 of preceding cycle along with GnRH-a, until the day of hCG
Eftekhari 2013 [16]	4 IU i.m. per day	From day 21 of previous cycle until the day of hCG injection
Bayoumi 2015 [17]	2.5 mg s.c. per day	From day 6 of hMG stimulation until the day of hCG
Bassiouny 2016 [18]	2.5 mg (equivalent to 7.5 IU) s.c. per day	From day 6 of hMG stimulation until the day of hCG triggering
Norman 2016 [19]	12 IU s.c. per day	From day 1 of IVF ovarian stimulation using recombinant follicle-stimulating hormone administration to day of oocyte maturation

15.3 Recommendations from the Guidelines

Based on the current evidence, mainly on the meta-analysis published in 2009 and the updated Cochrane systematic review in 2010, the updated NICE 2013 guideline concluded that there is no clear evidence of a benefit in supplementing IVF cycles routinely with growth hormone. Although there may be a positive effect in the use of GH supplementation in poor responders, studies have been weak and heterogeneous. Therefore, it recommended not to use growth hormone as adjuvant treatment in IVF protocols [23].

In a more recent narrative review of evidence on ovarian stimulation provided for the World Health Organization, the authors summarized that there is low-quality evidence to support use of growth hormone as adjuvant treatments during IVF for poor responders [24]. Overall there appears to be little value to its use given the expense of additional treatment.

References

1. Spiliotis BE. Growth hormone insufficiency and its impact on ovarian function. *Ann N Y Acad Sci.* 2003;997:77–84.
2. Homburg R, Singh A, Bhide P, Shah A, Gudi A. The re-growth of growth hormone in fertility treatment: a critical review. *Hum Fertil (Camb).* 2012;15: 190–3.
3. Lucy MC. Growth hormone regulation of follicular growth. *Reprod Fertil Dev.* 2011;24: 19–28.
4. Homburg R, Eshel A, Abdalla HI, Jacobs HS. Growth hormone facilitates ovulation induction by gonadotrophins. *Clin Endocrinol (Oxf).* 1988;29: 113–7.
5. Owen EJ, Shoham Z, Mason BA, Ostergaard H, Jacobs HS. Cotreatment with growth hormone, after pituitary suppression, for ovarian stimulation in in vitro fertilization: a randomized, double-blind, placebo-control trial. *Fertil Steril.* 1991;56: 1104–10.
6. Bergh C, Hillensjo T, Wikland M et al. Adjuvant growth hormone treatment during in vitro fertilization: a randomized, placebo-controlled study. *Fertil Steril.* 1994;62: 113–20.
7. Zhuang GL, Wong SX, Zhou CQ. The effect of co-administration of low dosage growth hormone and gonadotropin for ovarian hyperstimulation in vitro fertilization and embryo transfer. *Zhonghua Fu Chan Ke Za Zhi.* 1994;29: 471–4, 510.
8. Dor J, Seidman DS, Amudai E et al. Adjuvant growth hormone therapy in poor responders to in-vitro fertilization: a prospective randomized placebo-controlled double-blind study. *Hum Reprod.* 1995;10: 40–3.
9. Suikkari A, MacLachlan V, Koistinen R, Seppala M, Healy D. Double-blind placebo controlled study: human biosynthetic growth hormone for assisted reproductive technology. *Fertil Steril.* 1996;65: 800–5.
10. Kotarba D, Kotarba J, Hughes E. Growth hormone for in vitro fertilization. *Cochrane Database Syst Rev.* 2000(2):CD000099.
11. Guan Q, Ma HG, Wang YY, Zhang F. Effects of co-administration of growth hormone(GH) and aspirin to women during in vitro fertilization and embryo transfer (IVF-ET) cycles. *Zhonghua Nan Ke Xue.* 2007;13:798–800.
12. Kucuk T, Kozinoglu H, Kaba A. Growth hormone co-treatment within a GnRH agonist long protocol in patients with poor ovarian response: a prospective, randomized, clinical trial. *J Assist Reprod Genet.* 2008;25: 123–7.
13. Kolibianakis EM, Venetis CA, Diedrich K, Tarlatzis BC, Griesinger G. Addition of growth hormone to gonadotrophins in ovarian stimulation of poor responders treated by in-vitro fertilization: a systematic review and meta-analysis. *Hum Reprod Update.* 2009;15: 613–22.
14. Kyrou D, Kolibianakis EM, Venetis CA et al. How to improve the probability of pregnancy in poor responders undergoing in vitro fertilization: a systematic review and meta-analysis. *Fertil Steril.* 2009;91: 749–66.
15. Duffy JM, Ahmad G, Mohiyideen L, Nardo LG, Watson A. Growth hormone for in vitro fertilization. *Cochrane Database Syst Rev.* 2010(1):CD000099.
16. Eftekhar M, Aflatoonian A, Mohammadian F, Eftekhar T. Adjuvant growth hormone therapy in antagonist protocol in poor responders undergoing assisted reproductive technology. *Arch Gynecol Obstet.* 2013;287: 1017–21.
17. Bayoumi YA, Dakhly DM, Bassiouny YA, Hashish NM. Addition of growth hormone to the microflare stimulation protocol among women with poor ovarian response. *Int J Gynaecol Obstet.* 2015;131: 305–8.
18. Bassiouny YA, Dakhly DM, Bayoumi YA, Hashish NM. Does the addition of growth hormone to the in vitro fertilization/intracytoplasmic sperm injection antagonist protocol improve outcomes in poor responders? A randomized, controlled trial. *Fertil Steril.* 2016;105:697–702.
19. Norman RJ, Alvino H, Hart R, Rombauts L, the LIGHT investigators. A randomised double blind placebo controlled study of recombinant human growth hormone

- (r-HGH) on live birth rates in women who are poor responders. *Hum Reprod.* 2016;31 (Suppl 1):i37.
20. Hart RJ, Rombauts L, Norman RJ. Growth hormone in IVF cycles: any hope? *Curr Opin Obstet Gynecol.* 2017;29: 119–25.
 21. Tapanainen J, Martikainen H, Voutilainen R et al. Effect of growth hormone administration on human ovarian function and steroidogenic gene expression in granulosa-luteal cells. *Fertil Steril.* 1992;58: 726–32.
 22. Younis JS, Simon A, Koren R et al. The effect of growth hormone supplementation on in vitro fertilization outcome: a prospective randomized placebo-controlled double-blind study. *Fertil Steril.* 1992;58: 575–80.
 23. The National Institute for Health and Care Excellence. Fertility problems: assessment and treatment. Clinical guideline [CG156]. 2013.
 24. Farquhar C, Marjoribanks J, Brown J et al. Management of ovarian stimulation for IVF: narrative review of evidence provided for World Health Organization guidance. *Reprod Biomed Online.* 2017.

Mitochondrial Transfer Technology for Improving Older Eggs

J Szeptycki, D. Nayot, and R.F. Casper

16.1 Introduction

Mitochondria are unique organelles, having their own DNA, and play a significant role in energy production. Generating 90 percent of the body's ATP, they are essential to sustain and support life and organ function. Aside from their ATP-related functions, mitochondria are intimately involved in most major metabolic pathways used by each individual cell to grow, biosynthesize, breakdown and recycle molecular building blocks and are involved in programmed cell death or apoptosis. Mitochondria make energy, yet this simple requirement is achieved by a complex and intricate cascade of events known as the electron transport chain.

For mitochondria, missteps along this chain, mutations in DNA replication or environmental stresses or exposure to higher concentrations of reaction chain byproducts (free radicals or reactive oxygen species [ROS]), may result in undesirable pathologies. Mitochondrial diseases vary in presentation, but are recognizably associated with parts of the body having high energy demands, including the endocrine system as a whole. Inevitably, with biological aging comes a decrease in both quantity and quality of mitochondria, which is believed to be a key contributing factor to many of the age-related diseases commonly seen.

The mitochondria form one of the critical components of the cell and are distributed in the cell cytoplasm. The number of mitochondria per cell is highly variable and largely based on cell-specific energy demands. Within the interior matrix region of each mitochondrion lies a double-stranded circular DNA or mitochondrial DNA (mtDNA), which genetically codes for essential subunits of the respiratory chain complexes. Human mtDNA is circular, 16.6 kB long and contains 37 genes (encoding 2 ribosomal RNAs, 22 transport RNAs and 13 proteins). Mitochondria can carry several copies of mtDNA [1], which together with the nuclear DNA comprise the genome of the cell. Another defining feature of mtDNA is their relatively absent repair mechanisms in comparison to nuclear DNA, which accounts for increased mutations and deletions over time.

16.1.1 Maternal Inheritance of mtDNA

The concept of maternal inheritance of mtDNA as the decades-old consensus among reproductive biologists remains debated. The process of spermiogenesis includes gradual mtDNA copy elimination to a tenth of its original value. On average, the mature mammalian sperm contains 50–75 mitochondria and as few as 10 copies of mtDNA [2]. There is evidence that mtDNA copy number is negatively correlated with semen parameters, including sperm motility, concentration, morphology, progression and motion characteristics essential for fertilization [3].

In contrast, the typical mammalian oocyte is estimated to contain 30,000 (10^3) to 1,000,000 (10^6) mitochondria, and the human oocyte in particular is estimated to contain about 30,000 mitochondria and about 200,000 copies of mtDNA at the metaphase II (MII) stage [4]. The increased mtDNA copy number in these cells is related to their fertilizability [5].

Upon natural fertilization, there is no question that paternal mitochondria enter the ovum. As such, the notion of maternal inheritance of mtDNA may be a consequence of detection limits of technology or a selective pressure as the result of the dilution of paternal mtDNA. The maternal inheritance of mtDNA is fundamentally preserved at the cellular level. Mechanisms of autophagy as well as nuclease-dependent and ubiquitin-proteasome tagging systems provide evidence of selective inactivation or destruction of the paternal mtDNA in the developing embryo; however, the result is not absolute [6]. There is increasing evidence of paternal leakage of mtDNA in offspring. Although the majority of claims evidencing paternal inheritance in mammals and specifically humans have been refuted and attributed to laboratory artefact, there is at least one documented case in humans [7].

In the case of assisted reproductive technologies (ART), the complete sperm body, including the mitochondria-rich mid-piece, is introduced directly into the oocyte during intracytoplasmic sperm injection (ICSI). Paternal mtDNA has been detected in 4–8 cell stage abnormal embryos [8]; to our knowledge no paternal mtDNA has been detected in infants born after ICSI [9].

16.2 The Ageing Oocyte

Irrespective of race and ethnicity, the trend of women delaying child bearing until later reproductive years continues to grow. The challenge is that IVF success rates are inversely proportional to age, progressively decreasing from a national average of 40 percent cumulative live birth rate per egg retrieval at mid-30s to a less than 15 percent in early 40s [10]. By age 45, the only reasonable option is the use of donor eggs but many women in their early 40s opt to accept the lower chance of becoming pregnant to preserve their genetic heritage. Egg donation success rates confirm poor egg quality rather than endometrial receptivity as the primary obstacle in age-related decline in fertility. Understanding the basis for decreased oocyte competence in advanced maternal age patients is significant in infertility treatment.

During the reproductive lifetime, the natural loss of oocytes through apoptosis begins in utero and progresses at the same rate until menopause [11]. At the same time, the reproductive system is exposed to stochastic events, including the environment and other non-genetic factors. Adaptability to internal and external stresses is limited and may result in cumulative damage and epigenetic changes. These epigenetic changes including DNA methylation and histone modifications (e.g. methylation, acetylation, ubiquitination) may interfere with the regulation of gene expression in preovulatory ageing oocytes and most notably mitochondrial function.

Given the high energy requirements of oocyte maturation and early cleavage stage embryos, mitochondria, as the primary source of bioenergy, are critical for developmental competence. As embryonic mitochondrial replication does not occur until after the hatched blastocyst stage, maturation, fertilization and early embryonic growth are dependent upon the function of the mitochondrial pool present at ovulation. The critical minimal threshold theory for mitochondrial content at the mature (MII) oocyte phase is echoed by the evidence

that there is amplification of mitochondria quantity as the oocyte matures through folliculogenesis and that the mature oocyte has the highest mitochondrial concentration by several factors than any other type of cell in the body. Mitochondrial dysfunction is recognized as a significant factor in the phenotype of the ageing oocyte.

Mitochondrion exposure to extreme stress (elevated Ca^{2+} and ROS, nucleotide depletion, reduced membrane potential) can activate mechanisms initiating apoptosis and mitophagy. Inadequate numbers of mitochondria, quality and efficiency and concomitant increase in mtDNA mutation could lead to meiotic spindle disruption and segregation errors that may delay or terminate final maturation [12]. Similarly, mitochondrial dysfunction is a causative factor in poor embryonic development, implantation failure and birth defects.

As women age, both the oocyte quantity (ovarian reserve) and quality (competence) are negatively impacted, resulting in less efficient results in IVF treatment cycles. Investigation into therapeutic intervention and treatments aiming to improve mitochondrial function or repair are crucial in successful IVF outcomes. As such, under the purview of appropriate ethics and risk assessment, mitochondrial-based rejuvenation technologies have been explored.

Oocyte and embryo aneuploidy are directly related to maternal age and mitochondrial activity [13] as the rate of oocyte aneuploidy increases from approximately 25 percent at age <35, increasing to 80–90 percent by age 40, 95 percent by age 43 and nearly 100 percent in women >45. This relationship is also observed in the oocytes of advanced-age female bovine, hamsters and mice. The majority of age-related oocyte aneuploidy is believed to be related to meiosis I errors (non-disjunction or premature sister chromatid separation) of the oocyte that is prior to fertilization. The aetiology of this meiotic aberration has been postulated to be related to meiotic spindle dysfunction (mitochondria aggregate around the spindle during the first meiotic division), but ultimately a result of energy depletion likely of mitochondrial origin. The point in time of non-disjunction or premature sister chromatid separation is critical, as the current mitochondrial transfer techniques are aimed at optimizing IVF outcomes in older oocytes by targeting MII oocytes or pronuclear embryos. There is no evidence to support that aneuploid mature oocytes can be self-corrected even with adjuvant healthy mitochondria since meiotic errors will affect all of the cells in the embryo. Thus, in essence, mitochondrial transfer techniques attempt to maintain a euploid oocyte and provide it optimal energy as it progresses through fertilization, blastocyst formation and until implantation where embryonic mitochondrial replication ensues. On the other hand, intervention that may improve mitochondrial function prior to meiosis of the oocyte (such as during folliculogenesis or in vitro maturation) may theoretically improve the oocyte euploid yield during natural or assisted reproductive technologies.

16.3 Prior to Mature Oocyte

16.3.1 Mitochondrial Focused Supplements

As mentioned above, mitochondrial replication does not occur from mature oocyte to hatching blastocyst. Therefore, the amount and competence of mitochondria present in a mature oocyte are critical for reproductive success, leading to a minimal threshold theory. Studies support that human oocytes with a lower mtDNA count are more likely to be present in both older patients and oocytes that failed to fertilize, which is consistent with

other studies noting that a decrease in ATP production increases the embryo aneuploidy rate likely through meiotic non-disjunction (an energy-rich process) [14]. The benefit of mitochondrial transfers or addition of high-quality mitochondria during IVF in older patients may be limited as the mature oocyte is at significant risk of already being aneuploid to start with. However, supplements – mitochondrial cofactors and antioxidant therapies – may play a vital role in optimizing the functional mitochondrial load prior to fertilization (e.g. during folliculogenesis). Many fertility practitioners routinely implement some form of supplementation (whether by diet or pharmaceutical) for several weeks prior to an upcoming IVF cycle in the hope of improving oocyte and sperm quality.

Mitochondrial dysfunction secondary to cumulative oxidative stress is believed to contribute to age-related morbidities, one of which is fertility. Of interest, the mitochondria residing within the oocyte are essentially adjacent to the electron transport chain, which is the prime producer of ROS. On a cellular level, supplements such as alpha lipoic acid, resveratrol, L-carnitine and coenzyme Q10 have shown some benefit in improving mitochondrial function and even reproductive outcomes [15]. The list of supplements is quite exhaustive, but some of the better studied supplements also include vitamin C, vitamin E, zinc, selenium, melatonin, N-acetyl-cysteine (NAC), glutathione and L-arginine.

The vast majority of evidence comes from animal models or in vitro studies, as there is a paucity of clinical studies highlighting the role of mitochondrial cofactors and antioxidant therapy for women to improve IVF success [16]. Apart from the type of supplement that may be helpful, there is even less known about their optimal regimen, the dosage and duration required to achieve such results. Needless to say, there is also a significant role for antioxidant therapy pretreatment in men (as spermatozoa are particularly susceptible to oxidative stress), as well as their incorporation into embryo culture media. To this point it must be stressed that all exogenous supplementation has the potential to be deleterious ('the dose makes the poison' – Paracelsus); the use of mitochondrial cofactors and antioxidant therapies should be guided by evolving clinical evidence.

The more preventative approach to dealing with the negative impact of oxidative stress is by reducing their generation. Optimizing lifestyle habits, such as smoking cessation, exercising regularly and maintaining ideal body weight, along with limiting environmental toxicants, has a positive impact on baseline mitochondrial function and reproduction.

16.3.2 In Vitro Maturation

In vitro maturation (IVM) is a proven clinical technique to collect immature oocytes (germinal vesicle (GV) or MI) and mature them in culture prior to performing standard fertilization techniques with subsequent embryo development. Although the clinical efficiency of IVM is dwarfed compared to IVF, it may play a limited role in a subset of patients, such as those with a very high ovarian reserve at real risk of ovarian hyperstimulation syndrome (OHSS) or perhaps in patients where ovarian stimulation is contraindicated. Regardless of its clinical utility, it does present an interesting opportunity to manipulate immature oocytes as they are at a stage prior to completion of the first meiotic division – a critical event that results in the majority of oocyte aneuploidy. Future technologies focused on mitochondrial transfers or inserting adjuvant healthy mitochondria may have more potential impact on immature oocytes.

16.4 Mature Oocyte and Onwards

16.4.1 Origin of Transfer Technologies

Clear differences in development, implantation and spontaneous loss rates in embryos derived from young and aged oocytes paved the way to preliminary investigations into the aged oocyte rejuvenation technologies. Any manipulation at the time of IVF is essentially aiming to target euploid mature oocytes in the hope of minimizing mitotic errors leading to a viable euploid blastocyst.

The earliest studies (1980s) related to oocyte rejuvenation originated in the mouse embryo whereby transfer of donor ooplasm from healthy donor eggs into developmentally blocked strains restored a degree of developmental competence [17].

Similar studies in non-human models confirmed the importance of the ooplasmic domain in meiotic maturation and preimplantation development as transfer of GV stage cytoplasts led to poor development, arrest and decreased implantation rate. These results were foundational in confirming the importance of a population of ‘factors’ which are critical for normal and successful maturation of oocytes in preparation for fertilization.

Although unknown at the time, cytoplasmic transfer involved the transfer of a myriad of beneficial cytoplasmic factors, which are currently defined as various proteins, mRNA and organelles including what have largely become recognized as restorative mitochondria.

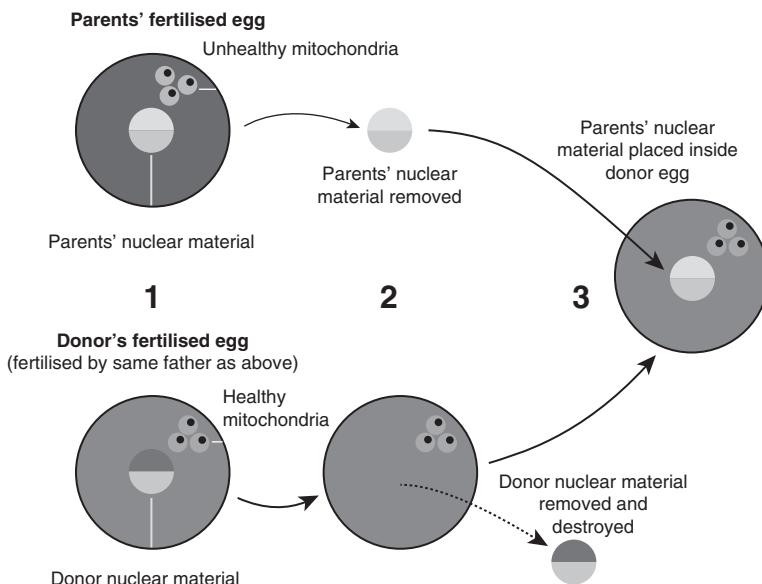
Initially receiving intense scrutiny, it wasn’t until the 1990s that clinical application of this cytoplasmic rescue technology began. Transfer of donor ooplasm from healthy donor eggs at MII stage into patient MII eggs was accomplished by (i) electrofusion of an ooplasmic donor fragment to a patient egg or (ii) direct injection of a small amount of ooplasm directly into a patient egg. Fusion technology revealed a modest increase in fertilization rate, with enhanced pronuclear anomalies and developmental arrest, whereas direct ooplasm injection revealed significantly higher fertilization rate in addition to successful pregnancy outcomes [18]. Similar ooplasm transfer experiments also restored normal growth in the embryos of patients otherwise exhibiting developmental failure [19]. An important determination was an ooplasm volume transfer threshold of ~15–20 percent beyond which was largely detrimental to the oocyte.

At the time, several successful pregnancies were achieved worldwide. As some of the children showed both maternal and donor mtDNA (heteroplasmy), concern was raised (“three parent IVF”), as in mice it was shown that similar mixing of mtDNA led to serious defects [20]. As a result of the potential risk to offspring, to be described later, third-party cytoplasmic (not autologous cytoplasmic) transfer was suspended in the United States and Canada.

Continued investigation into mitochondrial transfer technologies to prevent heritable mitochondrial diseases ensued. However, rather than introducing a small portion of healthy mitochondria and associated mtDNAs, an alternative thought was to isolate and transfer only the desired nuclear material into a healthy, enucleated oocyte or zygote leaving behind the deleterious mtDNA that would otherwise surely result in children born with severe mitochondrial disease.

16.5 Heterologous Mitochondrial Transfer Technologies

Using the same technology to prevent heritable mitochondrial disease could potentially also improve the mitochondrial cellular environment for older patients struggling with

**Figure 16.1**

Pronuclear transfer.
Repair is done after
fertilization

Source: www.theguardian.com/science/2013/jun/28/uk-government-ivf-dna-three-people

infertility, creating a host cellular environment with improved mitochondria. Subsequent attempts to remedy aged oocytes involved microsurgical nuclear transfer techniques otherwise referred to as (i) pronuclear transfer (PNT) or (ii) spindle transfer (ST).

The concept of the PNT method for the purposes of mtDNA replacement was first accomplished in a mouse model and further tested in polynucleic fertilized zygotes [21]. In this method mitochondrial transfer is accomplished post fertilization (Figure 16.1). In a simplified presentation the intended parents' zygote pronuclei are isolated and transferred into a healthy donor zygote which has previously undergone enucleation. Almost all unhealthy mtDNA are left behind in the original parental cytoplasm and the resultant embryo will be implanted into the intended mother anticipating to be unaffected by inherited mitochondrial disease.

Despite a degree of success with the procedure, a concern is that it requires the destruction of an embryo (the host enucleated embryo). A possible solution to avoid embryo destruction is to transfer nuclear material at a pre-fertilization stage.

The ST approach is performed at the time point when the nuclear material is assembled into a meiotic spindle (Figure 16.2). The spindle along with associated metaphase chromosomes is isolated and transplanted into the cytoplasm of a donated, unfertilized oocyte that has undergone enucleation. Free of mutated mtDNA, the constructed oocytes can undergo intracytoplasmic sperm injection for the purposes of fertilization and subsequent embryo transfer. This procedure has been successful in non-human primates [22].

Mitochondrial transfer protocols are technically challenging and require advanced micromanipulation skills. Care and consideration of the sensitivity of the chromosomal material cannot be underestimated in successful outcome.

These techniques are not without controversy. Contrary to the field of clinical ART which has been established with little dependency on clinical trials or animal-based

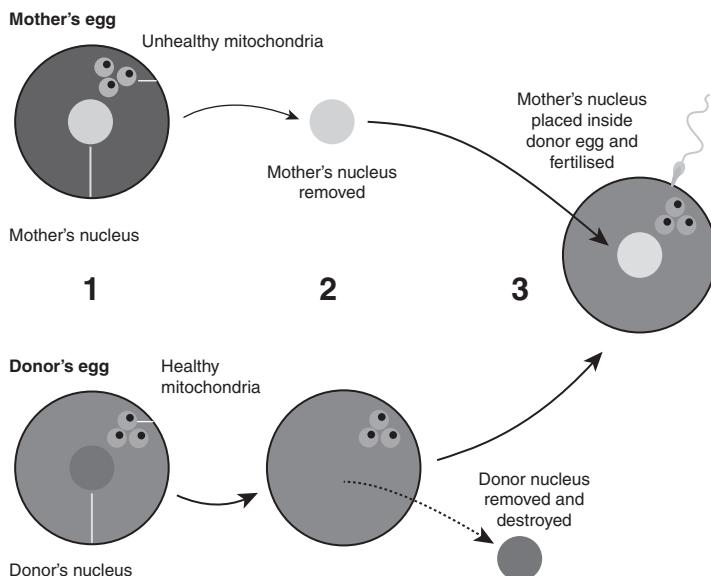


Figure 16.2 Spindle transfer. Repair is done before fertilization
Source: www.theguardian.com/science/2013/jun/28/uk-government-ivf-dna-tree-people

research, the safety and efficacy of germ line therapies present a major challenge in validating proof-of-concept.

16.5.1 Heteroplasmy and Risk Assessment

In the transfer techniques, failure of mitochondrial gene replacement may arise due to inadvertent transfer of mutated mtDNA bound to external structures such as pronuclei or spindles. Some children born using donor mtDNA exhibited mitochondrial heteroplasmy, whereby the cell of the offspring contains more than one mtDNA genotype [23]. As the control of mitochondrial function is mitigated by intricate communication between nuclear and mitochondrial genomes, infusion of third-party mtDNA with deletions and/or mutations could increase risk for heritable mitochondrial disease. Metabolic abnormalities in offspring may be due to incompatibilities in response to nuclear signals between the subpopulations of mtDNA.

Attempts to address these concerns revealed technological limitations in mutant DNA carryover detection below 2 percent, and adding even more complexity, varying distribution of mtDNA may occur within the same organism. Of significance, clinical manifestation of mtDNA disease required a threshold of 60 percent mutant mtDNA or higher [24]. Therefore, it is unlikely that low levels of carryover would manifest disease in children. Studies using distant nuclear and mtDNA haplotypes in rhesus monkey revealed no abnormalities in health and development, further validating the safety and efficacy of this technique [25]. Still, the long-term effects on the health of offspring remain unknown.

16.5.2 Regulatory, Ethical and Moral Considerations

Permanent changes in mtDNA through ST and PNT qualify as germ line gene therapy. The true challenge is to chart an appropriate preclinical course for effective evaluation of safety

in patients. The Human Fertilization and Embryology Authority (HFEA) position statement concludes that current evidence does not explicitly define these techniques as unsafe. As such, ST and PNT technologies are approved in cases of serious mitochondrial disease under specific construct. In as much, their permitted application is ongoing as it is to be conducted under specialized guidance and requires long-term follow-up of offspring.

As previously mentioned, a 2001 USA ban restricts cytoplasmic transfer of third-party mtDNA requiring lengthy clinical studies prior to widespread application. Canada has mirrored the actions of the US FDA in suspending cytoplasmic transfer of third-party mtDNA. Proponents of these technologies arguably view this denial of germ line treatment as unethical in severe mitochondrial disease cases, since replacing mutated mtDNA by ST or PNT does not include hazardous genome alterations as with recombinant DNA vectors.

Discussions surrounding donor mtDNA have utilized the term three-parent IVF giving consideration to parental claims to offspring. These have been largely disregarded as the donor mtDNA comprises less than 1 percent of the aggregated offspring DNA, and the nuclear DNA is solely from the intended parents.

Moral issues concerning mtDNA exchange have likewise been broadly reviewed by the Nuffield Council on Bioethics in the United Kingdom. A compressed report cites social advantage and moral benefit to individuals living free from mitochondrial disease and remarks that in effect such novel strategies are as compelling as medications and should be utilized by families [26].

Compelling evidence does exist supporting the concept of mitochondrial transfer as 2016 marked the birth of the world's first reported "three parent IVF" baby (although one may argue that earlier donor cytoplasmic transfer pregnancies should also be considered to have DNA from three sources). The debate surrounding donor mitochondrial technologies continues as recent attention has turned towards risk avoidance.

In doing so, two measures should be considered:

- (1) Mitochondria used in regeneration should be autologous.
- (2) Mitochondria should be of germ line lineage and have minimal deletions and/or mutations.

16.6 Autologous Mitochondrial Transfer

All cells in the body contain mitochondria (excluding erythrocytes), but the mitochondria within the oocyte are unique in appearance (small, spherical and with few cristae) in comparison to somatic cells (Figure 16.3).

Interestingly, although mitochondria replication is quiescent from fertilization to hatching blastocyst stage, there are significant structural changes that take place during this time to reflect the typical mitochondria seen in somatic cells in comparison to oocytes. Therefore, using oocytes or oocyte precursor cells as the source of surplus mitochondria is quite attractive. Furthermore, having an autologous tissue-specific mitochondrial source eliminates the concern of heteroplasmy.

Advent of a novel mitochondrial transfer technique circumvents the unresolved donor issues through autologous mtDNA transfer. The AUGMENTSM Treatment (OvaScience, Inc., Waltham, MA) involves the delivery of autologous germ line egg precursor cell (also called oogonial stem cells or female germ line stem cells (FGSC))–derived mtDNA to the oocyte during conventional intracytoplasmic sperm injection. Providing germ line quality mitochondria from the patient's own egg precursor cells is hypothesized to increase the

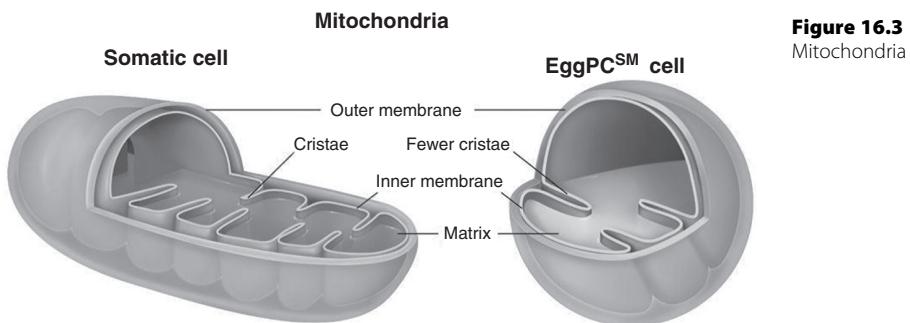


Figure 16.3
Mitochondria

proportion of high-quality, homoplasmic mtDNA in the oocyte and provide the necessary ATP to support early embryonic development in otherwise compromised oocytes [27]. This technology is designed to provide an additional opportunity for women with compromised or poor quality eggs (such as commonly noted in advanced maternal age women) to use their own eggs during IVF. The egg precursor cells are isolated via antibody-mediated cell sorting using an antibody specific for Ddx4 (also known as VASA) from a biopsied section of the ovarian cortex obtained by laparoscopy (Figure 16.4).

The AUGMENT treatment is commercially available; the Toronto Center for Advanced Reproductive Technologies (TCART) IVF laboratory produced the first embryo leading to a successful pregnancy outcome using this technology. Initial experience in a small, select number of patients with different and challenging diagnoses, ages and prior IVF history is encouraging, reporting pregnancy rates from 23 to 53 percent [28]. Still, across clinics, despite reports of egg precursor cells in both mouse and humans, sceptics claim little to no evidence supporting the existence of germ line stem cells and an overall lack of data to support ‘proof-of-concept’. Understandably, the egg precursor cell concept challenges the central dogma that the primordial follicle pool cannot be replenished after birth [29]. This is in contrast to males where the existence of spermatogonial stem cells within the testis is undisputed. In female mice, FGSCs have been isolated by several research groups, and even more intriguingly, they have been shown to be capable of developing into mature oocytes and embryos and producing viable offspring [30].

The hypothesis that replenishment of available mitochondrial stores during microinjection “boosts” developmental competence does not account for the incidence of oocyte aneuploidy in the ageing oocyte as previously described. Despite success in achieving an increased cohort of developmentally competent embryos in aged women, the resulting embryos may be genetically abnormal.

Aside from egg precursor cells, another potential source of autologous mitochondria is sibling ('sister') oocytes. Analogous to the donor ooplasm transfers, sacrificing sibling oocytes to improve the mitochondrial content of targeted oocytes may have the potential to improve reproductive outcomes. As proof-of-concept, pig oocytes known to have mtDNA deficiency increased blastocyst development after mitochondrial supplementation from sibling oocytes [31]. This strategy has yet to be attempted in humans, as sacrificing a subset of mature oocytes may ultimately decrease the overall chance of reproductive success from the IVF cycle. Perhaps the use of immature oocytes (MI or GV) that are routinely

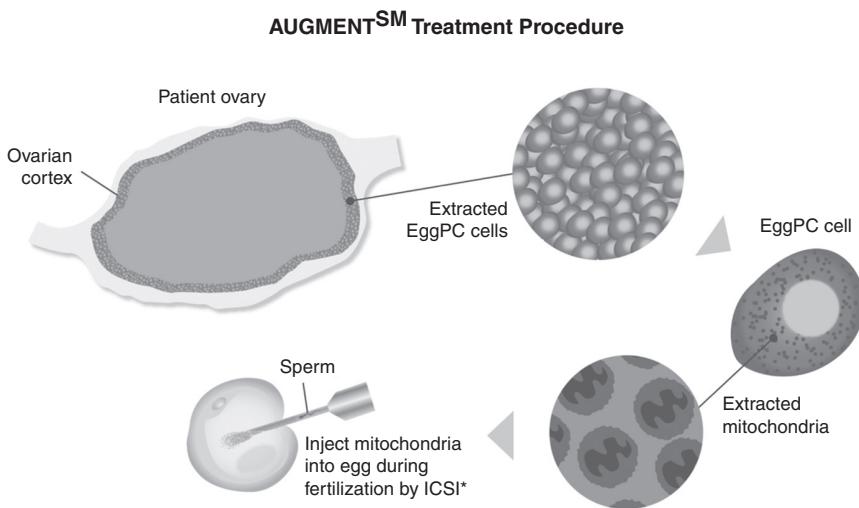


Figure 16.4 AUGMENTSM treatment procedure

retrieved and discarded during an IVF cycle would be more appropriate, but the quality of the mitochondria being isolated may be compromised.

16.7 Mitochondria within the Blastocyst

The role of mitochondria in reproduction is continuing to be addressed. Real-time polymerase chain reaction (PCR) allows the quantification of mtDNA copy number as an estimate of relative mitochondrial mass. Although the majority of studies suggest that a higher mtDNA within the oocyte (and even the cumulus-oocyte complex) is associated with improved reproductive outcomes, the opposite has been recently noted when assessing trophectoderm blastocyst biopsies. A recent, large retrospective study identified that euploid embryos (confirmed by pre-implantation genetic screening using next generation sequencing (NGS) or a comparative genomic hybridization (CGH) with a higher mtDNA level were less likely to implant [32]. An elevated mtDNA was also more likely to be seen in aneuploid embryos in comparison to euploid embryos [33]. The paradoxical finding in the blastocyst remains unexplained, but a prominent hypothesis is that the increased mtDNA is a reflection of cellular stress; the developing embryo is compensating by mitochondrial biogenesis in an attempt to produce more ATP. Although the clinical utility of mtDNA quantification as an independent assessment tool to complement pre-implantation genetic screening (MitoGrade; Reprogenetics; MitoScore; Igenomix) is gaining traction and increasing our understanding of the role of mitochondria, this is downstream to mitochondrial transfer techniques and in the realm of diagnostics rather than therapeutics.

16.8 Conclusion

Mitochondria are critical for cellular function and are believed to be a key component in normal reproduction from the evolution of a primordial follicle through folliculogenesis, oocyte maturation, fertilization and embryo development. The age-related

decline in fecundity among women is directly related to oocyte quality, which is hypothesized to be a function of declining mitochondrial function. As no new mitochondria are synthesized until the blastocyst stage, a mature oocyte requires a critical threshold of mitochondria to carry on with the energy-rich process of fertilization and rapid cell divisions of embryogenesis. It is therefore plausible that treatments focused on mitochondrial rejuvenation or optimization may ultimately improve age-related fertility rates. Currently only supplements (mitochondrial cofactors and antioxidants), along with reducing exposure to oxidative stress to the gametes, may improve oocyte mitochondrial function during folliculogenesis. In the future, perhaps with the aid of in vitro maturation techniques, mitochondrial therapies may be able to influence immature oocytes in vitro prior to the critical first meiotic division. Unfortunately, as oocytes age, the aneuploidy rate exponentially rises, resulting in higher incidence of subfertility/infertility along with miscarriage rates. All current reproductive-focused mitochondrial techniques (ooplasm donation from 'sister' or donor oocytes, ST, PNT or AUGMENT) are executed on mature oocytes during an IVF cycle. As mature oocytes have already undergone the first meiotic division, in principle mitochondrial techniques are likely only able to minimize mitotic errors. In their current form mitochondrial techniques are less likely to benefit older oocytes that are already at a significantly higher risk of being aneuploid at the completion of meiosis – that is prior to the therapeutic intervention. It is now clear that mitochondrial function is integral for reproductive success, and the realm of mitochondrial diagnostics and therapeutics is in its infancy. Mitochondrial technology may one day be able to overcome much of the age-related infertility, thereby allowing a readjustment of the biological clock.

References

1. Cavelier L, Johannisson A, Gyllensten U. Analysis of mtDNA copy number and composition of single mitochondrial particles using flow cytometry and PCR. *Exp. Cell Res* 2000; **259**, 79–85.
2. May-Panloup P, Chrétien M-F, Savagner F et al. Increased sperm mitochondrial DNA content in male infertility. *Hum Reprod* 2003; **18** (3): 550–556. doi:10.1093/humrep/deg096
3. Tian et al. Association of DNA Methylation and Mitochondrial DNA Copy Number with Human Semen Quality. *Biol Reprod.* 2014 September 10.
4. Van Blerkom J. Mitochondrial function in the human oocyte and embryo and their role in developmental competence. *Mitochondrion*. 2011 September; **11**(5): 797–813. doi:10.1016/j.mito.2010.09.012. Epub 2010 October 7
5. Reynier P, May-Panloup P, Chretien MF et al. Mitochondrial DNA content affects the fertilizability of human oocytes. *Mol. Hum. Reprod.* 2001; **7**, 425–429.
6. Sato Miyuki, Sato Ken. Maternal inheritance of mitochondrial DNA by diverse mechanisms to eliminate paternal mitochondrial DNA. *Biochimica et Biophysica Acta (BBA) – Molecular Cell Research* 2013 August; **1833**:8, 1979–1984.
7. Schwartz M, Vissing J. Paternal inheritance of mitochondrial DNA. *N Engl J Med.* 2002; **347**: 576–580.
8. St John J, Sakkas D, Dimitriadis K et al. Failure of elimination of paternal mitochondrial DNA in abnormal embryos. *Lancet* 2000; **355**:200.
9. Houshmand M, Holme E, Hanson C, Wennerholm UB, Hamberger L. Is paternal mitochondrial DNA transferred to the offspring following intracytoplasmic sperm injection? *J Assist Reprod Genet* 1997; **14**:223–227.
10. Canadian Assisted Reproductive Technologies Register (CARTR Plus). 62nd Annual Meeting – Toronto. September

- 22–24, 2016. <https://cfas.ca/wp-content/uploads/2015/08/BORN-CFAS-CARTR-Plus-presentation-Oct-2016-for-website-1.pdf>. www.sartcorsonline.com/rptCSR_PublicM ultYear.aspx?reportingYear=2014
11. Hansen KR, Knowlton NS, Thyer AC. A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause. *Human Reprod.* 2008; **23**: 699–708.
 12. Tsutsumi M, Fujiwara R, Nishizawa H et al. Age-related decrease of meiotic cohesins in human oocytes. *PLoS ONE* 2014; **9** e96710.
 13. Bentov Y, Yavorska T, Esfandiari N, Jurisicova A, Casper RF. The contribution of mitochondrial function to reproductive aging. *Journal of Assisted Reproduction and Genetics* 2011; **28**, 773–783.
 14. Kristensen SG¹, Pors SE¹, Andersen CY¹. Improving oocyte quality by transfer of autologous mitochondria from fully grown oocytes. *Hum Reprod.* 2017; March 2:1–8. doi:10.1093/humrep/dex043. [Epub ahead of print]
 15. Babayev E, Seli E. Oocyte mitochondrial function and reproduction. *Curr Opin Obstet Gynecol.* 2015 Jun; **27**(3): 175–181.
 16. Agarwal A, Durairajanayagam D, du Plessis SS. Utility of antioxidants during assisted reproductive techniques: an evidence based review. *Reprod Biol Endocrinol.* 2014 November 24; **12**:112.
 17. Muggleton-Harris A, Whittingham DG, Wilson L. Cytoplasmic control of pre-implantation development in vitro in the mouse. *Nature* 1982; **299**:460–462.
 18. Levron J¹, Willadsen S, Bertoli M, Cohen J. The development of mouse zygotes after fusion with synchronous and asynchronous cytoplasm. *Hum Reprod.* 1996 June; **11**(6):1287–1292.
 19. Cohen J, Scott R, Alikani M et al. Ooplasmic transfer in mature human oocytes. *Mol Hum Reprod.* 1998 March; **4**(3):269–280.
 20. Sharpley MS, Marciniak C, Eckel-Mahan K et al. Heteroplasmy of mouse mtDNA is genetically unstable and results in altered behavior and cognition. *Cell.* 2012 October 12; **151**(2):333–343.
 21. Craven L, Tuppen HA, Greggains GD et al. Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA disease. *Nature.* 2010 May 6; **465** (7294):82–85.
 22. Tachibana M, Amato P, Sparman M et al. Towards germline gene therapy of inherited mitochondrial diseases. *Nature.* 2013 January 31; **493**(7434):627–631.
 23. Brenner CA, Barritt JA, Willadsen S, Cohen J. Mitochondrial DNA heteroplasmy after human ooplasmic transplantation, *Fertility and Sterility.* 2000; **74**(3), 573–578.
 24. Amato P, Tachibana M, Sparman M, Mitalipov S. Three-Parent IVF: Gene replacement for the prevention of inherited mitochondrial diseases. *Fertility and Sterility.* 2014; **101**(1):31–35. doi:10.1016/j.fertnstert.2013.11.030.
 25. Tachibana M, Amato P, Sparman M et al. Towards germline gene therapy of inherited mitochondrial diseases. *Nature.* 2013 January 31; **493**(7434):627–631.
 26. Brenner CA, Kubisch, HM, Pierce, KE. The role of the mitochondrial genome in assisted reproductive technologies and embryonic stem cell-based therapeutic cloning. *Reproduction, Fertility and Development.* 2005; **16**(7), 743–751.
 27. Nakada K, Hayashi JI. Transmitochondrial mice as models for mitochondrial DNA-based diseases, *Experimental Animals.* 2011; **60**, 421–431.
 28. www.ovascience.com/news/article/ovascience-augment-fertility-treatment-shows-improved-pregnancy-rates-in-women
 29. Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature.* 2004 March 11; **428**(6979), 145–150.
 30. Zou K, Yuan Z, Yang Z et al. Production of offspring from a germline stem cell line derived from neonatal ovaries. *Nat Cell Biol.* 2009; **11**, 631–636.

31. Cagnone GL^{1,2}, Tsai TS^{1,2}, Makanji Y^{1,2}. Restoration of normal embryogenesis by mitochondrial supplementation in pig oocytes exhibiting mitochondrial DNA deficiency. *Sci Rep.* 2016 March 18;6, 23229. doi:10.1038/srep23229.
32. Ravichandran K, McCaffrey C, Grifo J et al. Mitochondrial DNA quantification as a tool for embryo viability assessment: retrospective analysis of data from single euploid blastocyst transfers. *Hum Reprod.* 2017 April 6:1–11. doi:10.1093/humrep/dew070. [Epub ahead of print]
33. Fragouli E, Spath K, Alfarawati S et al. Altered levels of mitochondrial DNA are associated with female age, aneuploidy, and provide an independent measure of embryonic implantation potential. *PLoS Genet* 2015;11:e1005241.

In Vitro Maturation

Roger Hart and Melanie Walls

17.1 Introduction

In vitro maturation (IVM) of oocytes is an assisted reproductive technique whereby oocytes are collected from a patient in an immature form, often after limited or even no exogenous stimulation, and then are matured in the laboratory prior to insemination. However, this definition is open to dispute as a recent publication asserts that IVM should relate to the collection of oocytes (*whether mature or otherwise*) from follicles 2–13 mm in diameter [1]. We would dispute this assertion, as it is our belief that IVM relates to the collection of immature oocytes without receiving a trigger injection (luteinizing hormone (LH) surge surrogate) and maturation of the oocytes within the laboratory.

The perceived benefits of this technique are that oocytes can be collected earlier in a menstrual cycle for a woman who requires to proceed to a cycle of chemotherapy treatment immediately; it avoids the use of costly ovarian stimulation drugs; it completely avoids the risk of ovarian hyperstimulation syndrome (OHSS) and the high oestrogen levels for a woman with an oestrogen-sensitive tumour or a significant pro-thrombotic tendency [2]. It is estimated that more than 3000 children have been born worldwide from this method, and although some clinics routinely perform IVM, it is still considered a research technique.

17.2 Background to IVM

In a normal menstrual cycle increasing oestradiol produced by the lead developing follicle initiates a pituitary surge of LH – the LH surge – which triggers a number of events, including resumption of meiosis and oocyte maturation. Synchrony of nuclear and cytoplasmic maturation is essential for oocyte competency and embryonic development post-fertilization. Nuclear maturation allows for the development and organization of the oocyte's nuclear material and progress to arrest after the second meiotic division, the metaphase II stage. Cytoplasmic maturation involves structural and functional modifications to support oocyte activation, fertilization and embryo development. IVM is the process where research has focused on trying to replicate this natural process of oocyte maturation and embryonic development processes in vitro. The research has consequently focused on ovarian stimulation (or absence of) protocols, the need for (or lack of) a 'trigger' injection and the development of specific culture conditions.

IVM was first demonstrated using a rabbit model by simply isolating oocytes from the follicular environment where oocytes from large follicles spontaneously matured, and was also attempted using an extract of the pituitary gland of a cow to stimulate oocyte maturation [3]. This approach was replicated in a human model, after oocytes collected from

unstimulated follicles underwent spontaneous maturation after ovarian biopsies were collected [4]. The first live birth was recorded in 1991, after oocyte maturation following collection of oocytes from 23 unstimulated ovaries and their culture in mature follicular fluid, resulting in a triplet pregnancy [5]. This was followed by a birth after oocyte collection using the transvaginal approach in 1994 [6]. The use of IVM has been relatively rare until the past decade due to the poor pregnancy rates achieved [7] and the requirement of the transfer of multiple embryos [8]. With the refinement of protocols, pregnancy rates equal to those of standard IVF cycles have been achieved [9]; however, a constant difficulty has been the inability to achieve satisfactory endometrial preparation, such that pregnancies from cryopreserved embryos have generally been much better than the transfer of fresh embryos [2].

With improvements in the laboratory aspects of IVM, there has been a renewed interest in the use of IVM for the process of fertility preservation for women with cancer [10–12] and for girls at risk of premature ovarian failure [13]. Ovarian tissue is assessed in the laboratory, and any follicles present undergo aspiration in an attempt to retrieve oocytes that can be subject to IVM with a view to preserving mature oocytes for the future.

17.3 Patients Suitable for IVM Treatment

17.3.1 Polycystic Ovary Syndrome (PCOS)

The ideal patient for an IVM protocol is a young woman with a high antral follicle count, as women with this feature of PCOS are particularly vulnerable to the condition of OHSS [14]. OHSS is a condition manifest by intravascular haemoconcentration and extravasation of fluid into the abdominal and pleural cavities. This leads to significant patient discomfort and medical compromise and puts patients at risk of a thrombotic event. However, we feel that to offer IVM to a woman of 37 years or older is not appropriate as the success rates of the treatment markedly diminish in our hands. This is perhaps because the older oocyte has a greater difficulty to synchronize nuclear and cytoplasmic maturation, consequently resulting in compromised blastocyst development [2, 15]. Another reason to advocate IVM treatment for a young woman with a reasonable antral follicle count is her geographical location, as a woman who potentially is at risk of OHSS is obliged to stay in close proximity to her treating unit until the risk of ovarian hyperstimulation syndrome subsides. This can be a significant impost on a woman who resides in a distant rural community. Hence, it is perhaps not surprising that a couple of the centres with a long track record of IVM treatment are referral centres for large regional populations in Western Australia and Quebec [2, 8], which has enabled them to develop their protocols and expertise.

It has to be acknowledged that with the recent advent of multiple strategies to significantly reduce the risk of OHSS for patients, it is interesting to consider whether the thrust to drive forward the development of OHSS protocols would have been as strong if these approaches existed a decade ago. The commonest approach employed to minimize the risk of OHSS is the use of an agonist trigger in an antagonist cycle and adopting a ‘freeze-all’ approach to the embryos, virtually eliminating the risk of OHSS for the majority of patients [16]. Other well-established strategies consist of the concurrent use of metformin, the use of an antagonist for ovarian down-regulation and cabergoline administered from the time of trigger, or pre-IVF ovarian drilling [17].

17.3.2 Oestrogen-Sensitive Tumours

Women with breast or endometrial cancer are understandably very concerned about any potential consequences of an elevated oestradiol concentration during a stimulated IVF cycle on their malignancy, despite reassuring evidence from studies of women with breast cancer undergoing standard IVF. However, most treating clinicians would aim to reduce the serum oestradiol concentration with the use of the adjunct letrozole (an aromatase inhibitor) or tamoxifen during an IVF cycle [18]. An alternative approach is to undergo IVM treatment, as follicles are aspirated at a maximal diameter of 12 mm; hence, the serum oestradiol concentrations approximate those found typically in mid-cycle for a woman in a spontaneous ovulatory cycle. Hence, this is very reassuring for the woman embarking on this approach. Before embarking on an IVM cycle, it is essential to ensure that the patient has an adequate antral follicle count, as she will generally have only half the number of mature oocytes available for cryopreservation as if she had embarked upon a standard IVF cycle [2]. A further benefit of an IVM approach, if appropriate, is that a short treatment regime is required to generate follicles of up to 12 mm in diameter, enabling a very short interval before oocyte retrieval, and limited delay, as well as minimal patient discomfort, prior to commencing chemotherapy. Furthermore, the stimulation can commence at any stage in the menstrual cycle and is eminently suited to a woman who has an irregular or absent cycles.

17.3.3 Thrombophilia

As for a woman with an oestrogen-sensitive tumour, she would be expected to be at a significant risk of a venous thrombo-embolic event during or just subsequent to an IVF cycle; hence, an IVM approach may reduce her risk of an adverse outcome. However, treatment should not be commenced in a woman with such a co-morbidity without the approval of an obstetric physician or a haematologist, as pregnancy itself would be expected to be a significant risk to the woman.

17.3.4 Minimal Delay for an IVF Cycle

As IVM cycles can be performed with either no or minimal ovarian stimulation at any stage of the menstrual cycle, it is possible to review a patient, and immediately commence treatment and collect oocytes within 3–5 days time frame, enabling a rapid progression to oncological management.

17.3.5 Fertility Preservation from Vaginal Oocyte Aspiration

As described earlier, an IVM approach is an excellent strategy to collect oocytes from a young woman or a woman with a polycystic ovarian morphology, prior to her commencing some gonadotoxic therapy, chemotherapy or radiotherapy for cancer, or immuno-suppressive treatment for a patient with a rheumatological condition. The reasons why it is an ideal approach for a young woman with cancer is that she may have limited time before the commencement of the oncology treatment, her malignancy puts her at an increased risk of a thrombo-embolic event and the discomfort and potential harm caused by ovarian hyperstimulation may complicate or compromise her oncology treatment.

17.3.6 Fertility Preservation from Ovarian Tissue at the Time of Ovarian Tissue Biopsy or Oophorectomy

Many women, particularly those with a haematological malignancy, have insufficient time to undergo an IVF cycle or even a short IVM cycle prior to the commencement of their oncology treatment, and hence the only option available to them is to undergo ovarian tissue freezing to offer the chance of fertility preservation [19]. This approach can also be suitable for an adolescent or premenarcheal girl, where ovarian stimulation and ultrasound monitoring (particularly, if vaginal) and oocyte retrieval are not appropriate. The indications for this approach may also be extended to include a girl at risk of premature ovarian failure, perhaps due to carrying the fragile X-expanded *FMR* gene [20], Turner syndrome [21], galactosaemia [22] or imminent gonadotoxic therapy [13]. The first reported live birth from this approach was a young patient, with advanced ovarian cancer, who had undergone fertility sparing surgery. Immature oocytes were recovered after oophorectomy and were fertilized after undergoing IVM. The embryos obtained were then cryopreserved and were later transferred to achieve a singleton healthy pregnancy [23].

We have reported the case of a young woman who required oophorectomy as part of the management of her advanced gynaecological malignancy. Due to vaginal extension of her disease, she could not undergo vaginal aspiration of follicles and hence was administered a few days of recombinant follicle-stimulating hormone (FSH), until she had a lead antral follicle of 11 mm size and then she underwent bilateral oophorectomy. Subsequently, 22 immature oocytes were retrieved from the ovaries in the laboratory by follicular aspiration. After 24 hours of culture, 15 oocytes advanced to the metaphase II stage, and after a further 24 hours of culture 3 more oocytes matured and hence 18 oocytes were cryopreserved for her future use [10].

17.4 Stimulation Protocols

Various approaches to the process of IVM stimulation protocols have been employed and documented and extensively debated in the literature [1]. Protocols differ with respect to the need for ovarian stimulation with FSH prior to oocyte retrieval; consider ‘ovarian priming’ to ensure a cohort of follicles starts to develop until the lead follicle is 10–12 mm in diameter at the time of follicular aspiration, versus the ‘natural cycle’ approach where follicular aspiration is performed without the use of FSH injections. Initial studies appeared to demonstrate that FSH priming in ovulatory women without a polycystic ovarian morphology did not improve retrieved oocyte numbers, their maturation rate or the embryo cleavage rate or embryo development [24]. However, a more recent study by the same group demonstrated that FSH priming improved the oocyte maturation potential and embryo implantation rates in women diagnosed with PCOS [25]. In more recent years, the use of FSH priming to enable the development of larger and a greater cohort of follicles has led to the highest reported success rates in IVM yet [26].

Furthermore, there exists further debate as to the requirement to administer to the patient a ‘trigger’ injection prior to follicular aspiration. While the use of the trigger is vigorously debated [1], the only randomized controlled trial of the ‘use’ versus ‘non-use’ of a trigger injection did not demonstrate any significant differences in embryological or clinical outcomes [27]. As protocols vary to a significant degree, each institution should audit their individual practice.

It is our opinion that IVM means that the whole process of oocyte maturation from germinal vesicle breakdown, the progression of meiosis, to the development of a metaphase II oocyte should be completed in the laboratory to satisfy the description of IVM. Our protocol is to transfer a single blastocyst in a subsequent frozen embryo transfer cycle, after the patient has undergone follicular priming with a few days of 150 IU of FSH, the aspiration of follicles at a maximum diameter of 12 mm, without the use of a trigger injection, and the maturation of the oocytes in the laboratory. In our hands, this has led to clinical pregnancy rates that are similar to those in a standard IVF cycle [2].

17.5 Oocyte Collection Procedure

Oocyte collection protocols for IVM vary, although they are very similar to the standard transvaginal oocyte aspiration procedure performed for IVF treatment, with slight modifications to collect oocytes from smaller follicles, while minimizing the potential harm to the cumulus–oocyte complexes. The collection procedure for IVM is important in order to maximize oocyte yield, while maintaining the integrity of the oocyte. Generally the number of oocytes collected is approximately half the number of follicles present [2].

References

1. Dahan MH, Tan SL, Chung J, Son WY. Clinical definition paper on in vitro maturation of human oocytes. *Hum Reprod.* 2016;31(7): 1383–6.
2. Walls ML, Hunter T, Ryan JP et al. In vitro maturation as an alternative to standard in vitro fertilization for patients diagnosed with polycystic ovaries: a comparative analysis of fresh, frozen and cumulative cycle outcomes. *Hum Reprod.* 2015;30 (1):88–96.
3. Pincus G, Enzmann EV. The comparative behavior of mammalian eggs in vivo and in vitro. *The Journal of Experimental Medicine.* 1935;62(5): 665–75.
4. Edwards RG. Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature.* 1965;208(5008): 349–51.
5. Cha KY, Koo JJ, Ko JJ et al. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. *Fertility and Sterility.* 1991;55(1):109.
6. Trounson A, Wood C, Kausche A. In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. *Fertility and Sterility.* 1994;62 (2):353.
7. De Vos M, Ortega-Hrepich C, Albuza FK et al. Clinical outcome of non-hCG-primed oocyte in vitro maturation treatment in patients with polycystic ovaries and polycystic ovary syndrome. *Fertil Steril.* 2011;96(4): 860–4.
8. Chian RC, Buckett WM, Too LL, Tan SL. Pregnancies resulting from in vitro matured oocytes retrieved from patients with polycystic ovary syndrome after priming with human chorionic gonadotropin. *Fertil Steril.* 1999;72(4): 639–42.
9. Junk SM, Yeap D. Improved implantation and ongoing pregnancy rates after single-embryo transfer with an optimized protocol for in vitro oocyte maturation in women with polycystic ovaries and polycystic ovary syndrome. *Fertil Steril.* 2012;98(4): 888–92.
10. Walls ML, Douglas K, Ryan JP, Tan J, Hart R. In-vitro maturation and cryopreservation of oocytes at the time of oophorectomy. *Gynecologic Oncology Reports.* 2015;13:79–81.
11. Uzelac PS, Delaney AA, Christensen GL, Bohler HC, Nakajima ST. Live birth following in vitro maturation of oocytes

- retrieved from extracorporeal ovarian tissue aspiration and embryo cryopreservation for 5 years. *Fertil Steril.* 2015;104(5): 1258–60.
12. Segers I, Mateizel I, Van Moer E et al. In vitro maturation (IVM) of oocytes recovered from ovariectomy specimens in the laboratory: a promising “ex vivo” method of oocyte cryopreservation resulting in the first report of an ongoing pregnancy in Europe. *J Assist Reprod Genet.* 2015;32(8): 1221–31.
 13. Ross C TK, Child T, Davies J, Becker C, Fatum M. Immature oocyte retrieval followed by in vitro maturation (IVM) and vitrification in combination with laparoscopic ovarian tissue cryopreservation (OTCP) for fertility preservation: UK pilot study. *Human Reproduction.* 2016; 31(Supplement 1): i335.
 14. Tang H, Hunter T, Hu Y et al. Cabergoline for preventing ovarian hyperstimulation syndrome. *Cochrane Database Syst Rev.* 2012(2):CD008605.
 15. Walls ML, Ryan JP, Keelan JA, Hart R. In vitro maturation is associated with increased early embryo arrest without impairing morphokinetic development of useable embryos progressing to blastocysts. *Hum Reprod.* 2015;30(8): 1842–9.
 16. Radesic B, Tremellen K. Oocyte maturation employing a GnRH agonist in combination with low-dose hCG luteal rescue minimizes the severity of ovarian hyperstimulation syndrome while maintaining excellent pregnancy rates. *Hum Reprod.* 2011;26(12): 3437–42.
 17. Boothroyd C, Karia S, Andreadis N et al. Consensus statement on prevention and detection of ovarian hyperstimulation syndrome. *Aust N Z J Obstet Gynaecol.* 2015;55(6): 523–34.
 18. Oktay K, Buyuk E, Libertella N, Akar M, Rosenwaks Z. Fertility preservation in breast cancer patients: a prospective controlled comparison of ovarian stimulation with tamoxifen and letrozole for embryo cryopreservation. *J Clin Oncol.* 2005;23(19): 4347–53.
 19. Stopp D, Cobo A, Silber S. Fertility preservation for age-related fertility decline, whilst maintaining the integrity of the oocyte. *Lancet.* 2014 October 4;384 (9950): 1311–9.
 20. Benard J, Calvo J, Comtet M et al. Fertility preservation in women of the childbearing age: Indications and strategies. *J Gynecol Obstet Biol Reprod (Paris).* 2016;45(5): 424–44.
 21. Huang JY, Tulandi T, Holzer H et al. Cryopreservation of ovarian tissue and in vitro matured oocytes in a female with mosaic Turner syndrome: Case Report. *Hum Reprod.* 2008;23(2): 336–9.
 22. van Erven B, Gubbels CS, van Golde RJ et al. Fertility preservation in female classic galactosemia patients. *Orphanet Journal of Rare Diseases.* 2013;8:107.
 23. Prasath EB, Chan ML, Wong WH et al. First pregnancy and live birth resulting from cryopreserved embryos obtained from in vitro matured oocytes after oophorectomy in an ovarian cancer patient. *Hum Reprod.* 2014;29(2): 276–8.
 24. Mikkelsen AL, Smith SD, Lindenberg S. In vitro maturation of human oocytes from regularly menstruating women may be successful without follicle stimulating hormone priming. *Human Reproduction.* 1999;14(7): 1847–51.
 25. Mikkelsen A, Lindenberg S. Benefit of FSH priming of women with PCOS to the in vitro maturation procedure and the outcome: a randomized prospective study. *Reproduction.* 2001;122 (4): 587–92.
 26. Junk SM, Yeap D. Improved implantation and ongoing pregnancy rates after single-embryo transfer with an optimized protocol for in vitro oocyte maturation in women with polycystic ovaries and polycystic ovary syndrome. *Fertility and Sterility.* 2012;98(4): 888–92.
 27. Zheng X, Wang L, Zhen X et al. Effect of hCG priming on embryonic development of immature oocytes collected from unstimulated women with polycystic ovarian syndrome. *Reprod Biol Endocrinol.* 2012;10:40.

Oocyte Banking through Vitrification

Zsolt Peter Nagy, Ching-Chien Chang, and Daniel B. Shapiro

18.1 Introduction to Technological Milestones in Embryology

The treatment of infertility has evolved significantly in the past four decades, starting with the successful conception and birth of the first IVF (in vitro fertilization) baby, Louise Brown, in 1978 [1]. The next major milestone in the development of IVF laboratory technology was introduced about a decade later, when scientists at Hammersmith Hospital in London combined the removal of cell(s) from an embryo with pre-implantation genetic testing (PGT). Under this broad acronym are two distinct types of testing, PGS (pre-implantation genetic screening) and PGD (pre-implantation genetic diagnosis). PGS counts chromosomes and determines the sex of an embryo, as well as detects structural alterations of chromosomes, while PGD refers to genetic testing for single-gene diseases like cystic fibrosis, muscular dystrophy and sickle cell anemia. Generally speaking, both procedures make it possible to identify genetically “normal” (and “abnormal”) embryos. For PGS, this means selecting only embryos that are euploid while for PGD this means selecting embryos that are homozygous normal or heterozygous unaffected (carrier only) for any specific genetic disease. The technology of genetic (chromosomal) testing itself has improved dramatically since its inception. Initially, hybridization techniques (FISH) were used to identify single genes and chromosome-specific markers. Variations in hybridization ultimately gave way to “next generation sequencing” (NGS) as the preferred technique for chromosome alterations. The popularity of the technique also led to a renaming of the procedure as “PGS 2.0” [2]. NGS is also relatively inexpensive, which has led to a worldwide increase in PGS as an adjunct to routine IVF treatment.

Approximately two years after the first successful PGT, a breakthrough in the treatment of male-factor infertility occurred. In 1992, scientists in Brussels, Belgium, achieved a live birth after using a micromanipulator apparatus to assist fertilization by injecting a single sperm directly into an egg. The technique, intracytoplasmic sperm injection (ICSI), expanded the number of couples who could expect success from IVF by making it possible for men with severely impaired spermatogenesis to initiate pregnancies [3]. The basic cell culture technique employed in “Conventional IVF” involves the placement of the egg with about 50,000 sperm under a droplet of oil. This technique worked reliably well in most patients but not in those with severe male-factor infertility who experienced higher rates of fertilization failure, lower rates of fertilization overall, and subsequently worse embryological outcomes with lower rates of pregnancy. The ICSI technique has overcome (almost) all male factor-related IVF failure. Because ICSI proved to be highly reliable at achieving fertilization and preventing occasional low or failed conventional insemination (which can

occur even with adequate sperm parameters), today it is used as a routine procedure in the majority of IVF treatments in most countries.

Successful cryopreservation predates both PGT and ICSI, but success rates with frozen embryos were generally poor in the early years of the technology. Despite the initial lack of efficiency with cryopreservation, embryo freezing has been a cornerstone of ART treatment since its introduction in 1983 [4]. Many IVF patients have “super-numerary” good quality embryos, which are not replaced in a fresh cycle and can be cryopreserved for future use. The initial cryopreservation technique, introduced in 1983, was based on a “slow-freezing” protocol. Slow-freezing is an automated procedure that drops temperature in a methodical and stepwise fashion over the course of several hours. The first pregnancy and live birth following oocyte cryopreservation was reported in 1986, using a similar “slow-freezing” technique [5]. However, while slow-freezing of embryos was relatively efficient (70–80 percent survival – with the exception of embryos that had also undergone a biopsy procedure) – slow-freezing of oocytes was not efficient at all (10–30 percent survival). This unfortunate reality made egg banking and fertility preservation untenable.

Two decades after the first successful pregnancy from a frozen embryo, the fundamentals of freezing technology experienced a revolution with the introduction of vitrification. Though vitrification is done manually and therefore has outcomes that are user-dependent, it is more rapid and is associated with superior survival, fertilization, and pregnancy rates than “slow-freeze.” The efficiency of vitrification, both in general terms and when compared to slow-freeze, makes it possible to freeze eggs for a variety of indications, including fertility preservation and donor oocyte banking. Additionally, egg freezing can be used for regular IVF patients who have religious or ethical concerns regarding embryo disposal and will elect to freeze eggs rather than inseminate them and risk having too many embryos “left over.” Patients living in countries with strict IVF regulations or patients whose partners fail to produce a sperm specimen can now comfortably choose to freeze eggs without putting their overall chance of pregnancy at risk.

18.2 Vitrification as a Standard of Cryopreservation

For the 50 years following the first successful semen freeze [6] in 1954, the dominant (possibly exclusive) technology for cryopreserving human gametes and embryos has been the slow-freezing procedure. Slow-freeze was the only known technique when embryos (1983) and subsequently eggs (1986) were successfully frozen [4]. For the 25 years following the first successful frozen embryo transfer in 1983, slow-freezing provided acceptable results for embryo cryopreservation at all different stages of embryo development up to blastocyst stage. Over the course of this time period, the same protocol was applied to attempt oocyte cryopreservation. Despite numerous efforts and multiple protocol modifications, very little progress was made in rates of oocyte survival or fertilization and only a handful of procedures resulted in live births. The main reason for the difference in efficiency between embryos and eggs is that the membrane properties of the oocyte are significantly different from those of the embryo, the egg being more fragile. Slow-freezing (also called equilibrium freezing) employs (relatively) low concentration of cryoprotectants that require a longer time to create equilibrium between intracellular and extracellular osmolality. This equilibrium needs to be maintained during the cooling stage, and therefore requires a very low cooling speed (approximately 0.3 degree Celsius per minute) [7]. Slow cooling is typically

done by automated freezers, without the need of operator assistance. Reproductive cells can be frozen using large volumes, and the protocol tolerates less precise handling or timing.

Vitrification (also called nonequilibrium freezing) on the other hand requires much higher concentration of cryoprotectants and much shorter incubation/exposure times. The most frequently used cryoprotective agent (CPA) for vitrification is the combination of dimethyl sulfoxide (DMSO) and ethylene glycol (also called permeating CPAs), with the addition also of sucrose (a non-permeating CPA). Alternative permeating and non-permeating CPA combinations may also be used (propylene glycol (PROH) /glycerol and glucose/ficoll). The most critical component of vitrification is the extremely high cooling (and warming) rate (ideally above 20,000 degree Celsius per minute) necessary to achieve successful vitrification. To obtain such high cooling/warming rates (to prevent harmful crystallization), very small volumes must be used when loading the oocyte to the vitrification carrier. Successful vitrification thus requires very accurate timing and precise handling, which makes this technology and its results much more operator-dependent than the more forgiving slow-freeze method. The benefits of vitrification, however, far outweigh the “handling challenges.” Vitrification provides superior outcomes compared to slow-freezing in all regards. Vitrification has better cryosurvival, but also is far superior at preserving oocyte physiology and ultrastructure (like the meiotic spindle), making fertilization and embryo development outcomes better comparable to fresh oocytes [8]. Vitrified-warmed (as well as frozen-thawed) oocytes are always inseminated using the ICSI technique [3] because cumulus and corona cells are typically removed prior to cryopreservation (thus conventional insemination is not an option after warming/thawing).

18.3 Indications for Oocyte Cryopreservation

There are several indications for cryopreserving oocytes with the major ones summarized in Box 18.1.

Box 18.1 The most frequent indications for oocyte cryopreservation

- Government restrictions/Legislation
- Emergency cycle management/failure to obtain sperm
- Ethical/ religious reasons (elective cycle management)
- Ovarian hyperstimulation syndrome (OHSS) (elective cycle management)
- Fertility preservation (medical or social)
- Donor egg banking

Legislation or restrictive IVF regulations may force patients undergoing IVF treatment to limit the number of oocytes inseminated; thus, supernumerary oocytes require cryopreservation to avoid disposal. A typical example for this scenario is Italy, where until recently the Italian government restricted IVF/ICSI to insemination of a maximum of three eggs at a time for any one IVF patient. This restriction led to a routine use of oocyte cryopreservation, resulting in enhanced experience with egg freezing. The depth of the Italian experience can be seen in the number of publications from Italian IVF centers [9].

Oocytes may be cryopreserved when unexpected circumstances prevent a male patient from producing a sperm sample on the day of egg retrieval. Although this situation is

unusual, the option to cryopreserve eggs, and inseminate them at a later time, helps reduce the pressure on the patient, as well as on the clinician.

Oocytes may also be cryopreserved as a part of “elective cycle management.” In these circumstances IVF patients will elect to limit the number of eggs for insemination for ethical or for religious reasons. Patients with these concerns typically have no qualms regarding disposal of eggs but are opposed to discarding viable frozen embryos.

The most “obvious” indication for oocyte cryopreservation is for fertility preservation. There are several medical conditions where the disease itself or the treatment for the disease will likely result in infertility by accelerating ovarian follicular atresia. The classic example of this scenario is a reproductive age woman with an early stage breast cancer who will have lumpectomy, radiation, and chemotherapy. For these patients (reproductive age), traditional ovarian stimulation followed by egg retrieval and subsequent vitrification is considered as the primary option. An alternative to oocyte cryopreservation is ovarian (cortex) cryopreservation, which can be used for patients in the prepubertal period, or for those who cannot delay medical treatment to allow the two weeks necessary for ovarian stimulation and egg collection. However, ovarian cortical cryopreservation is still considered an “experimental” procedure, and consequently it is performed far less and studied less well than oocyte cryopreservation.

Although somewhat debated, oocyte cryopreservation is increasingly performed for women without medical condition, mainly for the reason to serve as a “back up option” for age-related infertility (“social” fertility preservation). As society is changing/evolving, more and more women are delaying childbearing to more advanced “maternal age,” when natural fecundity is reduced (or becomes absent), and even the “conventional” infertility treatment may not be able to help, due to decreased/absent ovarian reserve and/or diminished egg “quality.” Cryopreserving oocytes at an early(ier) reproductive age maintains the reproductive potential of the person at the age when she “froze” her eggs. This provides the ability to conceive years or decade(s) later as efficiently as at the time the fertility preservation was performed. Oocyte cryopreservation for all these above-mentioned indications are more and more frequently performed, and it has become routine practice in most IVF centers [10].

Finally, the ability to cryopreserve oocytes efficiently provides another essential service, namely “donor egg-banking.” Oocyte donation, in countries where it is permitted with little or no restriction, traditionally has been performed “fresh,” that is, the egg donor was “stimulated” and the egg recipient was “synchronized” with the donor to be able to perform embryo transfer a few days after the egg retrieval was performed. While “synchronization” between donor and recipient was possible with careful planning and cycle monitoring, this approach has several drawbacks. The establishment of donor egg banks now provides an alternative to fresh egg donations with several significant advantages, listed in Table 18.1. The reasons for that are several. Donors typically produce many oocytes which in a fresh donation are typically all allocated to a single recipient. However, most recipients do not require a large number of eggs to fulfill their reproductive desires. Most recipients are of advanced maternal age, usually above 40, and they plan to have only one (more) child. A fresh egg donation cycle will usually result in more embryos than necessary to achieve a single live birth. Often, the majority of embryos from a donor cycle will be cryopreserved and then never used. Patients are often uncomfortable with their options for disposition of the extra embryos: continue to pay storage fees, donate to another couple, destroy them, donate them for scientific research, or use them themselves.

Table 18.1 Attributes of fresh and “frozen” egg donations (Donor Egg Bank)

	Fresh egg donation	Donor egg bank (“frozen” egg donation)
Synchronization (donor-recipient)	Required	Not required
Waiting time	Extended (several month/year)	No waiting time
Donor selection	Limited	Large donor pool
Quarantine	Not possible	Possible
No. of donor eggs/recipient	Usually large number (>10)	Usually smaller number [5–8]
Results	High pregnancy rates	High pregnancy rates*
“Supernumerary” embryo(s)	Usually high number	Usually low number
Cost	Very expensive	Moderately expensive**

* Some donor egg banks provide “guarantee programs” to safeguard against unexpected suboptimal outcomes related to limited egg number or unexpected lower egg “quality.” (This type of guarantee program is usually not provided for fresh egg donation.)

** Those donor egg banks that allocate eggs from one donor to several different recipients are able to reduce the costs, as donor costs are also “divided” among the different recipients.

Alternatively, in “Egg Banking” donation cycles the donor eggs are cryopreserved and divided among several recipients. In an egg banking model, the egg bank recruits donors and takes responsibility for the costs associated with recovering the donor eggs. Once recovered, the eggs will be frozen alone or in pairs to allow for efficient distribution of the eggs to multiple recipients. For instance, at “My Egg Bank,” the typical number of mature (metaphase II – M2) eggs obtained from a donor on average is 24–25 and as recipients typically receive 6 x M2 eggs in a “lot,” the average number of recipients per donor egg retrieval is four. The advantages to this approach are the ability to spread the cost of donor egg acquisition among several recipients, which also allows the IVF center to lower the cost of each donation cycle for recipients, and the ability to keep the “left-over embryo” problem to a minimum. The potential “down-side” of frozen egg donation relates to the number of eggs assigned/provided to a recipient. Periodically an “egg lot” will have poor survival rates or poor fertilization rates or poor embryology results, all of which lower the cumulative live birth rate per egg thaw procedure. To protect against unexpected lower egg quality and decreased chance of pregnancy, some egg banks offer different “guarantee programs” to the recipients, an option that is typically not used in fresh egg donation, though sub-optimal outcomes can happen there as well. The advantages of frozen egg donation through donor egg banks have led to a surge in demand for this less expensive option. In 2014, 30 percent of all donations were with “frozen” eggs in the United States, while just three years earlier nearly all (100 percent) of egg donations were performed fresh (<http://www.sart.org/>).

18.4 Clinical Management and Patient Preparations for Oocyte Cryopreservation

Clinical management of patients who are going through “regular” IVF treatment is unchanged when oocyte cryopreservation is performed for “Emergency Cycle Management” or for “Elective Cycle Management” (see Table 18.1). These patients’ primary goal is to have IVF treatment to achieve pregnancy as soon and as efficiently as possible. Oocyte cryopreservation for these patients is an “auxiliary” procedure that was either not planned at all (like in cases where unexpectedly no sperm was obtained) or was considered, but only as an “optional tool,” pending clinical or laboratory “variables” (risk of hyperstimulation, or having a large(r) number of oocytes, where cryopreserving some/or all eggs might be performed). For these reasons, the screening, testing, and ovarian stimulation for these patients are performed according to the established standards of the IVF clinic [11].

On the other hand, patients who do oocyte cryopreservation for fertility preservation might be handled/treated somewhat differently than “typical” IVF patients. The primary aim for these patients with fertility preservation is to maximize the number of eggs (mature eggs) retrieved, with no goal of achieving pregnancy anytime soon. For these reasons the ovarian stimulation protocol may be using higher doses than usual of gonadotropins (FSH and possibly LH) to stimulate the growth of more follicles. To prevent ovarian hyperstimulation (OHSS), these patients are typically put on gonadotrophin-releasing hormone (GnRH) antagonists medication (to prevent spontaneous ovulation), and when follicular cohort size is appropriate the final follicular maturation is triggered by GnRH agonist (instead of human chorionic gonadotropin – HCG). As several studies have demonstrated, GnRH agonist trigger is as efficient as HCG trigger; however, it prevents OHSS [12]. Using this ovarian stimulation protocol thus provides the possibility to maximize the number of oocytes retrieved (and cryopreserved), while making the intervention safe to the patient. Some of the fertility preservation patients with medical indications may not be able (or wanting) to wait to start their stimulation regimen as established by the “traditional” protocols (first day of menstrual cycle when using GnRH antagonist protocol). As it is understandable, most patients diagnosed with cancer would prefer to start the therapy as soon as possible. To accommodate this preference, “non-conventional” stimulation protocols including “random or luteal start” have been investigated and shown to be equally efficient as traditional “follicular start” protocols in their ability to stimulate follicular growth and retrieve (and freeze) oocytes within the shortest period of time [13].

The clinical management of patients who are volunteering as donors for cryo-egg banks (donor egg banks) is somewhat different. Firstly, donor applicants are “pre-selected” based on certain individual/physical/medical characteristics to comply with institutional and regulatory guidelines/requirements. For instance, in the United States, there is a very strict set of criteria, established by FDA that all donors (including egg donors) are required to pass through (especially regarding infectious disease testing); there are similar rules established in the European Union (EU tissue directives) and other countries as well. Among the different parameters tested/recorded, age, AMH (anti-Müllerian hormone), and basal antral follicle count (BAF) are considered as most predictive to “adequate” ovarian response for stimulation. At My Egg Bank (MEB), for instance, donor candidates are selected if they are younger than 30 years (and older than 21), have 2.0 (or higher) AMH, and at least 20 BAF. These (and other) parameters (BMI, educational level, medical/family history,

psychological evaluation, number and type of inheritable genetic diseases tested) may vary by egg bank, and will certainly vary much more by country. Those countries where there is no restriction in donor (financial) compensation are typically able to recruit more donors and select by more strict criteria, while countries with restrictions on donor compensation (no compensation/altruistic donation or only minimal compensation) usually have severe donor shortages (and very little possibility for donor selection). Stimulation of (highly selected) donors usually does not require high doses of gonadotropins – usually 225 IU–300 IU FSH (or combination of FSH and LH) is sufficient – especially if donors are not on contraceptive pills. Most donors will get trigger on day-11 or on day-12 of their stimulation, using always GnRH agonist (being on GnRH antagonist cycle) – which is (or should be) the standard for donor's stimulation protocol.

18.5 Outcomes Using Cryopreserved Oocytes

Outcomes with cryopreserved oocytes can vary greatly, depending on different variables. One of the most important variables has been the type of cryopreservation procedure. As several studies and meta-analyses have demonstrated, vitrification has been providing consistently and significantly superior outcomes compared to slow-freezing [8, 9, 14] – the reason why vitrification has become the “standard” cryopreservation procedure.

As one would expect, oocyte source (autologous versus donor) is strongly correlated with outcomes as donors are typically a “pre-selected,” younger-aged, high-responding group [14]. When looking at a more “homogenous” patient population, like IVF patients with “emergency” or “elective” cycle management, outcomes fall more within a narrow range. However, age remains the best predictor of success with oocyte cryopreservation. At our clinic, Reproductive Biology Associates (RBA), we conducted a study of 22 infertile patients undergoing IVF treatment where oocytes were collected transvaginally, vitrified/warmed, and then inseminated by ICSI. Outcomes were analyzed by grouping patients to “young” and “advanced” maternal age (11 patients in both groups), with results shown in Table 18.2. The data clearly demonstrate that there are differences based on age; with higher

Table 18.2 Vitrification outcomes according to age of regular IVF patients

Patient age (mean±SD)*	32.9 ±1.9	37.9 ±0.8
Survival rate	82.5%	76.4%
Fertilization rate	70.1%	62.9%
Day 3 good embryo rate*	55.6%	40.4%
Embryos transferred	2.18	2.64
Clinical pregnancy rate	63.6%	27.3%
Implantations rate	41.7%	20.7%
Oocyte to live birth rate	8.2%	3.3%

* P<0.05

Outcome of oocyte vitrification in a group of “young” compared to “mature age” women at RBA

maternal age, there was a trend toward lower oocyte survival, fertilization, embryo development, and implantation/pregnancy (Table 18.3).

One of the most significant questions regarding oocyte cryopreservation (with vitrification) is whether vitrified/warmed oocytes can provide outcomes comparable to those with fresh oocytes. There are some observational studies which indicate that oocyte vitrification can provide similar outcomes to those when fresh oocytes are used; thus, egg cryopreservation can be used as an elective or “emergency” (back up) procedure (and also serve as an efficient alternative for embryo cryopreservation) [10, 15–17]. Our own experience at RBA with regular IVF patients who elected to cryopreserve some of their oocytes (for ethical/religious reasons) and later returned to use them shows that laboratory and clinical outcomes are very similar (Table 18.3). Importantly, implantation and clinical pregnancy rates were the same, but interestingly there was a trend for a higher fertilization rate in the group with oocyte vitrification (Table 18.3), an outcome that has been observed on other occasions as well.

Another significant use of oocyte cryopreservation is for individuals who wish to preserve their fertility for the future, either for medical or for social reasons. While it is estimated that there have been (tens) of thousands of fertility preservation cycles performed worldwide (in the United States in 2014, close to six thousands fertility preservation cycles were reported to SART – www.sart.org); however, reported/published outcomes are scarce. This is understandable, as first, many of the patients who did cryopreserve their oocytes are likely not going to return to use them at all (oocyte vitrification for fertility preservation mainly serves as an “insurance policy” – collected in the hope that will never be needed), and secondly, most patients who decide to use their cryopreserved oocytes will do so only several years after they were vitrified; thus, fertility preservation results are not commonly

Table 18.3 Outcomes of fresh and vitrified-warmed oocytes on the same IVF patients who elected to cryopreserve some of their oocytes and returned subsequently to use those vitrified eggs (none of the outcome parameters were statistically different)

	Fresh cycle	Warming cycle
# Cycles	37	37
Avg. Age \pm S.D.	32.6 \pm 3.70	33.6 \pm 3.51
Avg. # Mill oocytes \pm S.D.	22.8 \pm 10.9	N/A
Avg. # Mill vitrified \pm S.D.	13.1 \pm 8.9	N/A
Avg. # Mill warmed \pm S.D.	N/A	9.6 \pm 7.48
Survival rate \pm S.D.	N/A	82.9%
Fertilization rate \pm S.D.	71.5%	77.9%
Blastocyst rate \pm S.D.	43.0%	49.8%
Avg. # embryos transferred	1.4	1.7
Implantation rate	16%	25%
Clinical pregnancy rate	33%	41%

obtainable. However, the few existing reports/publications indicate that outcomes of fertility preservation patients with vitrified-warmed oocytes are “satisfactory” and they are comparable to outcomes of IVF patients with “matching” age [10, 15, 18–20].

Finally, one of the most common uses of oocyte cryopreservation currently is for donor egg banking. As described earlier, oocyte donation with “Cryo-banked” oocytes has several advantages over fresh egg donation – an explanation for the increase in the number of “Donor Egg Banks,” which now provide a significant share for all egg donation treatment cycles. Initial studies provided by the first two vitrification-based donor egg banks (IVI in Spain and MEB-RBA in Atlanta/USA) demonstrate that results of oocyte donation through donor egg banking (using vitrified donor eggs) are excellent, and comparable to fresh oocyte donations [21, 22]. Interestingly, despite the fact that these two donor egg banks operate differently (in IVI all vitrified donor eggs are provided to a single recipient, and at MEB typically only six to seven donor eggs are provided to one recipient, which is typically about the quarter of the eggs retrieved from a donor), recipient outcomes (implantation and pregnancy rates) are very similar [23]. The first few years of experience at MEB show that results of recipient outcomes may vary depending on which IVF center performs the donor egg warming. However, overall results are adequate (Table 18.4). Importantly, many of the recipients have more than one “usable” embryo (quality of embryo is adequate to be transferred and/or to be cryopreserved), providing the possibility for embryo cryopreservation of “supernumerary” embryo(s). Initial observations on using these cryopreserved embryos demonstrate that their survival and implantation potential is excellent, despite them undergoing “double vitrification” (once at the MII stage and one more time at blastocyst stage or the cleavage stage) [24]. For these reasons, at RBA it is now the policy that recipients using MEB donor oocytes have elective single embryo transfer (eSET) to prevent the risk of multiple pregnancy. Since the introduction of this policy several years ago, it has been observed that cumulative pregnancy rates (eSET) followed by elective “frozen embryo transfer” (eFET) are actually higher than eDET (elective double embryo transfer) providing the highest chance of live birth with the lowest risk of multiple gestation – an objective that is the primary goal of all assisted reproductive treatment. Donor cryo-egg banking usually requires transportation of the vitrified oocytes from the “Egg Bank” to a “user” of the egg bank (which is typically an IVF clinic where no

Table 18.4 Recipient outcomes from My Egg Bank (2012–2015)

Donation cycles	1035
M2 eggs vitrified	23,060
M2 eggs vitrified / donor	22.3 +/−3.7
Recipient cycles	3,424
M2 eggs warmed	21,462 (6.3/R.)
M2 eggs warmed / recipient	6.3 +/−0.28
Survival rate	88%
Fertilization rate	78%
Clinical pregnancy rate	52%

donor egg cryopreservation is performed). However, transportation of vitrified eggs has raised questions on its “safety and efficacy.” Today, based on several years of experience (and several thousands of cases involving cryo-egg shipping), it is safe to state that the donor egg warming cycles which were performed at an IVF center different from the center where they were vitrified provides excellent results – thus proving that dry shipping of vitrified eggs has no impact on egg warming outcomes.

18.6 Safety of Oocyte Cryopreservation

The “overarching” question about every novel technology, besides its efficiency, is its safety. We do have sufficient evidence that vitrification is the most efficient way to cryopreserve oocytes (and embryos); but, “safety” data are still somewhat preliminary, especially when looking at children born. At the level of oocyte and embryo analyses, there are several studies demonstrating that vitrification does not affect significantly structure/sub-cellular morphology and the physiology of the oocyte or the derived embryo. It has been shown that metabolomic, proteomic, and genomic expression profile of vitrified-warmed oocytes (and derived embryos) is very similar to their fresh counterpart, and is significantly improved than after slow-freezing/thawing [25, 26]. As described earlier, because oocyte cryopreservation is performed typically after removing the surrounding cumulus and corona cells, this necessitates to perform insemination with the ICSI procedure. While ICSI is another “intervention” in addition to cryopreservation, it is by now well documented (after two decades of its introduction) that ICSI does not contribute to increased risks of assisted reproduction treatment. On the other hand, early studies (following slow-freezing) of oocyte cryopreservation have suggested that embryos derived from cryopreserved eggs may have a higher rate of chromosomal alterations. This was contributed to the fact that during slow-freezing (and more significantly during thaw of the slow frozen oocyte) the temperature-sensitive meiotic spindle apparatus (that is responsible for the separation of the centrally aligned chromosomes) may be damaged/ microtubules disassemble, leading to possible chromosome errors. During vitrification (and warming), however, microtubules are constantly maintained, ensuring a fully functional spindle. A recent study, analyzing chromosomal composition of embryos derived from vitrified-warmed (donor) oocytes, demonstrated that euploidy/aneuploidy rates were very similar to embryos obtained from fresh (not vitrified) donor oocytes [27] – providing further evidence on the safety of vitrification at the embryo level.

There are only very few studies published reporting on birth outcomes following oocyte vitrification. The largest study published to date is from Dr. Cobo and colleagues [28], who describe that obstetrical outcomes, as well as babies born after oocyte cryopreservation, are not different than after using “fresh” oocytes. These outcomes are similar to those results reported a few years earlier on a smaller number of babies born [29], and help to further assure the safety of oocyte vitrification.

An initial analysis of RBA’s internal data shows that vitrified donor oocytes generate the same live birth outcomes as using fresh donor oocytes (Table 18.5). As can be seen in Table 18.6, there was a trend to higher birth weights for infants born from vitrified oocytes; however, in the group of fresh oocytes there was a much higher multiple pregnancy/delivery rate than in the group with vitrified oocytes. Importantly, the number (rate) of birth defects was statistically not significantly different between the two groups, adding one more dataset

Table 18.5 Live birth data from RBA on recipients using either vitrified or fresh donor oocytes

	Fresh donor egg	Vitrified donor egg
No. of patients / deliveries	58	257
Recipient age (years)	39.9 ± 5.6	41.3 ± 4.5
Live births (infants born)	91	338
Term delivery 37 weeks	28	188
Infants with congenital anomalies	3 (3.3%)	5 (1.5%)
Birth weight (g) – All deliveries	2659.4 ± 690.9	2938.3 ± 770.0
Birth weight (g) – Term deliveries	3361.2 ± 677.2	3518.8 ± 585.2

that supports the safety of oocyte vitrification. It should be noted however that individual IVF centers' collection and publication of data though helpful in answering some safety questions regarding oocyte vitrification will not provide uniformly reliable information on the safety of the technique. In an ideal world, there would be national (and international) registry(ies), where these outcome data would be collected in a systematic and standardized manner.

18.7 Conclusions

Vitrification of oocytes (and embryos) provides excellent outcomes for both autologous and for donor oocyte treatment cycles. There is an overwhelming amount of scientific evidence that vitrification is the most effective approach for cryopreservation, and some initial data also support that it is safe to use.

Vitrification has provided several new options for patients undergoing IVF treatment, introducing elective (and "emergency"/"back up") oocyte cryopreservation. Oocyte vitrification on donor eggs now also provides a better alternative to fresh egg donation.

Additionally, vitrification can also provide options to preserve fertility for those who need it for either medical or for social reasons.

Vitrification has changed the way we work in an IVF clinic and it may change society as well by empowering women to be able to get pregnant at any time when they plan it.

References

- Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. *Lancet*. 1978;12;2:366.
- Sermon K, Capalbo A, Cohen J et al. The why, the how and the when of PGS 2.0: current practices and expert opinions of fertility specialists, molecular biologists, and embryologists. *Mol Hum Reprod*. 2016;22: 845–57.
- Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*. 1992;340:17–18.
- Trounson A, Mohr L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature*. 1983;305: 707–09.
- Chen C. Pregnancy after human oocyte cryopreservation. *Lancet*. 1986;1: 884–86.
- Bunge RG, Sherman JK. Fertilizing capacity of frozen human spermatozoa. *Nature*. 1953;172: 767–68.

7. Szeptycki J, Bentov Y. Cryopreservation of embryos and gametes: past, present, and future. In: Marco-Jimenez editor. *Cryopreservation in Eukaryotes*. IntechOpen, 2016.
8. Rienzi L, Gracia C, Maggiulli R et al. Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Hum Reprod Update*. 2017;23: 139–55.
9. Levi Setti PE, Porcu E, Patrizio P et al. Human oocyte cryopreservation with slow freezing versus vitrification. Results from the National Italian Registry data, 2007–2011. *Fertil Steril*. 2014;102:90–95.
10. Cobo A, Garcia-Velasco JA, Coello A et al. Oocyte vitrification as an efficient option for elective fertility preservation. *Fertil Steril*. 2016;105: 755–64.
11. Gardner DK. *Textbook of Assisted Reproductive Technologies: Laboratory and Clinical Perspectives*. 3rd edn. London: Informa Healthcare; 2009.
12. Engmann L, Benadiva C, Humaidan P. GnRH agonist trigger for the induction of oocyte maturation in GnRH antagonist IVF cycles: a SWOT analysis. *Reprod Biomed Online*. 2016;32: 274–85.
13. Cakmak H, Rosen MP. Random-start ovarian stimulation in patients with cancer. *Curr Opin Obstet Gynecol*. 2015;27: 215–21.
14. Nagy ZP, Anderson RE, Feinberg EC, Hayward B, Mahony MC. The human oocyte preservation experience (HOPE) registry: evaluation of cryopreservation techniques and oocyte source on outcomes. *Reprod Biol Endocrinol*. 2017;15:10.
15. Doyle JO, Richter KS, Lim J et al. Successful elective and medically indicated oocyte vitrification and warming for autologous in vitro fertilization, with predicted birth probabilities for fertility preservation according to number of cryopreserved oocytes and age at retrieval. *Fertil Steril*. 2016;105: 459–66.
16. Herrero L, Pareja S, Aragones M et al. Oocyte versus embryo vitrification for delayed embryo transfer: an observational study. *Reprod Biomed Online*. 2014;29: 567–72.
17. Chang CC, Elliott TA, Wright G et al. Prospective controlled study to evaluate laboratory and clinical outcomes of oocyte vitrification obtained in in vitro fertilization patients aged 30 to 39 years. *Fertil Steril*. 2013;99: 1891–97.
18. Cil AP, Bang H, Oktay K. Age-specific probability of live birth with oocyte cryopreservation: an individual patient data meta-analysis. *Fertility and Sterility*; 2013;100: 492–99.
19. Cobo A, Garcia-Velasco JA, Domingo J, Remohi J, Pellicer A. Is vitrification of oocytes useful for fertility preservation for age-related fertility decline and in cancer patients? *Fertil Steril*. 2013;99: 1485–95.
20. Garcia-Velasco JA, Domingo J, Cobo A et al. Five years' experience using oocyte vitrification to preserve fertility for medical and nonmedical indications. *Fertil Steril*. 2013;99: 1994–99.
21. Nagy ZP, Chang CC, Shapiro DB et al. Clinical evaluation of the efficiency of an oocyte donation program using egg cryo-banking. *Fertil. Steril.* 2009;92: 520–26.
22. Cobo A, Meseguer M, Remohi J, Pellicer A. Use of cryo-banked oocytes in an ovum donation programme: a prospective, randomized, controlled, clinical trial. *Hum Reprod*. 2010;25: 2239–46.
23. Cobo A, Remohi J, Chang CC, Nagy ZP. Oocyte cryopreservation for donor egg banking. *Reprod Biomed Online*. 2011;23: 341–46.
24. Cobo A, Castellò D, Vallejo B et al. Outcome of cryotransfer of embryos developed from vitrified oocytes: double vitrification has no impact on delivery rates. *Fertility and Sterility*. 2013;99: 1623–30.
25. Dominguez F, Castello D, Remohi J, Simon C, Cobo A. Effect of vitrification on human oocytes: a metabolic profiling study. *Fertil Steril*. 2013;99: 565–72.
26. Di Pietro C, Vento M, Guglielmino MR et al. Molecular profiling of human oocytes

- after vitrification strongly suggests that they are biologically comparable with freshly isolated gametes. *Fertil Steril.* 2010;94: 2804–07.
27. Forman EJ, Li X, Ferry KM et al. Oocyte vitrification does not increase the risk of embryonic aneuploidy or diminish the implantation potential of blastocysts created after intracytoplasmic sperm injection: a novel, paired randomized controlled trial using DNA fingerprinting. *Fertil Steril.* 2012;98: 644–49.
28. Cobo A, Serra V, Garrido N et al. Obstetric and perinatal outcome of babies born from vitrified oocytes. *Fertil Steril.* 2014;102: 1006–15.
29. Chian RC, Huang JY, Tan SL et al. Obstetric and perinatal outcome in 200 infants conceived from vitrified oocytes. *Reprod Biomed Online.* 2008;16: 608–10.

Fertilization and Complete Fertilization Failure in Human IVF

Emily A. Seidler, Lauren A. Murphy, Denis A. Vaughan, and Denny Sakkas

19.1 Introduction

In the realm of assisted reproductive technology (ART), much focus is put on the initial and final steps of the in vitro fertilization (IVF) process: the stimulation protocol and the embryo transfer. The laboratory processes, however, are also crucial to a successful outcome. The rate limiting step that is fertilization – either via traditional methods or via intracytoplasmic sperm injection (ICSI) – requires a deep understanding of the biologic and pathophysiologic processes. The majority of normal-appearing gametes in IVF laboratories fertilize as expected; however, it is the unexpected fertilization failures that cause both significant frustration for the provider and disappointment for the patient. This chapter will review the fundamental stages of human gamete fertilization as well as what may contribute to fertilization failure in IVF.

19.2 When and Where It All Goes Wrong at Fertilization

Although modern IVF laboratories can approach fertilization rates of 70–80 percent, total (and expected) fertilization failure still occurs even in patients with reasonable egg yields, and remains a frustrating obstacle for the patient and provider. This phenomenon can occur even in “healthy” appearing oocytes, that is, with a single polar body, normal cytoplasm and perivitelline space, and appropriate zona pellucida (ZP) thickness. Furthermore, ICSI can overcome only one etiology of fertilization failure: sperm penetration through the ZP and oolemma. In fact, following ICSI, human oocytes still fail to fertilize 25–35 percent of the time [1,2], and complete fertilization failure occurs at an estimated rate of 2–3 percent [3]. In our own clinic, when examining all homologous treatment cycles performed by either IVF or ICSI, the complete fertilization failure rates in all patients are approximately 3.9 percent. If we only evaluate patients who are both young (<38 years old) and had at least 5 eggs available, the failure rate is 1.3 percent (Figure 19.1).

It is not entirely clear why a relatively large cohort of “normal” appearing oocytes fail to fertilize, but several factors may be at play. For one, visual assessment of polar body extrusion is not sufficient to assess successful oocyte maturation – it is a far more complex process. Morphologic parameters of the oocyte, described earlier, certainly do not tell the whole story; there are likely many inherent oocyte-controlled events leading to fertilization failure that are not apparent microscopically. Moreover, the centriole is found within the sperm in humans [4] (as opposed to many other mammals, where it is found within the oocyte), which controls the cytoskeleton and may play a significant role in fertilization failure. For example, human sperm centrosomal function is low in abnormally shaped

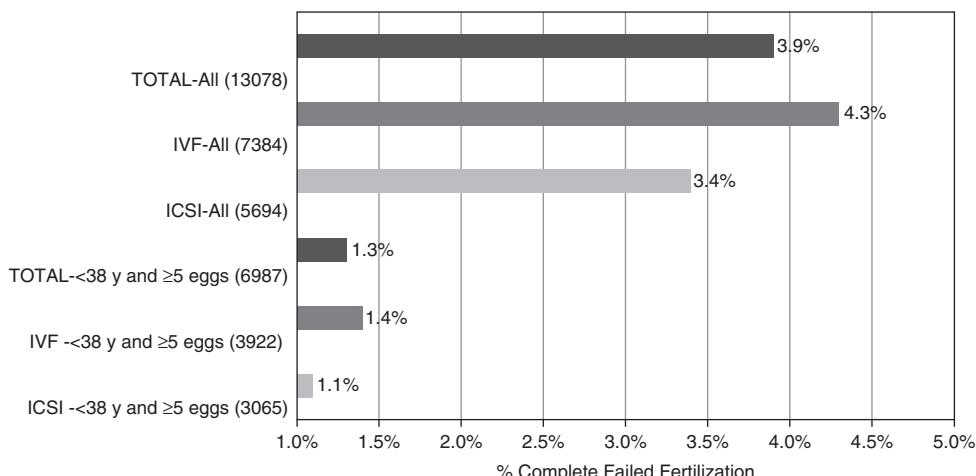


Figure 19.1 The number of non-donor cycles presenting with complete failed fertilization at Boston IVF between 2010 and 2017 for All IVF and ICSI patients with eggs retrieved and for All IVF and ICSI patients when the female partner was less than 38 and had > 5 oocytes collected. The number of cycles for each group is in parentheses

sperm [5]. Lastly, it is hypothesized that autoantibodies created against the ZP in some women may have an adverse outcome on IVF, specifically via fertilization failure [6].

It has been well established that oocyte quality and, in particular, the age of the female lead to a higher risk of IVF failure and miscarriage, and lower live birth rates [7,8]. When an IVF practitioner is confronted with an infertile couple, the onus falls largely on examining the female partner for hormonal and anatomical issues, whereas evaluation of male factor often simply consists of a semen analysis, including sperm concentration, motility, and morphology.

It is not uncommon to encounter the scenario where the workup for both male and female factor is unremarkable. When a patient carrying this diagnosis of unexplained infertility undergoes IVF and receives the unfortunate news of poor or no fertilization even though oocyte numbers and sperm parameters appeared reassuring, it is especially perplexing. Overall, an expected “complete fertilization failure” rate of 1–4 percent will occur after routine in vitro fertilization. Many clinics believe that fertilization failure can be resolved simply by performing ICSI. In fact, according to the Centres for Disease Control statistics, the overall utilization rate of ICSI in the USA is greater than 60 percent [9,10], which is considerably higher than documented rates of male factor infertility [10]. Furthermore, worldwide, the ICSI utilization rates are also reported to be approximately 70 percent [11]. This, in many cases, is a reflex to try to decrease the number of failed fertilizations. It is a common misconception that ICSI improves overall rates of complete fertilization failure, despite evidence to the contrary (Figure 19.1).

19.3 Can ICSI Overcome Complete Fertilization Failure?

Of course fertilization failure rates can be impacted by numerous factors, including female age and the number of oocytes collected. When examining our own Boston IVF data over

many years (2010–2017), we chose to eliminate the possible impact of age and oocyte number and examined only those couples in which the age of the female was <38 years and where ≥ 5 oocytes had been collected. The data indicate that, in this patient group, an overall fertilization failure rate of 1.4 percent is observed (Figure 19.1). This suggests that failed fertilization is far more likely to be related to a male factor component in approximately 1–2 percent of cases.

The routine semen analysis, therefore, fails to provide an accurate indication of whether a couple will experience failed fertilization. To date, no semen test has been identified that can predict whether a couple will experience fertilization failure. Two key questions therefore remain: How do we deal with these patients, and are there any markers that may indicate the likelihood of whether a particular couple will experience fertilization failure?

19.4 The Key Steps of Fertilization

To understand where fertilization may fail we first must examine the process itself. Broadly, successful fertilization requires two important processes: oocyte–sperm recognition and inhibition of polyspermy, which is the fertilization of an oocyte with more than one sperm.

19.4.1 Cumulus Cells

Initially, during fertilization, sperm encounters cumulus cells surrounding the oocyte. The extracellular matrix of cumulus cells is rich in the glycosaminoglycan hyaluronan, which forms a mesh-like network by crosslinking various proteins and proteoglycans [12]. This matrix is important for cumulus expansion or mucification [13]. Interestingly, it has long been thought that cumulus-free oocytes have lower fertilization rates *in vitro* when performing routine insemination; however, studies have shown that this is not the case [14]. It could therefore be argued that the inability of sperm to interact with the cumulus cells is not a major cause of fertilization failure.

19.4.2 Zona Pellucida

The ZP is a glycoprotein coat surrounding the oocyte that regulates the crucial processes of sperm binding and the acrosome reaction (AR). The human ZP is made up of four glycoproteins, ZP1–4, all of which are involved in oocyte–sperm binding. ZP1, ZP3, and ZP4 all help mediate the AR (see review by [15]). Arguably the most important element of the ZP is ZP2, which is necessary for oocyte–sperm recognition and penetration of the ZP, which was previously thought to be the responsibility of ZP3. Avella et al. [16] established a transgenic mouse model expressing human ZP4 that formed zonae pellucidae in the absence of mouse or human ZP2. Neither mouse nor human sperm bound to these ovulated eggs, and these female mice were found to be sterile after *in vivo* insemination or natural mating. The same phenotype was observed with truncated ZP2 that lacks a restricted domain within ZP251–149. These observations in transgenic mice document that the ZP251–149 sperm-binding domain is necessary for human and mouse gamete recognition and penetration through the ZP. The primary block to polyspermy is also due to the actions of ZP2.

Immediately following penetration of the first sperm, ovastacin within the oocyte cleaves ZP2 at the N-terminus, which hardens the ZP and prevents subsequent sperm from binding and penetrating the oocyte [17].

These events indicate how critical certain components are for fertilization to occur. It has yet to be established whether some patients possess mutations in these or other proteins involved in ZP recognition. Liu et al. [18] reviewed numerous publications and found that sperm defects associated with low sperm–ZP binding, or impaired ZP-induced AR, and sperm–ZP penetration are the major causes of fertilization failure, when all, or most, oocytes from a couple do not fertilize in standard IVF. Liu et al. [18] also found that there is a high frequency of defective sperm–ZP interaction in men with oligozoospermia ($<20 \times 10^6/\text{mL}$) and severe teratozoospermia (strict normal sperm morphology $<$ or $=5\%$). They reported that defective ZP-induced AR may cause infertility in up to 25 percent of men with idiopathic infertility. They also suggested that preclinical testing of these parameters may assist in triaging patients into ICSI without first failing routine IVF insemination. In relation to ZP binding deficiencies of sperm, the use of ICSI will normally overcome the failure to fertilize.

19.4.3 Oocyte Activation

The resumption of meiosis immediately following penetration of sperm into the oocyte is a critical step in successful fertilization. This reinitiation of meiosis, along with cortical granule exocytosis, leads to egg activation, or the early events in embryogenesis (review by [19]). Specifically, the entrance of a sperm head activates oocyte development by triggering a series of spikes in calcium levels in the cytosol. The main downstream target of calcium oscillations is CAMKII, which phosphorylates and inactivates other M-phase proteins, ultimately leading to resumption of meiosis. Later, the activity of MAPK decreases, which completes the process of egg activation, marked by pronuclear formation and initiation of interphase of the first cell cycle. In about 1 percent of sperm there are significant abnormalities in CatSper function present in IVF patients with normal sperm concentration and motility. Are these the sperm that fail to activate the oocyte even after ICSI? Interestingly, sperm with a near absence of CatSper failed to respond to activation of CatSper by progesterone and there was fertilization failure at IVF [20].

19.4.4 Pronuclear Formation

The sperm head contains the tightly packed haploid (1 n) chromosome. This condensed DNA must first be unpacked, or decondensed. Protamines surrounding the DNA strands expand and decondense via oocyte cytoplasm enzymes and a nucleic membrane forms; this represents the paternal pronucleus [21]. Concomitantly, a maternal pronucleus is also formed via the following steps: penetration of spermatozoa into the secondary oocyte, which triggers resumption and termination of meiosis II, thus creating a second polar body. The remaining maternal chromosomes are then encased by a separate nuclear membrane and decondensed in a similar fashion. Pronuclear formation is necessary for the subsequent DNA replication phase. DNA is duplicated before the beginning of each cell division, and is then distributed among the daughter cells. The whole process is strictly regulated. The timing of pronuclear formation and breakdown, DNA synthesis, and cleavage during the first cell cycle of human embryogenesis has been described in the past by Capmany and colleagues [22]. Pronuclei were formed between 3 and 10 hours post-

insemination (hpi; median 8 hpi). Synthesis or S-phase commenced between 8 and 14 hpi and was completed between 10 and 18 hpi. The mitosis M-phase was observed between 22 and 31 hpi (median duration 3 h), and cleavage to the 2-cell stage took place between 25 and 33 hpi. The use of time lapse has indicated that the timings at which second polar body extrusion (3.3–10.6 h), pronuclear fading (22.2–25.9 h), and length of S-phase (5.7–13.8 h) occurred successfully were linked to embryo implantation [23]. It has been well documented that even though sperm are able to enter the oocyte, either after routine insemination or after ICSI, many sperm fail to decondense [24, 25]. In the same study we found that sperm chromatin anomalies can influence decondensation after ICSI, leading to failure of fertilization [24]. It was subsequently reported that similar sperm chromatin packaging quality and sperm morphology assessments were useful clinical indicators of human fertilization failure. Moreover, it is believed that some immunofluorescence techniques could be used to provide a clear diagnosis of failed fertilization [26]. However, the use of these types of tests is still questionable [27], even though potential benefits have been described [18].

19.5 Methods of Improving Semen Analysis to Predict Fertilization Failure

As noted above, the ability to perform a direct test on sperm that will predict fertilization failure is yet to be established. A number of diagnostic tests, however, do exist which may help identify defective sperm. In the past two decades, numerous tests have been developed for the analysis of sperm nuclear DNA fragmentation (see review [28]). These tests include TdT-mediated-dUTP nick-end labeling (TUNEL), the COMET assay, chromomycin A3, in-situ nick translation, **DBD-FISH** (DNA breakage detection fluorescence *in situ* hybridization), sperm chromatin dispersion (SCD) test, and the sperm chromatin structure assay (SCSA). Although some data indicate that sperm DNA damage is associated with male infertility, as well as a significantly increased risk of pregnancy loss after IVF and ICSI, no studies have shown a strict correlation to fertilization failure. Indeed, in animal models, increasing sperm nuclear DNA damage does not decrease fertilization rates, but rather negatively affects embryonic and fetal development. Other techniques that have been developed to improve semen analysis and sperm selection include HA (hyaluronic acid) binding, deselection of apoptotic sperm, motile sperm organelle morphological examination (MSOME), and the hypo-osmotic swelling test (HOST)[29]. Of these tests, HA binding has been shown to correlate with zona binding [30]. However, one study examining the use of HA-bound sperm for ICSI in repeated poor fertilization patients with normal sperm parameters found it was not helpful [31]. Finally, the advent of molecular assessments may begin to provide some diagnostic potential. The work on sperm membrane potential, including CatSper [20,32], is starting to provide further insights into mechanisms that lead to failed fertilization.

19.6 Experimental Methods of Treating Fertilization Failure

Although a diagnostic method may not exist to predict fertilization failure, some methods of treatment can be used once a patient has experienced one or more fertilization failures after ICSI. The methods include artificial oocyte activation, polarization microscopy, or repeated ICSI. In recurrent fertilization failures, artificial oocyte activation can be achieved using calcium ionophore following ICSI to initiate the fertilization process [33]. Numerous

pregnancies have been reported using oocyte activation; however, its efficiency still remains unclear [34–39]. In one recent study, the authors stated that the data suggest that artificial oocyte activation using a ready-to-use compound is an efficient and safe method [3,38]. Polarization microscopy has also been used to visualize spindles during ICSI, which may help predict the optimal time to perform ICSI [40]. Finally, repeat cycles can again be treated with ICSI; however, the fertilization rate in most cases remains unchanged.

19.7 Conclusion

Two expectations remain for the treatment of failed fertilization. First, we need to identify a diagnostic test to intervene in the treatment of failed fertilization couples before they pursue costly IVF treatment. As outlined previously, due to the complex nature and multiple steps involved in fertilization, it will be difficult to find a single predictive marker, but the aim may be to have a panel of diagnostics that indicates susceptibility of a patient to failed fertilization. Furthermore, the use of treatments such as oocyte activation needs to be further investigated to ensure it is a safe treatment option. Continued research is needed in this vexing aspect of IVF treatment that affects between 1 and 2 percent of our patients.

References

1. Flaherty SP, Payne D, Swann NJ, Matthews CD. Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection. *Hum Reprod* 1995;10: 2623–29.
2. Flaherty SP, Payne D, Swann NJ, Matthews CD. Assessment of fertilization failure and abnormal fertilization after intracytoplasmic sperm injection (ICSI). *Reprod Fertil Dev* 1995;7:197–210.
3. Mahutte NG, Arici A. Failed fertilization: is it predictable? *Curr Opin Obstet Gynecol* 2003;15: 211–18.
4. Sathananthan AH. Paternal centrosomal dynamics in early human development and infertility. *J Assist Reprod Genet* 1998;15: 129–39.
5. Terada Y, Nakamura S, Simerly C et al. Centrosomal function assessment in human sperm using heterologous ICSI with rabbit eggs: a new male factor infertility assay. *Mol Reprod Dev* 2004;67: 360–65.
6. Gupta SK. Role of zona pellucida glycoproteins during fertilization in humans. *J Reprod Immunol* 2015;108:90–97.
7. Hook EB. Estimates of maternal age-specific risks of Down-syndrome birth in women aged 34–41. *Lancet* 7–3-1976;2: 33–34.
8. Speroff L. The effect of aging on fertility. *Curr Opin Obstet Gynecol* 1994;6: 115–20.
9. Boulet SL, Mehta A, Kissin DM et al. Trends in use of and reproductive outcomes associated with intracytoplasmic sperm injection. *JAMA* 2015;313: 255–63.
10. Center of Disease Control 2014. Assisted reproduction technology national summary report. 2017 page 5. <https://www.cdc.gov/art/pdf/2014-report/art-2014-national-summary-report.pdf> accessed September 14, 2018.
11. Dyer S, Chambers GM, de MJ et al. International committee for monitoring assisted reproductive technologies world report: Assisted reproductive technology 2008, 2009 and 2010. *Hum Reprod* 2016;31: 1588–609.
12. Russell DL, Salustri A. Extracellular matrix of the cumulus-oocyte complex. *Semin Reprod Med* 2006;24: 217–27.
13. Yerushalmi GM, Salmon-Divon M, Yung Y et al. Characterization of the human cumulus cell transcriptome during final follicular maturation and ovulation. *Mol Hum Reprod* 2014;20: 719–35.
14. Plachot M, Junca AM, Mandelbaum J et al. Timing of in-vitro fertilization of cumulus-free and cumulus-enclosed human oocytes. *Hum Reprod* 1986;1: 237–42.

15. Avella MA, Xiong B, Dean J. The molecular basis of gamete recognition in mice and humans. *Mol Hum Reprod* 2013;19: 279–89.
16. Avella MA, Baibakov B, Dean J. A single domain of the ZP2 zona pellucida protein mediates gamete recognition in mice and humans. *J Cell Biol* 6–23–2014;205: 801–09.
17. Burkart AD, Xiong B, Baibakov B, Jimenez-Movilla M, Dean J. Ovastacin, a cortical granule protease, cleaves ZP2 in the zona pellucida to prevent polyspermy. *J Cell Biol* 4–2–2012;197:37–44.
18. Liu DY, Garrett C, Baker HW. Clinical application of sperm-oocyte interaction tests in in vitro fertilization–embryo transfer and intracytoplasmic sperm injection programs. *Fertil Steril* 2004;82: 1251–63.
19. Sanders JR, Swann K. Molecular triggers of egg activation at fertilization in mammals. *Reproduction* 2016;152: R41–R50.
20. Williams HL, Mansell S, Alasmari W et al. Specific loss of CatSper function is sufficient to compromise fertilizing capacity of human spermatozoa. *Hum Reprod* 2015;30: 2737–46.
21. Sun QY, Schatten H. Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. *Reproduction* 2006;131:193–205.
22. Capmany G, Taylor A, Braude PR, Bolton VN. The timing of pronuclear formation, DNA synthesis and cleavage in the human 1-cell embryo. *Mol Hum Reprod* 1996;2:299–306.
23. Aguilar J, Motato Y, Escriba MJ et al. The human first cell cycle: impact on implantation. *Reprod Biomed Online* 2014;28: 475–84.
24. Sakkas D, Urner F, Bianchi PG, et al. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. *Hum Reprod* 1996;11: 837–43.
25. Kvist U. Sperm nuclear chromatin decondensation ability. An in vitro study on ejaculated human spermatozoa. *Acta Physiol Scand Suppl* 1980;486:1–24.
26. Esterhuizen AD, Franken DR, Becker PJ et al. Defective sperm decondensation: a cause for fertilization failure. *Andrologia* 2002;34:1–7.
27. Collins JA, Barnhart KT, Schlegel PN. Do sperm DNA integrity tests predict pregnancy with in vitro fertilization? *Fertil Steril* 2008;89: 823–31.
28. Sakkas D, Alvarez JG. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril* 3–1–2010;93: 1027–36.
29. Henkel R. Sperm preparation: state-of-the-art–physiological aspects and application of advanced sperm preparation methods. *Asian J Androl* 2012;14: 260–69.
30. Huszar G, Jakab A, Sakkas D et al. Fertility testing and ICSI sperm selection by hyaluronic acid binding: clinical and genetic aspects. *Reprod Biomed Online* 2007;14: 650–63.
31. Choe SA, Tae JC, Shin MY et al. Application of sperm selection using hyaluronic acid binding in intracytoplasmic sperm injection cycles: a sibling oocyte study. *J Korean Med Sci* 2012;27: 1569–73.
32. Brown SG, Publicover SJ, Mansell SA et al. Depolarization of sperm membrane potential is a common feature of men with subfertility and is associated with low fertilization rate at IVF. *Hum Reprod* 2016;31: 1147–57.
33. Kashir J, Heindryckx B, Jones C et al. Oocyte activation, phospholipase C zeta and human infertility. *Hum Reprod Update* 2010;16:690–703.
34. Sfontouris IA, Nastri CO, Lima ML et al. Artificial oocyte activation to improve reproductive outcomes in women with previous fertilization failure: a systematic review and meta-analysis of RCTs. *Hum Reprod* 2015;30: 1831–41.
35. Ebner T, Montag M, Montag M et al. Live birth after artificial oocyte activation using a ready-to-use ionophore: a prospective multicentre study. *Reprod Biomed Online* 2015;30: 359–65.

36. Sfontouris IA, Nastri CO, Lima ML et al. Artificial oocyte activation to improve reproductive outcomes in women with previous fertilization failure: a systematic review and meta-analysis of RCTs. *Hum Reprod* 2015;30: 1831–41.
37. Aydinuraz B, Dirican EK, Olgan S, Aksunger O, Erturk OK. Artificial oocyte activation after intracytoplasmic morphologically selected sperm injection: A prospective randomized sibling oocyte study. *Hum Fertil (Camb)* 2016;19: 282–88.
38. Miller N, Biron-Shental T, Sukenik-Halevy R et al. Oocyte activation by calcium ionophore and congenital birth defects: a retrospective cohort study. *Fertil Steril* 9–1-2016;106: 590–96.
39. Tavalaei M, Kiani-Esfahani A, Nasr-Esfahani MH. Relationship between potential sperm factors involved in oocyte activation and sperm DNA fragmentation with intra-cytoplasmic sperm injection clinical outcomes. *Cell J* 2017;18: 588–96.
40. Keefe D, Liu L, Wang W, Silva C. Imaging meiotic spindles by polarization light microscopy: principles and applications to IVF. *Reprod Biomed Online* 2003;7:24–29.

Physiology and Culture of the Early Human Embryo

David K. Gardner and Denny Sakkas

20.1 Introduction

If one is to culture any cell type, in order to be successful, it is a prerequisite to understand the physiology of the cell in question. What makes culture of the preimplantation human embryo so interesting (and challenging) is that the physiology of the embryo changes with development. Indeed, the starting point of development, the fertilized oocyte, exhibits a completely different physiology from the blastocyst prior to implantation. As shall be discussed, these two cell types are so far removed from each other with respect to their metabolism that they could be likened to comparing bone to active skeletal muscle. Reflecting these physiologies are differences in nutrient requirements as the fertilized oocyte develops and differentiates. In order to facilitate the development of a viable preimplantation embryo that will result in the birth of a healthy child, one needs to ensure that the embryo is able to maintain its stage-specific metabolic functions. Failure to do so will compromise not only development prior to transfer but post transfer as well.

Today there exist numerous embryo culture media for use in human IVF, which vary in their composition and complexity. Hence, there remains confusion regarding which medium or media to use, and even when to use them. Consequently, the physiology of the human embryo during its first week of development needs to be considered. Here the metabolic function of the preimplantation embryo is discussed, and the compositions of modern human embryo culture media reviewed, in order to assist the embryologist in making informed decisions about the conditions in which they grow patients' embryos.

20.2 Dynamics of Embryo Physiology

From fertilization to the formation of the blastocyst, the embryo undergoes several changes in its morphology, metabolic function, and transcription. Understanding these changes and the underlying physiology of the embryo has served us well in developing culture media that primarily support the metabolic functions of the developing embryo. Furthermore, any deviation from this stage-specific "metabolic blueprint" is associated not only in retarded development in culture but loss of viability, culminating in reduced pregnancy rates. Consequently, it is imperative to consider embryo physiology in order to optimize culture conditions that can support normal embryonic function and lead to a healthy live birth.

Culture media are not alone in its interaction with the developing embryo, as numerous factors external to the IVF laboratory also determine the resultant development, viability, and health of the preimplantation human embryo (Figure 20.1). These include patient age, genetics, aetiology of infertility, diet, smoking/drug habits, and ovarian stimulation. As ovarian stimulation overrides the dominant follicle, creating a heterogeneous pool of

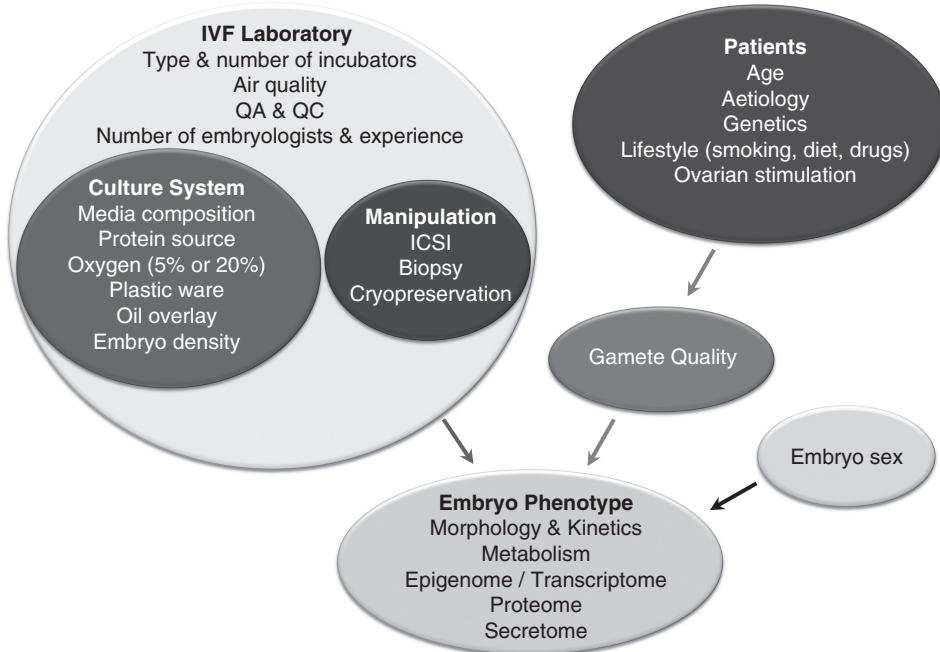


Figure 20.1. Impact of the IVF laboratory environment on human embryo phenotype. The IVF laboratory is comprised of several key components, one of which is the embryo culture system, which in turn is made up from several components, only one of which is the embryo culture media. Independent of the IVF laboratory, patient age and overall health impact the quality of gametes (both sexes), leading to an altered embryo phenotype. Furthermore, the use of exogenous gonadotropins to recruit follicles and induce ovulation has been implicated in the induction of epigenetic alterations in the resultant oocytes and embryos. Therefore, the use of hormones in human IVF may well be predisposing the resultant embryos to greater susceptibility to the laboratory environment. Finally, the gender of the embryo could also determine its interactions with the components of the IVF laboratory. QA, quality assurance; QC quality control. From [23] with permission.

oocytes, it increases the variability of gamete quality. The use of exogenous gonadotropins has been implicated in both the induction of epigenetic alterations in the resultant oocytes and embryos [1, 2] and also in changing the uterine environment [3]. All these factors contribute further variability and stress to the system, making it challenging to determine the precise effects of specific laboratory conditions [4]. Consequently, in order to successfully culture the human embryo conceived through IVF, one must ensure that a holistic approach is used in order to optimize all aspects of a patient's cycle.

20.3 Cleavage Stage Embryos

Prior to the maternal-zygotic transition, the cleavage stages are largely under the control of maternally derived proteins and mRNAs synthesized during oocyte maturation. The physiology of each blastomere is somewhat independent, and in many ways the cells of the cleavage stage embryo are more akin to unicellular organisms than one unified functioning entity [5]. Indeed, like unicellular organisms, the cleavage stage blastomeres utilize exogenous amino acids such as gly, ser, pro, ala, and tau to regulate intracellular functions such as maintenance of intracellular pH and osmotic control [6]. Of relevance to

this is that these very amino acids are among those present at high levels in oviduct fluid, and are not those typically found in those amino acids classified as essential for somatic cell culture.

With regard to energy requirements, the cleavage stage embryo does not have a high energy demand, as there is limited cell division, growth, or biosynthesis, culminating in an accumulation of ATP [7]. This high level of ATP allosterically inhibits the glycolytic pathway, restricting the ability to utilize glucose as a primary energy source. Rather than use glucose, the early preimplantation embryo uses pyruvate, lactate, and aspartate to support low levels of oxidative metabolism [8].

It is interesting to consider the recent interest in the rejuvenation of the aging human oocyte by the addition of exogenous mitochondria. This has been proposed as the ability of the added mitochondria to increase the energy (ATP) required for development. Given that the energy demands of the oocyte and cleavage stages are characterized by high levels of ATP (because it is not being used rapidly), perhaps it is worth considering that mitochondria have other cellular roles beyond energy production, and that should mitochondrial transfer be of benefit to an oocyte, then the effects could well be manifest through a non-metabolic mechanism [9].

20.4 Morulae and Blastocysts

Compaction of the individual blastomeres to form the morula represents a highly significant developmental milestone for the conceptus, in that it has created a transporting epithelium, capable of regulating its internal environment. Consequently, the embryo becomes less dependent upon external factors, such as specific amino acids, to regulate key intracellular processes such as pH.

Around the time of compaction, there is also a considerable increase in cell division and consequently an increase in the demand for both energy and biosynthesis. This demand for energy further increases dramatically with the formation of the blastocoel. The blastocoel is created through the action of Na/K ATPases situated on the basolateral membrane of the epithelium created at compaction [10]. As a result, there is a significant decrease in intracellular ATP levels (as the ATP is being utilized as fast as it can be produced), and a concomitant increase in the uptake of oxygen. As the levels of ATP fall, the inhibition on glycolysis is removed, and the embryo can readily utilize glucose as an energy source.

The metabolism of glucose by the developing blastocyst is rather intriguing, for although oxygen consumption increases, the blastocyst starts to produce significant amounts of lactate. At first analysis, one may conclude that the mitochondria of the blastocyst are dysfunctional, but this does not appear to be the case. Indeed, the mitochondria are functional; it is just rather that the blastocyst has another metabolic requirement from glucose other than ATP production. This idiosyncratic metabolism of the blastocyst is referred to as aerobic glycolysis and was first described in cancer cells by Otto Warburg in 1923, and hence this pattern of metabolism is also referred to as the Warburg Effect [11]. The significance of this type of metabolism to the mammalian blastocyst has been reviewed extensively recently [12]. Of note, a further reason for high levels of lactate production by the blastocysts could be that lactate is actually used by the blastocyst to create a distinct microenvironment around itself at peri-implantation [13]. Hence, rather than being simply an end-product of glycolysis, lactate is actually functioning as a signaling molecule, capable

of inducing angiogenesis, enhancing endometrial tissue remodeling, and facilitating immune modulation to prevent maternal rejection.

20.5 Using Metabolism to Assess Embryo Stress, Developmental Competence, and Viability

It was observed as early as 1970 that should an embryo deviate from its “metabolic blueprint” as development proceeds, then growth and viability are compromised [14]. Subsequent studies confirmed that by ensuring an embryo’s metabolism was similar to those developed *in vivo*, development was not only improved *in vitro* but also post transfer [15–18]. Given metabolic function is so important to development *in culture*, it makes a logical extension that analysis of metabolic function could reflect embryo viability. Several studies have gone on to show that not only is the rate at which glucose is consumed by the blastocyst related to transfer outcome, but also that the fate of the glucose, i.e. how much is converted to lactate, is related to pregnancy outcome [8].

20.6 Brief History of Embryo Culture

In order to understand the media available for use in clinical IVF today, it is important to understand how they were derived and what research underpins the culture solutions we use in our IVF laboratories. The history of modern embryo culture media dates back to the 1950s and 1960s in which several laboratories developed media to support the preimplantation mouse embryo (reviewed by [19]). These media were empirically based and in essence were balanced salt solutions, with bicarbonate to provide a stable pH when incubated in the presence of the appropriate level of carbon dioxide, supplemented with the carbohydrates pyruvate, glucose, and lactate. One of the first media developed specifically for use in human IVF was human tubal fluid medium (HTF) [20]. This medium, and subsequent derivatives of it such as P1 [21] are striking in their simplicity when compared to other media used in the formative years of human IVF, such as Ham’s F-10. Ham’s F-10 was originally designed as a tissue culture medium, and is highly complex, containing amino acids, vitamins, as well as copper, iron, and zinc. Hence, in the 1980s human embryos were being cultured in an extremely diverse range of culture media formulations, and to make matters more convoluted, these media were typically supplemented with serum [5,19]. Today IVF media are supplemented with protein either in the form of human serum albumin (HSA), derived from blood or in recombinant form, or protein added as serum albumin with additional globulins. Media can also be supplemented with the glycosaminoglycan, hyaluronan.

20.7 Carbohydrates

As discussed, the physiology and metabolism of the preimplantation human embryo is highly dynamic, with significant changes occurring in the nutrient requirements and biochemistry of the embryo at each successive stage. The switch from a metabolism based on pyruvate and lactate during the cleavage stages to one based heavily on glucose utilization has been well documented in several mammalian species, including the human [8]. What is highly significant is that the composition of the fluids within the human oviduct and uterus reflects the changing requirements of the human embryo, with oviduct fluid being characterized by relatively high levels of pyruvate and lactate, and low levels of glucose, whereas the levels of these nutrients in the uterus are inverted, with uterine fluid being

characterized by relatively high levels of glucose and lower pyruvate and lactate [22]. These nutrient gradients are not passive but regulate embryo development and affect viability [8], and although embryos can be cultured in a single medium formulation to the blastocyst stage, the physiologies/phenotypes of these blastocysts will be different to those developed under more natural conditions, that is, in sequential media [23]. It is proposed that cellular functions in embryos developed in sequential media will be less stressful, and hence could support a more normal embryo phenotype.

20.8 Amino Acids

Fluids of the female reproductive tract are characterized by high levels of specific amino acids, and yet, as discussed earlier, they were not present in the media based on simple salt solutions, such as HTF [20]. Once considered only as nitrogen sources for the embryo, extensive research has subsequently determined that amino acids are actually important regulators of several embryonic functions during the preimplantation period. Functions of amino acids during embryo development include buffers of intracellular pH, provision of osmotic protection, antioxidants, energy substrates, regulators of carbohydrate metabolism, biosynthetic precursors, signaling molecules, and regulators of differentiation [5]. It has been demonstrated that should a pronucleate oocyte be exposed to media lacking amino acids, then it loses its intracellular pool to the surrounding medium within minutes. It then takes the cell over six hours to regain its intracellular pool, resulting in significantly impaired embryo development [5]. It is therefore recommended that amino acids be in all collection, handling, culture, and transfer media, in order to reduce intracellular trauma.

20.9 Macromolecules

Typically, protein can be added to culture media in the form of HSA. Of note, serum resides in the annals of embryo culture, and no longer in culture media [19, 23]. HSA greatly facilitates gamete and embryo manipulation as well as negating the effects of toxins. Several commercially available serum products have been used to great success in replacing serum in human embryo culture systems, ranging from therapeutic albumin solutions to globulin-enriched albumin solutions such as Synthetic Serum Substitute (SSS), containing 84 percent HSA and 16 percent α - and β -globulins with less than 1 percent γ -globulin. It has been proposed that α - and β -globulins have a role in supporting embryo development in culture. Glycoproteins, which possess numerous hydroxyl groups, may confer benefit to the embryo by altering the solvent properties of the medium, making it more akin to the tubal environment. However, there have been no prospective randomized trials using such supplements. Although HSA is a relatively pure fraction, it is still contaminated with fatty acids, transition metals, and other small molecules, many of which are poorly characterized. Not only are there significant differences between sources of serum albumin, but also between batches from the same source. Furthermore, some HSA preparations contain the preservative sodium caprylate, which binds to the hydrophobic domains of the proteins and therefore cannot be removed by dialysis. The effects of such a preservative on embryo development have yet to be determined. Also, the processing of albumin can also lead to the introduction of known chemicals such as octanoic acid, which has been shown to be detrimental to embryo development [24].

Recombinant human albumin has been shown to be an effective replacement for serum-derived albumin in animal IVF and embryo culture, and its clinical efficacy validated in

a prospective randomized trial. It has been found to be equally as effective as human serum albumin in supporting embryo development in vitro and subsequent pregnancies [19]. Given the growing concerns surrounding the safety of human IVF, it is certainly timely to reconsider the inclusion of recombinant albumin to minimize risk and to increase consistency of function.

Hyaluronan, a glycosaminoglycan, is present in the female reproductive tract, and its concentration increases at the time of implantation in the mouse and can be readily obtained endotoxin- and prion-free from a yeast fermentation procedure. Hyaluronan works in synergy with albumin to promote embryo development and has also been shown to increase embryo cryotolerance in several mammalian species, including the human [5]. Perhaps of greatest significance, however, was the finding that the addition of hyaluronan to embryo culture medium significantly increases mouse blastocyst implantation and fetal development after transfer [25]. The inclusion of hyaluronan in transfer medium has since been adopted clinically [19].

20.10 Types of Media for Human Embryo Culture

Although media designed for somatic cell tissue culture were used in the early days of IVF, their use has all but discontinued today. Similarly, with the exception of a few clinics, those media formulated in the 1980s such as HTF are not commonly used (nor should their use be encouraged due to their lack of key regulators of embryonic function, such as amino acids) in clinical IVF.

Today there are now three media types available for human embryo culture:

1. Sequential media. These media were formulated around the levels of carbohydrates present in the human oviduct and uterus [22], and include stage-specific amino acids based on the analysis of embryo physiology, and were specifically designed to reduce intracellular stress in the developing human embryo, and to maintain the mammalian embryo's metabolic blueprint that is largely delineated before and after the 8-cell stage. They were based on data from the human female reproductive tract and from extensive analysis of embryo metabolic functions.
2. Monophasic media. A common clinically used version, Global medium, is based on the simplex medium, KSOM^{AA}. Simplex media were created using a computer software to generate successive media formulations based on the ability of mouse embryos to form blastocysts [26, 27]. Once a particular medium was formulated, tested, and blastocyst formation analyzed, the program generated new formulations for evaluation. This was done several times to create several media that supported high rates of mouse blastocyst development. The first iteration Simplex Optimized Media was termed SOM and later KSOM (which contains around 10 times more potassium than SOM). KSOM was subsequently modified by another laboratory to include amino acids (KSOM^{AA}) [28]. However, this last phase of medium development was based on independent studies on the mouse embryo [29] and did not involve the simplex procedure. No physiological or metabolic analyses were undertaken in the formulation of this medium. This medium formulation was then used to support human blastocysts in culture [30]. In such types of media, the embryo therefore has to adapt to its surrounding as it develops and differentiates.
3. Time-Lapse Media. The advent of time-lapse microscopy created a perceived need for uninterrupted culture (although as discussed, this approach is not physiological).

These media have been designed to cope with the extended build-up of ammonium from amino acid breakdown and metabolism, and the levels of nutrients balanced for the different requirements of the cleavage and blastocyst stages [31]. However, as these media are technically monophasic, the embryo will have to adapt during development.

The question is: “Which approach should we take to culture human embryos in our IVF laboratories?” Although the use of sequential media is based on embryo physiology, such media require the embryo to be moved from the first-phase medium to the second on day 3 of development. Unless performed using a gassed and humidified chamber, that is, an embryo islette designed for such procedures, one may incur transient shifts in temperature and pH. Consequently, and together with the increased use of time-lapse analysis, there has been a practical demand for uninterrupted culture. Although this latter approach does not take into account the dynamics of embryo physiology or the “metabolic blueprint” of the embryo, it does ensure that temperature and pH are constant throughout culture. So in consideration, have to be placed into context of how each IVF laboratory is set up, as well as how many patients are treated [4]. However, an interesting scenario in which the best of both worlds can reside is the use of a two-phase media system whereby the second phase is attained by adding the second medium directly to the first (as opposed to moving embryos from one medium to another), thereby creating sequential media “in situ”. This has the advantage of keeping any embryo-derived auto/paracrine factors within the medium drop, while diluting out any toxic products produced by the embryo. In the future, incorporation of microfluidic culture systems will allow the benefits of an uninterrupted monophasic culture system while incorporating aspects of sequential changes in media composition as the embryo reaches specific milestones [32, 33].

20.11 Embryo Culture Is More Than the Media Alone

Although the composition of the culture media selected is important, the process of embryo culture goes far beyond that of media formulations. In order to optimize embryo culture in the IVF laboratory, it is important to consider that the media are but one component of the embryo culture system, and their performance is affected by other aspects of the culture system. Hence, in order to maximize the functionality of culture media, one must take a holistic perspective of the IVF cycle in order to understand how other parameters impact the phenotype of the human preimplantation embryo. Figure 20.1 shows the factors and their interactions which impact the development and viability of the preimplantation human embryo [23].

20.11.1 Oxygen

The level of oxygen used to culture the human embryo varies significantly between IVF laboratories and countries [34]. Atmospheric oxygen (~20 percent) is significantly higher than the level of oxygen which the human embryos are exposed to *in vivo*, which is reported to be between 2 and 8 percent in the oviduct, and from 1.5 to 5 percent in the uterus [5, 19]. In all animal studies, it has been shown that embryo development and implantation post transfer are significantly decreased when embryos are cultured in atmospheric oxygen. Indeed, exposure to atmospheric oxygen has been shown to alter embryo developmental kinetics, transcription, histone remodeling and methylation patterns, the proteome, and metabolic state (reviewed in [4, 23]).

Examination through time-lapse of human embryo development under different oxygen levels shows that culture in atmospheric oxygen prior to compaction results in significantly slower embryo development compared to culture in 5 percent oxygen, with the timing of 4- to 8-cell being retarded in 20 percent oxygen [35]. Further, implantation and pregnancy rates are reduced following embryo culture in atmospheric oxygen [36, 37], culminating in a decrease in the live birth rate [37, 38]. Rather surprisingly, today around 75 percent of the world's IVF clinics employ atmospheric oxygen for some or all of the culture period [34], making it difficult to determine the impact of specific components within the IVF laboratory on embryo phenotype, as many have adverse interactions with atmospheric oxygen.

20.11.2 The Embryo as a Dynamic Component of the Culture System

When a human embryo is placed in a defined volume of medium, the said medium begins to change its composition due to the metabolic activity of the embryo and the production of embryo-derived factors. As well as the depletion of nutrients, the human embryo has the capacity to release the end products of their metabolism into the medium, the most noticeable of which are ammonium and lactate from the metabolism of amino acids and glucose respectively. It is the documented effects of ammonium on human embryo physiology that are perhaps the most concerning [39], which in turn is dependent upon the volume of the culture medium used per embryo, its frequency of renewal, and the oxygen concentration. The presence of 20 percent oxygen predisposes the embryo to other stressors present in the laboratory environment/culture system [23].

Interestingly, the volume of media per embryo (referred to as embryo density) varies widely between clinics, and its importance in human embryo development has yet to be resolved. In other species, embryo density can influence embryo development and blastocyst cell numbers, but data from the human have been contradictory. This confusion has probably arisen because optimum embryo density is dependent on other culture conditions, including oxygen, which suggests that individual clinics will need to optimize embryo density for their culture system [23].

Historically, human embryos have typically been cultured in groups, but with advances in techniques for embryo assessment and selection, around half of clinics worldwide now culture embryos individually [34]. This is of potential concern because data from animal models show that compared to group culture, single culture results in delayed cleavage divisions, fewer blastocysts, fewer cells per blastocyst and a reduction in inner cell mass (ICM), and more apoptotic cells. This is thought to be due to deprivation from paracrine signaling molecules. In addition, single embryos are more susceptible to other stresses in the culture system, such as atmospheric oxygen or peroxides from the oil overlay. However, there is limited evidence to indicate if single culture is detrimental to human embryos. Two studies have found no difference between grouped and single human embryos [40, 41], while another four have observed a reduction in development rates, the number of quality blastocysts, or pregnancy rate [42–45]. Again, these studies used a variety of culture systems, and only one compared group and single culture for the entire period from day 1 to blastocyst, which resulted in poorer morphology scores on days 4 and 5 [44]. However, single embryo culture can be improved through the use of micro-well dishes, such as those employed in time-lapse, which facilitate the creation of microenvironments [46].

20.12 Types of Incubator

It has been established for many years that the type of incubator, and the number of times the incubator door is opened, affects the development and viability of the embryo [4]. Should a standard box incubator be used, then it is important for the CO₂ sensor to be based on infra-red (IR) technology, rather than thermo-couple technology, as IR is not dependent upon chamber humidity and therefore ensures rapid gas recovery after the door has been opened. Today, more commonly used incubators are designed around bench top models, which have the advantage of direct heat transfer and continuous gassing. Similarly, the new time-lapse culture systems include such advantages, with the addition of the ability to quantitate embryo development every few minutes without disrupting the culture environment. Importantly, if a standard box incubator is properly maintained with appropriate QC, and the number of patients/door openings carefully managed, then they can provide an excellent culture environment. However, if an IVF clinic is performing a high volume of cycles, then newer incubation systems would be advocated. The benefits of including time-lapse into an incubator go beyond that of embryo selection, and the acquisition and application of such technology is considered integral to the optimal performance of an IVF laboratory of the twenty-first century (Box 20.1).

Box 20.1 Advantages to Embryo Culture Conferred by a Time-lapse Incubator

- Stable culture system, minimal drifts in temperature and pH
- Embryo de-selection
- Possible use of algorithms and AI for embryo selection
- Improved workflow in the IVF laboratory
- Analysis of blastocyst re-expansion after vitrification/warming
- Improved embryo assessment to time blastocyst biopsies
- Augments both quality control and quality management
- Training of embryologists and homogeneity of embryo scoring
- Referral for PGS as aberrant morphokinetics indicate possible aneuploidy
- Remote access to patients' embryo development
- Creation of embryo development videos for doctor / patient counseling
- Opens up many research possibilities and a means to improve media formulations

20.13 Advice for Successful Embryo Culture

When working with oocytes and embryos, it is important to minimize cellular trauma. Stressors to the preimplantation embryo tend to be cumulative in nature [4]. It is, therefore, paramount that a quality management system is in place in the IVF laboratory, so that all aspects of the IVF laboratory, including the culture system, have been thoroughly assessed, and further all contact supplies and equipment are performing within expected parameters [47]. It is imperative to work quickly and yet smoothly when handling embryos (pipetting

too quickly and abruptly can induce stress responses in the embryo itself). When transferring embryos from wash drops or from media to media, always use the minimum volume possible. To ensure this, use a pipette whose diameter is just larger than the embryo itself (day 1 to 3; around 200 μM); as the blastocyst expands, a larger pipette diameter will be required. Not only will this ensure more optimal performance of the media used, it takes less time.

20.14 Future of Embryo Culture Media

Although we have witnessed many major improvements over the past 20 years, there is still more to be done in this field. Focus has moved away from success rates, to safety and the long-term health of the children conceived through IVF. Although the impact of embryo culture on the human embryo phenotype has been documented [23], extensive analysis of large data sets over several years from the same program has revealed very limited impact of these changes on resultant birth weights of children [48]. Indeed, the parameter most associated with changes to outcome was cryopreservation. However, this must not lull us into a false sense of security, and long-term follow-up of all children conceived in vitro, especially after the introduction of a change in the culture system, is advocated.

Although the media we use today are more physiological than their predecessors, they still lack numerous factors present in the fluid of the female reproductive tract. Noticeable in their absence are cytokines and growth factors, whose abundance changes with the day of cycle and between fertile and infertile patients [49]. Further, the uterine environment that surrounds the embryo is highly hydrated and therefore does not behave like an aqueous solution. Although many studies have shown that individual factors can impact embryo development, in reality they are always present as a group, and it is therefore proposed that their true benefit will only be realized when at such time they are utilized together and not in isolation. Due to their ability to regulate several cell functions and differentiation, further research is warranted on their effects, especially at the epigenetic level.

Given the damaging effects of oxygen on embryo development, and that even if embryo culture is performed at reduced oxygen levels, gamete retrieval, preparation, ICSI, and embryo biopsy all occur in atmospheric oxygen, it would be appropriate to include anti-oxidants in media formulations. Similar to works on cytokines and growth factors, most analyses of antioxidant function have focused on individual antioxidants. Recently, the beneficial effects of a group of three antioxidants; acetyl-L-carnitine, N-acetyl-L-cysteine, and α -lipoic acid, have been shown not only to greatly improve mouse embryo development in vitro but to improve transfer outcome significantly. Of note, the beneficial effects of this group of antioxidants were manifest at both physiological and atmospheric oxygen levels, with the greatest effects being observed in the latter [50]. Clinical studies are currently underway to evaluate the effects of these antioxidants on human embryo development and viability.

Finally, just as the box-style tissue culture incubators have given way to bench-top/time-lapse incubation systems, these too will give way to the next generation of incubation systems, which will incorporate microfluidic chips as we progress inevitably toward laboratory automation. The next decade is envisaged to be dynamic and exciting in the world of embryo culture.

References

1. Fortier AL, McGraw S, Lopes FL et al. Modulation of imprinted gene expression following superovulation. *Mol Cell Endocrinol* 2014;**388**: 51–7.
2. Huffman SR, Pak Y, Rivera RM. Superovulation induces alterations in the epigenome of zygotes, and results in differences in gene expression at the blastocyst stage in mice. *Mol Reprod Dev* 2015;**82**: 207–17.
3. Simon C, Garcia Velasco JJ, Valbuena D et al. Increasing uterine receptivity by decreasing estradiol levels during the preimplantation period in high responders with the use of a follicle-stimulating hormone step-down regimen. *Fertil Steril* 1998;**70**: 234–39.
4. Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update* 2016;**22**: 2–22.
5. Gardner DK. Dissection of culture media for embryos: the most important and less important components and characteristics. *Reprod Fertil Dev* 2008;**20**: 9–18.
6. Lane M, Gardner DK. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reprod Fertil Dev* 2005;**17**: 371–78.
7. Leese HJ, Biggers JD, Mroz EA, Lechene C. Nucleotides in a single mammalian ovum or preimplantation embryo. *Anal Biochem* 1984;**140**: 443–48.
8. Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. *Fertility and Sterility* 2013;**99**: 1062–72.
9. Lees JG, Gardner DK, Harvey AJ. Pluripotent stem cell metabolism and mitochondria: beyond ATP. *Stem Cells Int* 2017;**2017**: 2874283.
10. Watson AJ, Kidder GM, Schultz GA. How to make a blastocyst. *Biochem Cell Biol* 1992;**70**: 849–55.
11. Warburg O. On respiratory impairment in cancer cells. *Science* 1956;**124**: 269–70.
12. Gardner DK, Harvey AJ. Blastocyst metabolism. *Reprod Fertil Dev* 2015.
13. Gardner DK. Lactate production by the mammalian blastocyst: manipulating the microenvironment for uterine implantation and invasion? *Bioessays* 2015;**37**: 364–71.
14. Bowman P, McLaren A. Cleavage rate of mouse embryos in vivo and in vitro. *J Embryol Exp Morphol* 1970;**24**: 203–07.
15. Quinn P, Wales RG. The relationships between the ATP content of preimplantation mouse embryos and their development in vitro during culture. *J Reprod Fertil* 1973;**35**: 301–09.
16. Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. *J Reprod Fertil* 1990;**88**: 361–68.
17. Gardner DK, Sakkas D. Mouse embryo cleavage, metabolism and viability: role of medium composition. *Hum Reprod* 1993;**8**: 288–95.
18. Lane M, Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Hum Reprod* 1998;**13**: 991–7.
19. Gardner DK, Lane M. Embryo culture systems. In: Gardner DK, Simon C, eds. *Handbook of In Vitro Fertilization*, 4th edition: CRC Press, 2017: 205–44.
20. Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil Steril* 1985;**44**: 493–98.
21. Carrillo AJ, Lane B, Pridman DD et al. Improved clinical outcomes for in vitro fertilization with delay of embryo transfer from 48 to 72 hours after oocyte retrieval: use of glucose- and phosphate-free media. *Fertil Steril* 1998;**69**: 329–34.
22. Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: metabolite analysis

- of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril* 1996;65: 349–53.
23. Gardner DK, Kelley RL. Impact of the IVF laboratory environment on human preimplantation embryo phenotype. *J Dev Orig Health Dis* 2017;8: 418–435.
 24. Leonard PH, Charlesworth MC, Benson L et al. Variability in protein quality used for embryo culture: embryotoxicity of the stabilizer octanoic acid. *Fertil Steril* 2013;100: 544–49.
 25. Gardner DK, Rodriegez-Martinez H, Lane M. Fetal development after transfer is increased by replacing protein with the glycosaminoglycan hyaluronan for mouse embryo culture and transfer. *Hum Reprod* 1999;14: 2575–80.
 26. Lawitts JA, Biggers JD. Optimization of mouse embryo culture media using simplex methods. *J Reprod Fertil* 1991;91: 543–56.
 27. Lawitts JA, Biggers JD. Culture of preimplantation embryos. *Methods Enzymol* 1993;225: 153–64.
 28. Ho Y, Wigglesworth K, Eppig JJ, Schultz RM. Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Mol Reprod Dev* 1995;41: 232–38.
 29. Gardner DK, Lane M. Amino acids and ammonium regulate mouse embryo development in culture. *Biol Reprod* 1993;48: 377–85.
 30. Biggers JD, Racowsky C. The development of fertilized human ova to the blastocyst stage in KSOM(AA) medium: is a two-step protocol necessary? *Reprod Biomed Online* 2002;5: 133–40.
 31. Morbeck DE, Baumann NA, Oglesbee D. Composition of single-step media used for human embryo culture. *Fertil Steril* 2017;107:1055–60 e1.
 32. Gardner DK. Mammalian embryo culture in the absence of serum or somatic cell support. *Cell Biol Int* 1994;18: 1163–79.
 33. Swain JE, Lai D, Takayama S, Smith GD. Thinking big by thinking small: application of microfluidic technology to improve ART. *Lab Chip* 2013;13: 1213–24.
 34. Christianson MS, Zhao Y, Shoham G et al. Embryo catheter loading and embryo culture techniques: results of a worldwide Web-based survey. *J Assist Reprod Genet* 2014;31: 1029–36.
 35. Kirkegaard K, Hindkjaer JJ, Ingerslev HJ. Effect of oxygen concentration on human embryo development evaluated by time-lapse monitoring. *Fertil Steril* 2013;99:738–44 e4.
 36. Catt JW, Henman M. Toxic effects of oxygen on human embryo development. *Hum Reprod* 2000;15 Suppl 2:199–206.
 37. Meintjes M, Chantilis SJ, Douglas JD et al. A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program. *Hum Reprod* 2009;24: 300–07.
 38. Gomes Sobrinho DB, Oliveira JB, Petersen CG et al. IVF/ICSI outcomes after culture of human embryos at low oxygen tension: a meta-analysis. *Reprod Biol Endocrinol* 2011;9:143.
 39. Gardner DK, Hamilton R, McCallie B, Schoolcraft WB, Katz-Jaffe MG. Human and mouse embryonic development, metabolism and gene expression are altered by an ammonium gradient in vitro. *Reproduction* 2013;146:49–61.
 40. Spyropoulou I, Karamalegos C, Bolton VN. A prospective randomized study comparing the outcome of in-vitro fertilization and embryo transfer following culture of human embryos individually or in groups before embryo transfer on day 2. *Hum Reprod* 1999;14:76–79.
 41. Rijnders PM, Jansen CA. Influence of group culture and culture volume on the formation of human blastocysts: a prospective randomized study. *Hum Reprod* 1999;14: 2333–37.
 42. Almagor M, Bejar C, Kafka I, Yaffe H. Pregnancy rates after communal growth of preimplantation human embryos in vitro. *Fertil Steril* 1996;66: 394–97.

43. Moessner J, Dodson WC. The quality of human embryo growth is improved when embryos are cultured in groups rather than separately. *Fertil Steril* 1995;64: 1034–35.
44. Ebner T, Shebl O, Moser M et al. Group culture of human zygotes is superior to individual culture in terms of blastulation, implantation and live birth. *Reprod Biomed Online* 2010;21: 762–68.
45. Rebollar-Lazaro I, Matson P. The culture of human cleavage stage embryos alone or in groups: effect upon blastocyst utilization rates and implantation. *Reproductive Biology* 2010;10: 227–34.
46. Kelley R.L., Gardner DK. In vitro culture of individual mouse preimplantation embryos: the role of embryo density, microwells, oxygen, timing and conditioned media. *Reprod Biomed Online* 2017;34: 441–454.
47. Mortimer ST, Mortimer M, *Quality and Risk Management in the IVF Laboratory*. Cambridge: Cambridge University Press, 2005.
48. Maas K, Galkina E, Thornton K, Penzias AS, Sakkas D. No change in live birthweight of IVF singleton deliveries over an 18-year period despite significant clinical and laboratory changes. *Hum Reprod* 2016;31: 1987–96.
49. Hannan NJ, Paiva P, Meehan KL et al. Analysis of fertility-related soluble mediators in human uterine fluid identifies VEGF as a key regulator of embryo implantation. *Endocrinology* 2011;152: 4948–56.
50. Truong TT, Soh YM, Gardner DK. Antioxidants improve mouse preimplantation embryo development and viability. *Hum Reprod* 2016;31: 1445–54.

Use of Medium Supplements for Oocyte and Embryo Culture

Cecilia Sjöblom and Harriet Swarman

21.1 Introduction

From the time of ovulation through fertilization, development and implantation, the female reproductive tract provides an optimal environment supporting growth and development of the embryo in a time-stage specific manner. Physical conditions such as pH and osmolality, together with the microenvironment of nutrients and metabolites, changes along the tract, presenting as a gradient rather than being static (Figure 21.1). The viability, growth and development of the oocyte and subsequent embryo are closely regulated by an array of signaling molecules, such as cytokines and growth factors, secreted from epithelial cells lining the oviduct and uterus [1]. These are synthesized under precise spatial and temporal controls driven predominantly by ovarian steroid hormones, and other factors of maternal origin [2] and also by factors in seminal plasma.

The conditions for human embryo culture in the in vitro fertilization (IVF) setting are constantly improving, but can still be considered as suboptimal when compared to the reproductive tract. As a result, the embryo viability and growth in vitro are believed to be compromised and these are most likely contributors to the high rates of implantation failure seen in human IVF. A major goal of ongoing research is to create in vitro culture conditions more similar to the conditions in the female reproductive tract, thereby fully supporting the growth of the embryo to the blastocyst stage. Embryo transfer has historically been done on day 2 or day 3, with selection of two or more cleavage stage embryos. In recent years there has been an increase in extended culture, to day 5, with the aim to select only one highly competent blastocyst for transfer and reduce the high number of multiple births resulting from IVF. Moreover, the transfer of blastocysts to the uterus is more physiological than transfer at early cleavage stages. However, prolonged culture puts further emphasis on the culture environment, and while modern culture medium can be seen as a complex mix of inorganic compounds, metabolites and amino acids, they are mostly void of the crucial signalling factors found in the reproductive tract [1, 2, 3].

21.2 Supplements to Support the Oocyte and IVM

In vitro maturation (IVM) of oocytes involves the culturing of immature oocytes or cumulus-oocyte complexes (COCs) such that they acquire full nuclear and cytoplasmic maturation, evident by successful preimplantation embryo development following fertilization. The use of IVM in assisted reproductive technology (ART) has had limited

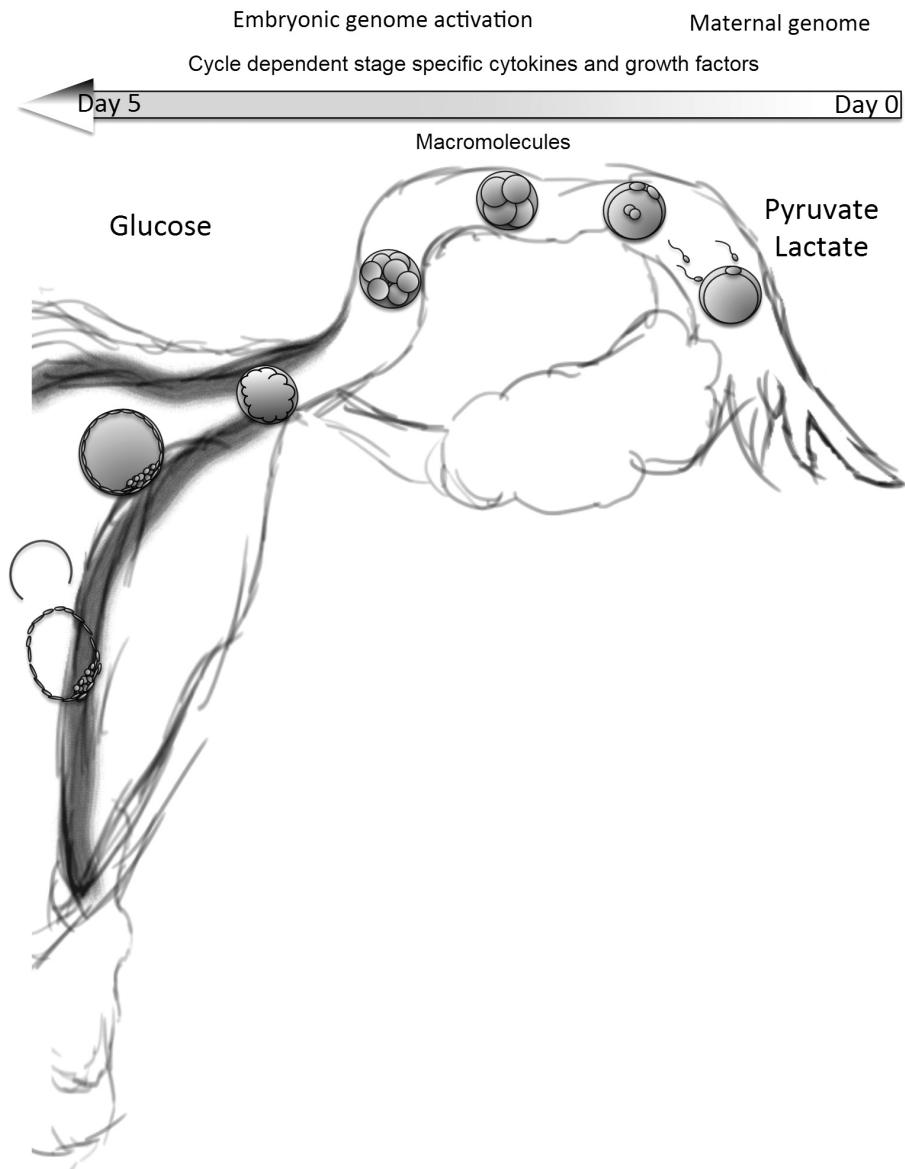


Figure 21.1 The female reproductive tract providing a cycle-dependent, precise environment for the developing embryo, presenting specific metabolites, cytokines, growth factors and macromolecules in a gradient rather than static manner (illustration by Ellie Rawley)

success, mainly due to the difficulty in successful supplementation of oocyte culture medium, allowing the oocyte to undergo full maturation. Importantly, the culture medium needs to reflect the *in vivo* follicular environment, which plays an important role in maintaining oocyte meiotic arrest and facilitating maturation (Figure 21.2) [4].

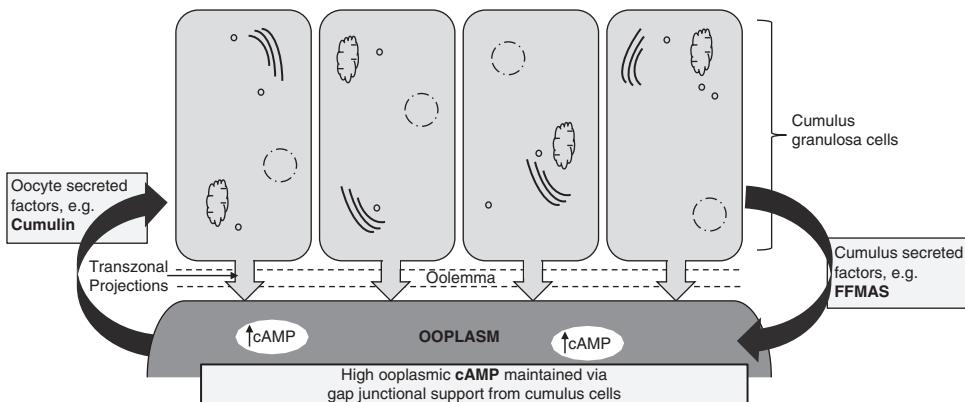


Figure 21.2 Paracrine oocyte- and cumulus cell-secreted factors stimulating oocyte growth and maturation, which have been added to IVM culture media. (adapted from Sutton et al.) [4]

21.2.1 Macromolecules

There has been debate as to whether the addition of protein, such as human serum albumin (HSA) and macromolecules, like polyvinyl pyrrolidone (PVP), can improve oocyte IVM. Protein supplements are difficult to quantify, making them less favourable supplements to oocyte culture media, although it is known that in animal models the addition of fetal calf serum (FCS) increases oocyte maturation, cleavage and blastulation rates compared to media without FCS [4]. PVP and polyvinyl alcohol (PVA) are synthetic organic alternative macromolecules that have been successfully combined with hormones and growth factors to improve blastulation rates, compared to media supplemented with protein only; however, PVP supplementation alone decreased blastulation rate in bovine oocytes [4]. Current indication in human IVM culture is that unquantifiable protein supplements could be replaced by non-biological macromolecules combined with quantified protein substrates to improve the success of IVM [5, 4].

21.2.2 FF-MAS

Follicular fluid meiosis activating sterol (FF-MAS) is a steroid secreted *in vivo* from cumulus cells to the oocyte; its concentration increases prior to oocyte maturation and ovulation. Addition of FF-MAS to IVM media has been shown to increase oocyte diameter in human oocytes, a key determinant of developmental status [6]. However, supplementation with FF-MAS remains controversial, as the mechanism by which it exerts its effect is not fully understood. It has also been shown that exposure of oocytes that were already large in diameter to FF-MAS resulted in atresia [6]. Evidence is conflicting and not yet substantial enough to indicate that FF-MAS supplementation has a true improvement on IVM culture media.

21.2.3 Meiotic Inhibitors

The oocyte remains arrested in prophase I of meiosis throughout maturation *in vivo*; only at the point of ovulation does the cell cycle resume maturation. It is important that the oocyte is maintained in meiotic arrest during IVM, until it acquires all the necessary nuclear and cytoplasmic machinery to be able to complete meiosis [5, 7]. The addition of cyclic adenosine monophosphate (cAMP) modulators, phosphodiesterase inhibitors (e.g. rolipram) and adenylate cyclase inhibitors (e.g. 6-(dimethylamino) purine (6-DMAP), roscovitine) all have a similar effect, in maintaining high ooplasmic cAMP levels which upholds meiotic arrest [7]. The control of cAMP levels has been shown to improve developmental competence in the oocyte, and it has been reported that some also have a downstream improvement on embryo development (such as roscovitine on bovine oocytes), whereas others show no effect (such as 6-DMAP in humans) [7].

21.2.4 Cumulin

In recent work, the addition of cumulin, a naturally forming dimer of oocyte secreted growth factors, growth differentiation factor-9 (GDF9) and bone morphogenic protein-15 (BMP15), increased quality and competence in porcine oocytes, more than doubling embryo production rates [8]. Clinical trials using supplementation of this factor in human oocyte culture media for IVM are currently ongoing, so it is unclear yet as to whether this will significantly improve oocyte developmental competence and embryo yield.

21.3 Supplements to Support Embryo Growth

21.3.1 Cytokines and Growth Factors

A woman's reproductive tract is known to express and secrete a number of cytokines and growth factors, with the embryo shown to express corresponding receptors for these. Adding these cytokines and growth factors to culture medium is known to promote human embryo growth and development. Many cytokines having regulatory effects on embryo development have been identified, and in most cases their receptors have been shown to be present in the embryo. Notably, these factors include most of the cytokines known to be produced by the uterine and oviduct epithelia during early pregnancy [1, 2]. The actions of cytokines are dependent on the developmental stage of the embryo, and may also vary somewhat between species and even among strains of mice. Factors such as insulin, insulin-like growth factors (IGFs), epidermal growth factors (EGFs), colony stimulating factor-1 (CSF-1) and transforming growth factor beta (TGF β) have all been shown to improve secretory activity and the proportion of mouse embryos that develop to the blastocyst stage and beyond. Conversely, a number of cytokines present in the tract, such as tumour necrosis factor alpha (TNF α) and interleukin 6 (IL-6), hold a regulatory function and exert inhibitory influences on inner cell mass (ICM) proliferation and embryo attachment and outgrowth respectively. Platelet-derived growth factor (PDGF), TNF α and basic fibroblast growth factor (bFGF) accelerate the development of bovine embryos. The embryo itself has been found to synthesize cytokines, including IL-1,

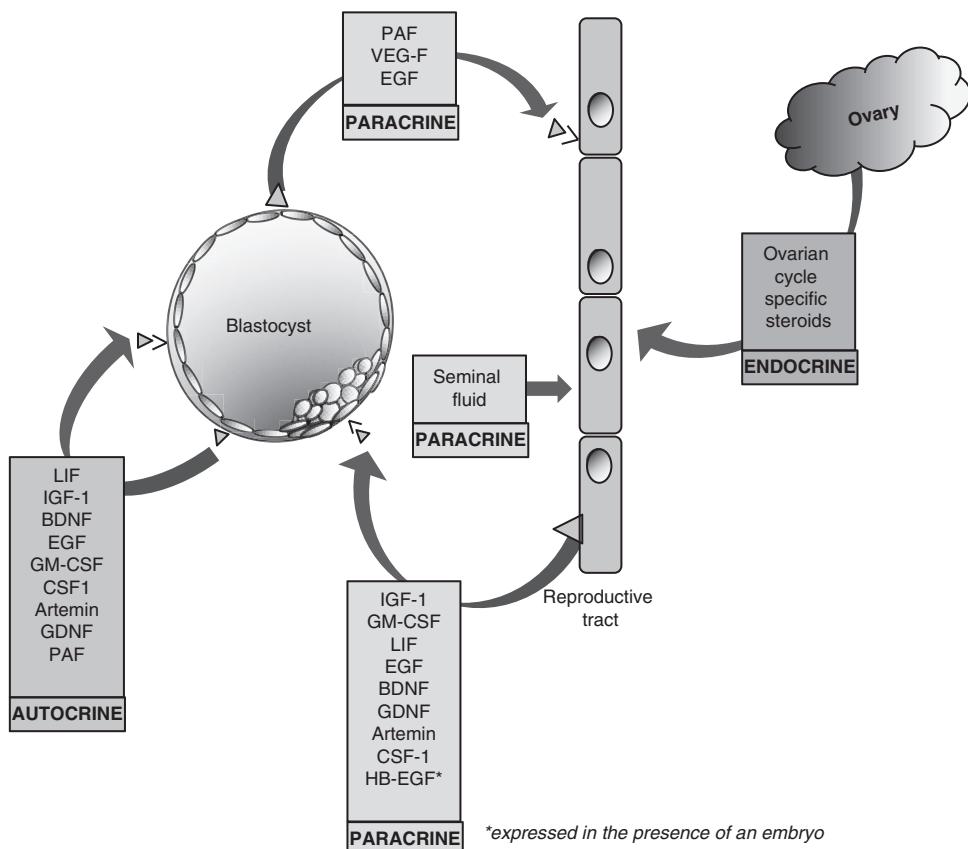


Figure 21.3 Autocrine, paracrine and endocrine factors regulating embryo development. (adapted with permission from Hardy & Spanos) [1]

leukemia inhibitory factor (LIF), TGF β and IL-6, which potentially have roles in autocrine pathways or providing signals to maternal tissues (Figure 21.3) [1, 2].

However, while a magnitude of cytokines and growth factors have been shown to have a proliferative effect on embryos in varying species, only a handful have been proven to have an effect on human embryo development and even fewer have been taken into clinical use.

21.3.1.1 Insulin, IGF-1, IGF-2 and GH

Insulin, the insulin-like growth factors 1 and 2 (IGF-1/IGF-2) together with growth hormone (GH) belong to a subfamily of the insulin/IGF/relaxin family. They are peptide hormones with high homology and share binding affinity to the same receptors. Insulin mediates signalling through binding to the homodimeric insulin receptor, while the IGFs mediate signalling by binding to the IGF-1 receptor, insulin and IGF-2 receptor [9]. IGF-1 is a mediator of the anabolic and mitogenic effect of GH. In plasma, the majority of IGFs form a complex with a family of binding proteins (IGFBPs), which

modulate the availability of free IGF-1 to tissues. While the liver secretes most IGF-1, many other tissues are known to secrete the peptide, with the secretory site a key determinant in its specific action [10].

GH is known to play an important role in the reproductive organs and is implicated in ovarian response, oocyte activation and maturation. GH has also been shown to improve treatment outcome in women with poor ovarian response. Addition of GH to IVM medium and embryo culture medium has been linked to improved embryo and blastocyst development, and a number of species express both the functional hormone and its receptor. The action of GH is thought to be through modulation of the metabolic profile, with embryos cultured in the presence of GH having a metabolic profile similar to embryos developed in vivo. There are no studies to date where GH has been added to human embryo culture medium [11].

Insulin and IGF-1 are secreted in the reproductive tract and are known to stimulate embryo growth and development in a number of species, and their respective receptors are expressed in early embryos. Insulin at extremely low concentrations (pico-molar levels) stimulates growth through its own receptor, promoting blastulation and, in particular, proliferation of the ICM. While results from adding insulin to human embryo cultures are inconclusive, it has historically been added to a number of commercially available embryo culture media. However, the high concentration used in both research and clinical medium (micro–nano-molar) could suggest that the effect of insulin is blocked through saturation of the receptor [9, 12].

The human embryo expresses all three receptors with binding affinity to IGF-1, namely the insulin receptor, IGF1R and IGF2R. Oviduct and uterine flushings from women both contain IGF-1 and the expression of the IGF-1 gene peaks in the secretory phase coinciding with fertilization, providing evidence that the factor is present during early embryo development. Supplementation of IGF-1 to media is known to benefit human embryo development, increasing the blastulation rate from 35 percent in the control to 60 percent in the treatment group. The effect of IGF-1 also included an increase in blastocyst cell number, due entirely to an increase of cells in the ICM [13]. There is further evidence that IGF-1 can act as a survival factor protecting preimplantation human embryos from apoptosis, with blastocysts displaying a significantly reduced number of apoptotic cells [1]. Though evidence supports the beneficial use of IGF-1 in embryo culture, there have been no studies to date to further investigate its use with regard to safety and long-term effect on offspring. IGF-1 is therefore not included in any off-the-shelf culture medium.

21.3.1.2 LIF

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine of the IL-6 family, which plays a regulatory role in a number of different cell systems. LIF action is mediated by binding to the LIF receptor comprising of a low-affinity LIF-binding chain and a high-affinity converter subunit, gp130 [14].

In mice, LIF is expressed throughout the reproductive tract and its secretion in the uterus is driven by factors in seminal plasma. It is suggested that LIF is involved in immunotolerance at the maternal–fetal interface, playing a crucial role during implantation. This is evidenced by transgenic mice, homogenous for LIF deficiency (LIF^{-/-}), which exhibit normal ovulation patterns, fertilization and embryo development; however, their blastocysts fail to implant. Implantation was restored when LIF was added exogenously to the mice and blastocysts from LIF^{-/-} mice also exhibit normal implantation when

transferred to wild-type mice. In humans, studies indicate that LIF is expressed in endometrial epithelial cells in a cycle-dependent manner reaching maximum levels in the secretory phase, with secretion being stimulated by human chorionic gonadotropin (hCG), TGF β and seminal fluid. Human blastocysts do express the LIF receptor, suggesting a role for LIF in the embryo–endometrial interaction and possibly in embryo development [2]. There is evidence that impaired LIF expression in women could be a cause of implantation failure.

Two parallel studies have provided conflicting evidence as to the benefit of adding LIF to human embryo culture medium. One study reported that supplementation of LIF to a complex serum-free human embryo culture medium increased the blastulation rate from 18 to 44 percent, with the effect being limited to blastocyst formation and no effect beyond blastulation being observed [14]. However, a separate study published at the same time failed to support these findings and reported no significant effect of LIF on embryo development over a range of different concentrations [15]. The opposing findings are likely due to the difference in experimental design, with the study failing to prove an effect having serum supplementation in both the test and control groups. Considering that serum is rich in LIF and would have exerted an effect on all test groups, the study where LIF was found to have an effect was done in a serum-free medium.

In a systemic approach, where women with recurrent unexplained implantation failure had twice daily subcutaneous injections of recombinant LIF for seven days after embryo transfer, no improvement in clinical pregnancy or live birth was seen. However, considering the role of LIF in implantation and embryo–endometrial interaction, it could be hypothesized that LIF could have an effect on implantation if added to the embryo transfer medium [16].

21.3.1.3 HB-EGF

Heparin-binding epidermal growth factor (HB-EGF) is a mitogenic cytokine of the EGF family and can be found in both an insoluble membrane-bound form and a soluble paracrine form. Actions are mediated through binding to the EGF receptor, which belongs to the tyrosine kinase superfamily of receptors. Both forms of HB-EGF and its receptor are present in the embryo and reproductive tract of a number of species and the membrane-bound form is known to play a role in blastocyst cell adhesion to the endometrium. In mice, HB-EGF is expressed in the uterine luminal epithelium in the presence of a blastocyst close to the time of implantation; however, it is not found in the pseudo-pregnant epithelium, suggesting that embryo–epithelial interaction is necessary for its expression. In women, the expression and secretion of HB-EGF changes throughout the menstrual cycle, with low levels in the proliferative phase, increasing over the secretory phase and coming to its peak at the time of implantation, being present at the apical surface of the luminal epithelium. The human embryo expresses both ligand and receptor for HB-EGF [17].

Culture of human embryos in the presence of HB-EGF has been shown to improve the proportion of embryos developing from day 2 through to the blastocyst stage, with blastulation rates increasing from 41 percent in the control group to 71 percent in the presence of HB-EGF. The resulting blastocysts from the HB-EGF supplemented cultures also exhibited a higher morphological score. The cleavage rate was not affected but the developmental competence, as assessed by hatching, showed a doubling in the number of embryos reaching the hatching stage as compared to control. Notably, the effect on blastulation and hatching was only exerted in good and fair quality embryos. Considering the hypothesis that HB-EGF

plays a role in implantation, blastocysts were cultured until day 14 on a Matrigel protein substrate surface with adherence and trophectoderm outgrowth being significantly improved in the presence of HB-EGF. While supplementing human embryo culture medium with HB-EGF resulted in blastocysts of high quality, the cell numbers and metabolic patterns, as measured by glucose and pyruvate uptake, were not significantly affected. Though convincing evidence supports the beneficial use of HB-EGF in embryo culture, there have been no studies to date to further investigate its use with regard to safety and long-term effect on offspring. HB-EGF is therefore not included in any off-the-shelf culture medium [18].

21.3.1.4 PAF

Platelet activating factor (PAF) is a phospholipid activator that plays a crucial role in platelet aggregation. Autocrine secretion of PAF has been confirmed in a number of species, including human, suggesting a role in preimplantation embryo development. An early clinical trial supplementing IVF medium with PAF from the time of oocyte insemination to transfer on day 2 showed no effect on embryo developmental parameters. Following embryo transfer, there was a significant increase in pregnancies with 17.5 percent in the PAF group compared to 10.3 percent in the control. While these numbers are low in today's perspective, the control group results were well on par with the national reported data at that time. Considering this initial study seemed promising, there have been no further studies investigating the effect of PAF on human embryo development and it is not included in any off-the-shelf culture medium [19].

21.3.1.5 GM-CSF

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a multifunctional cytokine originally identified as a product of activated T-lymphocytes. It stimulates proliferation, differentiation and activation of myeloid haematopoietic and vascular endothelial cells. GM-CSF is present in the reproductive tract and oestrogen-primed epithelial cells from both oviduct and uterus produce the cytokine. In mice, seminal fluid interacts with luminal epithelial cells in the uterus creating a surge in GM-CSF synthesis and release into the luminal compartment, coinciding with the preimplantation period. At the time of implantation, expression of GM-CSF is declining as a result of progesterone exerting an inhibitory effect; however, bioactive GM-CSF can be detected in placental and decidual tissues for the duration of pregnancy [2]. The role of GM-CSF as a regulator of embryo growth and development is supported by the cycle-dependent secretion pattern of GM-CSF seen throughout the tract. Expression and secretion of the cytokine in the oviduct peaks from the mid-follicular phase to the early to mid-luteal phase, and while being secreted by endometrial cells in the uterus, throughout the cycle there is a moderate increase in synthesis around the time of implantation. GM-CSF is also thought to play a key role in implantation, with ovine embryos exposed to GM-CSF having greater implantation potential, possibly through the increased trophectoderm expression of interferon-tau (IFN- τ) implicated with maternal recognition of pregnancy. The GM-CSF receptor is a heterodimer comprising of an α - and a β - subunit shared with IL-3 and IL-5. The ligand binds with low affinity to the α -chain followed by dimerization with the β -chain resulting in signal transduction. Mouse embryos are known to express the α - but not the β -chain of GM-CSF receptor.

Culture of embryos from a number of species in the presence of GM-CSF has been shown to improve embryo viability, growth, blastulation, hatching and implantation confirming a physiological role for GM-CSF as a regulator of preimplantation embryo development. GM-CSF is known to suppress apoptosis in haematopoietic precursor cells suggesting its role as a survival factor in embryos [20]. There is further evidence that GM-CSF plays a role in regulation of metabolism, with mouse embryos cultured in the presence of GM-CSF showing an increase in glucose uptake [2].

The human embryo expresses RNA and protein of GM-CSF receptor α -chain but not the β -chain, and expression of the α -subunit has been detected from the first cleavage through to the blastocyst stage [20]. Several studies supplementing GM-CSF to human embryo cultures have shown a more than doubling of the proportion of embryos developing to blastocysts, from 30 to 76 percent. The addition of GM-CSF also resulted in a morphokinetic improvement with the average blastocyst forming 14 hours earlier than those in the control group. These embryos were also more likely to hatch and exhibit cell outgrowth after attaching to protein-coated dishes [3]. The blastocysts cultured in the presence of GM-CSF contained 35 percent more cells, primarily due to an increase in the size of the ICM. Moreover, these blastocysts contained 50 percent fewer apoptotic nuclei and 30 percent more viable cells of the ICM. Neutralizing antibodies reactive with the GM-CSF receptor α -chain, but not antibodies to β -chain nor the β -chain-dependent antagonist E21 R, ablated the development-promoting effect of GM-CSF, confirming that GM-CSF acts on human embryos independent of the receptor β -chain [20].

Mouse embryos cultured in the presence or absence of GM-CSF were transferred to pseudo-pregnant females to assess the safety of GM-CSF exposure during the preimplantation period in regard to fetal development and offspring health. Embryos cultured in the presence of GM-CSF had a significantly higher implantation potential, subsequent fetal development and survival with a significant decrease in fetal resorption. While in vitro culture per se was associated with a significant reduction in a number of fetal and progeny health markers, this negative effect was alleviated by addition of GM-CSF, giving rise to fetuses that were similar in weight to those resulting from embryos developed *in vivo*. Furthermore, in vitro culture progeny exhibited an altered growth trajectory, including increased total body weight over time and relative fat mass in adulthood. Supplementation of GM-CSF to the in vitro cultures normalized these parameters [21].

Further safety studies included assessing the effect of GM-CSF supplementation on embryo ploidy confirming no increase in aneuploidies, rebuking fears that GM-CSF could rescue chromosomally abnormal embryos. A large clinical randomized controlled trial (RCT) using GM-CSF in a day 3 single embryo transfer setting found a significant difference in implantation rate after 12-weeks gestation, with the most profound effect of GM-CSF supplementation evident in the subgroup of patients experiencing recurrent miscarriage [2]. Apart from insulin, GM-CSF remains the only cytokine available in a commercial off-the-shelf medium (EmbryoGen, Origio, Denmark).

21.3.1.6 A Cocktail Approach to Cytokine/Growth Factor Supplementation

The presence of a large repertoire of different cytokines and growth factors in the reproductive tract during the time of preimplantation embryo development and implantation indicates that many different growth regulators may act in concert to orchestrate optimal embryo development. While a number of the cytokines and growth factors described to have an effect on human embryo development exert this effect through the same or similar

signalling pathways, it is clear that any future culture medium aiming to closely mimic the female reproductive tract would need to include a cocktail of different factors and supplements. This hypothesis has been tested in a large study supplementing human embryo cultures with seven autocrine/paracrine growth factors, EGF, IGF-1, GM-CSF, brain-derived neurotrophic factor (BDNF), CSF-1, artemin and glial cell-derived neurotrophic factor (GDNF) [17]. Receptors for all seven factors were confirmed in human embryos using RT-PCR and the ligand found to be paired with its receptor using immunofluorescent staining. Moreover, the expression of all seven factors was confirmed in the woman's reproductive tract using both RT-PCR and immunofluorescent staining. Supplementing human embryo culture medium from day 3 with the seven-factor cocktail resulted in a significant threefold increase in blastulation rate. Moreover, the blastocysts formed were of higher quality with a sevenfold increase in grade A blastocysts [17]. While the initial aim of the study was to refine culture conditions with a goal to improve the development of embryos after somatic cell nuclear transfer, it holds promise of remarkable future improvements in IVF embryo culture.

21.3.2 Macromolecule Supplements

Macromolecules play a role in supporting the embryo *in vivo* and historically sera from varying sources have been added to IVF medium to improve culture outcomes. Considering that serum contains a high concentration of growth factors and cytokines together with macromolecules such as albumin, the cultures could be seen as being an early form of the cocktail approach discussed earlier. However, the use of serum often resulted in inconsistencies in results; autologous patient serum was considered a safe option, but embryo development could be negatively affected by patient aetiology and general health. Human fetal cord serum proved a potent addition, but was not readily available for all clinics and safety was an obvious issue. Today, serum is not widely used in IVF and not considered safe or best practice [22].

With results more consistent than serum, modern embryo culture medium is supplemented with HSA, a 66.5 KDa macromolecule present in blood with a number of high affinity binding sites acting as a scavenger and a carrier of other molecules. In addition to being a scavenger and carrier of possible growth promoting molecules, the role of HSA in cultures includes acting as a nutrient, membrane stabilizer and pH buffer. It has been suggested that its scavenger properties can protect the embryo from toxic substances. There are also a number of serum replacement products used as an alternative to HSA in IVF medium; however, the manufacturers do not release the compositions of these products. A recent routine batch testing aiming to identify proteins in HSA and serum replacement preparations revealed that both HSA and replacement products contain physiological levels of a number of protein- and steroid hormones together with growth factors. The testing revealed differences between products and also between lots of the same product. It is possible that these differences contribute to lot variations seen in IVF culture medium [22]. A separate study found that preparations of HSA had the fewest unidentified components, while some of the replacement products had high concentrations of amino acids. It can be argued that the safest approach would be to use recombinant HSA together with other macromolecules such as hyaluronan. Most replacement products contain α - and β -globulins with the concentration varying between preparations and lots [23]. This is concerning considering that the safety of using these globulins has not been investigated.

Reflecting on these findings, where supplements with a somewhat random uncontrolled content of potent signalling molecules are being routinely used, in comparison to the careful approach of adding specific cytokines and growth factors, each IVF laboratory must assure that they are fully aware of the risks and consequences of any supplements used.

21.4 Medium Supplementation for Embryo Transfer

The use of adherence compounds such as hyaluronan and fibrin sealant in embryo transfer media has been employed to increase implantation rates following ART. Most studies have focused on the addition of hyaluronan, as the results from addition of fibrin sealant have suggested this supplement to have no effect on transfer outcome [24].

Hyaluronan is a glycosaminoglycan synthesized in the body; it is especially abundant along the female reproductive tract, where it is understood to provide a highly viscous environment. Production in the uterus increases significantly prior to implantation, and this has led to the idea of supplementing embryo transfer medium with this macromolecule, in an effort to increase the likelihood of the embryo implanting. Studies in humans have shown that an embryo transferred in medium containing functional concentrations of hyaluronan (e.g. 0.5mg/ml) has an 8.3 percent higher chance of pregnancy and live birth when compared to embryos transferred in the presence of very low or no hyaluronan [24]. This is encouraging data; however, meta-analysis of the same set of RCTs also suggested that the use of hyaluronan supplementation in transfer medium increases the risk of multiple birth by almost 11 percent [24]. It was not possible to discern from the data whether the increase in multiple implantation sites is as a result of the hyaluronan substitution or a result of multiple embryos being transferred.

Although the supplementation with hyaluronan improves embryo transfer outcome, the precise mechanism behind how it does this is currently unknown. Possible explanation of its effect comes from the fact that its addition to transfer medium increases viscosity and promotes cell-to-cell adhesion, thus aiding the initial apposition of the embryo to the lining of the uterus. Additionally, the increase in viscosity of transfer medium makes it more akin to endometrial fluid and may aid in the diffusion and mixing of the two liquids at transfer, ultimately dispersing the embryo onto the endometrial wall. Furthermore, the breakdown of hyaluronan results in increased angiogenesis in the uterus, which is a vital step in successful implantation. More recent work has indicated that addition of hyaluronan to transfer medium increased expression of MMP9 (matrix metallopeptidase-9) that transcribes an enzyme MYD88 (myeloid differentiation-88) involved in extracellular matrix degradation. The downstream effects of this enzyme encourage release of inflammatory cytokines, which help to create the prime microenvironment in the uterus to support implantation [25].

21.5 Summary and Conclusion

In the future embryo culture will most likely be supplemented with a number of molecules currently not included. An abundance of these have been thoroughly investigated in the animal model, many are yet to be discovered, some have been tested in a human model and very few in clinical trials. While results of these studies are encouraging, legal, financial and corporate strategic factors are likely to be the largest hurdles in moving IVF medium supplements from the research laboratory into the clinical embryology laboratory (Figure 21.4). It must be acknowledged that if we want to see a future with highly efficient, safe IVF culture medium

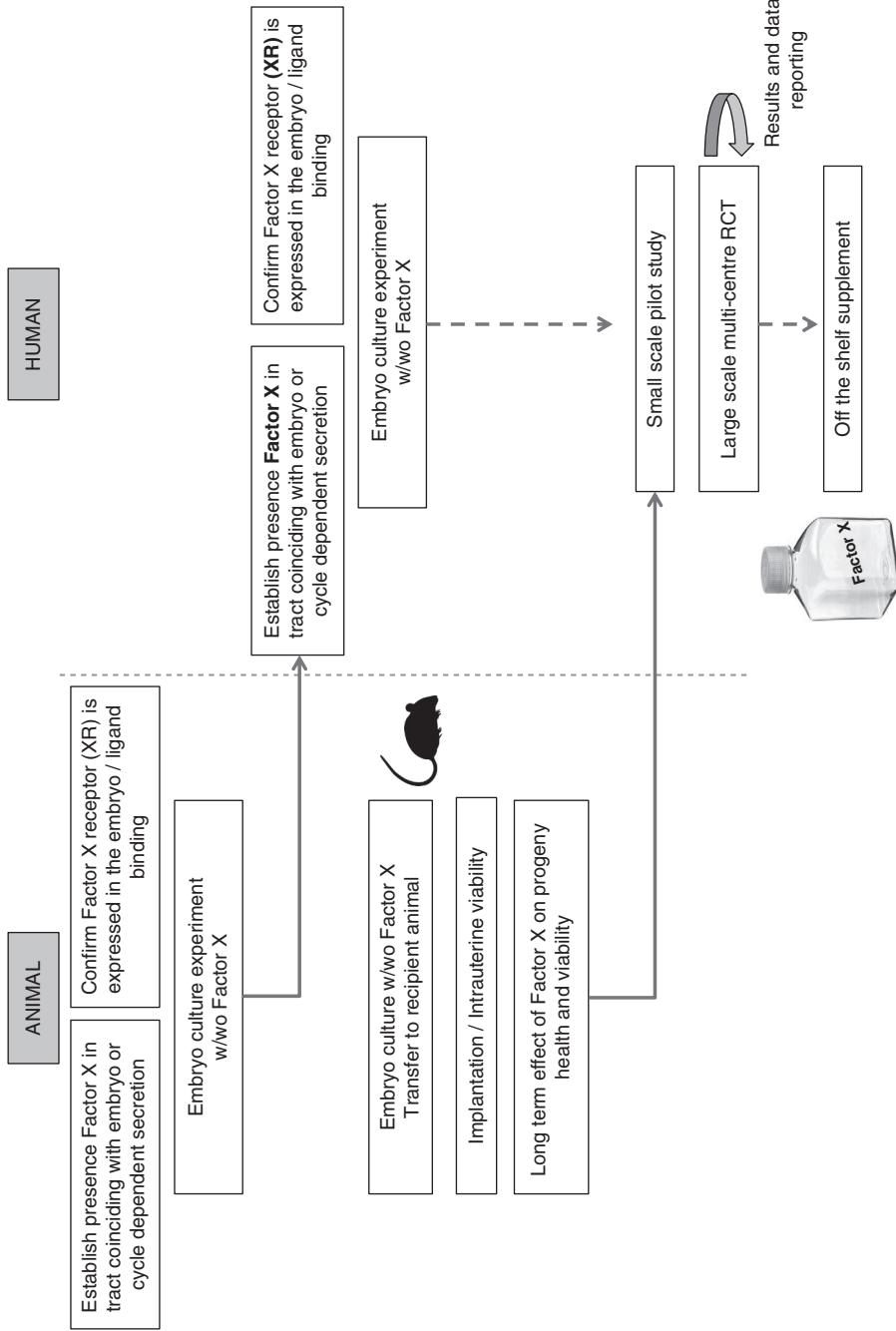


Figure 21.4 Validation process for introducing a new culture medium supplement, Factor X to IVF laboratories using both animal and human models. Full testing of novel media components is essential to ensure their safety, as well as efficacy, prior to use in embryology laboratories

then we also have to be prepared to pay a higher price for these products. The safety of any new supplements needs to be at the forefront of the agenda, taking into consideration the long-term health consequences of children born from IVF.

21.6 Conflict of Interest Statement

Cecilia Sjöblom is an inventor related to the use of GM-CSF in IVF culture medium; however, she receives no compensation (financial or other) in relation to this.

References

1. Hardy K, Spanos S. Growth factor expression and function in the human and mouse preimplantation embryo. *J Endocrinol.* 2002; **172**: 221–36.
2. Robertson SA, Chin PY, Schjenken JE, Thompson JG. Female tract cytokines and developmental programming in embryos. *Adv Exp Med Biol.* 2015; **843**: 173–213.
3. Sjöblom C, Wiklund M, Robertson SA. Granulocyte-macrophage colony-stimulating factor promotes human blastocyst development in vitro. *Hum Reprod.* 1999; **14**: 3069–76.
4. Sutton M, Gilchrist R, Thompson J. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Hum Reprod Update* 2003; **9**: 35–48.
5. Botigelli, RC, Razza EM, Pioltine EM, Nogueira MFG. New approaches regarding the in vitro maturation of oocytes: manipulating cyclic nucleotides and their partners in crime. *JBRA Assist Reprod.* 2017; **21**: 35–44.
6. Cavilla JL, Kennedy CR, Byskov AG, Hartshorne GM. Human immature oocytes grow during culture for IVM. *Hum Reprod.* 2008; **23**: 37–45.
7. Gilchrist RB, Thompson JG. Oocyte maturation: emerging concepts and technologies to improve developmental potential in vitro. *Theriogenology.* 2007; **1**: 6–15.
8. Mottershead DG, Sugimura S, Al-Musawi SL et al. Cumulin, an oocyte-secreted heterodimer of the transforming growth factor- β family, is a potent activator of granulosa cells and improves oocyte quality. *J Biol Chem.* 2015; **290**: 24007–20.
9. Kaye PL. Preimplantation growth factor physiology. *Rev Reprod.* 1997; **2**: 121–27.
10. Harvey MB, Kaye PL. Mediation of the actions of insulin and insulin-like growth factor-1 on preimplantation mouse embryos in vitro. *Mol Reprod Dev.* 1992; **33**: 270–75.
11. Hull KL, Harvey S. Growth hormone and reproduction: A review of endocrine and autocrine/paracrine interactions. *Int J Endocrinol.* 2014; **2014**: 1–24.
12. Kaye PL, Gardner HG. Preimplantation access to maternal insulin and albumin increases fetal growth rate in mice. *Hum Reprod.* 1999; **14**: 3052–59.
13. Lighten AD, Moore GE, Winston RM, Hardy K. Routine addition of human insulin-like growth factor-I ligand could benefit clinical in-vitro fertilization culture. *Hum Reprod.* 1998; **13**: 3144–50.
14. Dunglison GF, Barlow DH, Sargent IL. Leukaemia inhibitory factor significantly enhances the blastocyst formation rates of human embryos cultured in serum-free medium. *Hum Reprod.* 1996; **11**: 191–96.
15. Jurisicova A, Ben-Chetrit A, Varmuza SL, Casper RF. Recombinant human leukemia inhibitory factor does not enhance in vitro human blastocyst formation. *Fertil Steril.* 1995; **64**: 999–1002.
16. Brinsden PR, Alam V, de Moustier B, Engrand P. Recombinant human leukemia inhibitory factor does not improve implantation and pregnancy outcomes after assisted reproductive techniques in women with recurrent unexplained implantation failure. *Fertil Steril.* 2009; **1**: 1445–47.

17. Kawamura K, Chen Y, Shu Y et al. Promotion of human early embryonic development and blastocyst outgrowth in vitro using autocrine/paracrine growth factors. *PLoS ONE*. 2012; 7: e49328.
18. Martin KL, Barlow DH, Sargent IL. Heparin-binding epidermal growth factor significantly improves human blastocyst development and hatching in serum-free medium. *Hum Reprod*. 1998;13: 1645–52.
19. O'Neill C, Ryan JP, Collier M et al. Supplementation of in-vitro fertilisation culture medium with platelet activating factor. *Lancet*. 1989; 2; 769–72.
20. Sjoblom C, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor (GM-CSF) acts independently of the beta common subunit of the GM-CSF receptor to prevent inner cell mass apoptosis in human embryos. *Biol Reprod*. 2002; 67: 1817–23.
21. Sjoblom C, Roberts CT, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor alleviates adverse consequences of embryo culture on fetal growth trajectory and placental morphogenesis. *Endocrinology*. 2005; 146: 2142–53.
22. Meintjes M. Media composition: Macromolecules and embryo growth. In Smith GD, Swain JE, Pool TB (eds.) *Embryo Culture: Methods and Protocols, Methods in Molecular Biology*. 2012. Springer Science + Business Media, pp. 107–28.
23. Morbeck DE, Paczkowski M, Fredrickson JR et al. Composition of protein supplements used for human embryo culture. *J Assist Genet*. 2014; 31: 1703–11.
24. Bontekoe S, Johnson N, Blake D, Marjoribanks J. Adherence compounds in embryo transfer media for assisted reproductive technologies: summary of a Cochrane review. *Fertil Steril*. 2015; 6: 1416–17.
25. Buck C, Ruane P, Smith H, Brison D. Effects of hyaluronan-rich culture media on human embryo attachment and gene expression. *Proceedings of Fertility 2017*, Edinburgh, UK. 2017 P-065: 103.

Assisted Hatching – Fresh and Frozen

Katalin Kanyo, Sandor Cseh, and Janos Konc

22.1 Introduction

It is estimated that one out of six couples experience some delay in conception and an increasing number requires assisted reproductive treatment. It has been known for a long time that with increasing age, a woman's fertility decreases. But nowadays, the typical is that more and more women choose to delay starting a family year by year in developed countries for various social reasons. As more couples delay the commencement of childrearing, the age of those seeking infertility treatment increases which is a big disadvantage from the point of successful application of assisted reproductive techniques, since the age of the female partner is an important factor in the outcome of reproductive treatments.

The implantation rate of embryos resulting from in vitro fertilization cycles is estimated to be less than 20 percent and consequently up to 85 percent of transferred embryos do not implant. Among the reasons of the low implantation rate the most frequently mentioned factors are poorer oocyte and embryo quality, disturbed early embryo development, difficulties in hatching, decreased receptivity of the uterus, and embryo loss, sometimes resulting from chromosomal abnormalities. Studies report significantly reduced chances of pregnancy after the age of 37–40 years, and found that women >42 years of age had a <7 percent chance of pregnancy and there are very rarely observed viable pregnancies after 45 years of age.

22.2 Early Embryo Development and Blastocyst Formation

After fertilization, during the early cleavage stage of embryo development, when the individual blastomeres can no longer be counted accurately, the early embryo is called a morula. During the morula stage, blastomeres start to separate into two distinct populations, the inner and outer blastomeres. Blastomeres in the inner portion of the morula (embryoblastic cells) develop gap junctions while the outer blastomeres (trophoblastic cells) develop cell-to-cell adhesions known as tight junctions. After these events, fluid begins to accumulate inside the embryo. This fluid accumulation is believed to be brought about by an active sodium pump in the outer blastomeres of the morula that pumps sodium ions into the center portion of the morula. Therefore, the ionic concentration of the fluid, surrounding the inner cells, increases and induces water inflow through the zona pellucida into the embryo which begins to form the fluid-filled cavity called the blastocoel. When this distinct cavity is recognizable, the embryo is called a blastocyst.

22.3 Zona Pellucida Structure and Function

The zona pellucida is a 13–15 μm thick envelope (acellular matrix) which is composed of glycoproteins, carbohydrates, and zona pellucida-specific proteins. It has two layers

(bilayered), the outer is thick and the inner is thin but resilient. It is involved in sperm binding, because it contains a species-dependent sperm receptor. Furthermore, the zona pellucida triggers the acrosome reaction which is believed to support the penetration of the spermatozoa, through the zona pellucida into the oocyte. The successful penetration promotes the fusion of the egg and spermatozoa followed by the decondensation of the sperm head and the formation of the male pronucleus, which is the very first sign of fertilization. After fertilization, the zona pellucida undergoes biochemical modifications called zona hardening that prevents polyspermy. The zona pellucida loses its function and ruptures at the blastocyst stage in a process called hatching, a very important step of the pregnancy formation, since this event allows embryo implantation.

22.4 Hatching Process

As the blastocyst continues to grow, the blastomeres undergo mitosis and fluid continues to fill the blastocoel (cavity); therefore, the pressure within the embryo continuously increases. The trophoblastic cells start to produce proteolytic enzymes (lysins, proteases) that start to weaken the zona pellucida. Finally, the blastocyst itself begins to contract and relax inducing intermittent pressure pulses in the embryo, which together with the continuous growth and enzymatic degradation causes the zona pellucida to rupture. After that, the embryo squeezes out of the opening and escapes from the zona pellucida. From now on the blastocyst becomes completely dependent on the uterine environment for survival.

In natural conditions the hatching of the blastocyst is governed by the following factors: mechanical expansion and contraction/elasticity of the blastocyst, increasing blastomere number and pressure in the embryo, and thinning of the zona pellucida by enzymes produced by the trophoblastic cells, which are all involved in hatching and are essential for this process [1].

Hatching of the blastocyst is a critical step in the sequence of physiologic events culminating in implantation. A defect in the hatching process is considered a possible cause of implantation failure both in natural circumstances and in assisted reproduction. Four steps must be achieved before the embryo can attach/implant to the uterus/endometrium. They are development within the confines of the zona pellucida, hatching of the blastocyst from the zona pellucida, maternal recognition of the pregnancy, and formation of the extraembryonic membranes.

22.5 The Motivation behind the Clinical Use of Assisted Hatching

Both elasticity and thinning of the zona pellucida can be influenced adversely by advancing maternal age and in vitro culture conditions [1,2]. Data indicate that zona hardening may be initiated by cryopreservation as well [3,4]. Abnormalities in the hatching have been suggested as a possible explanation for the low implantation rates observed in some patients in advanced maternal age, poor prognosis and frozen–thawed in vitro fertilization and embryo transfer cycles (Cohen, 2007).

22.6 The Mechanism of Assisted Hatching

Assisted hatching procedures are based on creating an artificial opening or thinning of the zona pellucida, which is believed to support hatching and implantation of embryos that are

otherwise unable to escape from the zona pellucida. Assisted hatching involves the artificial thinning or breaching of the zona pellucida and has been proposed as one technique to improve implantation and pregnancy rates following in vitro fertilization. Yet, the effectiveness of assisted hatching to improve the outcome of fresh and frozen in vitro fertilization and embryo transfer cycles remains controversial. The assisted hatching procedure – in fresh and frozen cycles – is generally performed prior to embryo transfer on day 2, 3, 4, and rarely 5 or 6 after fertilization using various methods (see below).

The assisted hatching procedure may be associated with specific complications independent of the in vitro fertilization procedure itself, including lethal damage to the embryo or individual blastomeres, reducing the viability of the embryo planned to be transferred. The majority of assisted reproductive techniques are related to multiple embryos being transferred, and are dizygotic. Assisted hatching has been associated with an increased risk of monozygotic twin pregnancies. However, there are other studies with different observations indicating that there is no connection between assisted hatching and monozygotic twin pregnancies. However, the conclusion of the Practice Committee of the Society for Assisted Reproductive Technology and the Practice Committee of the American Society for Reproductive Medicine is that there is good evidence that assisted hatching is associated with multiple pregnancies, but there is insufficient evidence that it is associated with an increased risk of monozygotic twinning. The overall rate of monozygotic twin pregnancies in in vitro fertilization with assisted hatching is less than 1.0 percent [5,6].

22.7 Techniques of Assisted Hatching

The first technique that was used to assist the hatching process was a mechanical method [7]. Since then several approaches have been proposed, including the mechanical incision of the zona pellucida, chemical zona drilling and thinning with acidic medium, laser-assisted hatching, and more recently the piezo technology [2,7,8,9,10]. The mechanical and the chemical methods require technical skill to produce uniform, standardized micro holes using micropipettes mounted on micromanipulators. It is very important that the procedure be quick in order to minimize the time the embryo is not in the incubator and to prevent/reduce the variation in the temperature and pH that can be detrimental to embryo development. Micromanipulation is done on day 2, or later, on embryos after compaction has started and the adherence between the blastomeres has increased.

22.8 Mechanically Opening and Expanding the Zona Pellucida

Cohen (1989) was the first to describe the technique of assisted hatching using partial zona dissection to create an opening in the zona pellucida of the embryos being in the early cleavage stages [7]. During the procedure, the embryo is stabilized by a holding pipette and the zona pellucida is cut with a microneedle forming a lack of continuation in the zona pellucida. Finally, the embryo is released from the holding pipette. The partial zona dissection is a quick procedure, but the problem is that it produces openings with variable sizes that may not always be optimal.

The technique of artificial mechanical expansion of the zona pellucida tries to apply the effect of the natural expansion on the zona pellucida during the process of hatching. No thinning and no breaching of the zona pellucida are induced by this mechanical method.

Its rationale is based on that it expands or stretches the zona pellucida by injected hydrostatic pressure and through this effect assists the hatching process of the embryo. The observations of Cong et al. [11] showed that mechanically expanding the zona pellucida of cryopreserved day 3 embryos with injected hydrostatic pressure after thawing increases the implantation rate compared with control embryos [11]. They concluded that mechanical expansion of the zona pellucida is a potentially safe and effective method to improve the outcome of in vitro fertilization and embryo transfer cycles.

22.9 Chemical Techniques

Assisted hatching with acid solution (e.g. Tyrode solution) has been introduced by Cohen [8]. The embryo is stabilized by a holding pipette and a microneedle is directed to an area of the zona pellucida overlying the blastomere-free area (e.g. empty perivitelline space or where only extracellular fragments are located). From the microneedle the acidic solution is injected gently over a small area of the zona pellucida until it is breached. It is very important that suction must be applied immediately after breaching the zona pellucida in order to prevent excess acid entering the perivitelline space, because the acid is detrimental to the blastomeres adjacent to the drilled part of the zona pellucida. Therefore, after the zona dissolves on contact with the acidic solution, the embryo must be removed immediately and rinsed several times in normal solution (acid-free) to remove any trace of the acid.

22.10 Piezo Technology

The piezo technology has been introduced for zona pellucida drilling by Nakayama and colleagues [10]. Vibratory motion produced by a piezo-electric pulse is used to create a limited conical area in the zona pellucida while the embryo is stabilized by a holding pipette. Five to eight applications in adjacent areas may be used to produce a large-enough hole, which facilitates the complete hatching of the blastocyst.

22.11 Laser-Assisted Hatching

The successful use of noncontact microdrilling of mouse zona pellucida was first reported in 1996 [9]. The laser beam may either be directed using an optical lens tangential to the embryo through the zona pellucida in a noncontact mode, or the laser may be guided through an optical fiber touching the embryo in a contact mode. Nowadays, the technical advantages of the noncontact mode and the potential harmful mutagenic effects of the UV radiation have led to the general preference for the noncontact mode using the infrared 1.48 μm (1.480 nm) diode laser. First the laser was used to prepare a single full thickness hole through the zona pellucida. Later it turned out that thinning the zona pellucida is enough and it is not necessary to create a hole. Blake and colleagues demonstrated that significant increase in hatching in vitro could be achieved with laser-assisted zona thinning compared to preparation of a complete hole [12]. Their data indicated that higher clinical pregnancy rate was found when the laser was used to thin the zona on an extended area compared with producing a single full thickness hole. No ultrastructural degenerative alterations of the zona pellucida of oocytes and embryos, following laser-assisted zona drilling, have been

reported. Data of follow-up studies in our institute indicate and reassure that laser-assisted hatching has no harmful effects on the health status of children born from embryos treated with laser-assisted hatching procedures [13]. The final conclusion of the different studies is that the laser-assisted hatching is easy to perform and is a very precise procedure with high repeatability, and it does not have any negative impact on in vitro embryo development.

22.12 Clinical Application of Assisted Hatching

There is a sharp contradiction in the results observed in the different studies dealing with assisted hatching in fresh and frozen-thawed embryo transfer cycles. Because of the discrepancy in the outcomes between the studies, the results should be interpreted with caution. The variations in the assisted hatching techniques used, patients selected, and study designs make it very difficult to compare the results between studies and to pool data. Assisted hatching supports in vivo hatching of the blastocyst and therefore may improve pregnancy rates. Assisted hatching was reported to increase the outcome of in vitro fertilization – embryo transfer cycles in groups of patients with poor prognosis and/or poor embryonic morphology, with elevated FSH levels, with embryos having thicker zona pellucida, who were older and with a history of multiple in vitro fertilization failures. Thick zona pellucida may be associated with advanced female age, poor embryo scores, in vitro culture condition, and furthermore with cryopreservation [1,5, 6,14,15].

Repeated unexplained in vitro fertilization failure is associated with a reduced chance of pregnancy in subsequent treatment cycles.

On comparing the pregnancy rates of fresh in vitro fertilization cycles with poor prognosis patients (prior in vitro fertilization failures, two or more previous cycles) after using different techniques of assisted hatching, no difference was found in the efficacy of the methods, and all of them (laser, chemical, or microsurgical/mechanical) increased pregnancy rates [16]. Wellington and colleagues [17] found that assisted hatching was related to increased clinical pregnancy and multiple pregnancy rates in women with previous repeated failure of frozen-thawed embryos. However, assisted hatching is unlikely to increase clinical pregnancy rates when performed in fresh embryo transfer cycles and embryos are transferred to unselected or nonpoor prognosis women or to women with advanced age [17]. Conversely, data of Valojerdi and colleagues [18] revealed that laser-assisted hatching is of no benefit in patients with recurrent implantation failure (for >2 cycles). The pregnancy and implantation rates after laser-assisted hatching in women of all age groups with repeated failure for at least two cycles were lower than those in the control group, but these differences were not statistically significant [18]. The final conclusion of their study is that assisted hatching cannot be performed as a routine strategy in the in vitro fertilization laboratory and more research is needed to see more clearly the advantages of assisted hatching, and in which groups of patients.

It is well established that the success rate of in vitro fertilization decreases with advancing maternal age. One of the explanations is the spontaneous zona hardening due to age-related endocrine changes and/or the absence of lysins from surrounding tissues, which may act on embryos in vivo [1]. Immediately we have to add to this that other factors may be also involved in the fact that with increasing age women's fertility decreases. Besides zona hardening, other factors could be karyotypically abnormal embryos or decline in uterine receptivity. This is the reason why – although assisted hatching may benefit older women – it is unlikely to restore their fertility rate (pregnancy) to that of younger women. Maybe this

is the explanation why Baruffi in a prospective randomized study found no difference in the pregnancy rates in a population aged <37 years, and with no previous implantation failure in assisted hatching fresh cycles [19]. Moreover, Frydman and colleagues observed that laser-assisted hatching did not improve the outcome of fresh *in vitro* fertilization–embryo transfer cycles in women aged >37 years [20]. Similarly, Valojerdi found no statistically significant difference in the pregnancy and implantation rates of older (>37 years of age) versus younger (<37 years of age) patients after laser-assisted hatching [18]. In contrast, there are data indicating that assisted hatching dramatically increases the implantation and pregnancy rates in older women. Assisted hatching may be clinically useful in patients with poor prognosis (>2 failed cycles) and poor embryo quality, furthermore with advanced female age (>38 years of age) (Schoolcraft et al., 1995; Mansour et al., 2000; [18]).

It is well documented that cryopreservation induces alteration in the glycoprotein matrix of the zona pellucida leading to zona hardening similarly to *in vitro* culture and/or advanced female age. Assisted hatching may overcome this problem and increase implantation and pregnancy rates in frozen–thawed embryo transfer cycles. However, similarly to *in vitro* fertilization–embryo transfer fresh cycles, the results obtained after assisted hatching in frozen–thawed embryo transfer cycles are also very conflicting [15]. Assisted hatching with chemical zona drilling in frozen–thawed cycles has been shown to increase pregnancy rates in retrospective studies [3]. However, in other studies, using similar techniques, no advantage of the procedure could be detected in cryopreserved cycles [21,22]. In a randomized study Ng and colleagues [23] failed to show also any beneficial effect of laser-assisted hatching in the pregnancy rates of patients with advanced age following the transfer of cryopreserved embryos. They were able to detect only a trend toward increased pregnancy rates.

These observations are in contrast with ours, as we found in the frame of a clinical study focused on the use of a laser zona thinning method on day 3 cryopreserved embryos with the hypothesis that it may be a useful tool for improving pregnancy rates, especially in advanced female age in frozen–thawed *in vitro* fertilization and embryo transfer cycles [24]. In this study, the results of 413 cryopreserved embryo transfer cycles were evaluated. Out of the 413 embryo transfer cycles, in 203 laser-assisted hatching was applied on the embryos. In the control group, none of the transferred embryos were treated with laser. Comparing the laser + and laser – groups in the overall patient population, we could detect that laser-assisted hatching supports (33.3% vs. 27.4%, respectively, $P = 0.08$) the formation of clinical pregnancy. The statistical analyses, however, show only a trend but the results were not significantly different. When we compared the pregnancy rates of the laser + and laser – groups, based on the age of the patients, we found that laser-assisted hatching increased significantly the pregnancy rate in patients over the age of 37 (≤ 38) (18.3% vs. 11.3%, respectively, $P = 0.03$). In younger patients, under age of 37 we could see only a nonsignificant trend of increased pregnancy rate in the laser-assisted hatching + group (28.4% vs. 23.6%, respectively) [24]. In younger patients (<38 years), however, our data clearly show that patients' age had a positive supportive effect on clinical pregnancy establishment, and younger patients got pregnant more easily ($P = 0.003$) compared with older patients. Valojerdi et al. [18] found also that laser-assisted hatching improved the pregnancy rates in patients with frozen–thawed embryos but they observed no effect in patients with advanced female age [18]. Our results obtained in frozen–thawed cycles show that laser-assisted hatching increases pregnancy rates. The patient population was quite similar in the two groups, likewise the embryo characteristics, and the applied

cryopreservation and embryo culture procedures, thus precluding any bias that may have influenced our results. Similarly to our results, other studies also found that laser-assisted hatching significantly increased pregnancy rates in cryopreserved cycles with slowly frozen and vitrified embryos, compared with fresh cycles [25,26]. The possible explanation of the less successful application of laser-assisted hatching in frozen-thawed cycles could be that the size of the zona pellucida thinning may have an effect on the results. Zhang and colleagues compared the effect of the size of the zona pellucida thinning (40 um vs. 80 um) made by laser on the outcome of frozen-thawed embryo transfer cycles. They found that the size of the thinning by laser may influence the implantation and pregnancy rates (they obtained increased implantation and pregnancy when 80 um thinning was made) following frozen-thawed cleaved embryo transfer [26]. The data of Hiraoka and colleagues' study showed that in vitrified-warmed embryo transfers the size of zona pellucida thinning area by laser-assisted hatching impacts the clinical pregnancy rate and that half of zona pellucida thinning significantly increases the results compared with quarter of zona pellucida thinning [27].

In summary, besides increasing age, in vitro culture and cryopreservation may also induce zona hardening which may disturb the hatching of the blastocyst. Our data and others' studies show that assisted hatching may be useful for improving pregnancy rates of frozen day 3 embryo transfers with recurrent pregnancy failure patients whose age is over 37 years. In patients lesser than 37 years of age we could detect only a trend toward increased pregnancy rates in the laser-assisted hatching + group compared to laser-assisted hatching – group. It was very interesting that we also found that the number of transferred embryos has a strong positive effect on pregnancy formation, but only in patients lesser than 37 years of age. Over 37, this positive effect of embryo number on pregnancy could not be detected. Considering the promising published data and to overcome the negative effect of zona hardening (which is very typical in elder woman and cryopreservation makes the situation more difficult) the application of laser-assisted hatching on cryopreserved embryos as a routine strategy in frozen embryo transfer cycles should be considered, especially in patients with advanced female age and poor prognosis and recurrent pregnancy failure. There are several reports with data indicating that laser-assisted hatching is considered to be the best technique now, in regard to safety and efficacy.

22.13 Conclusions

The conflicting results obtained in the field of assisted hatching shows that the routine use of assisted hatching in case of every patient would be inappropriate in view of the lack of evidence for universal benefits and the potential risks. However, there are several studies supporting the hypothesis that assisted hatching provides benefits in certain circumstances, such as it improves clinical pregnancy rates in patients with poor prognosis or who have had two or more prior failed in vitro fertilization and embryo transfer cycles or in older woman (>38 years of age). The resolution of the Practice Committee of the Society for Assisted Reproductive Technology and the Practice Committee of the American Society for Reproductive Medicine reflects also this standpoint since it is included in its report that the observations of different studies show that assisted hatching may improve the clinical pregnancy rates of specific populations. Improvement may be found in the clinical pregnancy rate among women with previous unsuccessful in vitro fertilization-embryo transfer cycles in their history after

application of assisted hatching in the next, new cycle. Similarly, assisted hatching significantly increased the clinical pregnancy rate in women with older age or poor prognosis, but not in women with good prognosis [5].

In 2014, the Practice Committee of the Society for Assisted Reproductive Technology and the Practice Committee of the American Society for Reproductive Medicine published its newest resolution (the first was published in 2008; see above) connected to the routine application of assisted hatching in human-assisted reproduction. Their conclusion in the revised resolution is that there is good evidence that assisted hatching improves clinical pregnancy rates in poor prognosis patients, including those with prior failed in vitro fertilization cycles. However, they recommend that assisted hatching should not be used routinely for all patients undergoing in vitro fertilization. There is still insufficient evidence that assisted hatching improves live birth rates. Assisted hatching appears to be associated with an increased risk of multiple pregnancies, but there is insufficient evidence that it is associated with an increased risk of monozygotic twin pregnancies [6]. Further investigations are needed to make clear and prove the role of assisted hatching in increasing the outcome of in vitro fertilization and embryo transfer cycles with advanced female age and frozen embryos.

References

1. Cohen J. (2007): Manipulating embryo development. In: *Human Preimplantation Embryo Selection* (Eds.: Elder K. and Cohen J). Informa Healthcare, Informa UK Ltd.,
2. Cohen J., Alikani M., Trowbridge J., Rosenwaks Z. (1992): Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis. *Hum Reprod* 7. 685–91.
3. Cohen J., Lindheim S., Sauer M. (1999): Assisted hatching causes beneficial effects on the outcome of subsequent frozen embryos transfers of donor oocyte cycle. *Fertil Steril* 72 (Suppl. 1). S5.
4. Elhussieny A., El Mandoub M., Hanafi S., Mansour GM., El-Kotb A. (2013): Effect of laser assisted hatching on outcome of assisted reproductive technology. *Open J Obstet Gynecol* 3. 18–23.
5. The Practice Committee of the Society for Assisted Reproductive Technology and the Practice Committee of the American Society for Reproductive Medicine. (2008): The role of assisted hatching in in vitro fertilization: A review of the literature. A Committee opinion. *Fertil Steril* 90. S196–98.
6. The Practice Committee of the Society for Assisted Reproductive Technology and the Practice Committee of the American Society for Reproductive Medicine. (2014): The role of assisted hatching in in vitro fertilization: a guideline. *Fertil Steril* 102. 348–51.
7. Cohen JH., Mather H., Wright G. et al. (1989): Partial zona dissection of human oocytes when failure of zona pellucida penetration is anticipated. *Hum Reprod* 4. 435–42.
8. Cohen J., Elsner C., Kort H. (1990): Impairment of hatching process following IVF in the human and improvement of implantation by assisted hatching using micromanipulation. *Hum Reprod*. 5. 7–13.
9. Rink K., Delacretaz G., Salathe RP. (1996): Non-contact microdrilling of mouse zona pellucida with an objective-delivered 1.48 um diode laser. *Laser surg Med* 18. 52–62.
10. Nakayama T., Fujiwara H., Yamada S. et al. (1999): Clinical application of a new assisted hatching method using a piezo-micromanipulator for morphologically low quality embryos: in poor-prognosis infertile patients. *Fertil Steril* 71. 1014–18.
11. Cong F., Tao L., Ben-Yu M., Guang-Lun Z., Canquan Z. (2010): Mechanically expanding the zona pellucida of human frozen thawed embryos: a new method of assisted hatching. *Fertil Steril* 94. 1302–1307.

12. Blake DA., Forsberg AS., Johansson BR., Wikland M. (2001): Laser zona pellucida thinning-alternative approach to assisted hatching. *Hum Reprod* **16**. 1959–64.
13. Kanyo K., Konc J. (2003): A follow-up study of children born after diode laser assisted hatching. *Eur J Obstet Gynecol Reprod Biolo* **110**. 176–80.
14. Mansour, RT., Rhodes, CA., Aboulgar, MA., Serour, GI., Kannal, A. (2000): Transfer of zona-free embryos improves outcome in poor prognosis patients: a prospective randomized controlled study. *Hum Reprod* **15**. 161–64.
15. Carney SK., Das S., Blake D. et al. (2012): Assisted hatching on assisted conception (in vitro fertilisation) (IVF) and intracytoplasmic sperm injection (ICSI). *Cochrane Database Syst Rev*. 2012 December 12;12:CD001894. doi: 10.1002/14651858.CD001894.pub5.
16. Feng HL., Herslag A., Scholl GM., Cohen MA. (2009): A retrospective study comparing three different assisted hatching techniques. *Fertil Steril* **91**. 1323–25.
17. Wellington PM., Rocha IA., Ferriani RA., Nastri CO. (2011): Assisted hatching of human embryos: a systematic review and meta-analysis of randomized controlled trials. *Human Reprod Updates* **17**. 438–53.
18. Valojerdi MR., Eftekhari YP., Karimian L., Ashtiani SK. (2008): Effect of laser zona pellucida opening on clinical outcome of assisted reproduction technology in patients with advanced female age, recurrent implantation failure, or frozen-thawed embryos. *Fertil Steril* **90**. 84–91.
19. Baruffi R., Mauri A., Petersen C. et al. (2000): Zona thinning with noncontact diode laser in patients aged < 37 years with no previous failure of implantation: a prospective randomized study. *J Assist Reprod Genet* **17**. 557–60.
20. Frydman N., Madoux S., Hesters L. et al. (2006): A randomized double-blind controlled study on the efficacy of laser zona pellucida thinning on live birth rates in cases of advanced female age. *Hum Reprod* **21**. 2131–35.
21. Edirisinghe W., Ahnonkitpanit V., Promviengchai S. et al. (1999): A study failing to determine significant benefits from assisted hatching: patients selected for advanced age, zona thickness of embryos, and previous failed attempts. *J Assist Reprod Genet* **16**. 294–301.
22. Primi M., Senn A., Montag M. et al. (2004): A European multicentre prospective randomized study to assess the use of assisted hatching with a diode laser and the benefit of an immunosuppressive/antibiotic treatment in different patient populations. *Hum Reprod* **19**. 2325–33.
23. Ng E., Naveed F., Lau R. et al. (2005): A randomized double blind controlled study of the efficacy of laser assisted hatching on implantation and pregnancy rates of frozen-thawed embryo transfer at the cleavage stage. *Hum Reprod* **20**. 979–85.
24. Kanyo K., Zeke J., Kriston R. et al. (2016): The impact of laser-assisted hatching on the outcome of frozen human embryo transfer cycles. *Zygote* **24**. 42–47.
25. Ebner T., Moser M., Tews G. (2005): Possible applications of a non-contact 1.48 um wavelength diode laser in assisted reproduction technologies. *Hum Reprod Update* **11**. 425–35.
26. Zhang XJ., Yang YZ., Lv Q. et al. (2009): Effect of the size of zona pellucida thinning by Laser assisted hatching on clinical outcome of human frozen-thawed embryo transfers. *Cryo Letters* **30**. 455–61.
27. Hiraoka K., Hiraoka K., Horiuchi T. et al. (2009): Impact of the size of the zona pellucida thinning area on vitrified-warmed cleavage-stage embryo transfers: a prospective, randomized study. *J Assist Genet* **26**. 515–21.

Assessing Embryo Morphology to Enhance Successful Selection and Transfer

Basak Balaban and David K. Gardner

23.1 Introduction

Improvements in embryo culture systems have not only resulted in significant increases in implantation and pregnancy rates but also assisted the routine culture of viable human embryos in the IVF laboratory for up to six days [1–4]. This opportunity provided the patient the possibility of transferring embryos at any stage during the preimplantation period. Whichever stage of development and day of transfer is adopted as routine for a particular clinic, the transfer of just one embryo to the uterus following IVF has become a priority objective of assisted human reproduction in order to significantly decrease multiple pregnancy rates and associated medical complications. As single embryo transfer becomes the “standard-of-care” in human IVF, the means of assessing embryo viability become more important. Successful selection of the most viable embryo will reduce time to first pregnancy and facilitate the ranking of the remaining embryos that undergo cryopreservation, in order to ensure the subsequent transfer of the more viable embryo first.

Morphological inspection of embryos has been the most commonly used technique of embryologists to assess development and select embryos for transfer. Since the beginning of human IVF, various grading systems have been developed with the aim of quantitating embryo development, viability and implantation potential. As embryo development is highly dynamic, assessing embryos at specific time points on each day has been adopted and key morphological characteristics of the embryo linked to developmental potential [5]. With the advent of time-lapse technology, we have been able to further expand the times at which the morphology of the embryo can be viewed and recorded, providing even greater detail about how an embryo’s appearance relates to developmental potential. Here we consider the appearance of each developmental stage in detail.

23.2 Pronucleate Oocyte

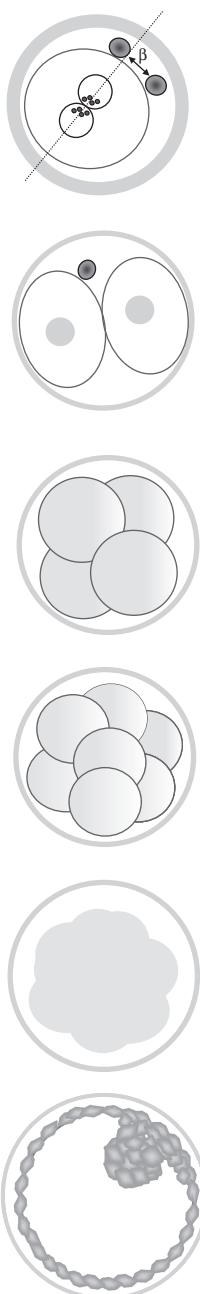
Following fertilization, the embryo must undergo the extrusion of the second polar body, decondensation of the sperm head, pronuclei formation and increase in their subsequent size, and finally the breakdown of the pronuclear membrane. Subsequently, nucleolar precursor bodies within the pronuclei materialize and move. First reports of early events during the embryo’s first cell cycle using time-lapse videography detailed waves of cytoplasmic granulation which exhibited 20–53 minute periodicity, the length of which related to embryo quality. The appearance, growth and subsequent abuttal of the pronuclei, and concomitant nucleolar movement events were then characterized. Analysis of the nucleolar precursor bodies, considering their number and distribution, determined that pronuclear morphology based on the alignment and number of nucleoli within each nucleus could be

used to predict the developmental capacity of human embryos [6, 7]. Subsequent studies investigated the predictive value of pronuclear morphology assessment for cleavage stage as well as blastocyst stage embryo transfers, and revealed that pronuclear morphology may be correlated with the blastocyst development, implantation potential, and chromosomal status of the resultant embryo [8]. The Istanbul consensus on embryo assessment proposed a three-category grading system for pronuclei with symmetry (equivalent to Z1 and Z2 as suggested by Scott's pronuclear scoring system [6]): Category 1 for good quality, symmetrical pronuclei (all other arrangements, including peripherally sited pronuclei as suggested previously by Scott's pronuclear scoring system), Category 2 representing medium-quality pronuclei (with zero or one nucleolar precursor body as suggested by Scott's pronuclear scoring system), and Category 3 representing the lowest quality pronuclei [9, 10]. Pronuclear orientation, in addition to morphology, together with the positions of the polar bodies, has also been examined. Analysis of pronucleate oocytes facilitates the calculation of the angle β , representing the angle between a line drawn through the axis of the pronuclei and the position of the farthest polar body, with an increase in angle β being associated with poor embryo development. These parameters have been reinvestigated using time-lapse technology; the timing of second polar body extrusion, pronuclear fading, and length of S-phase were all associated with successful implantation [11]. Due to the fact that pronuclear orientation is a highly dynamic event, the Istanbul consensus group (Alpha and ESHRE special interest group of embryology) decided not to include it in the static morphological evaluation scheme.

Figure 23.1 shows the key morphological parameters associated with successful IVF outcome. Even though pronuclear morphology may provide valuable information about the quality of the oocyte itself and the viability potential of the fertilized oocyte, evidence-based data show that changes in pronuclear stage are highly time-dependent. So, standardizing the time of fertilization through ICSI or the use of 1 h insemination periods will increase the efficiency of morphometric analysis. It is therefore important to either stick firmly to designated times for observation or take advantage of time-lapse evaluation.

23.3 Cleavage Stage Embryo

The percentage and localization of fragmentation, evenness of the blastomeres, multi-nucleation, and rate of cleavage are features typically assessed to evaluate the quality of a cleavage stage embryo on day 2 and day 3. What are the key morphological parameters during the cleavage stages that are associated with subsequent embryo viability and implantation potential post transfer? The common characteristics of embryos with high viability include 4 or 5 blastomeres on day 2, and at least 7 blastomeres on day 3 after fertilization, with <20 percent fragmentation and no signs of multinucleation. Rienzi et al. [12] suggested a cumulative classification scheme based on the most essential morphological features that should be examined to evaluate a cleavage stage embryo. Morphological parameters selected were cleavage rate, blastomere symmetry, cytoplasmic appearance, extent of fragmentation, and blastomere nucleus status. Such a grading scheme effectively predicted blastocyst formation and implantation. Day 1 PN morphology and nucleolar precursor body (NPB) ratio, day 2 cell number, blastomere symmetry and nucleation, and the ability to cleave from day 2 to day 3 appear to be the six most significant factors associated with subsequent fetal development.



Ideal features shared by pronucleate oocytes with high viability:
 (i) Number of nucleolar precursor bodies (NPB) in both pronuclei never differed by more than 3
 (ii) NPB are always polarized or not polarized in both pronuclei but never polarized in one pronucleus and not in the other
 (iii) Angle β from the axis of the pronuclei and the furthest polar body is less than 50°

Ideal features shared by 2-cell embryos with high viability:
 (i) Mononucleated blastomeres
 (ii) Equal cell size
 (iii) < 20% fragmentation

Ideal features shared by 4-cell embryos with high viability:
 (i) Mononucleated blastomeres
 (ii) Equal cell size
 (iii) < 20% fragmentation

Ideal features shared by day 3 embryos with high viability:
 (i) Mononucleated blastomeres
 (ii) Equal cell size
 (iii) < 20% fragmentation
 (iv) At least 7 blastomeres

Ideal features shared by morulae with high viability:
 (i) Visibly compacted cells denoted by the slight reduction in overall size of the embryo and increase in space between the embryo and zona pellucida
 (ii) Lack of fragments

Ideal features shared by blastocysts with high viability:
 (i) Expanded blastocoel cavity by day 5
 (ii) Well formed ICM clearly composed of many cells
 (iii) Cohesive epithelium made up from many cells in the TE
 (iv) Signs of the zona pellucida thinning

Figure 23.1
 Key morphological parameters of embryos with high viability potential

Multinucleation, defined as more than a single interphase nucleus, is a key morphological feature of the cleavage stages, and its presence is associated with reduced viability and implantation potential. Reported multinucleation rates per treatment with classical morphological evaluation vary from at least 44 percent of the patients up to 80 percent.

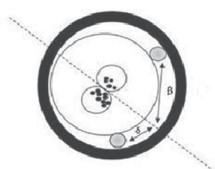
Interestingly, these differences may be attributed to differences in times at which embryos were assessed. Mechanisms leading to multinucleation are proposed to include karyokinesis without cytokinesis, partial fragmentation of nuclei, or defective migration of chromosomes at mitotic anaphase. Multinucleation has been correlated with uneven cell size, a higher degree of fragmentation and numbers of blastomeres on days 2 and 3, and a higher risk of chromosomal abnormalities [13]. In a retrospective study of 5982 embryos in 619 blastocyst-stage transfers, Yakin et al. [14] reported that the detection of multinucleated blastomeres either on day 2 or day 3 indicates a poor prognosis for blastocyst formation. Further, no good quality blastocyst was observed from an embryo which had more than one multinucleated blastomere. Consequently, the presence of multinucleated blastomeres in human embryos should be recorded, and this morphological trait should be included in grading schemes [13]. Time-lapse monitoring is a valuable way to identify cases of multinucleation, given that using a static grading scheme (the Istanbul consensus grading scheme) will only identify 20–28 percent of all embryos with multinucleated blastomeres.

Time to first cleavage has been shown to relate to embryo quality, and is also related to transfer outcome. Hardarson and colleagues determined that early cleaving embryos are more likely to cleave uniformly, which is highly correlated with low incidence of chromosomal errors [13]. Assessment of early cleavage should therefore be performed at a standardized timing (26–28 h post insemination (PI)), as embryos cleaving earlier than 20 h PI have poorer prognosis. Evaluation of early cleavage can also be used to detect zygotes that cleave directly into three or more cells, which is associated with chromosomal abnormality [15].

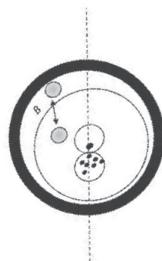
Several studies have considered the relative weight of such cleavage stage morphological parameters and revealed that cell number, equal blastomere size, and the number of mononucleated blastomeres on day 2 were the most predictive markers of blastocyst development and implantation potential. Rienzi and colleagues reported that cell number is the most critical factor determining the viability of the embryo, not only on day 2 but also on day 3, inferring that the optimal development can be defined as 4 or 5 cells on day 2 at 44–46 h PI, and more than 6 cells (7–9 cells) on day 3 at 66–68 h PI [12]. In agreement with such reports, the latest Cochrane review on cleavage stage versus blastocyst stage embryo transfer suggested that the most favorable group for blastocyst transfers was the good prognosis patient group that had higher numbers of eight-cell embryos on day 3 [16]. Evidence-based data clearly demonstrate that several morphological features of the cleavage stages are associated with implantation potential and/or successful blastocyst formation.

Figures 23.1 and 23.2 exemplify key morphological features of the cleavage stage embryo. Even though cell number is a key determinant for quality and viability of the embryo, cell size/symmetry and multinucleation are also important features to examine the developmental potential [17].

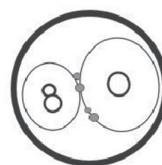
Even though medium to low viability embryos may have some similar morphological features of those embryos with high viability, such as polarized nuclei at the pronuclear stage, mononucleated blastomeres with <20 percent fragmentation during the cleavage stages, and even an expanded blastocoel cavity on day 5 of development, some other aspects of their morphology may not be similar with those embryos of higher viability. For example, in the pronuclear stage even though both pronuclei can be aligned, developmental capacity may be reduced due to other characteristics, such as differences in the number of nucleolar precursor bodies differing by more than 3, or the angle β from the axis of the pronuclei and the farthest polar body being greater than 50°. Similarly, in the cleavage stages, although the



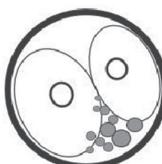
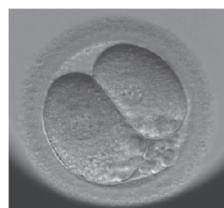
Sub-optimal features shared by pronucleate oocytes with medium/low viability:
 (i) Number of nucleolar precursor bodies (NPB) differ by more than 3
 (ii) Angle β from the axis of the pronuclei and the furthest polar body is more than 50°



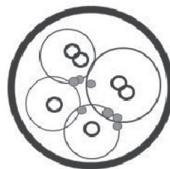
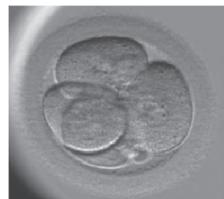
Sub-optimal features shared by pronucleate oocytes with medium/low viability:
 (i) Number of nucleolar precursor bodies (NPB) differ by more than 3
 (ii) Distribution of NPB being polarized in one pronuclei, whereas it's non-polarized in the other



Sub-optimal features shared by 2-cell embryos with medium/low viability:
 (i) Multinucleated blastomeres
 (ii) Unequal cell size



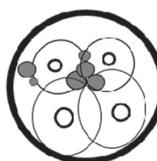
Sub-optimal features shared by 2-cell embryos with medium/low viability:
 (i) Unequal cell size
 (ii) 20% fragmentation



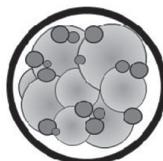
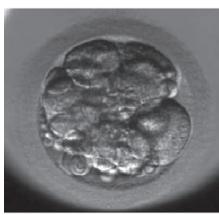
Sub-optimal features shared by 4-cell embryos with medium/low viability:
 (i) Multinucleated blastomeres
 (ii) Unequal cell size

Figure 23.2 Key morphological parameters of embryos with medium to low viability potential

blastomeres may have <20 percent fragmentation, they may have other characteristics correlated with reduced viability, such as multinucleation and/or unequal size. Finally, in



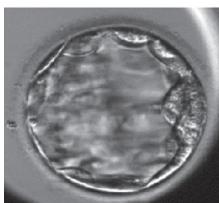
Sub-optimal features shared by 4-cell embryos with medium/low viability:
 (i) Unequal cell size



Sub-optimal features shared by day 3 embryos with medium/low viability:
 (i) Unequal cell size
 (ii) 35% fragmentation



Sub-optimal features shared by morulae with medium/low viability:
 (i) Non-participation of all cells in compaction
 (ii) 20% fragmentation



Sub-optimal features shared by blastocysts with medium/low viability:
 (i) Loosely formed ICM composed of few cells
 (ii) Loosely formed epithelium made up from few cells in the TE

Figure 23.2 (cont.)

the blastocyst, although the blastocoel may be well expanded, the cells in the inner cell mass and trophectoderm, may be poorly developed, reflecting lower implantation capacity.

23.4 Morula

Compaction of an embryo is one of the key developmental events during the preimplantation period and has a profound effect on how the embryo develops in culture [1, 18–20]. For this reason, it is logical to assess the morphology of the embryo on day 4 during this critical phase of development, which can now be accessed in detail and better understood through time-lapse imaging. During compaction, the blastomeres establish tight junctions to

facilitate the formation of the first transporting epithelium of the conceptus. This event is a prerequisite for the embryo to control its internal environment and to facilitate the formation of the blastocoel. In addition, upon compaction the embryo increases its ability to regulate changes in external pH, and should the embryo decompact it loses this ability. Based on these, compaction could be considered as an essential parameter to score given its significance to embryo physiology.

Using a grading system whereby embryos were scored from 1 to 4 in order to classify morula development, it was found that the assessment on day 4 was of value for embryo selection, with the highest grade morulae having significantly higher post-thaw survival, and were transferred at a higher frequency than embryos with lower grades [21]. Ebner and coworkers examined the degree of cellular compaction on day 4, and determined that those embryos which were fully compacted gave rise to significantly more, and higher quality, blastocysts [22]. Other similar four-step grading systems published also demonstrated that there was a trend to lower pregnancy rates with decreasing score and that embryos scoring the lowest grade failed to establish a pregnancy. In one study, morulae exhibited similar pregnancy rates to day 5 transfers [23].

Further data examining the efficient morphological features of the morula stage embryo are needed. Dynamic evaluation of the compaction event will facilitate understanding the critical key features affecting the implantation potential of a morula stage embryo. Figure 23.1 shows key morphological parameters of the morula stage embryo.

23.5 Blastocyst

Analysis of blastocyst morphology initially focused on the rate of expansion, and the determination of cell allocation to the two lineages of the inner cell mass (ICM) and trophectoderm (TE). An advantage of assessing the blastocyst is that one is assessing the embryo proper (cleavage stage morphology reflects primarily oocyte competence). Determining the rate of expansion alone has physiological value, as the formation of the blastocoel is energetically demanding, and hence the embryo must possess a degree of metabolic competency to create the ionic gradient required to allow fluid to accumulate within the morula and coalesce to form a blastocoel. Analysis of the degree of expansion can be difficult after a blastocyst collapses. Interestingly, the thickness of the zona reflects each blastocyst's "expansion history", as an expanding blastocyst will induce changes to the shape of the surrounding zona, which does not resort back to its original form when the blastocoel collapses.

Gardner and Schoolcraft [24] developed a comprehensive alphanumeric system, designed to incorporate the degree of blastocoel expansion, as well as the quality and cell number of the two cell lineages. Herein, blastocyst expansion rate is characterized by numbers from the early blastocyst to the hatched stage. Analysis of both the ICM and TE quality can then be performed for expanded blastocysts in which both cell types are clearly evident. ICM and TE quality are characterized by three grades, A to C. ICM grades: A, a tightly packed ICM with many cells; B, loosely grouped ICM with several cells; or C, very few cells and disorganized. TE grades: A, denoting TE with many cells forming a cohesive epithelium; B, few cells forming a loose epithelium; and C, very few large cells (see Figure 23.1 for the morphological characteristics of a 4AA blastocyst). Using this scoring system a strong correlation between the clinical outcome (implantation rates, pregnancy and twinning rates) and the morphological grade of blastocysts was established [25].

Subsequently, Ahlstrom and colleagues determined that although blastocyst expansion and ICM grade were significant predictors in both univariate and multivariate analyses, they were not predictors of live birth when analyzed by stepwise logistic regression, rather the TE was the most important determinant of successful transfer outcome [26]. Such observations have been further supported by Ebner and colleagues [27].

The physiology underlying the significance of the TE grade may reside in the significance of the TE during the initiation and progression of implantation, and that a healthy TE ensures acceptable implantation. A larger number of TE cells will be able to ensure greater signaling and interaction with the endometrium. Interestingly, although the TE produces human chorionic gonadotropin (hCG) as one of the earliest signals to the mother, it has recently been proposed that lactate production by the blastocyst, which will be derived primarily from the TE, has several key functions in regulating the implantation process [28]. These physiological roles endorse the importance of a good TE grade in predicting implantation potential.

In spite of the importance of the TE grade, it remains important to include the ICM grade in order to determine true blastocyst quality, as it transpires that all three parameters of the blastocyst (degree of expansion, ICM and TE quality) are significantly associated with pregnancy and live birth rates [29]. Transfer of blastocysts with an “A” (top quality) grade ICM further reduces the incidence of pregnancy loss. Further, studies have also determined that ICM grade is positively associated with birth weight [30]. Considering the quality of both the ICM and TE makes physiological sense as they do not exist in isolation, but rather coexist as a functional unit, as although it is the TE which creates a unique environment for the ICM by the synthesis of blastocoel fluid, it is the ICM itself that regulates the proliferation and activity of the TE [31].

23.6 Discussion

Data reviewed in this chapter highlight the importance of the inclusion of morphological assessment in the selection/deselection of embryos [28], despite the introduction of novel objective selection methods. Considering the highly dynamic structure of the human embryo, it appears logical that one should not consider appearance without including time as a variable. However, the converse is also true that it would be logical to include key morphological features of embryos (and not just times of division) into algorithms initially based on kinetics. Key morphological parameters that could be considered are multinucleation and the relative size/symmetry of blastomeres. As morphological evaluation has a degree of subjectivity, the use of computer-assisted embryo grading will in the future assist in reducing variability in assessment. The availability of grading systems that are clinically validated, standardized, and evidence-based by internationally recognized authorities (for example, Alpha and ESHRE [8]) will assist in reducing the subjectivity of morphological assessment. It is evident that at the cleavage and blastocyst stages good morphology is indeed associated with an increase in pregnancy and live birth rates [32].

Metabolic and proteomic data have revealed that differences in physiology exist between embryos of the same grade, and hence we still need to look beyond morphology. However, even with quantitative markers of developmental potential for euploid embryos, morphology and kinetics could still be of value in the deselection/selection process. Whereas morphology and kinetics are useful for embryo deselection, the analysis of embryo physiology through quantitative assays will facilitate embryo selection. In line with this, Lee and colleagues

reported a relationship between early cleavage events and subsequent blastocyst physiology and viability, supporting the concept of obtaining and using as much information as possible about each embryo prior to transfer [33]. However, it is still required to see prospective clinical studies performed on the efficiency of these techniques. Until such time, assessment of morphology remains a valuable tool as a routine selection method in clinical practice.

A recent study by Mastenbroek [34] questioned the necessity for any embryo selection, rather the authors proposed that “the path of embryo selection is turning into a dead-end in the quest for optimal IVF success rates,” and that in the “perfect world” the best that embryo selection can do is shorten the time to pregnancy. However, without any form of ranking of embryo viability, their approach involves successive cycles of cryopreservation that will inevitably lead to significant delays in conception and an increased number of miscarriages. As we face a potential paradigm shift, moving from fresh transfer, to cryopreserved cycles in order to replace the embryo into a non-stimulated uterus, we will still need to know the order in which embryos should be selected for transfer. Even if embryo selection is only used to decrease the time it takes for a couple to conceive, selection still remains critical. Patients do not consider a reduction in the time to achieve a pregnancy as non-consequential. Various studies have shown that the dropout rates after IVF treatment can reach up to 50 percent. Without some basis for selection for transfer, we condemn patients to successive cycles of retrievals, cryopreservation and replacements. Our major aim and mission when performing IVF to the patients is to facilitate their needs to have a healthy family sooner than later, not sooner or later [35].

References

1. Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update* 2015.
2. Swain JE. Optimal human embryo culture. *Semin Reprod Med* 2015;33: 103–17.
3. Gardner DK, Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF? *Hum Reprod Update* 1997;3: 367–82.
4. Quinn P. The development and impact of culture media for assisted reproductive technologies. *Fertil Steril* 2004; 81:27–29.
5. Rhenman A, Berglund L, Brodin T et al. Which set of embryo variables is most predictive for live birth? A prospective study in 6252 single embryo transfers to construct an embryo score for the ranking and selection of embryos. *Hum Reprod* 2015;30:28–36.
6. Scott LA, Smith S. The successful use of pronuclear embryo transfers the day following oocyte retrieval. *Hum Reprod* 1998;13: 1003–13.
7. Tesarik J, Greco E. The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. *Hum Reprod* 1999;14: 1318–23.
8. Balaban B, Yakin K, Urman B, Isiklar A, Tesarik J. Pronuclear morphology predicts embryo development and chromosome constitution. *Reprod Biomed Online* 2004;8:695–700.
9. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Reprod Biomed Online* 2011;22: 632–46.
10. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Human Reproduction* 2011;26: 1270–83.
11. Aguilar J, Motata Y, Escriba MJ et al. The human first cell cycle: impact on implantation. *Reprod Biomed Online* 2014;28: 475–84.
12. Rienzi L, Ubaldi F, Iacobelli M et al. Significance of morphological attributes of

- the early embryo. *Reprod Biomed Online* 2005;10: 669–81.
13. Hardarson T, Hanson C, Sjogren A, Lundin K. Human embryos with unevenly sized blastomeres have lower pregnancy and implantation rates: indications for aneuploidy and multinucleation. *Hum Reprod* 2001;16: 313–18.
 14. Yakin K, Balaban B, Urman B. Impact of the presence of one or more multinucleated blastomeres on the developmental potential of the embryo to the blastocyst stage. *Fertil Steril* 2005;83: 243–45.
 15. Hardarson T, Selleskog U, Reismer E et al. Zygotes cleaving directly into more than two cells after 25–27 h in culture are predominantly chromosomally abnormal. *Hum Reprod* 2006;21:i102.
 16. Glujoovsky D, Blake D, Farquhar C, Bardach A. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Database Syst Rev* 2012;7:CD002118.
 17. Gardner DK, Balaban B. Assessment of human embryo development using morphological criteria in an era of time-lapse, algorithms and OMICS: Is looking good still important? *Mol Hum Reprod* 2016 October;22(10):704–18.
 18. Biggers JD, Bell JE, Benos DJ. Mammalian blastocyst: transport functions in a developing epithelium. *Am J Physiol* 1988;255:C419–32.
 19. Gardner DK, Lane M, Schoolcraft WB. Physiology and culture of the human blastocyst. *J Reprod Immunol* 2002;55:85–100.
 20. Fleming TP, Johnson MH. From egg to epithelium. *Ann Rev Cell Biol* 1988;4: 459–85.
 21. Tao J, Craig RH, Johnson M et al. Cryopreservation of human embryos at the morula stage and outcomes after transfer. *Fertil Steril* 2004;82: 108–18.
 22. Ebner T, Moser M, Shebl O et al. Morphological analysis at compacting stage is a valuable prognostic tool for ICSI patients. *Reprod Biomed Online* 2009;18: 61–6.
 23. Feil D, Henshaw RC, Lane M. Day 4 embryo selection is equal to Day 5 using a new embryo scoring system validated in single embryo transfers. *Hum Reprod* 2008;23: 1505–10.
 24. Gardner DK, Schoolcraft WB. In vitro culture of human blastocyst In: Jansen R, Mortimer D, eds. *Towards Reproductive Certainty: Fertility and Genetics Beyond* 1999. Carnforth, UK: Parthenon Publishing 1999: 378–88.
 25. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril* 2000;73: 1155–8.
 26. Ahlstrom A, Westin C, Reismer E, Wikland M, Hardarson T. Trophectoderm morphology: an important parameter for predicting live birth after single blastocyst transfer. *Hum Reprod* 2011;26: 3289–96.
 27. Ebner T, Tritscher K, Mayer RB et al. Quantitative and qualitative trophectoderm grading allows for prediction of live birth and gender. *J Assist Reprod Genet* 2016;33:49–57.
 28. Gardner DK. Lactate production by the mammalian blastocyst: manipulating the microenvironment for uterine implantation and invasion? *Bioessays* 2015;37: 364–71.
 29. Van den Abbeel E, Balaban B, Ziebe S et al. Association between blastocyst morphology and outcome of single-blastocyst transfer. *Reprod Biomed Online* 2013;27: 353–61.
 30. Licciardi F, McCaffrey C, Oh C, Schmidt-Sarosi C, McCulloh DH. Birth weight is associated with inner cell mass grade of blastocysts. *Fertil Steril* 2015;103: 382–7 e2.
 31. Ansell JD, Snow MH. The development of trophoblast in vitro from blastocysts containing varying amounts of inner cell mass. *J Embryol Exp Morphol* 1975;33: 177–85.
 32. Oron G, Son WY, Buckett W, Tulandi T, Holzer H. The association between embryo quality and perinatal outcome of singletons

- born after single embryo transfers: a pilot study. *Hum Reprod* 2014;29: 1444–51.
33. Lee YS, Thouas GA, Gardner DK. Developmental kinetics of cleavage stage mouse embryos are related to their subsequent carbohydrate and amino acid utilization at the blastocyst stage. *Hum Reprod* 2015;30: 543–52.
34. Mastenbroek S, van der Veen F, Aflatoonian A,. Embryo selection in IVF. *Hum Reprod* 2011; 26: 964–66.
35. Gardner DK, Meseguer M, Rubio C, Treff NR. Diagnosis of human preimplantation embryo viability. *Hum Reprod Update* 2015;21: 727–47.

Evaluation of Embryo Quality

Time-Lapse Imaging to Assess Embryo Morphokinesis

Belén Aparicio-Ruiz and Marcos Meseguer

24.1 Introduction

In vitro fertilization (IVF) programs are every day closer to the goal of reducing multiple pregnancies while maintaining good clinical results. Concerns about the “epidemic” of multiple gestations have raised awareness of the risks associated not only to the mother (gestational diabetes, hypertension, and anemia) but also to the babies: extreme prematurity, low birth weight, children with neurological damage, etc., not to mention the psychological burden and suffering for the parents and the tremendous health costs that it entails. From the laboratory point of view, several achievements are worth mentioning as well: studies on embryo metabolism have led to the formulation of suitable culture media; in the early 1990s the introduction of intra cytoplasmic sperm injection (ICSI) revolutionized the treatment of male infertility and preimplantation genetic screening became the gold standard against the selection of aneuploid embryos. Vitrification came along, and preservation of fertility was no longer a utopia for modern women; the wave of the “omics” initiated an era of noninvasiveness to study human embryos in the laboratory and most recently the introduction of imaging systems allowed us to assess embryos in a different way: through their morphokinetics.

24.2 Time-Lapse Technology

Traditional embryo assessment is based on time-point evaluations. Through this approach, embryo categories are normally based on the number of blastomeres and nucleus, the percentage of fragments, the cell symmetry and the quality of the inner cell mass (ICM), and trophectoderm (TE). Even though great knowledge has been achieved through this approach, it has been demonstrated that embryo status can markedly change within a few hours. In addition, inter- and intra-observer variability is a commonly described problem probably due to the subjective nature surrounding traditional morphological assessment. In theory, increasing the number of observations could provide better information of the development of the embryo and therefore improve its assessment. However, increased handling and higher evaluation frequency will expose the embryo to undesirable changes in temperature, humidity, and gas composition [1].

Time-lapse systems (TMS) represent a solution to this problem. The two main advantages of these systems are (1) improved and stable culture conditions and (2) the determination of objective and accurate markers, both quantitative and qualitative. In addition, we should mention reduced handling and human risk; minimization of culture media, gas, and oil; detection of abnormal events that would normally occur between observations; reduced inter- and intra-observer variations and reduced number of hours needed by the embryologist in the laboratory [2].

Morphokinetics, defined as the combination of the embryo appearance (morphology) and the timing in which cellular events occur, has been introduced as a new concept to improve embryo selection. The use of this strategy could allow single embryo transfers (SET) without compromising the overall IVF success, becoming very attractive specially in European countries in which legislation is stricter about the number of embryos transferred.

24.2.1 Models in Market

There are different available options of TMS in the market. Some of them present all the items integrated in one single equipment, for example, EmbryoScope® (Vitrolife), Geri (Genea Biomedex), and Miri® TL (Esco Medical). Others offer the option of introducing a microscope inside an available incubator, for example, Primo Vision® (Vitrolife) and The Eeva™ Test (Merck-Serono). Figure 24.1 describes the clinical and technical features of all the TMS available in the market.

24.2.1.1 Embryoscope®

Embryoscope® is the world's most used TMS for observation of embryo development, while maintaining stable embryo culture conditions. It has been used in more than 300,000 patient treatments since 2009. The Embryoscope® incubator ensures stable incubation while automatically taking images of the developing embryos at defined intervals. This information is transferred to the embryoscope server so that the information can be accessed from conveniently accessed computer stations.

The last version of this incubator is Embryoscope+.

SYSTEM	ESD+	PRIMO VISION	EEVA	GERI	MIRI
DISHES					
Optics	Bright field	Bright field	Dark field	Bright field	Bright field
Embryos and dishes	16emb/dish 15dishes/system	16emb/dish 1dish/camera	12emb/dish 1dish/camera	16emb/dish 6dishes/system	14emb/dish 6dishes/system

Figure 24.1 Description of the first time-lapse systems available in the market

Embryoscope+ has more than double the patient capacity, compared to other bench-top TMSs. The Embryoscope+ can simultaneously acquire time-lapse videos from up to 15 patient EmbryoSlide culture dishes with up to 16 embryos making it ideal for clinics who wish to offer time-lapse to all of their patients.

To improve workflow, the culture dishes are automatically registered using a special patient barcode labeling system. The large capacity in combination with a small footprint and efficient workflow provides optimized usage of clinic resources.

A patient identification barcode system offers a flexible and efficient workflow automatically reading and registering entry of a new patient and re-entry of an existing patient slide.

Embryoscope+ provides a very stable and controlled incubation environment with a comprehensive and rigorous monitoring system. A unique isolated loading area design ensures that culture conditions in the internal incubation chamber are unaltered upon door opening to add or remove patients.

The wells are individually numbered and the culture dish contains four additional wells for flushing and rinsing.

24.2.1.2 Eeva™

Eeva™ stands for Early Embryo Viability Assessment Test, a noninvasive test that helps embryologists identify on day 3 which embryos are most likely to advance to the blastocyst stage.

The Eeva Scope fits into the conventional incubator and provides automatic dark-field image capture and cell-division tracking without intervention by the embryologist or excessive light exposure to the embryos.

The Eeva™ Test is clinically validated and is the first noninvasive device with FDA clearance to be used to aid embryo assessment. The background of the Eeva™ Test is a landmark study conducted at Stanford University, which identified cell division timings as key parameters that can predict which embryos will likely grow to blastocyst stage. The significance of distinct timing windows has been confirmed, with several scientific publications highlighting the robustness of morphokinetics to predict embryo development.

The Eeva™ Test uses a patented algorithm to predict embryo development and identify those most likely to become blastocysts.

When used adjunctively with traditional morphology, the Eeva Test can help to improve IVF outcomes by providing you with objective information on embryo viability.

Eeva™ Xtend algorithm considers five biological parameters identifying subtle but critical differences among embryos. The updated Eeva™ Xtend Algorithm now includes egg age, cell count, and Post P3 analysis – which measure cell activity after the four-cell stage. The Post P3 is the result of a proprietary analysis based on 74 computer-based attributes that are combined into one parameter.

Using this multidimensional prediction model, the new Eeva™ Test with Xtend algorithm gives each embryo a unique and objective grading output of their developmental potential, ranging from 1 (highest) to 5 (lowest).

24.2.1.3 Miri®

The Miri's overall design provides cultured embryos in a minimum-stress environment. The six chambers are also independent of each other; hence, any disruption such as temperature drop when opening the lid will have no impact on the rest of the system.

One of the advantages of Miri benchtop multiroom incubator is the speed of recovering temperature and gas parameters after opening a chamber.

Each chamber contains a heating optimization plate to facilitate heat transfer directly to the culture dishes. It has inserts to fit various dish sizes. The plate is removable for easy cleaning.

There is also a continuous pH monitoring system integrated to the Miri incubator. This technology used in combination with disposable sensors accurately and reliably monitors the pH of small volumes of fluids such as the media used during embryo culture without having to remove samples from the incubator.

The Miri® TL incubator has powerful annotation tools to help make decisions confidently. The Assist function automatically detects the first cleavages; one can make a side-by-side comparison and compare actual timings to ideal.

The Miri® TL has 12 temperature sensors (2 for each chamber) to ensure constant temperature stability. On top of that, each compartment has a separate individual PT1000 sensor and gas sample port specifically designed for independent and continuous validation of temperature and gas concentration. A Miri GA can be optionally connected for external validation. The Miri® TL also has a built-in pH measuring system for pH monitoring.

24.2.1.4 Primo Vision®

Primo Vision® is easy to install and use without disturbing the daily routine in the lab. The system integrates quickly into most clinics' routine, by utilizing the existing equipment and maintaining culture conditions.

It offers a modular structure that significantly lowers the investment hurdle.

The microscopes are operated by a central controlling unit, situated outside of the incubator. Connection between the microscopes and the controlling unit is provided by a 3-m long USB cable, supplying electricity to the microscopes only for a few seconds to capture images. The microscopes remain electromagnetically silent at all other times.

The Primo Vision® system uses a specially designed microwell group culture dish: each dish combines the benefits of group culture for up to 9 or 16 embryos while keeping them in place for individual monitoring.

Primo Vision® software is designed to provide an easy and reliable way to analyze, compare, and report the development of embryos. User-defined system settings provide complete freedom for embryologists to create individual profiles.

24.2.1.5 Geri®

Geri® is a benchtop incubator with six individual chambers, each designed to hold a single patient's embryos with individual sensors and a LED display so you can continuously monitor culture conditions. Each chamber has an individual microscope with high resolution camera, providing detailed time-lapse views of each embryo's development and is independently controlled to minimize lid openings and help maintain the optimum environment.

Geri® allows humidified cultivation through the insertion of customized water bottles. This unique feature helps to maintain the optimal medium osmolality even at day 5 or 6 of incubation. Humidity inside the chamber is tracked by independent sensors. The Geri® chamber is also designed to hold an extra dish, either for equilibration of the next sequential medium or for transfer.

Geri® features numerous fail-safe mechanisms in order to make sure the chamber's set points are reached as quickly as possible and stable culture conditions are maintained [5].

The two independent temperature-control systems have backup heating elements in each chamber to maintain temperature in case of failure.

A gas purge is performed whenever the instrument's lids are opened. The gas connections are designed so that a fault in one chamber will have no impact on any other [5].

Meanwhile, in the event of any variations in temperature or CO₂ levels, an alarm is triggered to notify users of the fault. This can be linked to external alarms so that lab professionals can know of any faults outside of working hours.

One microscope and camera per chamber provides more pictures in a shorter time frame than current benchtop time-lapse incubators on the market.

Images are captured every 5 minutes. Geri® can incubate six patients at once, with 16 embryos or fertilized eggs per dish. This means a total capture of 96 images across 11 focal planes. Settings for each chamber can be adjusted including camera focus, image contrast, and camera alignment.

As an additional safety feature, the separation of firmware driving the incubator and software driving the cameras means that any issue with the camera has no impact on incubation parameters.

The Geri® dish has 16 microwells, which are positioned in a circle to allow dish rotation.

The microwells are 500 µm in diameter to allow comfortable embryo addition or removal, and 400 µm deep to prevent embryos leaving microwell. The microwells on the Geri® dish are designed so that all embryos share the same medium.

24.3 Morphokinetic Studies

Over the last years there has been a special interest in this field. Many groups have focused their research on different morphokinetic parameters and with different conclusions that on the whole reach the same point: the utility of time lapse as a promising tool on embryo selection.

In 1997, Payne et al. were the first to develop time-lapse cinematography in order to manage an intermittent observation of the process of oocyte fertilization. Later on, the observation period was augmented while maintaining optimal culture conditions and nowadays TMS allow the complete observation of the entire process of embryo development in the IVF laboratory.

It is no longer a new technology or an experimental strategy subject because it has been supported by many studies and even prospective studies (with the difficulties the development of these kinds of studies cause).

Due to the increasing interest in this field and the number of publications over the last 20 years, the aim of the current review is to summarize the publications of Maybe reword since 2016 was not last year regarding the use of time-lapse imaging for noninvasive selection of viable embryos (Tables 24.1 and 24.2).

24.3.1 Previous Studies to Bear in Mind

Without underrating the significance of the other studies developed and published, we are going to go through some of the key and most relevant studies published during the last years before focusing on this year's publications.

Table 24.1

Objective	
Goodman et al.	To determine if the addition of morphokinetic data improves reproductive outcomes when all embryos are cultured in a closed system.
Petersen et al.	To determine if a generally applicable morphokinetic algorithm suitable for day-3 transfers originating from different culture conditions and fertilization methods can be developed for the purpose of supporting the embryologist's decision.
Huang et al.	To detail several additional dynamic features of blastocyst expansion in human embryos generated from donor oocytes.
Ahlstrom et al.	To assess the ability of patient characteristics and embryo morphology with morphokinetic variables to predict live birth after day 2 transfer.
Adaman et al.	To determine if an automated time-lapse test (TL-test) combined with traditional morphology for embryo selection and day-3 transfer result in improved clinical outcomes.
Kieslinger et al.	To determine if prospective embryo selection using the result from the Eava Test in combination with standard morphology increase the pregnancy rate of IVF and ICSI patients compared to embryo selection based on the morphology only.
Patel et al.	To identify the morphokinetic differences by analyzing the development of euploid and aneuploid embryos using time lapse and to check the accuracy of a previously described model for selection of euploid embryos based on morphokinetics.
Aparicio-Ruiz et al.	To correlate the different categories provided by a commercial diagnostic test with blastocyst formation, quality, implantation potential, and ongoing pregnancy (OPR) for the purpose of validating the automatic annotations and the classification algorithm.
Motato et al.	To describe the events associated with the blastocyst formation and implantation that occur in embryos during preimplantation development based on the largest sample size ever described with time-lapse monitoring.
Kirkegaard et al.	To study to what extent patient- and treatment-related factors explain the variation in morphokinetic parameters.
Liu et al.	To present a time-lapse deselection model involving both qualitative and quantitative parameters for assessing embryos on day 3.
Bodr et al.	To determine which morphokinetic variables are related to embryo gender in a cohort of consecutive live births obtained through single blastocyst transfer following mild ovarian stimulation.

In 2010, Wong et al. [3] analyzed kinetic parameters of 100 embryos that were cultured up to day 5 or 6 of development. Three parameters were found as predictors of blastocyst formation:

- **P1** – duration of the first cytokinesis (14.3+-6.0 min);
- **P2** – interval between the end of the first mitosis and the initiation of the second mitosis (11.1+-2.2 h); and
- **P3** – the synchrony between the second and third mitosis (1.0+-1.6 h).

Table 24.2

Objective	
Dai Canto et al.	Comparison of the morphokinetic behavior of embryos developed from oocytes matured in vivo and in vitro. Extended the morphokinetic analysis of these embryos by a comparison with embryos obtained in stimulated assisted reproduction technology cycles.
Tejera et al.	To evaluate correlations between oxygen consumption (oc) measurements before and after embryo cytokinesis, observing oc during embryo cleavages and combining that information with morphokinetics to relate to implantation potential.
Costa-Borges et al.	To evaluate the efficiency of using a continuous (one-step) protocol with a single medium for the human embryos in a time-lapse incubator.
Balakier et al.	To study the effect of human embryo multinucleation on the rate of aneuploidy, in vitro development, morphokinetics and pregnancy outcome.
Bar-El et al.	The purpose of the study was to explore the effect of blastomere biopsy for preimplantation genetic diagnosis (PGD) on the embryo's dynamics, further cleavage, development, and implantation.
Del Carmen Nogles et al.	To study the differences in the cleavage time between types of embryo chromosomal abnormalities and elaborate algorithm to exclude aneuploidy embryos, with preference for euploid embryos.
Wu et al.	To compare pregnancy rates and embryo quality on day 3 after fertilization and embryologist time utilized per processed embryo in two populations: poor prognosis patients and embryos from egg donor-recipient cycles.
Herzberger et al.	To evaluate time-lapse microscopy as a selection tool for single-embryo transfer (SET) on day 5, blastocyst stage.
Kong et al.	To investigate how different behaviors affect cell number and development potential of day-3 embryos by time-lapse imaging. Division behavior and morphokinetic parameters were analyzed in 5 groups of day-3 embryos with different cell numbers.
Bodri et al.	To ascertain the rate of blastocyst collapse observed by time-lapse monitoring in a retrospective cohort of unselected infertile patients undergoing single blastocyst transfer and to determine its association with live birth.
Demirel et al.	To see if oocytes retrieved from an ovary with an endometrioma would develop into embryos with aberrant timings of cleavage and poorer morphologic quality compared with sibling oocytes from the contralateral ovary with no endometrioma in the same patient.
Milewski et al.	To create a model to predict the implantation of transferred embryos based on information contained in the morphokinetic parameters of time-lapse monitoring.
Reviews	Leung et al. 2016, Castelló et al. 2016, Gardner et al. 2016, Mandawala et al. 2016, Martins et al. 2016, Meseguer 2016.

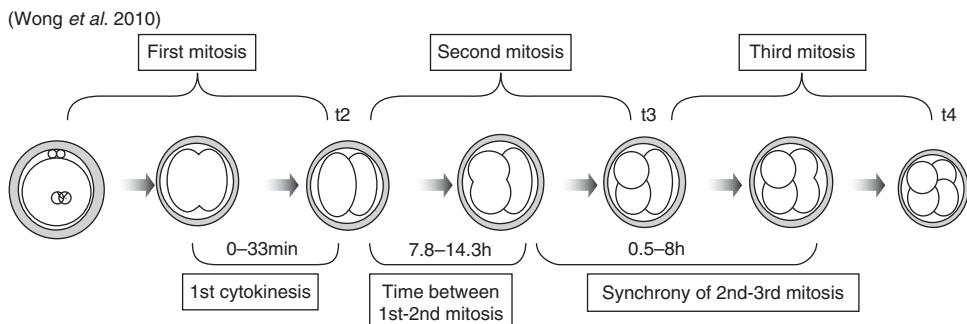


Figure 24.2 Description of the morphokinetic variables used for the first model of embryo selection based on blastocyst formation potential reported by Wong et al. Together with the drawings of the cleavage embryos, optimal time-ranges were included

The authors concluded that embryo development to the blastocyst stage could be predicted with 94 percent sensitivity and 93 percent specificity after using those parameters. Embryos with one or more values outside these ranges were expected to arrest (Figure 24.2).

They also collected single embryos for gene expression analysis and revealed that embryos with P1, P2, and P3 outside of the optimal ranges exhibited abnormal RNA patterns for embryo cytokinesis, micro RNA (miRNA) biogenesis, and maternal mRNA reserve, suggesting that embryo fate may be predetermined and inherited very early in development (by the 4-cell stage).

Meseguer et al. [4] published a study where several parameters were correlated with embryo implantation. The study was based on 247 embryos with known implantation (KID) and it developed a hierarchical model that subdivided embryos into six categories from A to F. Four of these categories (A–D) were further subdivided into two sub-categories (+) or (−). The hierarchical classification procedure starts with a morphological screening of all embryos in a cohort to eliminate those embryos that are clearly NOT viable (i.e. highly abnormal, atresia or clearly arrested embryos). Those embryos that are clearly not viable are discarded and not considered for transfer (category F). Next step in the model is to exclude embryos that fulfill any of the three exclusion criteria: (i) uneven blastomere size at the 2-cell stage, (ii) abrupt division from one to three or more cells or (iii) multinucleation at the 4-cell stage (category E). The subsequent levels in the model follow a strict hierarchy based on the binary timing variables t5, s2, and cc2. First, if the value of t5 falls inside the optimal range (48.8–56.6 h), the embryo is categorized as A or B. If the value of t5 falls outside the optimal range (or if t5 has not yet been observed at 64 h), the embryo is categorized as C or D. If the value of s2 falls inside the optimal range (≤ 0.76 h) the embryo is categorized as A or C depending on t5; similarly, if the value of s2 falls outside the optimal range, the embryo is categorized as B or D depending on t5. Finally, the embryo is categorized with the extra plus (+) if the value for cc2 is inside the optimal range (≤ 11.9 h) (A+/B+/C+/D+) and is categorized with a minus (−) as (A−/, B−/, C−/, D−) if the value for cc2 is outside the optimal range (Figure 24.3).

Validation of Meseguer's algorithm from 2011 came along with a triple-blind randomized prospective controlled trial published by Rubio et al. [5]. In this study, 405 patients were included in the control group in which embryos were selected purely on

(Meseguer et al. 2011)

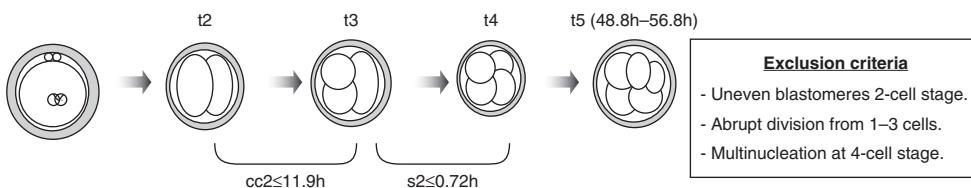


Figure 24.3 Description of the morphokinetic variables used for the first model of embryo selection based on implantation potential reported by Meseguer et al. Together with the drawings of the cleavage embryos, optimal time-ranges were included

(Basile et al. 2014)

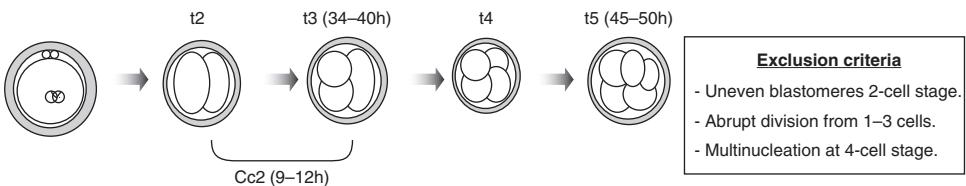


Figure 24.4 Description of the morphokinetic variables used for the first model of embryo selection based on aneuploidy risk reported by Basile et al. Together with the drawings of the cleavage embryos, optimal time-ranges were included

morphology and 438 patients were included in the study group in which embryos were selected based on the algorithm. Implantation rates were significantly higher in the study group (44.9%; 95% CI, 41.4–48.4) versus the control group (37.1%; 95% CI, 33.6–40.7). In addition, the ongoing pregnancy rate was also higher in the study group versus the control group (54.5%; 95% CI, 49.6–59.2 vs 45.3%; 95% CI, 40.3–50.4). The authors concluded that morphokinetic variables allow us to reject embryos with lower implantation while distinguishing those with higher implantation probabilities. Selecting embryos through kinetic markers may therefore improve reproductive outcomes.

Basile et al. [6] continued the study by Meseguer et al. [4] and published an improved version of the algorithm by studying a larger dataset of embryos from four different IVF clinics. For that aim a sequential approach was adopted by the author. During Phase 1 of the study an algorithm was developed taking in consideration morphokinetic data of 754 KID embryos that were selected for transfer based only on conventional morphological criteria. The new algorithm included the variables t3, cc2 and t5 in combination with morphology and exclusion criteria (DC, UBS, and MN) and classified embryos from A to E according to their implantation potential (Figure 24.4).

3). Subsequently, during Phase 2 of the study, the predictive ability of this new algorithm was tested by applying it for embryo classification in a different group of IVF patients (885 cycles). Considering only cycles with known implantation (100% or 0% implantation, $n = 1137$), a significant decrease in IR was observed as embryo categories decreased from A to E. More specifically: “A” 32%, “B” 28%, “C” 26%, “D” 20%, and “E” the lowest 17% $P<0.001$.

(Conaghan *et al.* 2013)

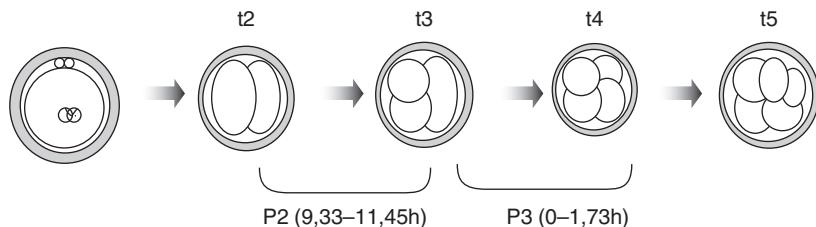


Figure 24.5 Description of the morphokinetic variables used for the first model of embryo selection reported by Conaghan *et al.* based on automatic annotations. Together with the drawings of the cleavage embryos, optimal time-ranges were included

Conaghan *et al.* [7] accomplished a two-phase multicenter study to develop and validate an algorithm to predict blastocyst formation. A total of 1,727 embryos were monitored by an automatic cell tracking software. The time between cytokinesis 1 and 2 (P2) and the time between cytokinesis 2 and 3 (P3) turned out to be the strongest parameters in the prediction model. The results indicated a higher probability of usable blastocyst formation when both P2 and P3 were within specific cell division timing ranges (P2, 9.33–11.45 h; and P3, 0–1.73 h) and a low probability when either P2 or P3 were outside the specific cell timing ranges. Through this model, the authors observed that usable blastocysts could be predicted with a specificity of 84.2% (95% CI = 78.7%–88.5%), sensitivity of 58.8% (95% CI = 47.0%–69.7%), positive predictive value (PPV) of 54.1% (95% CI = 42.8%–64.9%) and negative predictive value (NPV) of 86.6% (95% CI = 81.3%–90.6%). By comparison, the same prediction based on morphology alone was achieved with a specificity of 52.1% (95% CI = 39.7%–64.6%), sensitivity of 81.8% (95% CI = 70.6%–92.9%), PPV of 34.5% (95% CI = 31.5%–37.5%), and NPV of 90.9% (95% CI = 87.3%–94.5%). The authors concluded that the use of this algorithm significantly improved the specificity (84.2% vs 52.1%; $P < .0001$) and PPV (54.1% vs 34.5%; $P < .01$) of usable blastocyst predictions enabling embryologists to better discriminate which embryos would be unlikely to develop to blastocyst. Therefore, they recommend the adjunctive use of this algorithm to improved embryo selection (Figure 24.5).

The Conaghan model was tested retrospectively by a different group using a set of 1,519 transferred embryos with known clinical outcome [8]. According to the algorithm, embryos were classified into usable and non-useable. The difference in implantation rate between the usable group and the whole cohort was 30 percent indicating that implantation rates could increase using this model. In addition, the percentage of non-useable embryos that resulted in implantation was 50.6 percent causing concern regarding the discarding of viable embryos. Even though the Conaghan model was developed for blastocyst formation and the endpoint of this study was clinical outcome, the authors expressed that implanted embryos should derive from the usable embryo group and not from the non-useable group (or at least not in such a high proportion). The possible explanation, according to the authors, could be that the model is based on narrow time intervals.

24.3.2 Brief Overview of Morphokinetic Studies Published in 2016

Some groups have focused on the development of different algorithms.

Petersen et al. [9] presented a morphokinetic algorithm suitable for day 3 and concluded that it can be used independently of culture conditions and fertilization method providing predictive power.

Huang et al. [10] detailed several additional dynamic features of blastocyst expansion in human embryos generated from donor oocytes showing that it might be useful in ranking embryos.

Del Carmen Nogales et al. [11] elaborated an algorithm to exclude aneuploid embryos observing that morphokinetics are affected by chromosome aneuploidy and further analysis of the chromosome content reveals higher differences when the complexity in the chromosome disorders is increased.

Milewski et al. [12] created a model to predict the implantation of transferred embryos based on morphokinetic parameters of time-lapse monitoring and concluding that, embryo quality is not the only factor responsible for implantation, and, thus, the power of prediction of the considered model is not as high as in models for blastocyst formation.

Liu et al. [13] presented a time-lapse deselection model involving both qualitative and quantitative parameters for assessing embryos on day 3 that predicts day 3 embryo implantation potential and is applicable to all IVF embryos regardless of insemination method by using pronuclei fading (PNF) as the reference starting time point.

Motato et al. [14] proposed two multivariable models to classify embryos according to their probabilities of blastocyst stage and implantation in the largest dataset ever reported of human blastocysts.

Others have moved a step further focusing on different embryo behaviors that can only be analyzed using time lapse.

Kong et al. [15] investigated how different division behaviors affect cell number and developmental potential of day 3 embryos by time-lapse imaging. After excluding embryos with abnormal division behaviors, the developmental potential, implantation rate and live birth rate of day-3 embryos increased with cell number.

Bodri et al. [16] analyzed the rate of blastocyst collapse observed by time-lapse monitoring and determined its association with live birth concluding that blastocyst collapse pattern should not be evaluated alone without taking into account morphokinetic variables that are stronger predictors of reproductive outcome.

The aim of other groups has been to compare this new technology with conventional evaluation and to describe and certify the value provided by this new tool.

Adamson et al. [17] showed how the noninvasive TL-test adds valuable information to traditional morphologic grading demonstrating improved implantation rates in patients receiving day-3 embryo transfers based on the combined use of a TL-test alone and traditional morphology.

Kieslinger et al. [18] presented how embryo selection using the Eeva Test plus standard morphology on day 3 results in comparable pregnancy rates as conventional morphological embryo selection.

Aparicio-Ruiz et al. [19] correlated the different categories provided by a commercial diagnostic test with blastocyst formation, quality, implantation potential and ongoing pregnancy (OPR) for the purpose of validating the automatic annotations and the classification algorithm, independently from clinical features of the patient or day of transfer.

Goodman et al. [20] developed a study to determine if the addition of continuous morphokinetic data improves reproductive outcomes when all embryos are cultured in a closed system observing no significant improvement but an association of absence of multinucleation, timing of blastulation, and morphokinetic score with blastocyst implantation rates.

Herzberger et al. [21] evaluated time-lapse microscopy as a selection tool for single-embryo transfer on blastocyst stage showing no decrease in pregnancy rates compared to DET. However, transfers of two embryos increased the rate of multiple pregnancies.

Ahlstrom et al. [22] focused on fresh day-2 transfers observing that early cleavage in combination with fragmentation grade at 43–45 h should be considered when selecting between good quality embryos.

Costa-Borges et al. [23] presented that human embryos can be successfully cultured continuously from day 0 onward in single medium with no need to renew it on day 3 without affecting embryo morphokinetics or development and offering more stable culture conditions, as well as practical advantages and reduced costs for the IVF laboratory.

Other groups have used time lapse in different research fields.

Dal Canto et al. [24] compared the morphokinetic behavior of embryos developed from oocytes matured *in vivo* and *in vitro* suggesting only marginal differences in their morphokinetics.

Tejera et al. [25] evaluated correlations between oxygen consumption (OC) measurements before and after embryo cytokinesis, observing OC during embryo cleavages and combining that information with morphokinetics to relate to implantation potential suggesting that these measurements can be used as markers of embryo quality.

Selection of euploid embryos and even embryo gender has also been the target of some groups.

Patel et al. [26] observed that keeping track of time interval between two stages can help us recognize aneuploid embryos at an earlier stage and prevent their selection for transfer.

Balakier et al. [27] observed that most multinucleated embryos have the capacity for self-correction during early cleavage divisions and can develop into euploid blastocysts resulting in healthy babies.

Bar-El et al. [28] analyzed morphokinetic parameters to explore how blastomere biopsy interferes with the dynamic sequence of developmental events delaying the compaction and the blastulation of the embryos.

Bodri et al. [29] suggested that several expanded blastocyst stage morphokinetic parameters are associated with female embryo gender.

Some groups decided to focus their morphokinetic studies in specific patient populations.

Wu et al. [30] studied two populations: poor prognosis patients and embryos from egg donor-recipient cycles. In poor prognosis patients no differences in day-3 embryo scores, implantation and clinical pregnancy rates between EmbryoScope™ and standard embryology were observed. The EmbryoScope™, however, more than doubled embryology staff time. In egg donor cycles, embryos grown in the EmbryoScope™ demonstrated significantly poorer day-3 quality.

Demirel et al. [31] studied whether the oocytes retrieved from an ovary with an endometrioma would develop into embryos with aberrant timings of cleavage and poorer morphologic quality compared with sibling oocytes from the contralateral ovary with no

endometrioma in the same patient, concluding that removal of endometriomas before IVF is not a necessity in terms of better oocyte quality and development.

Kirkegaard et al. [32] studied to what extent patient-, and treatment-, related factors explain the variation in morphokinetic parameters underlining the importance of treating embryos as dependent observations and suggesting a high risk of patient-based confounding in retrospective studies.

24.4 Conclusions

Static observations obtained from standard microscopes have contributed significantly to the knowledge of embryo development; however, it is becoming more challenging to identify embryos with the highest implantation potential due to the static and notoriously subjective character of this type of morphological evaluation. The study of embryo kinetics through time-lapse technology has given rise to new markers for embryo selection representing a new and exciting powerful tool for viewing cellular activity and embryogenesis in a coherent and uninterrupted manner, otherwise not available through standard microscopy. The present chapter presents an overview of the most recent studies that describe the use of this new technology in the IVF laboratory. It is our opinion that standard morphological assessment should remain the gold standard to initiate embryo evaluation; however, if possible, it should be complemented with the detection of kinetic markers known to improve clinical results. This new approach will allow the embryologists to perform a more accurate and objective embryo selection and therefore the goal of a single embryo transfer slowly becomes more tangible.

References

1. Aparicio B, Cruz M, Meseguer M. Is morphokinetic analysis the answer? *Reproductive Biomedicine Online* 2013;27(6): 654–63.
2. Basile N, Caiazzo M, Meseguer M. What does morphokinetics add to embryo selection and in-vitro fertilization outcomes? *Curr Opin Obstet Gynecol* 2015 June;27(3):193–200.
3. Wong CC, Loewke KE, Bossert NL et al. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol* 2010;28(10): 1115–21.
4. Meseguer M, Herrero J, Tejera A et al. The use of morphokinetics as a predictor of embryo implantation. *Human Reproduction* 2011;26(10): 2658–71.
5. Rubio I, Galán A, Larreategui Z et al. Clinical validation of embryo culture and selection by morphokinetic analysis: a randomized, controlled trial of the EmbryoScope. *Fertil Steril* 2014;102(5): 1287–94. e5.
6. Basile N, Vime P, Florena M et al. The use of morphokinetics as a predictor of implantation: a multicentric study to define and validate an algorithm for embryo selection. *Hum Reprod* 2015 February;30(2): 276–83.
7. Conaghan J, Chen AA, Willman SP et al. Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: results from a prospective multicenter trial. *Fertil Steril* 2013;100: 412–9.
8. Kirkegaard K, Campbell A, Agerholm I et al. Limitations of a time-lapse blastocyst prediction model: a large multicentre outcome analysis. *Reproductive BioMedicine Online* 2014;2: 156–8.
9. Petersen BM, Boel M, Montag M, Gardner DK. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on Day 3. *Hum Reprod* 2016 October;31(10): 2231–44.
10. Huang T, Chinn K, Kosasa T, Ahn H, Kessel B. Morphokinetics of human

- blastocyst expansion in vitro. *Reproductive BioMedicine Online* 2016;33: 659–67.
11. Nogales MDC, Bronet F, Basile N et al. Type of chromosome abnormality affects embryo morphology dynamics. *Fertil Steril* 2017;107: 229–35.
 12. Milewski R, Czerniecki J, Kuczyńska A, Stankiewicz B, Kuczyński W. Morphokinetic parameters as a source of information concerning embryo developmental and implantation potential. *Ginekol Pol* 2016;87(10): 677–84.
 13. Liu Y, Chapple V, Feenan K, Roberts P, Matson P. Time-lapse deselection model for human day 3 in vitro fertilization embryos: the combination of qualitative and quantitative measures of embryo growth. *Fertil Steril* 2016;105(3): 656–62. e1.
 14. Motato Y, de los Santos, María José, Escriba MJ et al. Morphokinetic analysis and embryonic prediction for blastocyst formation through an integrated time-lapse system. *Fertil Steril* 2015.
 15. Kong X, Yang S, Gong F et al. The Relationship between cell number, division behavior and developmental potential of cleavage stage human embryos: a time-lapse study. *PloS one* 2016;11(4): e0153697.
 16. Bodri D, Sugimoto T, Serna JY et al. Blastocyst collapse is not an independent predictor of reduced live birth: a time-lapse study. *Fertil Steril* 2016;105(6): 1476–83 . e3.
 17. Adamson GD, Abusief ME, Palao L et al. Improved implantation rates of day 3 embryo transfers with the use of an automated time-lapse-enabled test to aid in embryo selection. *Fertil Steril* 2016;105(2): 369–75. e6.
 18. Kieslinger DC, De Gheselle S, Lambalk CB et al. Embryo selection using time-lapse analysis (Early Embryo Viability Assessment) in conjunction with standard morphology: a prospective two-center pilot study. *Hum Reprod* 2016;31: 2450–57.
 19. Aparicio-Ruiz B, Basile N, Albalá SP et al. Automatic time-lapse instrument is superior to single-point morphology observation for selecting viable embryos: retrospective study in oocyte donation. *Fertil Steril* 2016;106(6): 1379–85. e10.
 20. Goodman LR, Goldberg J, Falcone T, Austin C, Desai N. Does the addition of time-lapse morphokinetics in the selection of embryos for transfer improve pregnancy rates? A randomized controlled trial. *Fertil Steril* 2016;105(2): 275–85. e10.
 21. Haikin Herzberger E, Ghetler Y, Tamir Yaniv R et al. Time lapse microscopy is useful for elective single-embryo transfer. *Gynecological Endocrinology* 2016;32:1–3.
 22. Ahlstrom A, Park H, Bergh C, Selleskog U, Lundin K. Conventional morphology performs better than morphokinetics for prediction of live birth after day 2 transfer. *Reproductive Biomedicine Online* 2016;33:61–70.
 23. Costa-Borges N, Bellés M, Meseguer M et al. Blastocyst development in single medium with or without renewal on day 3: a prospective cohort study on sibling donor oocytes in a time-lapse incubator. *Fertil Steril* 2016;105(3): 707–13.
 24. Dal Canto M, Novara PV, Coticchio G et al. Morphokinetics of embryos developed from oocytes matured in vitro. *J Assist Reprod Genet* 2016;33(2): 247–53.
 25. Tejera A, Castelló D, de los Santos, Jose Maria et al. Combination of metabolism measurement and a time-lapse system provides an embryo selection method based on oxygen uptake and chronology of cytokinesis timing. *Fertil Steril* 2016 July;106(1): 119–26.
 26. Patel DV, Shah PB, Kotdawala AP et al. Morphokinetic behavior of euploid and aneuploid embryos analyzed by time-lapse in embryoscope. *Journal of Human Reproductive Sciences* 2016;9(2):112.
 27. Balakier H, Sojecki A, Motamedi G, Librach C. Impact of multinucleated blastomeres on embryo developmental competence, morphokinetics, and aneuploidy. *Fertil Steril* 2016 July;106: 119–26.
 28. Bar-El L, Kalma Y, Malcov M et al. Blastomere biopsy for PGD delays embryo

- compaction and blastulation: a time-lapse microscopic analysis. *J Assist Reprod Genet* 2016 November;33:149–1457.
29. Bodri D, Kawachiya S, Sugimoto T et al. Time-lapse variables and embryo gender: a retrospective analysis of 81 live births obtained following minimal stimulation and single embryo transfer. *J Assist Reprod Genet* 2016;33(5): 589–96.
30. Wu Y, Lazzaroni-Tealdi E, Wang Q et al. Different effectiveness of closed embryo culture system with time-lapse imaging (EmbryoScope TM) in comparison to standard manual embryology in good and poor prognosis patients: a prospectively randomized pilot study. *Reproductive Biology and Endocrinology* 2016;14(1):49.
31. Demirel C, Bastu E, Aydogdu S et al. The Presence of endometrioma does not impair time-lapse morphokinetic parameters and quality of embryos: a study on sibling oocytes. *Reprod Sci* 2016 August;23(8): 1053–57.
32. Kirkegaard K, Sundvall L, Erlandsen M et al. Timing of human preimplantation embryonic development is confounded by embryo origin. *Hum Reprod* 2016 February;31(2): 324–31.

Metabolomic Screening of Embryos to Enhance Successful Selection and Transfer

Paul J. McKeegan and Roger G. Sturmy

25.1 Introduction

A key role of cellular metabolism is the provision of energy, in the form of adenosine triphosphate (ATP). In the case of the embryo, energy demand is dynamic and highly regulated to facilitate developmental milestones such as fertilization, cleavage, embryonic genome activation (EGA), and blastocoel formation. Broadly, ATP demand increases throughout embryo development, most dramatically at the blastocyst stage to provide enough ATP for the Na^+/K^+ ATPase to pump fluid into the forming blastocoel and satisfy energy demands as the embryo initiates significant protein synthesis and true growth. However, embryos have considerable metabolic plasticity, in that they can adapt to sub-optimal conditions by shifting reliance from one energy substrate to another. In addition, an individual embryo has a unique pattern, or profile, of metabolism, which may be measured noninvasively through determination of the composition of spent culture medium. The extent to which metabolic profile maps on to key aspects of development, including viability, has been the subject of significant research, offering the alluring prospect of a noninvasive biomarker of embryo health.

While morphology and morphokinetics are the most commonly used methods of predicting oocyte and embryo quality in the clinic, there is a mature body of literature from numerous groups that suggest that noninvasive methods of metabolic profiling can offer accurate predictions of developmental capacity. Metabolic assays report on factors which cannot be seen by morphological assessment. For example, individual or groups of metabolites and substrates, such as amino acids, carbohydrates, or lipid, have been shown able to relate to blastocyst rate [1,2], implantation rate and pregnancy rate [3], levels of aneuploidy [4], and embryo sex [5]. However, emerging metabolomic methods may prove even more powerful.

25.2 What Is Metabolomics?

Metabolomics is distinctive from metabolic studies, despite the similar sounding names. This similarity emerges from a common root *metabole*, which derives from the ancient Greek μεταβολλειν and post-classical Latin *metabola*, which directly translates as “to change.” Metabolomics and metabolic assays share an inherent commonality in that they are related to the study of metabolism; however, there are critical differences in the details. **Metabolomics** refers to a collection of methods which attempt to assay the metabolome in part or in its entirety. The metabolome may be defined as the entire complement of compounds which are consumed and released by a tissue in a given time; in practical terms, this is often through one experimental procedure. In contrast to **metabolomics**,

metabolic assays measure a comparatively narrow range of specific substrates and pathways: for example, the glycolytic conversion of glucose to lactate.

In the case of embryos, the data produced from studies of metabolic activity reflect many factors, including, but not limited to epigenetic modification, gene expression, protein production, media composition, endogenous substrate availability, redox balance, culture conditions, and any possible environmental contaminants. It therefore reports, to an extent, on the transcriptome, epigenome, and proteome, since the metabolome is a downstream product of these. In ART, metabolomics can be regarded as the study of all measurable factors that are consumed and released by the gamete or embryo due to normal metabolism during development and stress responses. However, in order to fully exploit metabolomics data in an ART setting, a detailed knowledge of metabolic function is required. Much of our knowledge in this area has been arrived at through noninvasive assays.

25.3 Non-Invasive Assays

The majority of data collected to date on oocyte and embryo metabolism is from non-invasive, nontoxic assays. These approaches measure consumption and release of energy substrates and metabolites from culture media. Typically, oocytes or embryos are cultured singly in small droplets of culture media for a defined period of time. The spent media can then be analyzed and compared to control media drops which were prepared and incubated in the same way as the assay drops, but without the presence of oocytes or embryos. Studies exploiting these methods have generated a wealth of data on the turnover of single or groups of substrates, such as amino acids, glucose, pyruvate, and lactate, over the defined culture period. Such methods are powerful because they yield quantitative data on consumption or production of substrates under investigation. These approaches are, however, indirect, meaning that while we can measure depletion or appearance of compounds, we cannot easily track the fate of each molecule through the various pathways available to it. This can be done using radiolabeling to quantify the fate of radiolabeled C¹³-glucose, but using radioactive materials is clearly not suitable for clinical applications.

25.4 Sample Selection

Most publications tend to report analyses of consumption and release of metabolites and energy substrates from either oocyte or embryo culture media. This is an appealing approach, since it is a minimally invasive and nontoxic approach to assess each oocyte/embryo individually, making use of spent culture droplets that would be otherwise discarded. However, such approaches tend to rely on culturing embryos individually. Moreover, in the case of oocytes it is necessary to denude the cells of cumulus cells causing significant biological stress.

An added complication to performing noninvasive assays based on metabolic principles is that many commercially available media are not suitable as substrate concentrations are too high to detect changes. This is compounded by a reluctance to make available the precise composition of the medium. Many basic scientists and clinicians have called for ART media companies to share this information with the user, and the potential application of metabolomic technologies is another major impetus for this to happen [6].

25.5 Oocyte Media

Oocyte metabolic profiles differ depending on morphological quality and can provide information about oocyte-cumulus interactions. However, care must be taken in defining research questions and sample selection. For oocyte-specific metabolic profiles, denuded oocytes must be incubated individually for the assay in modified culture media, removing cumulus-oocyte substrate transfer. Measuring oocyte-cumulus complex (OCC) metabolism may be more physiologically relevant, but it is difficult to account for what contribution the oocyte makes to substrate consumption/release and to control for the varying number of cumulus cells. However, denuded oocytes and conjugate cumulus cells have different metabolic phenotypes, for example, denuded oocytes consume pyruvate, while OCCs have more balanced uptake of pyruvate and glucose [7,8,9,10]. For amino acid profiling, traditional serum-free *in vitro* maturation medium must be diluted with a salt solution to reduce amino acid concentrations to levels low enough to detect changes [11].

25.6 Embryo Media

Embryo culture media does not need to support somatic cells and hence can be simpler than oocyte media, comprising several salts, metal ions, and energy substrates. In the research lab, media is often made in-house and hence component concentrations are known and can be easily modified for consumption and release experiments. However, this feature is a challenge for the translation of metabolite-based assays into the clinical situation. Despite these challenges, significant data on embryo metabolism have been generated.

25.7 Energy Substrates

Probably the most widely used methods in the study of oocyte and embryo metabolism are based on those developed by Leese and Barton [12]. First established using fluorescent microscopy and hand-made nanolitre pipettes, this method has more recently been optimized to use a fluorometric plate reader, a much more high-throughput and user-friendly approach [2,13,14] with potential clinical application. Regardless of the detection method, the technique relies on the enzymatic metabolism of the substrate under investigation being coupling of the oxidation of NAD(P) to NAD(P)H. The corresponding change in the ratio of NAD(P):NAD(P)H can be detected by fluorescence. These classes of assays have been used to study metabolism of glucose, lactate, pyruvate, creatine, triglyceride, and glutamine [2,15,16,17].

Using these kinds of methods, we have been able to construct a contemporary picture of early embryo metabolism *in vitro*. In summary, there is an increase in pyruvate consumption throughout development, with a sharp rise in glucose consumption and lactate release at blastocyst stage. In addition to this, there is good evidence to link the range of several individual substrates to embryo viability in a range of species. Glucose is positively correlated to human embryo viability and grade [18], whereas intermediate pyruvate consumption during early cleavage stages predicts blastocyst development [19,20].

25.8 Amino Acids

Amino acids are a class of compounds that comprise an amine and carboxyl group. Although there are a large number of compounds that may be characterized as amino acids, the term is most usually applied to 20 specific members that are the basic chemical units of proteins. Each of these 20 proteinaceous amino acids has a unique side chain, or R group. In addition to serving as the building blocks of proteins, amino acids have diverse additional functions, including as energy substrates, osmolytes, anti-oxidants and electron carriers to name but a few. As a consequence, they are commonly added to embryo culture medium in varying ratios and concentrations. Their addition to embryo culture medium is seen as a significant contributor to improved outcomes of ART.

The measurement of amino acid depletion and/or appearance by embryos is typically carried out using high performance liquid chromatography (HPLC; see Sturmy et al. [21]). Early data using these approaches indicated that amino acid metabolism by early embryos may relate to a number of embryo characteristics, including sex [5], aneuploidy [4], oxidative stress [22], and, crucially, developmental capacity (1,3). Moreover, there is good evidence that such methods can be applied to oocytes, with Hemmings et al. [23] reporting that amino acid profile (AAP) of human oocytes reflects a number of features, including developmental competence and hormone regimen in superovulation. The method is appealing as a possible biomarker for embryo selection, since it requires relatively small amounts of media (1 μ l) and is highly sensitive, able to detect changes in amino acid concentrations in drops that contain single embryos or oocytes. However, translation to a clinical setting has been challenging, due to the timescales needed to perform assays, as well as the need for a number of organic solvents which are unsuitable for use in a clinical IVF environment.

25.9 Oxygen

While oocytes and embryos appear as glycolytic tissues, most ATP is still provided via oxidative phosphorylation (OXPHOS). OXPHOS occurs in the mitochondria and involves the coupling of electron transfer from TCA cycle intermediates FADH₂ and NADH to the synthesis of ATP from ADP and inorganic phosphate (Pi). The electrons flow through an electron transport chain or “respirasome” comprising four multiprotein complexes. The final electron acceptor in this chain is oxygen (O₂), forming water (H₂O). The energy released by this electron flow is used to transport protons (H⁺) from the mitochondrial matrix into the intermembrane space, forming an electrochemical gradient. This is dissipated by the spontaneous flow of protons through the F0 ion channel component of ATP synthase, releasing free energy to drive the mechanical rotation of the F1 head component of ATP synthase and the formation of ATP from ADP + Pi. This process is near ubiquitous in eukaryotic systems and is the principle means of generating metabolic energy.

There are two main ways to determine mitochondrial activity. The most simple approach is the measurement of the proton gradient in vitro by fluorescent staining with probes sensitive to inner mitochondrial membrane potential ($\Delta\psi_m$). Such approaches have been used extensively in the study of mammalian embryo development. In perhaps the best examples of using mitochondrial dyes to probe embryo metabolic function, Van Blerkom et al. [24] demonstrated how mitochondria play an important role in determining cell location during formation of the blastocyst. These roles were linked intrinsically to the

polarity of the mitochondria. However, despite the enormous value of mitochondrial dyes in basic research these probes are invasive, insomuch as they are taken up by the cell and hence have limited direct clinical relevance.

The second main way to measure mitochondrial function is by determining oxygen consumption. As described, oxygen acts as the terminal electron acceptor of the electron transport chain and so depletion of oxygen is directly linked to mitochondrial function. Oxygen consumption rate (OCR) was first reported on rabbit ova by Fridhandler et al. [25]. Subsequent to this, OCR of oocytes and embryos has been determined by a number of researchers, in a range of species. Of the more notable studies, Tejera et al. [26] reported OCR is a marker of quality for oocytes in humans, building on data from Scott et al. [27], who reported that embryo OCR is affected by ovarian stimulation treatment, maternal age and FSH concentration. A number of noninvasive techniques have aimed to measure oxygen consumption of embryos as sensitively as possible. Nanorespirometry exploits miniaturized Clark-type oxygen electrodes to measure the gradient of oxygen concentrations created when an embryo is cultured in a static column of culture media. The technique can measure OCR of single oocytes or embryos and does not affect the viability of embryos [28]. However, this technique is impractical in the clinic as it is technically challenging, time-consuming and ethical policies require that a separate probe must be used for each patient. Recently, a chip-based system for monitoring embryo respiration has been developed [29]. This is a highly relevant step that enables the determination of OCR by single human embryos, cultured in commercially available embryo culture medium.

25.10 Omic Approaches

Applying metabolomics to ART allows us to move away from single-target approaches towards a better understanding of overall metabolism. A hallmark of metabolomic approaches is that a very large number of metabolites and substrates are investigated concurrently. There are several approaches, including targeted and non-targeted methods. However, in carrying out metabolomics studies on oocytes and early embryos, there are a number of significant challenges that must be overcome. Unlike the more mature omic-methodologies of genomics, transcriptomics, and proteomics, the diversity of targets that make up the metabolome is vast, across different chemical characteristics such as varying hydrophobicity, charge, size, and abundance, which may differ by orders of magnitude. These challenges are amplified by the volume of data generated by metabolomics work streams, meaning that data interrogation and interpretation often requires a systems-based approach. However, considerable effort to overcome these challenges is ongoing, and given the amount of data that may be generated by metabolomic approaches, the prospect of noninvasive screening biomarker for embryo selection remains tantalizingly possible.

25.11 Vibrational Spectroscopy

Many of the metabolomics techniques presently available are unsuitable for clinical application in their current form. However, rotational-vibrational spectroscopy (often referred to as vibrational spectroscopy) has been viewed as particularly promising. In simple terms, vibrational spectroscopy determines the vibrational interaction of a species in response to electromagnetic radiation. Typically, this radiation is in the form of infrared (IR) light. IR covers a wavelength of $10,000\text{--}10\text{ cm}^{-1}$ but may be further subdivided into near-IR $10,000\text{--}4,000\text{ cm}^{-1}$ (NIR) and Fourier Transform IR (FTIR), as

well as Raman spectroscopy. Molecules are typically analyzed in the gas phase. Vibrational spectroscopy is mostly concerned with using IR to measure changes in vibrational energy level in molecules, while rotational spectroscopy relies on microwave radiation to measure transitions to higher rotational energy levels. These energy levels are discrete, defined measurable packets of energy. Data are resolved into lines to form a spectrum for data interpretation. Raman spectroscopy is widely used to generate an identifying “fingerprint” for chemical species. It relies on “Raman” scattering of light from the visible, near-IR, or near-UV spectra. The laser beam interacts with vibrations within the system, shifting the laser photons to higher energy levels. These methods were originally restricted to analyses of small molecules, but advances over the last 30 years have allowed analysis of mixtures of complex biomolecules.

There was initial excitement at the early reports of the application of spectroscopic approaches in the selection of viable embryos, due, in large part, to the findings reported by Seli et al. [30,31]. In both retrospective and prospective studies, early data demonstrated clear association between metabolic signatures and reproductive potential. This was subsequently validated with a degree of independence [32]. Rather than targeting individual metabolites, a “viability index” was devised that predicted outcome based on the overall profile. However, disappointingly, in a randomized controlled trial, the approach was unable to reproduce links between spectroscopic profiles of embryo culture medium and reproductive outcome. A more sensitive targeted approach may be more successful, which will be informed by our continued increased understanding of metabolic processes during the first stages of development.

25.12 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) spectroscopy is a bioanalytical method that detects compounds based on the magnetic resonance properties, or spin, of the nuclei of atoms. Not all atoms contain nuclei with an appropriate resonance for exploitation in NMR; however, hydrogen and carbon are among those that do. Consequently, NMR is suitable for the measurement of organic compounds. Although the underlying biophysics is complex, in broad terms, when placed in a magnetic field, nuclei with an appropriate spin value align themselves to the field. Radio waves are then applied, causing the nuclei to change energy state. The change in energy state may then be detected and generate a spectrum, which is specific for a given compound. Unlike IR Spectroscopy, NMR is able to identify compounds and allow a degree of quantification. NMR is attractive since it may be used to analyze solids or liquids and so is directly applicable for spent droplets of embryo culture medium.

There are reports of the application of NMR in the analysis of the metabolism of embryos. Pudakalakatti et al. [33] assessed the concentration of a number of compounds in spent human embryo culture medium and were able to show that reduced levels of implantation were associated with embryos that had a lower ratio of pyruvate: alanine. Subsequent studies have indicated associations with genetic instability in embryos and concentrations of a range of compounds in spent medium, all measured by NMR spectroscopy [34]. Importantly, Wallace et al. [35] generated data indicating that, on the basis of NMR spectroscopy, it was possible to identify differences in metabolite ratios of spent medium between embryos that were able to implant with those that did not. However, by contrast, Nadal-Desbarats et al. [36] reported that no significant differences were detectable between the NMR spectra of embryos that implanted and those that did not. These data are

important in further contributing to the evidence base that metabolic activity of embryos offers insight into their phenotype and viability; however, the sensitivity of current proton-NMR technologies seems insufficient for robust clinical analyses.

25.13 Lipidomics and Mass Spectrometry

In recent years, the composition of the lipid store and regulation of lipid metabolism has become a significant area of interest [37,38]. The reduction in fertility with dyslipidemia, due in part to the global obesity epidemic, as well as conditions such as metabolic syndrome, diabetes mellitus, and polycystic ovarian syndrome, implies that greater understanding of the link between lipid metabolism, fertility, and adult health might be important for contemporary and future fertility treatment [39]. The oocyte has a relatively large lipid store in the form of triglyceride (TG), a nonpolar esterified lipid class formed of three fatty acids bound to glycerol. This TG is a vital energy store during oocyte maturation and early embryo development. However, it is not yet possible to assess total TG by noninvasive means. The total amount of TG and the fatty acid composition reflects the fatty acid composition of the follicular fluid, itself dependent on blood plasma content. Oocyte fatty acid composition is directly dependent on diet and health. In addition to these key roles in the oocyte and embryo, lipids also have several other vital roles in mammalian cells, including key components of the membrane, cell signaling, and posttranslational modification of proteins.

An extension of metabolomics, lipidomics refers to the full characterization of lipid species, including their biological roles [40]. The complexity of physiological lipid mixtures and the specific chemical properties of lipid classes require an analytical approach distinct from that of other biomolecules.

Lipidomics, or lipid profiling, often combines chromatography with tandem MS/MS. First, complex mixtures of lipids are separated by chromatography, with different types of chromatography used dependent on the lipid class of interest; for example, thin layer chromatography can be used to separate lipids based on class which are then analyzed by MS. Liquid chromatography offers greater resolution, normal phase HPLC can separate based on lipid class, while reverse-phase HPLC can separate based on carbon chain length and the number of double bonds present, allowing resolution of lipids of similar chain lengths based on the degree of unsaturation. Coupling a normal-phase fractionation to a reverse phase resolution offers a more complete profile of lipids [41]. Gas chromatography (GC) can also be used for lipidomic studies and can be particularly useful for study of fatty acid metabolism. Tandem or “hyphenated” MS/MS involves performing an initial mass spectrometry analysis to separate species based on mass/charge ratios, followed by a second mass spectrometry analysis to fragment and identify the species separated in the first MS.

Data are slowly emerging on the potential of lipidomic approaches in reproductive biology. Vilella et al. [42] discussed the possibility of utilizing lipidomic approaches in identifying endometrial receptivity. Proof of principle was recently demonstrated by Belaz et al. [43], who, using a bovine model, were able to identify differences in the phospholipid composition in the uterus, which retrospectively correlated with uterine receptivity. This emerging technology will undoubtedly generate significant data in the coming years.

25.14 The Future of Omics-Technologies

A recent Cochrane systematic review [44] was unable to demonstrate any selective advantage of using metabolomic approaches compared to using conventional selection tools. However, importantly, the review concluded that this failure to demonstrate association was due, in large part, to inherent bias in many of the studies included, which meant that the evidence available on the value of metabolomics approaches for embryo selection is of poor quality. Despite this, however, embryo metabolism is unquestionably related closely to physiology and can provide a window into a range of aspects of phenotype. There remain a number of credible reports that link metabolism with viability, and these have emerged from various laboratories. Thus, with a concerted, coordinated approach with continued effort and innovation, combined with well-designed, properly executed studies, the prospect of a marker of embryo viability on the basis of metabolism remains achievable.

References

1. Houghton, F.D., Hawkhead, J.A., Humpherson, P.G. et al., 2002. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Human Reproduction* (Oxford, England), **17**(4), pp. 999–1005.
2. Guerif, F., McKeegan, P.J., Leese, H.J. & Sturmy, R.G., 2013. A simple approach for COnsumption and RElease (CORE) analysis of metabolic activity in single mammalian embryos. *PloS one*, **8**(8), p. e67834.
3. Brison, D.R., Houghton, F.D., Falconer, D. et al., 2004. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Human Reproduction* (Oxford, England), **19** (10), pp. 2319–24.
4. Picton, H.M., Elder, K., Houghton, F.D. et al., 2010. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. *Molecular Human Reproduction*, **16**(8), pp. 557–69.
5. Sturmy, R.G., Bermejo-Alvarez, P., Gutierrez-Adan, A. et al., 2010. Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. *Molecular Reproduction and Development*, **77**(3), pp. 285–96.
6. Sunde, A., Brison, D.R., Dumoulin, J.C.M. et al., 2016. Time to take human embryo culture seriously. *Human Reproduction*, **31** (10), pp. 2174–82.
7. Zuelke, K.A. & Brackett, B.G., 1992. Effects of luteinizing hormone on glucose metabolism in cumulus-enclosed bovine oocytes matured in vitro. *Endocrinology*, **131**(6), pp. 2690–96.
8. Downs, S.M. & Utecht, A.M., 1999. Metabolism of radiolabeled glucose by mouse oocytes and oocyte-cumulus cell complexes1. *Biology of Reproduction*, **60**(6), pp. 1446–52.
9. Downs, S.M. & Hudson, E.D., 2000. Energy substrates and the completion of spontaneous meiotic maturation. *Zygote* (Cambridge, England), **8**(4), pp. 339–51.
10. Sutton, M.L., Gilchrist, R.B. & Thompson, J.G., 2003. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Human Reproduction Update*, **9**(1), pp. 35–48.
11. Hemmings, K.E., Leese, H.J. & Picton, H. M., 2012. Amino acid turnover by bovine oocytes provides an index of oocyte developmental competence in vitro1. *Biology of Reproduction*, **86**(5), p. 165.
12. Leese, H.J. & Barton, A.M., 1984. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *Reproduction*, **72**(1), pp. 9–13.

13. Maruthini, D., Harris, S.E., Barth, J.H. et al., 2014. The effect of metformin treatment in vivo on acute and long-term energy metabolism and progesterone production in vitro by granulosa cells from women with polycystic ovary syndrome. *Human Reproduction*, **29**(10), pp. 02–2316.
14. Marei, W.F.A., De Bie, J., Mohey-Elsaeed, O. et al., 2017. Alpha-linolenic acid protects the developmental capacity of bovine cumulus-oocyte complexes matured under lipotoxic conditions in vitro†. *Biology of Reproduction*, **96**(6), pp. 1181–96.
15. Gardner, D.K. & Leese, H.J., 1987 April. Assessment of embryo viability prior to transfer by the noninvasive measurement of glucose uptake. *Journal of Experimental Zoology*, **242**(1), pp. 103–5.
16. Ferguson, E.M. & Leese, H.J., 1999. Triglyceride content of bovine oocytes and early embryos. *Reproduction*, **116**(2), pp. 373–78.
17. Forsey, K.E., Ellis, P.J., Sargent, C.A., Sturmey, R.G. & Leese, H.J., 2013. Expression and localization of creatine kinase in the preimplantation embryo. *Molecular Reproduction and Development*, **80**(3), pp. 185–92.
18. Gardner, D.K., Wale, P.L., Collins, R. & Lane, M., 2011. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. *Human Reproduction*, **26**(8), pp. 1981–86.
19. Conaghan, J., Hardy, K., Handyside, A.H., Winston, R. M., & Leese, H.J., 1993. Selection criteria for human embryo transfer: a comparison of pyruvate uptake and morphology. *Journal of Assisted ...*, **10**(1), pp. 21–30.
20. Leese, H.J., Guerif, F., Allgar, V. et al., 2016. Biological optimization, the Goldilocks principle, and how much is lagom in the preimplantation embryo. *Molecular Reproduction and Development*, **83**(9), pp. 748–54. doi:10.1002/mrd.22684.
21. Sturmey, R.G., Brison, D.R., Leese, H.J., 2008 October. Symposium: innovative techniques in human embryo viability assessment. Assessing embryo viability by measurement of amino acid turnover. *Reprod Biomed Online*, **17**(4), pp. 486–96.
22. Gardner, D.K. & Wale, P.L., 2013. Analysis of metabolism to select viable human embryos for transfer. *Fertility and Sterility*, **99**(4), pp. 1062–72.
23. Hemmings, K.E., Maruthini, D., Vyjayanthi, S. et al., 2013 April. Picton HM. Amino acid turnover by human oocytes is influenced by gamete developmental competence, patient characteristics and gonadotrophin treatment. *Hum Reprod*, **28**(4), pp. 1031–44.
24. Van Blerkom, J., Cox, H. & Davis, P., 2006. Regulatory roles for mitochondria in the peri-implantation mouse blastocyst: possible origins and developmental significance of differential DeltaPsim. *Reproduction (Cambridge, England)*, **131**(5), pp. 961–76.
25. Fridhandler, L., Hafez, E.S.E. & Pincus, G., 1957. Developmental changes in the respiratory activity of rabbit ova. *Experimental Cell Research*, **13**(1), pp. 132–39.
26. Tejera, A., Herrero, J., de Los Santos, M.J. et al. 2011. Oxygen consumption is a quality marker for human oocyte competence conditioned by ovarian stimulation regimens. *Fertility and Sterility*, **96**(3), pp. 618–23.e2.
27. Scott, L., Berntsen, J., Davies, D. et al., 2008. Human oocyte respiration-rate measurement – potential to improve oocyte and embryo selection? *Reproductive BioMedicine Online*, **17**(4), pp. 461–69.
28. Lopes, A.S., Larsen, L.H., Ramsing, N. et al., 2005. Respiration rates of individual bovine in vitro-produced embryos measured with a novel, non-invasive and highly sensitive microsensor system. *Reproduction (Cambridge, England)*, **130**(5), pp. 669–79.
29. Kurosawa, H., Utsunomiya, H., Shiga, N. et al. 2016. Development of a new clinically applicable device for embryo evaluation which measures embryo oxygen

- consumption. *Human Reproduction* (Oxford, England), **31**(10), pp. 2321–30.
30. Seli, E., Sakkas, D., Scott, R. et al., 2007. Noninvasive metabolomic profiling of embryo culture media using Raman and near-infrared spectroscopy correlates with reproductive potential of embryos in women undergoing in vitro fertilization. *Fertility and Sterility*, **88**(5), pp. 50–1357.
31. Seli, E., Vergouw, C.G., Morita, H. et al., 2010. Noninvasive metabolomic profiling as an adjunct to morphology for noninvasive embryo assessment in women undergoing single embryo transfer. *Fertility and Sterility*, **94**(2), pp. 535–42.
32. Vergouw, C.G., Botros, L.L., Judge, K. et al., 2011. Non-invasive viability assessment of day-4 frozen–thawed human embryos using near infrared spectroscopy. *Reproductive BioMedicine Online*, **23**(6), pp. 769–76.
33. Pudakalakatti, S.M., Uppangala, S., D’Souza, F. et al., 2013. NMR studies of preimplantation embryo metabolism in human assisted reproductive techniques: a new biomarker for assessment of embryo implantation potential. *NMR in Biomedicine*, **26**(1), pp. 20–27.
34. D’Souza, F., Pudakalakatti, S.M., Uppangala, S. et al. 2016. Unraveling the association between genetic integrity and metabolic activity in pre-implantation stage embryos. *Scientific Reports*, **6**, p. 37291.
35. Wallace, M., Cottell, E., Cullinane, J. et al., 2014. ^1H NMR based metabolic profiling of day 2 spent embryo media correlates with implantation potential. *Systems Biology in Reproductive Medicine*, **60**(1), pp. 58–63.
36. Nadal-Desbarats, L., Veau, S., Blasco, H. et al., 2013. Is NMR metabolic profiling of spent embryo culture media useful to assist in vitro human embryo selection? *Magnetic Resonance Materials in Physics, Biology and Medicine*, **26**(2), pp.193–202.
37. McKeegan, P.J. & Sturmy, R.G., 2012. The role of fatty acids in oocyte and early embryo development. *Reproduction, Fertility, and Development*, **24**(1), pp. 59–67.
38. Dunning, K.R., Russell, D.L., Robker, R.L. et al., 2014. Lipids and oocyte developmental competence: the role of fatty acids and oxidation. *Reproduction*, **148**(1), pp. R15–R27.
39. Leary, C., Leese, H.J. & Sturmy, R.G., 2015. Human embryos from overweight and obese women display phenotypic and metabolic abnormalities. *Human Reproduction* (Oxford, England), **30**, pp. 122–32.
40. Lagarde, M., Géloën, A., Record, M., Vance, D. & Spener, F., 2003. Lipidomics is emerging. *Biochimica et Biophysica Acta (BBA) – Molecular and Cell Biology of Lipids*, **1634**(3), p. 61.
41. Roberts, L.D., McCombie, G., Titman, C. M. & Griffin, J.L., 2008. A matter of fat: An introduction to lipidomic profiling methods. *Journal of Chromatography B*, **871**(2), pp. 174–81.
42. Vilella, F., Ramirez, L.B. & Simón, C., 2013. Lipidomics as an emerging tool to predict endometrial receptivity. *Fertility and Sterility*, **99**(4), pp. 1100–06.
43. Belaz, K.R.A., Tata, A., França, M.R. et al. 2016. Phospholipid profile and distribution in the receptive oviduct and uterus during early diestrus in cattle. *Biol Reprod*, **95**(6), pp.1–11.
44. Siristatidis, C., Sergentanis, T.N., Vogiatzi, P. et al., 2015. In vitro maturation in women with vs. without polycystic ovarian syndrome: A systematic review and meta-analysis F. Qu, ed. *PLOS ONE*, **10**(8), p.e0134696.

Preimplantation Genetic Screening of Embryos for IVF

Joyce Harper and Carmen Rubio

26.1 Introduction

If a couple present with a known genetic or chromosomal abnormality that could be passed on to their children, a genetic counselor should discuss their reproductive options. The majority of couples would undertake prenatal diagnosis, either amniocentesis or chorionic villi sampling, and more recently non-invasive prenatal testing (NIPT). For all three options, the test is performed on an established pregnancy and if the results show that the fetus is affected, the couple has to decide if they wish to continue with the pregnancy or undergo a termination.

Preimplantation genetic diagnosis (PGD) offers an alternative for these couples, where the genetic test is performed on the preimplantation embryo. In PGD, couples go through routine IVF/ICSI procedures to generate embryos *in vitro*, the embryos are biopsied, and the genetic test is performed on the biopsied cells. PGD therefore allows embryos that are free from the genetic disease to be transferred to the uterus, so the pregnancy is started knowing that the fetus is unaffected with the disease.

The first PGD cycles were performed in the late 1980s and involved embryo sexing for patients carrying X linked diseases [1]. With improvements in diagnostic techniques, PGD was extended to single gene defects and chromosome abnormalities.

In the mid-1990s, two teams applied PGD technology to certain groups of patients going through IVF with the aim of determining which embryos were chromosomally normal ([2] and [3]). This technique is called preimplantation genetic screening (PGS) or PGD-aneuploidy (PGD-A). PGS has been applied to women of advanced maternal age, those with repeated implantation failure, patients with repeated miscarriage where the karyotypes of the parents is normal, young egg donors, male factor infertility, and in some cases patients with no indication. PGS (version 1) was initially performed on cleavage stage embryos using fluorescent *in situ* hybridization (FISH), but 11 randomized controlled trials (RCTs) showed that the technique was ineffective, probably due to the high level of chromosomal mosaicism seen in cleavage stage embryos and the inefficiency of the FISH technique [4]. PGS version 2.0 mainly uses blastocyst biopsy and genetic tests that enable analysis of all chromosomes, including array comparative genomic hybridization (aCGH) and genome sequencing. Four studies have indicated that PGS v2 may improve outcomes in mainly good prognosis patients.

In this chapter, we will detail the history of PGS, the methods used, and what the future holds.

26.2 Embryo Biopsy

There are few studies evaluating the efficacy and safety of any stage of biopsy, but it is thought that removing any cells from the embryo may have an effect on implantation. There are three stages when cells can be biopsied from embryos for PGD and PGS: polar body, cleavage, and the blastocyst stage [5]. All three stages have advantages and disadvantages, but the majority of PGS cycles worldwide are now performed using blastocyst biopsy [6].

To ensure an efficient and cost-effective PGS service, in most cycles the biopsied embryos are frozen so that the samples can be batched for a cheaper diagnosis and to allow more time for analysis. In some cases of PGS, embryo banking is used. This is where the couple go through multiple cycles of egg collection and vitrification of embryos prior to biopsy. Batching of embryos ensures a sufficient number of embryos are available before biopsy and diagnoses are carried out, increasing the chance of finding a chromosomally abnormal embryo for transfer.

26.2.1 Polar Body Biopsy

For polar body biopsy, both the first and the second polar bodies are required [5]. These can be removed either simultaneously or sequentially. Simultaneous biopsy is easier to perform and is less invasive to the embryo, but it does not differentiate between polar body 1 and 2 and sometimes the first polar body may have degenerated by the time the embryo is biopsied. Sequential biopsy ensures better integrity of the polar bodies. Sequential biopsy involves the biopsy of the first polar body on the day of the egg collection and biopsy of the second polar body at fertilization.

The most common method to remove the polar bodies is to use a laser to make a small hole in the zona through which the polar bodies can be aspirated.

The advantages of polar body biopsy are that the maternal chromosomes are analyzed and these are more likely to be involved with aneuploidy. Also polar body biopsy gives up to four days for the diagnosis if a blastocyst transfer is being performed. This can make the management of the PGS cycle much easier, especially if the diagnosis is being done in another location.

The disadvantages of polar body biopsy are that the technique is time-consuming to the embryology lab due to the timing of the release of the second polar body, and paternal genes and chromosomes cannot be analyzed, post zygotic mosaicism cannot be detected, and the polar bodies are small and fragile to handle. It can also be expensive as there are numerous polar bodies to analyze. If a patient has 10 eggs collected, there will be 20 samples. For blastocyst biopsy, we would expect to obtain five blastocysts from 10 eggs, so a quarter of the samples need to be analyzed in blastocyst biopsy.

Polar body biopsy has been popular in countries that do not allow later stages of biopsy due to ethical restraints.

26.2.2 Cleavage Stage Biopsy

Cleavage stage biopsy is performed on day 3 when the embryo is about 6–8 cells [5]. Usually a laser is used to drill a hole in the zona pellucida and one blastomere is aspirated. Tyrodes Acid and mechanical methods have been used to make a hole in the zona, but are rarely used these days as the laser is quicker and more efficient. Blastomeres at this stage are starting to

undergo compaction and so $\text{Ca}^{2+}\text{Mg}^{2+}$ medium is used to help break the junctions between the cells to make the biopsy easier.

The advantages of cleavage stage biopsy are that both maternal and paternal chromosomes can be examined and the procedure is relatively straightforward to perform. The disadvantages are cell lysis due to compaction, a shorter time for diagnosis and chromosome mosaicism which is highest in cleavage stage embryos from between 5 and 90 percent depending on age, embryo quality, culture conditions, and stimulation.

26.2.3 Blastocyst Stage Biopsy

Blastocyst biopsy is the most common stage of biopsy for PGS. As PGS is a selection method, growing embryos to the blastocyst stage is already one level of selection. It is also cost-effective for the reasons stated above.

There are two ways of removing trophectoderm cells. The hole can be made on day 3 and the embryo left to develop. During this time, some of the embryo will start to expand through the hole and these cells can easily be removed on day 5 of development. This is a quick method but problems can arise if the inner cell mass starts to herniate through the hole. The second method involves drilling the hole on the morning of day 5, ensuring that the hole is away from the inner cell mass. The trophectoderm cells can either be left to herniate or gently aspirated through the hole. In both methods, either a laser or mechanical means is used to separate the trophectoderm cells from the rest of the blastocyst.

The advantage is that there are normally about five cells available for the diagnosis and thus it is cheaper as there are less cells to biopsy and less to analyze. The disadvantages are that there might not be any blastocysts to biopsy and there is a shorter time for analysis unless vitrification is used.

26.2.4 Blastocoel Fluid Biopsy

There has been some success in aspirating a small amount of blastocoel fluid and using this for the genetic testing [7]. The advantage of this is that it does not remove any of the cells from the embryo. The disadvantage is that the technique has not been validated.

26.3 Genetic Testing

26.3.1 PGS Version 1.0

For many years, PGS involved the use of FISH. The first reports from the mid-1990s used just three FISH probes, but as the technique developed and commercial probes became available, an increasing number of FISH probes were used, increasing from 3 to 5 and up to 12. Some groups used 5 FISH probes in two or three rounds of re-probing to allow analysis of more chromosomes. However the technique was done, the techniques became less efficient when more probes were used [8].

Initial studies were mostly retrospective analysis, but from 2005 onwards, RCTs started to report that the PGS treatment group did not show an increase in live birth rate. In 2007, the first RCT was published that showed a significant decrease in live birth rate after PGS. By 2010, 11 RCTs mainly using cleavage stage biopsy and FISH for patients of advanced maternal age showed no benefit and many societies produced position statements that PGS using FISH and cleavage stage biopsy should stop [4]. It was suggested that PGS

version 1 did not work because of the high levels of mosaicism seen at cleavage stages and the inefficiency of the FISH procedure. However, several aspects related to good practice could explain the optimal results achieved by some groups [9]. Additionally, two RCTs with FISH have shown improved live birth rates with day-3 biopsies compared to the transfer of untested blastocyst [10].

At this time, new developments in the field of genetic testing using array comparative genomic hybridization came onto the market to allow the development of PGS version 2.0.

26.3.2 PGS Version 2.0

PGS v2.0 applies to diagnostic techniques that analyze all chromosomes. This includes aCGH, quantitative fluorescence-polymerase chain reaction (QF-PCR), and next generation sequencing (NGS).

aCGH technology allows for the analysis of chromosome copy number variations in DNA from an embryo compared to a reference sample. A single blastomere or 4–6 trophectoderm cells are amplified (whole genome amplification [WGA]), and the amplified DNAs from each embryo and controls are labeled with different fluorescent probes, combined, and then hybridized onto a slide containing specific bacterial artificial chromosome (BAC) probes across the length of the chromosomes with 1Mb coverage. Chromosome loss or gain is revealed by the color adopted by each spot after hybridization. Fluorescence intensity is detected using a laser scanner and specific software for data processing from which whole chromosome aneuploidy and sub-chromosomal structural imbalances can be analyzed.

Several studies have used QF-PCR for comprehensive chromosome screening and two small studies have been reported [11,12]. These studies have shown an increase in implantation rates with blastocyst biopsy compared to blastocyst transfer without aneuploidy screening.

The decrease in the cost of genome sequencing has positioned NGS as one of the most promising platforms for the study of not only aneuploidies but also small gene duplications/deletions and genetic disorders [13]. For NGS, most extended protocols share the first steps of the aCGH protocols, starting with WGA. This is followed by barcoding, in which the different samples are labeled with unique sequences, in a way that they can be mixed later and sequenced as they can be individually identified. This barcoding process allows pooling from 24 to 96 biopsies in a sequencing run, optimizing the cost per sequenced embryo. After sequencing, each sequence is aligned with a reference human genome and copy number variations for whole chromosomes and small deletions/duplications are established by the specific software. The depth of sequencing is also an important aspect to consider, especially for the simultaneous study of aneuploidies and gene disorders, which would need high coverage on those regions of interest.

In addition, NGS can detect lower mosaicism degrees in trophectoderm biopsies than previous technologies [14]. However, the current bioinformatic analysis of sequencing data finds difficulties to clearly discriminate low degree mosaicism from the experimental noise related to the quality and quantity of the biological samples and amplification artifacts. There is consensus among most groups to report mosaicism over 30 percent and to consider transfer of embryos with low mosaicism if there are not totally euploid embryos available.

26.4 Indications

The main goals for most of the indications are not only to increase implantation and pregnancy rates but also to decrease miscarriages and the risk of aneuploid offspring, as well as to decrease the time to conceive. More recently, the cost-efficiency per healthy baby at home has been also considered with blastocyst biopsy and NGS; the cost of PGS would not be a limitation any more [15].

The following are the most common current indications:

26.4.1 Advanced Maternal Age (AMA)

Maternal age is a major factor in the prevalence of aneuploidy. Most of the groups have traditionally considered couples as AMA if the woman is over 38 years of age, despite a trend to lower the cut-off to 35 years. Without the ability to screen for aneuploidy, AMA patients with a high percentage of aneuploid embryos may be subjected to multiple unsuccessful embryo transfers for months, some of which may end in distressing miscarriages and associated medical risks. With the introduction of NGS, the cost of PGS is becoming increasingly affordable and enables embryo chromosome analysis in IVF at a lower cost.

None of the RCTs in AMA patients using PGS 1.0 showed clinical improvement for these patients (reviewed by [16]). However, some more recent studies have shown a decrease in miscarriage rates and delivery rates per fresh embryo transfer [10,15].

26.4.2 Recurrent Miscarriage (RM)

The definition of RM varies by country, and is considered as the occurrence of 2, 3, or more consecutive miscarriages with a gestational age up to 14 weeks. For the purpose of PGS, other causes of miscarriage should be discarded before indicating this treatment, with a proper infertility work-up. Despite no RCTs having been conducted for this indication, retrospective studies have reported significantly higher implantation rates in RM couples with 2–4 previous miscarriages, more so when they had suffered previously aneuploid miscarriages. A systematic review suggested that the miscarriage rate might be lower after undergoing PGS [17].

26.4.3 Repetitive Implantation Failure (RIF)

RIF is defined as three or more failed IVF attempts or failed IVF treatments after the cumulative transfer of more than 10 good-quality embryos. One RCT with PGS 1.0 concluded no significant differences in clinical pregnancy rates [18]. An RCT including more chromosomes showed a clear trend towards a better live birth rate with PGS [10]. More recently, a retrospective study with aCGH has shown the impact of different factors that could affect clinical outcomes, with the best prognosis observed in younger patients with a sperm concentration lower than 10 million sperm/mL, and with more than 15 MII oocytes. The number of previous failed cycles only increases the probability of couples producing embryos with a complex division pattern but did not impact their overall clinical implication [19].

26.4.4 Severe Male Factor Infertility (MF)

Severe oligozoospermia has been related to increased risk of sperm aneuploidy. Also, it has been studied how different types of sperm chromosomal aneuploidies are translated in the embryos, following a similar pattern, with increased trisomy for sex chromosome in sperm samples with increased sex chromosome disomies and higher triploidy rates in embryos from sperm samples with increased diploidy rates [20].

In blastocyst biopsies, a significant increase in the occurrence of sex chromosome abnormalities was observed compared to embryos derived from normal semen samples. Aneuploidy rates in embryos derived from sperm with normal parameters were not significantly different, whether ICSI or standard insemination was used to achieve fertilization. These results highlight severe male factor infertility as a possible indication for PGS [21].

26.4.5 Previous Trisomic Pregnancy (PTP)

Some studies suggest that a history of a trisomic pregnancy is associated with an increased risk of a further aneuploid conception. The incidence of chromosomal abnormalities in preimplantation embryos was reported to be significantly higher in individuals with a previous aneuploid conception [22].

26.4.6 Good Prognosis Patients

Trophectoderm biopsy using aCGH has also been applied in good-prognosis patients with a high potential to increase live birth rates and to decrease multiple pregnancies by enabling euploid single-embryo transfer (SET). The first pilot RCT on a small number of patients comparing SET at blastocyst stage with and without aCGH in good-prognosis patients showed an aneuploidy rate of 44.9 percent among biopsied blastocysts, with a significantly higher clinical pregnancy rate in the comprehensive chromosome screening group (70.9% vs 45.8%, $p = 0.017$). There were no twin pregnancies. This study revealed the limitations of SET when conventional morphology was used alone, even in patients without an increased risk for aneuploidy, since the CGH arrays group implanted with greater efficiency and yielded a lower miscarriage rate than those selected without CGH arrays [23]. Two other small RCTs were published subsequently recruiting good prognosis patients undergoing assisted reproduction. In one of them, the study group consisted of single euploid blastocyst transfer, whereas the control group underwent double blastocyst transfer after routine care for embryo selection. The clinical outcomes include a similar ongoing pregnancy rate between groups [11]. In the same year, another trial was published by Scott and colleagues [12]. In summary, these three RCTs showed that the use of PGS, compared with embryo selection based on morphology criteria alone, significantly improved clinical implantation rate in good prognosis patients undergoing ART. These findings were valid regardless of the technology used, such as aCGH or quantitative polymerase chain reaction (qPCR).

26.5 The Future

26.5.1 Biomarkers of Implantation and Embryo Viability

Recent studies have suggested the association of mitochondrial DNA (mtDNA) copy number with implantation potential and mtDNA has been proposed as a biomarker of

embryo viability [24]. However, controversial results have been published so far, using different technologies for mtDNA quantification. Levels of mtDNA in euploid and aneuploid embryos showed a statistically significant difference by NGS and by qPCR, but maternal age and implantation were not significantly different according to mtDNA [25,26].

For a future application of mtDNA as a biomarker in IVF, RCTs need to be performed.

26.5.2 Non-Invasive PGS

Non-invasive PGS by analyzing spent culture media with array CGH or NGS is promising but still has high false negative rates due to the presence of maternal DNA, mostly coming from the granulosa cells surrounding the oocytes and embryos. Therefore, optimization of the technique would be needed before its clinical implementation [27,28].

26.6 Conclusions

Different factors contribute to patient-perceived determinants when choosing to accept or decline PGS, including cost, religion, ethical values, social and family support, provider influences, and past reproductive experience of the patient [29].

Some authors with the current trend toward single-embryo transfer could argue that failure to investigate the chromosomal constitution of the preimplantation embryo to be transferred may raise ethical questions of its own [30].

More recently, some authors have proposed the possibility of transferring some types of mosaic embryos with the result of healthy newborns [31]. However, the authors are cautious and stated that additional clinical data must be obtained before this approach could be extended, since miscarriage rates are much higher and pregnancy rate can decrease by three times compared to the transfer of euploid embryos. There is an ongoing discussion to understand the need of balancing the risks of discarding a competent embryo versus transferring an embryo that may ultimately have a lower implantation potential, considering obstetrical and neonatal outcomes.

As with any adjunct treatment in IVF, before routine clinical practice, RCTs are needed to show that the technique is providing an improvement to the treatment outcome which will normally be an increase in the live birth rate [32]. Thousands of patients went through PGS v1.0 which has now been shown to be invalid. And tens of thousands of patients have gone through PGS v2.0 annually, but we only have a handful of RCTs, all of which are underpowered. It is essential that clinics performing any new ART technology engage in well-designed studies to ensure the procedure is of benefit to the patients who are paying for the procedures and that the demographics of the patients in the studies are taken into consideration when applying to a different patient population.

References

1. Handyside AH, Kontogianni EH, Hardy K, Winston RM. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature* 1990;344(6268): 768–70.
2. Munné S, Dailey T, Sultan KM, Grifo J, Cohen J. The use of first polar bodies for preimplantation diagnosis of aneuploidy. *Human Reprod* 1995;10: 1015–21.
3. Verlinsky Y, Cieslak J, Freidline M et al. Pregnancies following pre-conception diagnosis of common aneuploidies by fluorescent in situ hybridisation. *Mol. Hum Reprod*. 1995;10: 1923–27.
4. Harper, J., Coonen, E., De Rycke, M. et al. What next for preimplantation genetic

- screening (PGS)? A position statement from the ESHRE PGD Consortium steering committee. *Hum. Reprod.* 2010; **25**: 821–23.
5. Harton, G, Magli, C, Lundin Kersti et al. JC ESHRE PGD consortium/embryology special interest group-best practice guidelines for polar body and embryo biopsy for preimplantation genetic diagnosis/screening (PGD/PGS). *Human Reproduction* 2010;**26**:41–46.
 6. De Rycke, M, Belva, F, Goossens, V et al. ESHRE PGD Consortium data collection XIII: cycles from January to December 2010 with pregnancy follow-up to October 2011. *Hum Reprod* 2015;**30**: 1763–89.
 7. Gianaroli L, Magli MC, Pomante A et al. Blastocentesis: a source of DNA for preimplantation genetic testing. Results from a pilot study. *Fertil Steril* 2014 December; **102** (6): 1692–9.
 8. Ruangvutilert P, Delhanty JDA, Rodeck C, Harper JC. Relative efficiency of FISH on metaphase and interphase nuclei from non-mosaic trisomic or triploid fibroblast cultures. *Prenatal Diagnosis*. 2000; **20**: 159–62.
 9. Rubio C, Giménez C, Fernández E et al. Spanish interest group in preimplantation genetics, Spanish society for the study of the biology of reproduction. The importance of good practice in preimplantation genetic screening: critical viewpoints. *Hum Reprod*. 2009; **24**: 2045–47.
 10. Rubio C, Bellver J, Rodrigo L et al. Preimplantation genetic screening using fluorescence in situ hybridization in patients with repetitive implantation failure and advanced maternal age: two randomized trials. *Fertil Steril*. 2013; **99**: 1400–07.
 11. Forman EJ, Hong KH, Ferry KM et al. In vitro fertilization with single euploid blastocyst transfer: a randomized controlled trial. *Fertil Steril*. 2013; **100**:100–07.
 12. Scott RT Jr, Upham KM, Forman EJ et al. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: a randomized controlled trial. *Fertil Steril*. 2013; **100**:697–703.
 13. Vera-Rodríguez M, Michel CE, Mercader A et al. Distribution patterns of segmental aneuploidies in human blastocysts identified by next-generation sequencing. *Fertil Steril*. 2016; **105**: 1047–55.
 14. Ruttanajit T, Chanchamroen S, Cram DS et al. Detection and quantitation of chromosomal mosaicism in human blastocysts using copy number variation sequencing. *Prenat Diagn*. 2016; **36**: 154–62.
 15. Rubio C, Bellver J, Rodrigo L et al. In vitro fertilization with preimplantation genetic diagnosis for aneuploidies in advanced maternal age: a randomized, controlled study. *Fertil Steril*. 2017; **107**: 1122–29.
 16. Mastenbroek S, Twisk M, van der Veen F, Repping S. Preimplantation genetic screening: a systematic review and meta-analysis of RCTs. *Hum Reprod Update*. 2011; **17**: 454–66.
 17. Musters AM, Repping S, Korevaar JC et al. Pregnancy outcome after preimplantation genetic screening or natural conception in couples with unexplained recurrent miscarriage: A systematic review of the best available evidence. *Fertil Steril*. 2011; **95**: 2153–57.
 18. Blockeel C, Schutyser V, De Vos A et al. Prospectively randomized controlled trial of PGS in IVF/ICSI patients with poor implantation. *Reprod Biomed Online*. 2008; **17**: 848–54.
 19. Garcia-Herrero S, Rodrigo L, Mateu E et al. Médecine thérapeutique/Médecine de la reproduction, gyn_ecologie et endocrinologie. *John Libbey Eurotext*, 2014; **14**: 112–19.
 20. Rodrigo L, Mateu E, Mercader A et al. New tools for embryo selection: comprehensive chromosome screening by array comparative genomic hybridization. *Biomed Res Int*. 2014; **2014**:517125.
 21. Coates A, Hesla JS, Hurliman A et al. Use of suboptimal sperm increases the risk of aneuploidy of the sex chromosomes in preimplantation blastocyst embryos. *Fertil Steril*. 2015; **104**: 866–72.
 22. Al-Asmar N, Peinado V, Vera M et al. Chromosomal abnormalities in embryos

- from couples with a previous aneuploid miscarriage. *Fertil Steril.* 2012;98: 145–50.
23. Yang Z, Liu J, Collins GS et al. Selection of single blastocysts for fresh transfer via standard morphology assessment alone and with array CGH for good prognosis IVF patients: results from a randomized pilot study. *Mol Cytogenet* 2012;5:24.
 24. Ravichandran K, McCaffrey C, Grifo J et al. Mitochondrial DNA quantification as a tool for embryo viability assessment: retrospective analysis of data from single euploid blastocyst transfers. *Hum Reprod.* 2017;32: 1282–92.
 25. Treff NR, Zhan Y, Tao X et al. Levels of trophectoderm mitochondrial DNA do not predict the reproductive potential of sibling embryos. *Hum Reprod.* 2017;32: 954–62.
 26. Victor AR, Brake AJ, Tyndall JC et al. Accurate quantitation of mitochondrial DNA reveals uniform levels in human blastocysts irrespective of ploidy, age, or implantation potential. *Fertil Steril.* 2017;107:34–42.
 27. Feichtinger M, Vaccari E, Carli L et al. Non-invasive preimplantation genetic screening using array comparative genomic hybridization on spent culture media: a proof-of-concept pilot study. *Reprod Biomed Online.* 2017; pii: S1472-6483(17)30147–5.
 28. Liu W, Liu J, Du H et al. Non-invasive preimplantation aneuploidy screening and diagnosis of beta thalassemia IVSII654 mutation using spent embryo culture medium. *Ann Med.* 2017;49: 319–28.
 29. Gebhart MB, Hines RS, Penman A, Holland AC. How do patient perceived determinants influence the decision-making process to accept or decline preimplantation genetic screening? *Fertil Steril* 2016;105: 188–93.
 30. Hens K, Dondorp W, Handyside AH et al. Dynamics and ethics of comprehensive preimplantation genetic testing. A review of the challenges. *Hum Reprod Update* 2013;19: 366–75.
 31. Greco E, Minasi MG, Fiorentino F. Healthy babies after intrauterine transfer of mosaic aneuploid blastocysts. *N Engl J Med.* 2015;373:2089–90.
 32. Harper, J, Jackson, E, Sermon, K et al. (2017). Adjunts in the IVF laboratory: where is the evidence for ‘add-on’ interventions? *Human Reproduction*, 2: 485–91.

Cleavage-Stage Transfer or Blastocyst Transfer?

Evangelos G. Papanikolaou and Tatiana Chartomatzidou

27.1 Introduction

Since the first successful in vitro embryo-cultured pregnancy, the field of IVF is evolving rapidly. Important improvements have been accomplished in the field of embryo culture, embryo transfer and cryopreservation techniques.

Regarding in vitro conditions, a milestone in the history of IVF is the evolution of sequential media [1], which allowed the extension of the embryo culture further than the first three days. As an embryo evolves from the cleavage to the blastocyst stage, it has different metabolic needs in an in vitro environment. These stage-dependent requirements are fulfilled by a change in the composition and the concentration of the media. The suitability of a medium is determined by the amino acid and glucose concentration. A cleavage-stage embryo (until day 3) shows different metabolic needs compared to a blastocyst (from day 5), as on day 4 the embryo starts compacting and its genome activates, resulting in increasing demand of nutrients [2]. As a consequence of the extension of the embryo culture, new questions began to arise regarding the day on which the embryo transfer is more likely to achieve a pregnancy. Due to the new sequential media, Edwards and colleagues managed to achieve the first IVF pregnancy after blastocyst embryo transfer in 1995 [3]. Since then, the day of the embryo transfer is a matter of thorough research, often leading to controversial data. In order to consider the ideal day of the embryo transfer, many factors need to be analyzed. Studies focus on results obtained on the quality of the embryos transferred, the pregnancy and live birth rates, miscarriages, and other obstetric parameters.

27.2 Natural Conception

Assisted reproductive technology (ART) has evolved to a level that shows great results, after thorough research that focuses on how in vitro techniques can mimic the in vivo conditions of natural conception. During the follicular phase of the menstrual cycle, the increasing amounts of estrogens result in ceasing of bleeding and thickening of the endometrium. In the first days, the rise of follicle stimulating hormone (FSH) stimulates few ovarian follicles. After a complicated interaction between a complex of hormones and the developing follicles in the ovary, only one or two of them become dominant. In the middle of the menstrual cycle, the surge of the Luteinizing hormone (LH) causes ovulation, as the dominant follicle releases an oocyte. The mature oocyte is released from the ovarian follicle to the oviduct. At this time, if no fertilization occurs, the oocyte can live only for 24 hours, as after that period, it will disintegrate. Otherwise, a spermatozoon fertilizes the egg in the

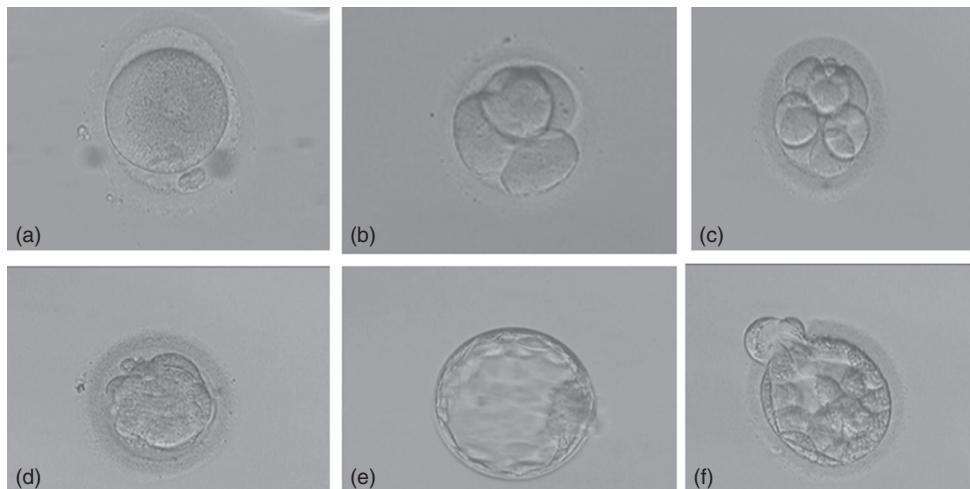


Figure 27.1 (a) day 1 after ICSI: fertilized egg, the two pronuclei can be observed, (b) day 2: four-cell cleavage stage embryo, (c) day 3: 8-cell cleavage stage embryo, (d) day 4: morula, (e) day 5: blastocyst stage embryo, (f) day 6: hatching blastocyst. Photos provided by Assisting Nature IVF Clinic

ampulla of the fallopian tube. Immediately, after the fertilization the process of embryogenesis starts.

During the first day after the fertilization the nuclei of the two gametes can be observed as pronuclei (Figure 27.1a). The total 46 chromosomes of a normal fertilized egg undergo changes, before the first mitotic division. The first mitotic division of the zygote leads to the formation of the first two cells. This point marks the beginning of the cleavage process of the embryo. Mitotic divisions occur every 12–24 hours and as a result – after the first two cells – an embryo at this stage consists of four cells, eight cells, and so on. During the cleavage stage, the embryo increases the number of its cells, but overall does not increase in size. All the divisions that happen from the time that the embryo consists of two cells to the time of the eight cells occur during the first three days after fertilization, as the embryo passes through the oviduct to reach the uterus (Figure 27.1b and 27.1c). The eight-cell embryo reaches the uterus only during day 4 after fertilization, as it has formed a compacted morula (Figure 27.1d). At this point the cellular differentiation begins and the embryo – during day 5 – is formed as a blastocyst (Figure 27.1e). A blastocyst-stage embryo consists of cells of the outer layer, the trophoblast and the inner cell mass. Between these two cell categories, is formed a cavity, which is called blastocoel. The trophectoderm cells will develop into the placenta and the inner cell mass will become the fetus. The increasing size of the blastocyst causes hatching of the embryo out of the zona pellucida (Figure 27.1f), which then will disintegrate. Hatching occurs during day 6–7 in the uterus environment and during day 8–9, the blastocyst is attached to the endometrium by the trophoblast, in order to implant. By this time the endometrium is suitably prepared by hormones in order to be receptive to the implantation of the blastocyst. The period of the receptivity is short and is called implantation window. This period is defined by the actions of oestrogen and progesterone. If the endometrium cells are not properly formed to allow adherence of the embryo, the blastocyst is flushed out of the uterus and no pregnancy is achieved.

27.3 The Conventional Cleavage Stage (Day 2 or 3) Embryo Culture and Transfer

In the initial years of IVF, the modus operandi was to culture the produced embryos up to the cleavage stage and then to perform the embryo transfer. The reasons were: first, it was mimicking the natural conception where the embryo descends to the uterus in the middle of day 3 to day 4 of its life. Secondly, the culture media was difficult to produce and therefore it already was a great success to have media up to cleavage stage, and research had been focused on how to optimize these conditions. Third, a lot of research was done on the morphology of cleavage-stage embryos and how this was related to pregnancy outcome and therefore all embryologists were focused on how to optimize their day 2 or day 3 selection and not how to extend to blastocyst stage. Fourth, embryologists were reluctant to extend embryo culture to blastocyst stage, as this strategy was more time-consuming, meaning more working hours and even weekends. Fifth, doctors were also reluctant to transfer on day 5 as they were afraid how to explain to patients that sometimes no embryo would be available for transfer.

For all these reasons, the cleavage-stage culture and embryo transfer were very popular. On the other hand, research into blastocysts was really innovative, with a lot of criticism and there were even obstacles within teams in order for it to be adopted [4].

27.4 Reasons to Extend Embryo Culture to Day 5 (Blastocyst Stage)

27.4.1 Embryo Quality Assessment

The morphology of an embryo is considered as an indicative parameter for its quality and as a consequence its implantation and pregnancy potential. The earliest time that morphological grading of embryos is available is day 2. A right assessment includes noting the blastomere size, the cell number, the fragmentation rate, and the cleavage speed, but generally at cleavage stage, there are limited morphological criteria for the embryos. Many reports have studied the morphological quality of cleavage embryos and its correlation with the implantation rate. It has been shown that there is no significant difference in the implantation rate of cleavage-stage embryos regarding the number of the blastomeres or the compaction degree. On the other hand, the most important parameter is considered the fragmentation rate of the embryo on day 3.

As it is obvious, one of the most important advantages of extending the embryo culture to the blastocyst stage is that it allows a more detailed assessment and therefore, a more successful embryo selection. Extending the culture to day 5 is considered a way of identifying the embryos with the highest developmental potential, since not all embryos can reach the blastocyst stage. As a result, on day 5 only the best embryos will be developed and consequently selected. In addition, selecting embryos at a later developmental stage during day 5 allows assessment with more objective criteria, which can lead in the selection of the top-quality embryos. Moreover, the selection of an embryo on day 5 relies on the developmental stage and on morphological parameters. Information obtained by day 3 embryo assessment is combined with information from the quality of the embryo on day 5. The fragmentation rate on day 3 seems to be significantly important in the implantation potential of the advanced blastocysts that are transferred on day 5. Specifically, it has been

shown that blastocysts with fragmentation rate >10 percent on day 3 have a lower implantation rate. On the other hand, when the fragmentation rate on day 3 is limited to less than 10 percent, the implantation rate of the advanced blastocysts reaches 50 percent. Additional cytogenetic data show also that highly fragmented embryos are more likely to be chromosomally abnormal [5].

Moreover, another important indicative parameter is the speed of a cleavage-stage embryo to form a blastocyst. During day 5, an embryo can be observed as a morula, an early blastocyst or an expanding blastocyst, which reflects the speed of the embryo development. Ideally, blastocysts with clear inner cell mass and an expanded cavity on day 5 are considered suitable for transfer, as these factors indicate an embryo which is more likely to implant and result in a pregnancy. As a consequence, blastocyst scoring according to a systematic scoring system [6] is a useful tool for selection of the embryos with the highest implantation potential, as top-quality expanded blastocysts show a higher implantation potential and a better pregnancy outcome, compared to full blastocysts [7,8]. Based on the previous outcomes, the fragmentation rate on day 3 and the developmental stage on day 5 are two parameters that should be taken into account when more than one blastocyst is available for transfer, in order to achieve the highest implantation and pregnancy potential.

27.4.2 Aneuploidy Rate

Although extended culture is a way of “natural selection” of the best embryos, there is still the possibility of chromosomal abnormalities, even in cases when the morphological criteria indicate an embryo is of top quality [9]. Embryos that are chromosomally abnormal may be present in a cleavage-stage culture, as well as in a blastocyst-stage culture. The difference, according to the studies, is the proportion of these embryos in each case. It has been shown that a great proportion of cleavage-stage embryos that are morphologically normal show chromosomal abnormalities after PGD examination. In particular, even 59 percent of top-quality day-3 embryos may be genetically abnormal [10]. As a result, it is proposed that transferring more day-3 embryos increases the chances of transferring the normal ones [4]. Despite the obvious rationality, this strategy opposes the current trend of single embryo transfer and its benefits, increasing the risks of a multiple pregnancy.

Regarding the blastocyst-stage embryos, it is profound that only the most promising embryos can reach this stage in an in vitro culture. Although, there is still the possibility for some of the blastocysts to be abnormal, the proportion of these embryos is lower, than in cleavage-stage cases, since this is only 35 percent [10]. Consequently, choosing to transfer an embryo at day 5 reduces the risk of choosing an aneuploid blastocyst, increasing in this way the chances of a pregnancy.

27.4.3 Embryo and Uterus Interaction

Besides the morphological criteria available, the first subject that must be taken into account is to investigate the advantages and the disadvantages of the extension of the embryo culture from day 3 to day 5, regarding the implantation potentials. The uterine environment that occurs under the superovulation is considered premature for the early stage embryos, due to the increase of the estrogens. Since naturally an embryo reaches the uterus only at the stage of morula (during day 4), a transfer at day 3 may be stressful for the embryo, decreasing the implantation rate. The progesterone rise during the late follicular phase has a detrimental

effect on the implantation of day-3 embryos, while day-5 embryos seem to be less affected. As a conclusion, the out-of-phase factors are considered inhibitory for the cleavage-stage embryos, as they result in development inhibition of the embryos, leading to the risk that the embryo will not reach the blastocyst stage to implant. In addition, blastocyst-stage embryos seem to interact less with the out-of-phase endometrium, which usually recovers by the time of the implantation window [7].

Moreover, another advantage for the blastocysts is that at the time of transfer the low uterine pulsatility diminishes the chances of embryo expelling [11], allowing the chances of implantation to increase.

27.4.4 In Vitro Conditions

However, despite all the advantages that were previously mentioned, extending the culture to day 5 may cause plenty of other unexpected results, too. In an ART cycle, embryos develop in in vitro culture conditions that simulate, but definitely are not equal to, the in vivo situation. This fact leads to the risk of losing embryos – eventually normal – in extended cultures that otherwise would have survived if they were transferred to the uterus [12]. As a result, an embryo transfer on day 5 risks the number of the available embryos. In the worst scenario, there is also the possibility of no available blastocyst to transfer, resulting in psychological and further financial implications to the couple. On the other hand, a day-3 embryo transfer provides eventually more available cleavage-stage embryos; but at the same time there are higher chances of aneuploidy and negative interaction with the altered endometrium [7], reducing the possibility of a potential pregnancy, as mentioned before. Moreover, the extra 48 hours of embryo culture requires more laboratory work load. Leaving aside the additional laboratory expenses, an extended culture to day 5 requires excellent quality control in the laboratory, in order to achieve optimal results. Otherwise, suboptimal lab conditions may even lead to lower embryo development or embryonic arrest.

27.5 Evidence and Problematics Regarding Blastocyst Stage Culture

27.5.1 Implantation and Clinical Pregnancy Rates

Implantation and clinical pregnancy rates are some of the first factors analyzed, assessing the success of ART cycles. These are the parameters that are indicative of the day that an embryo should be transferred, in order to achieve a pregnancy. An implantation rate is considered as the number of the fetal sacs observed divided by the number of embryos transferred. Various studies support the fact that the implantation rate is significantly higher in cases of blastocyst transfer during day 5 [13,14,15,11] (Table 27.1). But since nowadays implantation rate is not considered a useful outcome, most studies focus on the clinical pregnancy rate. Previous studies have also shown that there is a significant higher rate of clinical pregnancy after transfer on day 5, compared to day 3 [7,15] (Table 27.2). In accordance to the previous outcomes, more recent meta-analysis of 27 RCTs (4031

Table 27.1 Implantation rate after blastocyst and cleavage-stage embryo transfer

Study	Blastocyst (n/N)	Cleavage (n/N)	OR (fixed) 95% CI	P value
Papanikolaou et al., 2005	37.3% (59/158)	20.6% (35/170)	2.29	P <0.001
Mangalraj et al., 2009	40.16 (49/122)	11.43 (35/306)		P = 0.000
Kaur et al., 2014	35.17%	21.35%		P <0.01
Aziminekoo et al., 2015	14.5%	12.1%		P =0.535
Fernández-Shaw et al., 2015	42.8 % (36/84)	28.1% (20/71)		P =0.058

Table 27.2 Clinical pregnancy rate after blastocyst and cleavage-stage embryo transfer

Study	Blastocyst (n/N)	Cleavage (n/N)	OR (fixed) 95% CI	P value
Papanikolaou et al., 2005	52.5% (42/80)	32.1% (27/84)	2.33 (1.23–4.40)	P =0.008
Papanikolaou et al., 2006 (results after SET)	33.1% (58/175)	23.3% (41/176)	1.42 (1.01–2.00)	P = 0.04
Mangalraj et al., 2009	62% (31/50)	29.76% (25/85)		P = 0.000
Kaur et al., 2014	44% (66/150)	29.33% (44/150)		P < 0.01
Fernández-Shaw et al., 2015	53.4% (31/58)	34.7% (16/46)		P=0.057
Aziminekoo et al., 2015	33.3% (19)	279% (17)		P = 0.519
Glujokovsky et al., 2016 (Meta-analysis)	39–46%	36%	1.30 (1.14–1.47)	

women/ couples) showed higher clinical pregnancy rate in the blastocyst group as well, after fresh embryo transfer (OR 1.30, 95% CI 1.14–1.47) [11]. At the same time, there are also studies that show no significant differences between the implantation rate after day-3 or day-5 embryo transfer [16,17]

In addition to higher clinical pregnancy rates, extending embryo culture to day 5 allows a blastocyst biopsy procedure for embryo chromosome screening. In these cases, the embryos are frozen at this stage, until the PGS results are out. Recent RCTs have shown that transfers following such treatments show improved implantation and pregnancy rates [18,19,20].

27.5.2 Ongoing Pregnancy and Live Birth Rates

An ongoing pregnancy is defined as a pregnancy that is confirmed by ultrasound scan and continued for at least 21 weeks [21]. According to previous research, the rate of ongoing pregnancies is significantly lower after cleavage embryo transfer and higher after embryo transfer at the blastocyst stage [7,16,17]. The same significant difference is observed in the case of live birth rates, as well, showing a positive correlation between blastocyst transfer and delivery rates [1,7,11,12]. In addition, the meta-analysis of Glušovský and colleagues [11] on 13 RCTs (1630 women) favored the live birth rates after blastocyst embryo transfer in fresh cycles, over cleavage stage, although there was low-quality evidence to clarify this subject significantly (OR; 1.48, 95% CI: 1.20– .82).

27.5.3 Miscarriage Rate

The analysis of miscarriage rate is important in assessment of IVF treatment results. Previous studies have shown that the early pregnancy loss rate is higher after single day-3 embryo transfer, compared to single blastocyst transfer (26.8% versus 17.2%, $P = 0.017$) [14]. More recent studies, although, show that there is no significant difference in miscarriage rates, between day-3 or day-5 embryo transfer [7,17,11].

27.5.4 Monozygotic Twins – Multiple Pregnancies

Assisted reproduction technology has allowed many infertile couples to achieve pregnancy. The IVF treatments – especially in the initial steps of this field – have led to dizygotic twins or multiple pregnancies, due to the transfer of more than one embryo, in order to maximize the chances of success. This fact has been a matter of debate, since multiple pregnancies carry major risks related to the mother and the fetal health. The extension of the embryo culture, which allows limiting the number of the transferred embryos, due to better quality assessment, can minimize the risk of multiple pregnancies [1]. In the past, some studies have shown correlation between day-5 transfer and multiple pregnancies [13,14], but more recent research has proven that there is no significant difference, in the rate of this case between day-3 or day-5 embryo transfer [7,17,11]. On the contrary, it has been even shown that in some cases, multiple pregnancies seem to occur less after day-5 embryo transfers [15]. Minimizing the number of the fetuses has led IVF to the elective single embryo transfer (e-SET). E-SET is achieved usually after blastocyst culture, since the extension of the culture allows the selection of the most promising embryos.

On the other hand, despite the single embryo transfer, there has been an increased incidence of monozygotic twins. Studies show that the risk of monozygotic twins is 2.25 times higher after IVF treatments, than in natural conception [22]. On the other hand, other studies show no significant difference in the probability of monozygotic twins between cleavage stage or blastocyst embryo transfer [23]. The increased monozygotic twinning after IVF may be a result of multiple causes, related to the embryo culture. Research in this field has correlated monozygotic twinning with ovarian stimulation, alterations in the zona pellucida or changes in the composition of the culture media [22].

27.5.5 Cancellation of the ART

Cancellation of ART after egg retrieval may be a result of no available embryos to transfer. The rate of cancellation is significantly higher when the embryo transfer is committed on day 5 [7,11]. This is a result of the extension of the embryo culture. The number of the embryos that are available to transfer on day 3 is expected to be higher compared to day 5. Since, the time that is required for a cleavage-stage embryo to reach blastocyst stage is essential in an in vitro environment, many embryos arrest and only the embryos with the highest developing potential survive to day 5. As a consequence, there are more embryos available for cleavage-stage embryo transfer, but only those with the best quality can reach blastocyst stage. Hence, there is a higher probability of no embryos to transfer, if day 5 embryo transfer is chosen. Naturally, the most crucial factor that leads the decision for the day of the transfer in this case is the number and the quality of the embryos that are available after the ART treatment.

27.5.6 Cryopreservation of Surplus Embryos

After an ART treatment, despite a fresh embryo transfer, the surplus embryos are cryopreserved, in order to be transferred in a future cycle. The modern and more effective method of cryopreservation, vitrification, gives a woman the opportunity to have more transfers after frozen-thaw cycles treatment. The advantage of the embryo vitrification is that it allows a future embryo transfer, in order to achieve a second pregnancy, or after a failed ART, but this time the woman does not have to undergo the medical treatment once again. In accordance with the above, regarding the extension of the embryo culture to day 5 and the reduction of the available embryos, a lower rate of embryo cryopreservation is observed for blastocysts, rather than cleavage-stage embryos [7,11].

27.5.7 Cumulative Pregnancy Rate

A “cumulative pregnancy rate” is defined as “the probability of a couple to achieve a live birth after an egg retrieval” with fresh or frozen cycles. This fact is informative and indicative for couple counseling, as it illuminates the chances of having a live birth, especially when a cycle fails. Studies have shown controversial results on this topic. According to recent meta-analysis [11] there is no significant difference in cumulative pregnancy rates, after cleavage or blastocyst transfer. On the other hand, there are results that show higher rates in cleavage transfer groups, due to the extension of the embryo culture. According to other studies [11,12], the cumulative pregnancy rate is lower in cases of day 5 embryo transfers. The explanation is based on the fact that embryos which do not reach blastocyst stage are discarded, in contrast to the cleavage stage, when they usually are vitrified as good-quality embryos. However, other studies conducted have shown that the rate is irrespective to the day of the transfer, but only in cases of women less than 35 years. On the contrary, for women older than 40 years, results have shown that cumulative pregnancy rate is better on day-5 transfers [24].

27.5.8 Obstetric and Perinatal Outcomes

Despite the undeniable advantages of blastocyst embryo transfer that have been shown by numerous studies, it is considered necessary to examine the obstetric and perinatal outcomes of these pregnancies, as well. Recent meta-analyses have shown a high relative risk between blastocyst transfer and preterm delivery (less than 37 weeks), as well as with very preterm (less than 32 weeks) delivery [25,26,12]. A hypothesis that explains this incidence is that an extended embryo culture can result in genetic and epigenetic changes [14] in trophectoderm cells that can lead to abnormal implantation and as a consequence to preterm delivery. This hypothesis is also supported by animal studies [27]. In contrast with the previous studies, there are also reports that show no significant difference in preterm deliveries between day-3 and day-5 embryo transfers in cases of SET in women less than 40 years old, as well as other obstetric and perinatal outcomes such as birth weight, preeclampsia or other neonatal complications [28].

Additionally, studies have shown that blastocyst transfer is also correlated with “large for gestation age” offsprings [29,30], but with inadequate or non-objective data. As a result, more research in this field is considered necessary.

Another recent meta-analysis has shown higher congenital anomalies correlated to blastocyst stage embryo transfer, compared to the babies born after cleavage transfer [25]. In addition, other reports have suggested an altered sex ratio after blastocyst transfer, as the extension of the culture is reported to lead to a male:female ratio of 1.29 [14,31]. The higher percentage of male embryos may be explained by the fact that they develop faster compared to the female embryos and as a result are more likely to be chosen by the embryologists to get transferred.

All the previous outcomes underline the importance of further investigation, regarding possible obstetric and perinatal outcomes, connected to IVF treatments. More, large studies and meta-analysis can be useful tools in order to enlighten hidden aspects of IVF and help couples, not only to have a baby, but also to guide them in a healthy live birth, through a low-risk pregnancy.

27.6 Deciding the Day of the Transfer

According to the current available literature, there are obvious advantages for embryo transfer on day 5. Meanwhile, some advantages have been reported in favor of cleavage embryo transfer, over blastocyst transfer, in some cases. As a result, it seems quite rational, the decision of the day of the transfer to be taken, according to each treated couple personally. Physicians should firstly consider which couples would benefit from day-3 or day-5 embryo transfer. The extension of the embryo culture should be chosen as a treatment to selected couples, since despite its benefits, it may lead to no available embryos to transfer. It is obvious that if a small number of embryos are available on day 3, it is possible that none will reach the blastocyst stage. Consequently, the ART cycle could be cancelled due to embryo arrest. So, it is found that extension of the culture is suggested to couples with a minimum number of embryos on day 3, as the more cleavage-stage embryos will subsequently lead to more blastocysts. According to [32], patients with more than three cleavage-stage embryos are suitable for a blastocyst transfer. Based on other more recent studies, there is a correlation between the minimum number of cleavage-stage embryos and a woman’s age, in order to achieve a pregnancy after blastocyst transfer. Younger women with good ovarian response and at least three good-quality cleavage-stage embryos can be

offered extended culture. In addition, since the clinical pregnancy rates are higher, fewer embryos shall be transferred. In this way, in good prognosis patients, physicians can achieve better pregnancy rates eventually, as risk of multiple pregnancy is minimized [15]. On the other hand, in cases of poor responders, shortening the duration of the embryo culture and subsequently, maximizing the number of available embryos can achieve higher pregnancy rates [33]. This problem is solved, as a threshold of at least four quality embryos on day 3 has been suggested. In cases of women with at least four embryos on day 3, a day-5 embryo transfer is acceptable, despite the age, as they have at least one available blastocyst. In these cases, a higher ongoing pregnancy rate was obtained for women of all ages and a higher rate of ongoing and cumulative pregnancy was obtained for women older than 35 years. As a result, a blastocyst embryo transfer shows improvement in pregnancy rates for all patients, but is more significant for patients older than 35 years [17].

27.7 Current and Future Aspects

IVF treatments have helped many couples, presenting with fertility problems, to fulfill their dream of a baby. Despite the fact that ART has made great steps during the years, it still seems far from showing an ideal efficacy. Until now, the field of IVF focused mainly on the ovarian stimulation, efficient embryo culture, and endometrial quality in order to achieve a pregnancy. Moreover, regarding the embryo assessment, the emergence of time-lapse incubators can achieve a non-distorted embryo culture, which may lead to better quality embryos. Ensuring the maintenance of the ideal conditions in an embryo culture is of great importance, as they are crucial factors in determining further survival and development of the embryos. These conditions are maintained in special incubators that allow continuous control and stability of all the important parameters, such as temperature, pH, humidity and concentrations of O₂ and CO₂ in the embryo atmosphere. A time-lapse incubator contains an integrated video system that allows the continuous digital imaging of the embryos. In this way, embryo cultures can be assessed in a detailed and constant manner, since the fertilization day and until the time of the embryo transfer. Consequently, time-lapse incubators offer the advantage of a substantial limitation in embryo handling and condition disruption. In addition, these kinds of incubators allow the imaging of important embryonic developmental stages, such as the appearance of the pronuclei and the cleavage/blastocyst stage. Moreover, embryologists have the chance to evaluate new morphokinetic values of embryos, to optimize the embryo selection. The whole point to this new approach is to minimize all the external factors that can affect the embryo, besides its own genetically specified quality, in order to correctly choose the day of the transfer.

Although, the right embryo assessment has increased the rates of pregnancy and live birth events in IVF, there are still other key issues to be discovered. A new perspective in the field is based on the idea that embryo selection is not just a “beauty contest.” Apart from all the morphological criteria previously analyzed, there are plenty of other parameters at a molecular level. Therefore, embryo selection nowadays can be based not only on the morphological data, but on more informative data, obtained by new molecular and cytogenetic technologies. The introduction of the OMICS technology can provide in the near future a huge amount of information. The emergence of all these new techniques can reveal unknown factors involved in infertility, especially in cases of couples with idiopathic infertility [34]. The OMICS technologies include research on epigenomics, genomics,

transcriptomics, proteomics, and metabolomics. In other words, these technologies focus on obtaining information from the level of the DNA and how it is affected by environmental factors, to the level of functions of transcription and translation. In that way, we can evaluate the potential of a cell, based on its genes and analyze its real function, based on the results of transcriptomics, proteomics and metabolomics [34]. Moreover, this technology can be applied not only on the gametes and the embryos, but it could be helpful to evaluate the endometrium, too. Except from the histologic evaluation of the endometrium that has been used the last decade, transcriptome studies can be applied to evaluate the endometrium during the different phases of the menstrual cycle and determine the mechanisms and the time of the implantation window [35,36].

Apart from the idealization of the embryo culture conditions and the selection of the top-quality embryos that can achieve a pregnancy, it is found that IVF has still many things to discover regarding its efficacy. In order to assess the success of ARTs and standardize the optimum choices on each case, more RTCs have to be conducted. Studies with enough data, high-quality evidence and meta-analyses are crucial in order to investigate whether the current trends in IVF can contribute to the increase of its potential and help the dream of a baby to come true.

References

1. Gardner DK and Lane M. Alleviation of the “2-cell block” and development to the blastocyst of CFI mouse embryos: role of amino acids, EDTA and physical parameters. *Hum Reprod* 1996; **11**:2701–12.
2. Gardner DK. Development of serum-free media for the culture and transfer of human blastocysts. *Hum Reprod* 1998; **13 Suppl 4**: 218–25.
3. Edwards RG, Brody S. *Principles and Practice of Assisted Human Reproduction*. WB Saunders, Philadelphia, 1995.
4. Blake DA, Proctor M, Johnson NP. The merits of blastocyst versus cleavage stage embryo transfer: a Cochrane review. *Hum Reprod* 2004; **19**:795–780.
5. della Ragione T, Verheyen G, Papanikolaou EG et al. Developmental stage on day -5 and fragmentation rate on day -3 can influence the implantation potential of top-quality blastocysts in IVF cycles with single embryo transfer. *Reprod Biol Endocrinol* 2007; **5**:2.
6. Gardner DK and Schoolcraft WB. In vitro culture of human blastocysts. In: Jansen R, Mortimer D (eds.) *Toward Reproductive Certainty: Fertility and Genetics Beyond*.
7. Papanikolaou EG, Kolibianakis EM, Tournaye H et al. Live birth rates after transfer of equal number of blastocysts or cleavage-stage embryos in IVF. A systematic review and meta-analysis. *Hum Reprod* 2008; **23**:91–99.
8. Wilson M, Hartke K, Kiehl M et al. Transfer of blastocysts and morulae on day 5. *Fertil Steril* 2004; **82**:327–33.
9. Fragouli E, Alfarawati S, Spath K, Wells D. Morphological and cytogenetic assessment of cleavage and blastocyst stage embryos. *Mol Hum Reprod* 2014; **20**, 117–26.
10. Staessen C, Platteau P, Van Assche E et al. Comparison of blastocyst transfer with or without preimplantation genetic diagnosis for aneuploidy screening in couples with advanced maternal age: a prospective randomized controlled trial. *Hum Reprod* 2004; **19**:2849–58.
11. Glujsovsky D, Farquhar C, Quinteiro Retamar AM, Alvarez Sedo CR, Blake D. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Database of Systematic Reviews* 2016, Issue 6. Art. No.: CD002118.

Parthenon Publishing, Carnforth, UK, 1999, pp. 378–88.

12. Maheshwari A, Hamilton M, Bhattacharya S. Should we be promoting embryo transfer at blastocyst stage? *Reproductive BioMedicine Online* 2016; **32**:142–46.
13. Papanikolaou EG, D'haeseleer E, Verheyen G et al. Live birth rate is significantly higher after blastocyst transfer than after cleavage-stage transfer when at least four embryos are available on day 3 of culture. *A randomized prospective study*. *Human Reprod* 2005; **20**:3198–203.
14. Papanikolaou EG, Camus M, Fatemi HM et al. Early pregnancy loss is significantly higher after day 3 single embryo transfer than after day 5 single blastocyst transfer in GnRH antagonist stimulated IVF cycles. *Reprod Biomed Online*. 2006; **12**:60–65.
15. Kaur P, Swarankar ML, Maheshwari M, Acharya V. A comparative study between cleavage stage embryo transfer at day 3 and blastocyst stage transfer at day 5 in IVF/ICSI on clinical pregnancy rates. *Indian Journal of Clinical Practice* 2014; **24**: 663–67.
16. Aziminekoo E, Mohseni Salehi MS, Kalantari V et al. Pregnancy outcome after blastocyst stage transfer comparing to early cleavage stage embryo transfer. *Gynecol Endocrinol* 2015; **31**:880–84.
17. Fernández-Shaw S, Cercas R, Brana C, Villas C, Pons I. Ongoing and cumulative pregnancy rate after cleavage-stage versus blastocyst-stage embryo transfer using vitrification for cryopreservation: impact of age on the results. *J Assist Reprod Genet* 2015; **32**:177–84.
18. Yang Z, Liu J, Collins GS et al. Selection of single blastocysts for fresh transfer via standard morphology assessment alone and with array CGH for good prognosis IVF patients: results from a randomized pilot study. *Mol Cytogenet* 2012; **5**:1–8.
19. Forman EJ, Hong KH, Ferry KM et al. In vitro fertilization with single euploid blastocyst transfer: a randomized controlled trial. *Fertil Steril* 2013b; **100**: 100–07. e101.
20. Scott RT Jr, Upham KM, Forman EJ et al. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: a randomized controlled trial. *Fertil Steril* 2013a; **100**: 697–703.
21. Kedem A, Haas J, Geva LL et al. Ongoing pregnancy rates in women with low and extremely low AMH levels. A multivariate analysis of 769 cycles. He B, ed. *PLoS ONE*. 2013; **8**:e81629.
22. Vitthal S, Gelbaya TA, Brison DR, Fitzgerald CT, Nardo LG. The risk of monozygotic twins after assisted reproductive technology: a systematic review and meta-analysis. *Hum Reprod Update*. 2009; **15**: 45–55.
23. Papanikolaou EG, Fatemi H, Venetis C et al. Monozygotic twinning is not increased after single blastocyst transfer compared with single cleavage-stage embryo transfer. *Fertil Steril* 2010; **93**: 592–97.
24. Abuzeid MI, Bolonduro O, La Chance J et al. Cumulative live birth rate and assisted reproduction: impact of female age and transfer day. *Facts Views Vis Obgyn* 2014; **6**: 145–49.
25. Dar S, Lazer T, Shah PS, Librach CL. Neonatal outcomes among singleton births after blastocyst versus cleavage stage embryo transfer: a systematic review and meta-analysis. *Hum Reprod Update*. 2014; **20**:439–48.
26. Maheshwari A and Bhattacharya S. Elective frozen replacement cycles for all: ready for prime time? *Hum Reprod* 2013; **28**:6–9.
27. Rizos D, Lonergan P, Boland MP et al. Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality. *Biol Reprod* 2002; **66**:589–95.
28. Oron G, Esh-Broder E, Son WY, Holzer H, Tulandi T. Predictive value of maternal serum human chorionic gonadotropin levels in pregnancies achieved by in vitro

- fertilization with single cleavage and single blastocyst embryo transfers. *Fertil Steril* 2015;103:1526–31.
29. Makinen S, Soderstrom-Anttila V, Vainio J, Suikkari AM, Tuuri T. Does long in vitro culture promote large for gestational age babies? *Hum Reprod* 2013;28:828–34.
 30. Zhu J, Lin S, Li M et al. Effect of in vitro culture period on birthweight of singleton newborns. *Hum Reprod* 2014; 29:448–54.
 31. Chang HJ, Lee JR, Jee BC, Suh CS, Kim S H. Impact of blastocyst transfer on offspring sex ratio and the monozygotic twinning rate: a systematic review and meta-analysis. *Fertil Steril* 2009;91:2381–90.
 32. Racowsky C, Jackson KV, Cekleniak NA et al. The number of eight-cell embryos is a key determinant for selecting day 3 or day 5 transfer. *Fertil Steril* 2000; 73:558–64.
 33. Kyrou D, Kolibianakis EM, Venetis CA et al. How to improve the probability of pregnancy in poor responders undergoing in vitro fertilization: a systematic review and meta-analysis. *Fertil Steril* 2009;91: 749–66.
 34. Egea RR, Puchalt NG, Escrivá MM, Varghese AC. OMICS: Current and future perspectives in reproductive medicine and technology. *J Hum Reprod Sci* 2014; 7:73–92.
 35. Talbi S, Hamilton AE, Vo KC et al. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology* 2006;147:1097–1121.
 36. Ruiz-Alonso M, Blesa D, Simón C. The genomics of the human endometrium. *Biochim Biophys Acta*. 2012;1822:1931–42.

Embryo Transfer Technique

Ragaa Mansour

28.1 Introduction

Successful implantation after IVF requires a viable embryo, a receptive endometrium, and an optimal embryo transfer (ET) technique.

Various IVF steps proceed successfully up to the ET stage in about 80 percent of the cases. However, only a small percentage of them achieve pregnancy. Over the past two decades major technical developments occurred in embryo culture techniques, increasing the percentage of viable in vitro grown embryos. Vitrification, comprehensive chromosomal screening, and preimplantation genetic testing are some of the revolutionary techniques in the IVF laboratory. However, the ET technique has remained with little changes.

Unfortunately, most clinicians consider the ET technique a simple procedure. To them, it only means a simple task of inserting the ET catheter into the uterine cavity and delivering the embryos. However, it is not as simple as it appears and it is easier said than done.

The ET technique has a great impact on the IVF results. It has been proven that pregnancy rates differ significantly among different individuals performing ET within the same IVF program [1]. However, when the ET technique is standardized, the probability of pregnancy is not dependent on the physician performing the ET [2].

ET is routinely performed using the transcervical route, which is basically a blind technique associated with multiple negative factors. In a survey of 80 IVF practitioners, standardization of the ET technique was considered the most important factor influencing the success rate in IVF [3]. Furthermore, it was estimated that poor ET technique may account for as much as 30 percent of all IVF failures. Individualizing embryo transfer training for timing and performance demonstrated that ET technique is easy to learn [4]. In a large study it was demonstrated that fellows can be trained to perform ET without compromising results [5].

Extra attention and time should be given to the procedure of ET so that it will be performed meticulously.

28.2 Potential Negative Factors Associated with ET

28.2.1 Uterine Contractions

Initiation of uterine contractions may lead to an immediate or delayed expulsion of the embryos and has been considered a big concern in ART. In a study on cows, “artificial embryos” in the form of resin spheres impregnated with radioactive gold were traced after ET. After 1.5 hr, a large proportion of the spheres were expelled from the uterus.

In human IVF, Fanchin et al. [6] noted that more uterine contractions at the time of ET were associated with a lower pregnancy rate. In a study by Woolcott and Stanger, it was observed that the embryos could move as easily toward the cervical canal as toward the fallopian tubes [7]. As a result, it is possible that the embryos may be expelled from the uterus partially or totally, after the transfer. In a study using radio-opaque dye, mimicking ET, it was found that the dye remained primarily in the uterine cavity in only 58 percent of cases. In another study using methylene blue, the dye was extruded in 42 percent of the cases after dummy ET.

28.2.2 Failure to Pass the Internal Cervical Os

It is crucial for a successful ET that the catheter pass through the cervical canal, and internal os to enter the uterine cavity. The ET catheter, especially soft ones, can be unnoticeably curved inside the cervical canal.

An important cause for failure of the catheter to pass the internal cervical os is the lack of alignment between the catheter (straight) and the utero-cervical angle (curved or acutely angulated). The acute degree of anteversion/retroversion or utero-cervical angulation and cervical stenosis are the most common reasons of difficult transfer.

Scarring of the lower uterine segment and cervix, distorted anatomy with fibroids, previous surgery or congenital anomalies may lead to very difficult ET.

28.2.3 Cervical Mucus

Plugging the tip of the catheter with mucus can cause embryo retention, especially with such a small volume of culture media to inject with the embryos.

The mucus can also drag the embryos outside during withdrawal of the catheter. It may also interfere with implantation if the mucus is pushed or injected in the uterine cavity. In an experimental dummy ET using methylene blue, it was demonstrated that the dye was extruded at the external os in a significantly higher rate when the cervical mucus was not removed.

In a large study by Nabi et al. [8], the authors reported that the embryos were much more likely to be retained when the catheter contained mucus or blood. Moreover, cervical mucus may be a source of bacterial contamination with subsequent lower pregnancy rates. It can also be contaminated with vaginal progesterone which has been demonstrated to significantly impair mouse embryo development. In a prospective randomized trial by Eskandar et al. [9], removal of cervical mucus prior to ET significantly improved the pregnancy rates. In another RCT by Visschers et al., this positive effect of mucus removal was not significant [10].

28.3 Optimizing the Embryo Transfer Technique

28.3.1 Evaluation of the Uterine Cavity

To ensure proper embryo placement, it is important to evaluate the uterine cavity before starting the IVF cycle by doing the following:

28.3.1.1 Dummy ET (mock transfer)

Performing a mock ET before the IVF cycle has been shown to significantly improve the pregnancy rate. This procedure is recommended to be performed before the start of the IVF cycle or immediately before the actual ET. At our center we usually do both.

In a retrospective study of 289 ETs, there was significant difference in ongoing pregnancy rates when mock ET was done before the IVF cycle compared to performing it at retrieval [11]. During the mock ET we can measure the length of the uterine cavity and evaluate its direction and the degree of cervico-uterine angulation. It is also a good test to choose the most suitable kind of catheter and discover any unanticipated difficulty such as pinpoint external os, cervical polyps or fibroids, stenosed cervix from previous surgery, or congenital anomalies. If cervical stenosis is diagnosed, it is advisable to perform cervical dilatation before starting the IVF cycle. The use of cervical laminaria one month before the IVF cycle is an adequate means of cervical dilatation. When cervical dilatation was done at the time of ovum pick-up, the pregnancy rate was very low.

28.3.1.2 Ultrasonographic Evaluation

The ultrasound (US) is a precise method to measure the length of the uterine cavity and the cervical canal. It is very important in evaluating the cervico-uterine angle. Ultrasonography is also essential in diagnosing the presence of fibroids and its encroachment on the uterine cavity or cervical canal.

The accuracy of measuring the uterine length using a catheter was determined with the US. The authors reported a discrepancy of ≥ 1.5 cm in approximately 19 percent of the patients and ≥ 1 cm in 30 percent of the patients.

28.3.1.3 Hysteroscopy

Hysteroscopy is considered the definitive diagnostic tool to evaluate any uterine cavity abnormalities suspected on hysterosalpingography or ultrasound.

28.3.2 Avoiding the Initiation of Uterine Contractions

The following precautions have to be taken to avoid the initiation of uterine contractions:

28.3.2.1 Avoid Touching the Uterine Fundus

It was demonstrated that touching the uterine fundus with the catheter stimulated uterine contractions. In an experiment by Lesny et al. [12] using ultrasonically visible material, the authors demonstrated that touching the fundus with the catheter initiated strong random uterine contractions relocating the contrast material. That is probably why early sources in IVF described the optimal location for embryo placement as between 0.5 and 1.0 cm from the fundus. Not touching the fundus was ranked high as a prognostic factor for IVF success in a recent survey [13].

It is a routine procedure for some IVF specialists to place the catheter approximately 0.5 cm or 1–1.5 cm below the fundus to avoid touching it.

Depositing the embryos in the mid-fundal area of the uterus improved the pregnancy rates. It was also found in another study that positioning of the catheter 2 cm from the fundus was superior to 1 cm from the fundus [14].

Therefore, individual measurement of the cervical canal and uterine cavity length is extremely important. However, the use of a fixed-distance technique greatly reduced the variation in pregnancy rates among physicians, probably due to reducing the rate of touching the fundus.

28.3.2.2 Soft Catheters

The value of soft ET catheters has been recognized since the beginning of IVF. The ideal catheter should be soft enough to avoid any trauma to the endometrium and malleable enough to find its way through the cervical canal into the uterine cavity. Soft ET catheter means a combination of physical flexibility, malleability, and smoothness of the tip [4]. Soft catheters usually have an outer rigid sheath which should be stopped short of the internal cervical os to benefit from the advantages of soft catheters. If the outer sheath is introduced, it will convert a “soft” catheter into a “stiff” catheter. The stimulus of the ET catheter passing through the internal cervical os can possibly cause the release of prostaglandins and uterine contractions.

Different kinds of catheters were studied and soft catheters were associated with improved pregnancy rates [4], and in other studies there was no significant difference. Two large prospective randomized studies have shown significantly higher ongoing clinical pregnancy rates using soft catheters as compared to rigid ones [15,16]. Changing from rigid catheters to soft ones has been associated with improved pregnancy rates. It is recommended to use soft catheters for ET of embryos with assisted hatching. A recent meta-analysis has shown that the clinical pregnancy rates were significantly better using soft ET catheters as compared to rigid ones [17]. Different soft catheters are more or less the same and the pregnancy rates associated with them are not significantly different.

28.3.2.3 Gentle Manipulations

Atraumatic delivery of embryos into the endometrial cavity is the primary goal of ET. As a rule, the ET procedure should be a simple and painless procedure, and gentle manipulations should be observed, even when introducing the vaginal speculum. Holding the cervix with a vullsellum should be avoided except in rare cases [14]. On a clinical study on humans, serial blood samples were collected in time intervals of 20 seconds during the ET procedure to measure the serum oxytocin concentrations [18]. When the tenaculum was used, there was a temporary elevation of oxytocin levels, which remained elevated until the end of ET. In another study on humans, the use of tissue forceps to hold the cervix was found to trigger uterine contractions.

Applying 1–2 mL of local anesthetic (1 percent procaine) to the anterior lip of the cervix through a fine needle before applying the tenaculum was very acceptable to the patients and did not affect the outcome. Technically difficult ETs were associated with reduced pregnancy rates in a number of studies. Difficult manipulations initiate uterine contractions with possible embryo expulsion. Despite all the precautions to ensure atraumatic ET, sometimes it can still be difficult, without having an adverse effect on the pregnancy rate or outcome (73 [19]).

28.3.2.4 Uterine Relaxing Substances

Serum progesterone levels on the day of ET correlate inversely with the frequency of uterine contractions. Starting progesterone on the day of oocyte pick-up to relax uterine contractility at the time of ET was suggested, although it did not improve the pregnancy rates as compared to starting it on the day of ET.

Sedation with 10 mg diazepam 30–60 minutes before ET did not make a difference in the pregnancy rates. However, it is given as a routine in some centers to alleviate the patient's anxiety.

Similarly, tocolytic agents or prostaglandin synthetase inhibitors did not have a positive effect. In some patients who experience severe stress and anxiety during ET, propofol anesthesia can be used and it was found to have no effect on the pregnancy rates. Nonsteroidal anti-inflammatory drugs to inhibit prostaglandin production was given (such as 10 mg piroxicam), and there was a significant improvement of the implantation and pregnancy rates [20]. In a prospective randomized study, oxytocin antagonist (atosiban) was given intravenously before ET and the results showed a significant improvement in the implantation and pregnancy rates, but a recent RCT failed to show a beneficial effect [21].

28.3.3 Removal of Cervical Mucus

Removing the cervical mucus before ET is advisable, to avoid the adverse effect mentioned previously. It can be removed by repeated gentle aspiration using a 1 cm³ syringe with its tip placed at the external cervical os or using a soft catheter.

The endocervix can be cleaned of mucus using a sterile cotton swab or small brush, then a small amount of culture media.

Vigorous cervical washing was reported in a retrospective study to improve the pregnancy rates; however, a prospective randomized study showed no significant difference [22]. Another large multicenter study showed no significant difference. In a large retrospective study including 470 ET procedures, it was found that the presence of macroscopic or microscopic blood and mucus did not affect the clinical pregnancy or implantation rates. In a Cochrane review no evidence of benefit was found when the cervical mucus was removed before ET [23].

28.3.4 Make Sure the ET Catheter Passed the Internal Cervical Os

The ultimate goal at the end of an IVF cycle is to safely deposit the embryos inside the uterine cavity. We have to be sure that the catheter has passed the internal os and is not kinked or curved inside the cervical canal. Performing the ET under US guidance makes it easy to confirm proper passage of the catheter. For clinicians performing ET without US guidance, soft catheters can sometimes be misleading. A simple test of rotating the catheter 360° can discover kinking of the catheter if it recoils.

One of the most common causes of failure to pass the ET catheter is the pronounced curvature or angulations of the cervico-uterine angle. Proper curving of the catheter to follow the cervico-uterine curvature should be done before loading the embryos into the catheter. That is why it is important to perform a dummy ET and revise the US picture of the uterus before loading the embryos. It has been reported that molding the ET catheter according to the US cervico-uterine angle improved the clinical pregnancy rate and diminished the incidence of difficult ET. Straightening the utero-cervical angle can be achieved with a full bladder before ET. This effect can be achieved indirectly when performing ET under US. However, in a Cochrane review no evidence of benefit with a full bladder was found [23].

Sometimes just a simple maneuver of the vaginal speculum can change the direction of the cervix and facilitate introduction of the ET catheter.

If the soft catheter cannot pass the cervical canal, a more rigid one should be used. Rigid catheters should be malleable to allow making the required curve to overcome the acute cervico-uterine angulations.

Using a malleable stylet to place the outer sheath correctly through the cervical canal before introducing the soft catheter did not have a negative impact on the implantation and pregnancy rates [24].

In difficult cases it may be necessary to hold the cervix with a vulsellum to stabilize the uterus during the introduction of the ET catheter. Holding the cervix with a vulsellum is painful and it should be done under local or general anesthesia.

In rare cases it is very difficult or even impossible to pass the catheter. More rigid stiffer catheters have to be used. Another system that can be used in difficult cases is the coaxial catheter. Cannulation of a resistant internal os can be done with the outer sheath, before introducing the soft inner catheter.

Cervical dilatation can be resorted to in cases of cervical stenosis. A short interval between cervical dilatation and ET can result in a very low pregnancy rate. Performing cervical dilatation before the IVF cycle resulted in easier ET and improved pregnancy rates.

Another helpful way in cases of cervical stenosis is to place a laminaria before the start of the IVF cycle, or to place the hygroscopic rods in the cervix prior to ovarian stimulation. Laminaria have been used routinely five to six weeks before ET to be left through the internal os for 24 hours, and was found to improve the incidence of easy ET in cases of difficult or stenotic cervix. Cannulation of the cervix under fluoroscopic guidance followed by dilatation was successfully tried in some tortuous and stenotic cervical canals. In the early days of IVF, the rate of difficult ET was high. In a report of 867 ET procedures, 1.3 percent were impossible, 3.2 percent were very difficult (manipulation >5 minutes or cervical dilatation), and 5.6 percent were difficult (93). Twenty-five years after this report, the current experience of most IVF centers makes the rate of impossible and difficult ET procedures extremely low. In these rare difficult cases transmyometrial surgical ET can be used [25].

Surgical ET has been used successfully achieving results comparable to the trans-cervical route.

The surgical technique is straightforward and requires no greater experience than that necessary for US-guided oocyte collection [26]. The surgical ET set is composed of a metal needle (like an oocyte pick-up needle) with a stylet and an ET catheter that fits in the needle after withdrawal of the stylet.

28.3.5 Prevention of Embryo Expulsion

Recently, a technique using the vaginal speculum to prevent embryo expulsion after ET was described. After introducing the ET catheter into the uterine cavity, the screw of the vaginal speculum is loosened so that its two valves press on the portio vaginalis of the cervix, occluding the cervical canal. After waiting for 30–60 seconds, the embryos are ejected and the catheter is withdrawn slowly. The speculum is kept in place gently pressing on the cervix for about 7 more minutes and then is removed. The results of this study reported a significant improvement in the implantation and pregnancy rates.

28.4 Embryo Transfer Using Ultrasound

Using ultrasound guidance for ET was reported by a number of investigators as simple and reassuring, and significantly improved the pregnancy rates. However, other investigators found no significant difference in the pregnancy rates when ET was performed under US guidance as compared to clinical touch ET [27]. A RCT by Kosmas et al. [28]

showed that US-guided ET does not offer any benefit in clinical outcomes. A recent large prospective RCT comparing transabdominal US guided ET with clinical touch ET guided with previous US uterine length measurement showed comparable results, but the clinical touch technique was better tolerated by the patient and easier to be performed by a single operator.

However, the results of a meta-analysis of eight prospective RCTs showed that the pregnancy and implantation rates were significantly better when US was used [29].

Another recent meta-analysis has reached a similar conclusion. A Cochrane review of 17 RCTs showed that ultrasound-guided ET increased the ongoing pregnancy rates compared to clinical touch [30]. For easier identification of the catheter, some kinds have an ultrasonically visible tip. Transrectal US can be used in obese women during ET.

28.5 Loading the Embryos in the ET Catheter

Putting the embryos in the ET catheter should start after completing the dummy ET and making sure that the dummy catheter passed the internal os. After the dummy ET, the suitable kind of ET catheter will be selected and flushed with tissue culture medium. Then the ET catheter is filled with ET culture medium and up to 10–15 µL will be aspirated first. The embryos are then aspirated in another 10–15 µL medium and moved in the catheter to stop away from the tip.

Using a continuous fluid column without air bubbles is recommended. The volume of fluid used for ET should be as small as possible to prevent flowing out of the embryos into the cervical canal or the fallopian tubes. A large volume (60 µL) of transfer media and a large air bubble in the catheter result in the expulsion of the embryos. A continuous fluid column of 30 µL without air bubbles is recommended.

Two prospective RCTs were performed to investigate the effect of the presence of air bubbles in the ET catheter, on the IVF outcome. The authors concluded that the presence of air bubbles had no negative effect. However, there is no definitive reason to support the enclosure of air spaces before and after the media containing the embryos in the ET catheter. It was suggested that the air bubbles mark the position of the embryos inside the catheter and protect against loss or entangling with mucus. It was also shown that injecting the air bubble after the fluid column resulted in a significant improvement in the pregnancy rate. However, it was demonstrated that the air bubble moved toward the cervix in 12.4 percent of the cases 60 minutes after ET and the pregnancy rates were lower as compared to cases when the air bubble remained in place or moved toward the fundus. When assessing the position of the air bubble after ET, it was demonstrated that when it was at the fundal half of the endometrial cavity, the pregnancy rate was significantly higher than when the bubble was in the lower half. More precisely, it was shown that air bubble flashes located <15 mm from the fundus were associated with significantly higher pregnancy rates as compared to >15 mm.

The protein concentration in the transfer media does not affect the result nor does it increase the viscosity. Hyaluronan enriched transfer medium has been recently used in a large randomized trial and showed significant improvement in implantation and pregnancy rates. However, another RCT showed no beneficial effect [31]. In patients with repeated IVF failures the use of hyaluronan improved the pregnancy rate. Recently, a Cochrane review was performed to determine whether ET medium enriched with adherence compounds have an impact on live birth rate in ART compared to regular ET medium

[32]. The review included 16 studies and the results showed that the clinical pregnancy rates and multiple pregnancy rates were significantly higher in hyaluronan group.

28.6 Infusion of hCG

It was recently published that intrauterine infusion with 40 µL of tissue culture media containing 500 IU of hCG significantly improved the clinical pregnancy rates and implantation rates [33]. This positive effect was confirmed by three other controlled randomized studies. However, two other randomized controlled studies did not show a beneficial effect. A recent meta-analysis showed a benefit of hCG injection before ET [34]. The conflicting results in the literature can be due to the variable degrees of purity of hCG, and the use of recombinant hCG could avoid such a problem. It could also be due to variable days of ET ranging from day 2 to 5. Moreover, hCG is a fragile molecule and it should be prepared right before its use and not kept overnight in the incubator. Another factor is to prevent its expulsion after injection by using the vaginal speculum to press on the portio vaginalis of the cervix.

28.7 Retained Embryos after ET

The ET catheter must be checked for retained embryos after ET. This problem occurs more frequently after a difficult ET, or when the catheter was filled with mucus or blood. It decreases the implantation rates. It is advisable to retransfer the retained embryos immediately.

The volume of tissue culture medium for the ET is another cause of retained embryos. It is advisable to aspirate approximately 10–15 µL of culture medium first before aspirating the embryos to ensure the presence of enough medium to push out the embryos. It is also important to keep the pressure on the plunger of the syringe after ejecting the embryos until complete withdrawal of the catheter to avoid re-aspirating the embryos.

It is also advisable to withdraw the catheter slowly after ejecting the embryos to avoid creating negative pressure and withdrawal of the embryos following the catheter. Leong et al. [35] reported that withdrawal of the catheter about 1 cm and brisk injection avoided retrograde flow of the transfer media along the catheter by “capillary action.” An automated device that generates a standardized injection speed was described [36]. It is a peristaltic pump system to ensure pumping a small volume of medium containing the embryos which needs further research to investigate its clinical value.

28.8 Bed Rest after ET

Bed rest after ET was originally practiced in most IVF centers for several hours for fear of mechanical expulsion of the embryos. Different investigators have reported that there is no need for bed rest after ET. The position of the embryos was ultrasonically traced after ET immediately in a standing position. The authors reported that standing shortly after ET did not play a significant role in the final position of embryos. It has also been reported in a prospective controlled trial of 406 patients that immediate ambulation following the ET has no adverse effect on the pregnancy rate. The same conclusion was reached in a Cochrane review [37]. It was even found in a RCT that bed rest after ET negatively affects the pregnancy rate [38].

The so-called endometrial cavity is only a potential space and not a real cavity. The ET catheter only separates the opposed endometrial surfaces, and once the catheter is removed,

the endometrial surfaces re-oppose. Then, the embryos and fluid injected into the potential space are relocated by the endometrial and myometrial peristalsis as well as the surface tension between the fluid and solid interface. It is believed that the embryos generally implant near where they were deposited. Dummy ET was performed using small micro-spheres immediately before hysterectomy. The uterine cavity was then inspected and the microspheres were found within 1 cm from the site of deposition. In another study by Baba et al., it was found that 26 of 32 gestational sacs, seen by US, were in the area where the air bubble was seen immediately after ET [39]. Therefore we should assure patients that studies did not show benefit of bed rest after ET; however, patient's anxiety is an important factor to respect and we should allow some time of bed rest.

28.9 Duration of ET

The time interval from loading the embryos in the catheter to depositing them in the uterine cavity should be kept at a minimum to prevent prolonged exposure of the embryos to ambient temperature, light or other factors. A long time of more than 60 seconds or 120 seconds has been shown to lower the pregnancy and implantation rates. On the other hand, another study showed no adverse effect of the duration of the procedure on the results, even with transfers lasting up to 7.5 minutes. The prolonged time of ET procedure may be a marker of difficult ET; therefore, variables for delayed ET should be separated. The time interval between ejecting the embryos and withdrawal of the catheter was studied. Immediate withdrawal was compared to 30-second waiting, and there was no significant difference in the pregnancy rate. In a mock ET it was demonstrated that rapid injection of the fluid containing the embryos was associated with an increased rate of collapse and shrinkage of the embryos. It is advisable to inject slowly to avoid traumatizing the embryos.

28.10 ET Technique as a Cause of Ectopic Pregnancy

The risk of ectopic pregnancy following IVF was estimated to be 5 percent in a multicenter study undertaken with 1163 pregnancies. In a recent review the prevalence of ectopic pregnancy secondary to ART ranged between 2.1 and 8.6 percent of all pregnancies [40]. The simultaneous ectopic and intrauterine pregnancy, known as heterotopic pregnancy, was reported to be about 1 percent of patients during assisted conception (158). This figure is much higher than in natural conception.

The distance from the fundus to the tip of the ET catheter was studied in relation to the ectopic rate. The authors reported a decrease in the ectopic rate associated with an increased distance between the fundus and the tip of the catheter. The mid-fundal technique resulted in a lower percentage of ectopic pregnancy and did not negatively affect the pregnancy rate.

Ectopic pregnancy was 3.9 times more frequently associated with difficult ET than with an easy procedure.

Another factor in the etiology of ectopic pregnancy in IVF is the size of the uterus. It was reported that the ectopic pregnancy rate was significantly higher in women with a uterine cavity length less than 7 cm. ET technique can also be a cause of ectopic pregnancy due to forcing the embryo(s) through tubal ostia by hydrostatic pressure or by using a large volume of ET medium. The speed of the transfer was also implicated in inducing ectopic pregnancy. It was recommended to be performed slowly over 10 seconds.

Finally it was found that the uterine contractions in the early luteal phase are generally cervico-fundal in origin and it may be the cause for some ectopic pregnancies in IVF.

As a result of uterine peristalsis the embryos could move as easily toward the cervical canal as toward the fallopian tubes.

28.10.1 Concluding Remarks

The embryo transfer technique is the final and most critical step in ART. Individual training and monitoring of doctors is essential to standardize the technique. Several factors have been studied to develop guidelines for optimizing the ET technique. The evidence based factors include: (a) soft catheters, (b) ultrasonic guidance, (c) dummy ET, (d) curving the ET catheter according to the cervico-uterine angulations, (e) not touching the fundus and atraumatic technique, (f) small volume of medium to deposit the embryos, (g) the inclusion of hyaluronan in the ET media, and (h) hCG injection during the dummy ET. Other factors that are based on clinical experts' recommendations include: (a) gentle manipulation, (b) removing cervical mucus, (c) slow withdrawal of the catheter to avoid negative pressure, (d) minimizing the time of the procedure.

28.11 Description of an ET Procedure

- The patient is instructed to be fasting as the need for general anesthesia may arise.
- The patient/couple is informed of the fertilization rate, the number of embryos selected for the transfer and if there are extra embryos for cryopreservation. The patient should be assured of the simplicity of the procedure to alleviate anxiety and stress; if still anxious, oral valium can be given.
- The file of the patient is revised to see the US picture of the uterus and the comments on the previous dummy ET, particularly the length of the uterine cavity and the degree and direction of the utero-cervical angulation.
- The patient is put in the lithotomy position and the cervix is visualized using Cusco's speculum.
- The cervix and vaginal vaults are wiped with sterile gauze and tissue culture media to remove excess cervical mucus and vaginal secretions.
- The cervical mucus at the external os is aspirated gently and repeatedly using a 1 cm³ syringe.
- A dummy ET is performed using a sterile soft ET catheter. Only the soft inner catheter is introduced to pass the internal os and the rigid outer sheath is stopped short of the internal os. To make sure the soft catheter is not kinked inside the cervical canal, the catheter is rotated 360°, then left alone resting on the hand. If it recoils, it indicates that the catheter is bent. The catheter can be withdrawn to be reintroduced again after modifying the position of the cervix by manipulating the vaginal speculum (the degree of opening and how far it is introduced). If the soft catheter cannot be introduced, a more rigid and malleable one can be tried. The rigid malleable catheter can be molded to follow the curvature of the cervico-uterine angle (reported previously in US picture). Sometimes the catheter needs to be moved gently in different directions until its tip passes the internal os. The catheter curvature may need to be increased in order to overcome the acute angulation of the cervico-uterine angle. In almost all cases, it is possible to introduce the rightly curved rigid catheter.
- If the above maneuvers fail, the procedure is stopped and the patient is put under general anesthesia. General anesthesia is given in the form of propofol 2 mg/kg as an induction dose and anesthesia is maintained by inhalation of isoflurosome 1.5 percent and oxygen

100 percent through a face mask. The previous trial is repeated with or without a vulsellum to support the cervix. As a last resort, a rigid introducer may be used. In extremely rare cases, neither the rigid nor the metal introducer can pass the cervical canal and enter the internal os. If this happens, you can either resort to transmyometrial surgical ET or you can cryo-preserve the embryos and postpone the ET for later after doing cervical dilatation or hysteroscopic cannulation of the cervix.

- After introduction of the dummy ET catheter 40 μ L ET medium containing 500 IU hCG is injected intrauterine.
- The embryologist will start loading the embryos in a new catheter similar to the one that was introduced in the dummy ET.
- The ET catheter is flushed with tissue culture medium, and then filled with ET medium. About 10–15 μ L of transfer medium is aspirated first in the ET catheter, then the embryos are aspirated in another 10–15 μ L medium, to withdraw the embryos away from the tip of the catheter.
- The loaded ET catheter is introduced gently through the cervical canal to pass the internal os, then advanced slowly till the mid-uterine cavity and stopped 1–2 cm short of the uterine fundus.
- The screw of the vaginal speculum is loosened so that the two valves of the speculum gently press on the portio vaginalis of the cervix. At this moment some patients experience suprapubic heaviness or discomfort. After one minute, when this complaint disappears, the catheter is withdrawn about 0.5 cm, then the embryos are ejected and pressure is kept on the plunger of the syringe while slowly withdrawing the catheter out (the inner catheter and outer sheath simultaneously). The ET catheter is returned to the laboratory to check for the presence of any retained embryos. If found, retransfer is done immediately. The speculum is kept in place for an average of about 7 minutes and then removed.
- If the ET is done under US guidance, the protocol and tips are beautifully described by Schoolcraft (77).

References

1. Karande VC, Morris R, Chapman C, Rinehart J, Gleicher N. Impact of the “physician factor” on pregnancy rates in a large assisted reproductive technology program: do too many cooks spoil the broth? *Fertil Steril.* 1999 June;71(6):1001–09.
2. van Weering HG, Schats R, McDonnell J, Hompes PG. Ongoing pregnancy rates in in vitro fertilization are not dependent on the physician performing the embryo transfer. *Fertil Steril.* 2005 February;83(2):316–20.
3. Salha OH, Lamb VK, Balen AH. A postal survey of embryo transfer practice in the UK. *Hum Reprod.* 2001 April;16(4):686–90.
4. López MJ, García D, Rodríguez A et al. Individualized embryo transfer training: timing and performance. *Hum Reprod.* 2014 July;29(7):1432–37.
5. Eaton JL1, Zhang X2, Barnes RB2. Embryo transfer by reproductive endocrinology fellows vs attending physicians: are live birth rates comparable? *Am J Obstet Gynecol.* 2014 November;211(5):494.e1–5.
6. Fanchin R, Righini C, Olivennes F et al. Uterine contractions at the time of embryo transfer alter pregnancy rates after in-vitro fertilization. *Hum Reprod.* 1998 July;13(7):1968–74.
7. Woolcott R, Stanger J. Potentially important variables identified by transvaginal ultrasound-guided embryo transfer. *Hum Reprod.* 1997 May;12(5):963–66.
8. Nabi A, Awonuga A, Birch H, Barlow S, Stewart B. Multiple attempts at embryo transfer: does this affect in-vitro fertilization

- treatment outcome? *Hum Reprod.* 1997 June;12(6):1188–90.
9. Eskandar MA, Abou-setta AM, El-Amin M, Almushait MA, Sobande AA. Removal of cervical mucus prior to embryo transfer improves pregnancy rates in women undergoing assisted reproduction. *Reprod Biomed Online* 2007;14:308–13.
 10. Visschers BA, Bots RS, Peeters MF, Mol BW, van Dessel JH. Removal of cervical mucus: effect on pregnancy rates in IVF/ICSI. *Reprod Biomed Online* 2007;15: 310–15.
 11. Katiyari KO, Bates GW, Robinson RD, Arthur NJ, Propst AM. Does the timing of mock embryo transfer affect in vitro fertilization implantation and pregnancy rates? *Fertil Steril* 2007;88:1462–64.
 12. Lesny P, Killick SR, Tetlow RL, Robinson J, Maguiness SD. Embryo transfer—can we learn anything new from the observation of junctional zone contractions? *Hum Reprod.* 1998 June;13(6):1540–46.
 13. Kovacs GT. What factors are important for successful embryo transfer after in-vitro fertilization? *Hum Reprod.* 1999 March;14 (3):590–92.
 14. Coroleu B, Barri PN, Carreras O et al. The influence of the depth of embryo replacement into the uterine cavity on implantation rates after IVF: a controlled, ultrasound-guided study. *Hum Reprod.* 2002 February;17(2):341–46.
 15. van Weering HG, Schats R, McDonnell J et al. The impact of the embryo transfer catheter on the pregnancy rate in IVF. *Hum Reprod.* 2002 March;17(3):666–70.
 16. McDonald JA, Norman RJ. A randomized controlled trial of a soft double lumen embryo transfer catheter versus a firm single lumen catheter: significant improvements in pregnancy rates. *Hum Reprod.* 2002 June;17(6):1502–06.
 17. Abou-Setta AM, Al-Inany HG, Mansour RT, Serour GI, Aboulghar MA. Soft versus firm embryo transfer catheters for assisted reproduction: a systematic review and meta-analysis. *Hum Reprod.* 2005 Nov;20(11):3114–21.
 18. Dorn C, Reinsberg J, Schlebusch H et al. Serum oxytocin concentration during embryo transfer procedure. *Eur J Obstet Gynecol Reprod Biol.* 1999 November;87 (1):77–80.
 19. Tur-Kaspa I, Yuval Y, Bider D et al. Difficult or repeated sequential embryo transfers do not adversely affect in-vitro fertilization pregnancy rates or outcome. *Hum Reprod.* 1998 September;13(9): 2452–55.
 20. Moon HS, Park SH, Lee JO, Kim KS, Joo BS. Treatment with piroxicam before embryo transfer increases the pregnancy rate after in vitro fertilization and embryo transfer. *Fertil Steril.* 2004 October;82(4): 816–20.
 21. Ng EH, Li RH, Chen L et al. A randomized double blind comparison of atosiban in patients undergoing IVF treatment. *Hum Reprod.* 2014 December;29(12):2687–94.
 22. Sallam HN, Farrag A, Ezzeldin F, Agameya A, Sallam AN. The importance of flushing the cervical canal with culture medium prior to embryo transfer. *Fertil Steril* 2000;74(3)suppl 11: S64–S65.
 23. Derk RS, Farquhar C, Mol BW, Buckingham K, Heineman MJ. Techniques for preparation prior to embryo transfer. *Cochrane Database Syst Rev.* 2009 October 7;(4):CD007682. doi:10.1002/14651858.CD007682.pub2. Review.
 24. Nielsen IK, Lindhard A, Loft A, Ziebe S, Andersen AN. A Wallace malleable stylet for difficult embryo transfer in an in vitro fertilization program: a case-control study. *Acta Obstet Gynecol Scand.* 2002 February;81(2):133–7.
 25. Groutz A, Lessing JB, Wolf Y et al. Comparison of transmyometrial and transcervical embryo transfer in patients with previously failed in vitro fertilization—embryo transfer cycles and/or cervical stenosis. *Fertil Steril.* 1997 June;67 (6):1073–76.
 26. Sharif K, Afnan M, Lenton W et al. Transmyometrial embryo transfer after difficult immediate mock transcervical

- transfer. *Fertil Steril.* 1996 May;65(5): 1071–74.
27. García-Velasco JA, Isaza V, Martínez-Salazar J et al. Transabdominal ultrasound-guided embryo transfer does not increase pregnancy rates in oocyte recipients. *Fertil Steril.* 2002 September;78(3):534–39.
 28. Kosmas IP, Janssens R, De Munch L, et al. Ultrasound-guided embryo transfer does not offer any benefit in clinical outcome: a randomized controlled trial. *Hum Reprod* 2007;22:1327–34.
 29. Buckett WM. A meta-analysis of ultrasound-guided versus clinical touch embryo transfer. *Fertil Steril.* 2003 October;80(4):1037–41.
 30. Brown J, Buckingham K, Abou-Setta AM, Buckett W. Ultrasound versus ‘clinical touch’ for catheter guidance during embryo transfer in women. *Cochrane Database Syst Rev.* 2010 January 20;(1): CD006107. Review
 31. Karimian L, Rezazadeh VM, Baghestani AR, Moeini A. A prospective randomized comparison of two commercial embryo transfer medium in IVF/ ICSI cycles. *Hum Reprod* 2004;19 (Suppl1):i52.
 32. Bontekoe S, Blake D, Heineman MJ, Williams EC, Johnson N. Adherence compounds in embryo transfer media for assisted reproductive technologies. *Cochrane Database Syst Rev.* 2010 July 7; (7):CD007421.
 33. Mansour R, Tawab N, Kamal O et al. Intrauterine injection of human chorionic gonadotropin before embryo transfer significantly improves the implantation and pregnancy rates in in vitro fertilization/intracytoplasmic sperm injection: a prospective randomized study. *Fertil and Steril.* 2011;96(6):1370–4.
 34. Ye H, Hu J, He W, Zhang Y, Li C. The efficacy of intrauterine injection of human chorionic gonadotropin before embryo transfer in assisted reproductive cycles: Meta-analysis. *J Int Med Res.* 2015 December;43(6):738–46.
 35. Leong M, Leung C, Tucker M, Wong C, Chan H. Ultrasound-assisted embryo transfer. *J In Vitro Fert Embryo Transf.* 1986 December;3(6):383–85.
 36. Groeneveld E, de Leeuw B, Vergouw CG et al. Standardization of catheter load speed during embryo transfer: comparison of manual and pump-regulated embryo transfer. *Reprod Biomed Online.* 2012 February;24(2):163–69.
 37. Abou-Setta AM, Peters LR, D’Angelo A et al. Post-embryo transfer interventions for assisted reproduction technology cycles. *Cochrane Database Syst Rev.* 2014 August 27;8:CD006567.
 38. Gaikwad S, Garrido N, Cobo A, Pellicer A, Remohi J. Bed rest after embryo transfer negatively affects in vitro fertilization: a randomized controlled clinical trial. *Fertil Steril.* 2013 September;100(3):729–35.
 39. Baba K, Ishihara O, Hayashi N et al. Where does the embryo implant after embryo transfer in humans? *Fertil Steril.* 2000 January;73(1):123–25.
 40. Refaat B, Dalton E, Ledger WL. Ectopic pregnancy secondary to in vitro fertilisation-embryo transfer: pathogenic mechanisms and management strategies. *Reprod Biol Endocrinol.* 2015 April 12;13:30.

Vitrification of Embryos for IVF

Juergen Liebermann

29.1 Brief Background

29.1.1 Purposes of Cryopreservation of Human Embryos in General

Since the announcement in 1972 of the survival of mouse embryos after cryopreservation at -196°C [1] the impact of cryopreservation on the growth and improved efficiency of assisted reproduction in humans has become increasingly appreciated. Moreover, routine in vitro blastocyst culture cryopreservation has also been shown to increase overall pregnancy rates, while allowing for better selection of embryos. Indeed it is possible to achieve implantation and pregnancy rates with frozen-thawed embryos as high as those achieved with fresh embryos. With more reliable cryopreservation techniques, lower numbers of embryos are now being transferred, resulting in less high-order multiple pregnancies, as well as increased healthy implantations.

29.1.2 The Vitrification Component in Particular

The major disadvantage of using cryostorage is that it can lead to crystallization of water, and thereby can create new and unwanted physical and chemical events that may injure the cells that are being preserved. As early as 1985, ice-free cryopreservation of mouse embryos at -196°C by vitrification was reported in an attempted alternative approach to cryostorage. Since then, vitrification techniques have entered more and more the mainstream of animal and human reproduction as an alternative cryopreservation method to traditional slow-cooling/rapid-thaw protocols. In addition, the last few years have seen a significant resurgence of interest in the potential benefits of vitrification protocols and techniques in human-assisted reproductive technologies. The radical strategy of *vitrification* results in the total elimination of ice crystal formation by establishing a glassy or vitreous state, both within the cells being vitrified (intracellular) and in the surrounding solution (extracellular). The physical definition of vitrification is the solidification of a solution at low temperature, not by ice-crystallization, but by extreme elevation in viscosity during cooling [2, 3]. In addition, recent publications have shown the relatively greater importance of warming rate over cooling rates with regard to the survival of oocytes subjected to a vitrification procedure [4–5].

The earliest attempts using vitrification as an ice-free cryopreservation method for embryos were first reported in 1985 [6]. In 1999 and 2000 successful pregnancies and deliveries after vitrification and warming of human oocytes were reported [7, 8]. Since that time, and because it seems to be that oocytes as well as blastocysts appear to be

especially chill-sensitive cells in ART, blastocysts appear to have received a significant boost in survival rates by avoiding ice-crystallization through use of vitrification [9].

It is important to mention that cryopreservation interferes without doubt with the intracellular and extracellular homeostasis and therefore poses a risk for inducing spindle abnormalities, chromatid nondisjunction, fertilization errors, and abnormal mitotic divisions during embryonic development. In addition, specific subcellular compartments containing accumulated transcripts and proteins necessary for spindle formation, epigenetic control, and chromosome alignment are also mandatory for proper spindle functionality. Although it is far too early to draw conclusions about the risk of imprinting mutations and birth defects after vitrification of oocytes and embryos, the current literature is reassuring. An increased incidence of imprinting diseases in live births after cryopreservation of oocytes and preimplantation embryos has not been identified [10].

29.1.3 Cryoprotectant Agents

One “drawback” of vitrification considered by embryologists not familiar with the vitrification technique, is the use of high concentrations of cryoprotectants, which does potentially mean that vitrification solutions are potentially more toxic than their counterpart solutions used for conventional slow-freezing. This is necessitated by the practical limit for the rate of cooling, and the biological limit of tolerance of the cells for the concentration of toxic cryoprotectants being used to achieve the cryopreserved state. It is important to note that recently published papers [11–13] have shown that the use of relatively high concentration of cryoprotectants such as 15 percent (v/v) ethylene glycol (EG) used in an equimolar mixture with dimethylsulfoxide (DMSO) had no negative effect on the perinatal outcomes from thawed blastocyst transfers following vitrification when compared with those from fresh blastocyst transfers.

Cryoprotective agents (CPA) are essential for the cryopreservation of cells. Basically two groups of cryoprotectants exist: [1] permeating (e.g., glycerol, EG, DMSO) and [2] non-permeating (e.g., disaccharides, proteins and polymers) agents. The key component of a vitrification solution is the permeating agent. These compounds are hydrophilic non-electrolytes with a strong dehydrating effect. Furthermore, these CPAs are able to depress the “freezing point” of the solution. Additionally, these highly permeating cryoprotectants are also more likely to diffuse rapidly out of the cells so that the cells quickly regain their original volume upon warming, thus preventing osmotic injury. The second component of a vitrification solution is the non-permeating CPAs such as disaccharides, for example, sucrose, which does not penetrate the cell membrane, but helps to draw out more water from cells by osmosis, and therefore lessens the exposure time of the cells to the toxic effects of the permeating CPAs. The non-permeating sucrose also acts as an osmotic buffer to reduce the osmotic shock that might otherwise result from the dilution of the cryoprotectant after cryostorage. Furthermore, permeating agents are able to bind with intracellular water, and therefore water is very slowly removed from the cell. During warming, using a high extracellular concentration of sucrose (e.g., 1.0 M) counterbalances the high concentration of the cryoprotectant agents in the cell, as it reduces the difference in osmolality between the intra- and extracellular compartments. The high sucrose concentration cannot totally prevent the cell from swelling, but it can reduce the speed and magnitude of swelling [14, 15].

29.1.4 Artificial Collapsing and Assisted Hatching

Most important, residual fluid in the blastocoel can reduce post-warming survival of embryos. Vanderzwalmen et al. [16] showed that survival rates in cryopreserved, expanded blastocysts could be improved by artificial reduction of the blastocoel cavity, and others consider that blastocoel collapse is necessary pre-vitrification on whichever day the blastocyst forms [16, 17] and the most effective strategy for promoting both permeation of cryoprotectant and dehydration [18].

Artificial collapse (AC) of the blastocoel can be performed using different techniques, such as microneedles, sucrose solutions or lasers [19–21]. In 2003 and 2004, two groups independently reported a beneficial effect by applying AC to blastocysts prior to vitrification. Son et al. [19] observed an improved clinical pregnancy rate of 48 percent, and an implantation rate of 29 percent with the use of AC. Hiraoka et al. [20] collapsed day-5 and day-6 blastocysts by manually pipetting embryos until they collapsed, and achieved a clinical pregnancy rate of 50 percent, with an implantation rate of 33 percent after warming. Moreover, Mukaida et al. [21] found that the survival rate of vitrified blastocysts was negatively correlated with the size of the blastocoel. They speculated that a large blastocoel may disturb the efficacy of vitrification. After applying AC, the survival improved from 86 percent to 97.2 percent. Moreover, their pregnancy rate went up from 34.1 percent to 60.2 percent, with an implantation rate of 46.7 percent. Iwayama et al. [22] used a laser pulse, or osmotic shock resulting from exposure of the whole embryo to sucrose, and the implantation rate was significantly higher in both groups compared to the control group without AC (59.7% and 49.3% vs 34.2%). Furthermore, Hur et al. [23] looked at the effect of AC, achieved using a 29-gauge needle or laser pulse, on clinical outcomes in fresh transfers, and they observed a significant increase in the clinical pregnancy in the study group compared to the control group (58.8 vs 39.0 percent). All publications mentioned, including Liebermann and Conaghan [24], conclude that AC has a beneficial effect both in frozen blastocyst transfers, and for overall cumulative pregnancy and implantation rates. A recent study by Kazemi et al. [25] assessed the effect of pre-vitrification blastocoel fluid reduction on the survival, hatching rate, and the expression of genes related to apoptosis (*Tp53*), pluripotency (*Pou5f1*, *Nanog*), and differentiation (*Cdx2*, *Eomes*, *Gata6*) in mouse blastocysts. The survival rate of treatment groups was similar to the control group, whereas the hatching rate of artificial collapse/vitrified blastocysts was significantly higher than vitrified blastocysts. Quantitative reverse-transcription PCR analysis revealed a considerable reduction in the expression of *Cdx2*, *Eomes*, *Gata6*, *Grb2*, and *Tp53* transcripts following artificial collapse/vitrification in comparison to the vitrification-alone group; the abundance of *Pou5f1* and *Nanog*, however, did not change. The authors concluded that artificial collapse of the blastocoel cavity before vitrification leads to relatively normal expression of apoptosis and development-related genes plus higher hatching rates.

Because of the post-thaw phenomena called “zona hardening” [26] and based on recently published data [27] all thawed embryos underwent assisted hatching using a laser by removing about a third of the zona pellucida.

Warming of blastocysts occurred 1–2 hours prior to transfer. Both natural and hormone replacement cycles were used to increase the receptivity of the endometrium. Progesterone was supplemented on day 15 of the cycle and blastocysts were warmed on day 6 of progesterone supplementation.

29.2 Brief Overview of Method

29.2.1 Materials

1. HSV (High Security Vitrification Kit (Catalog # 022137; CryoBioSystem).
2. Heat sealer (CryoBioSystem).
3. Polycarbonate micropipettes, 275 μm end hole (Origio Inc. MXL3-275).
4. Brady labeler (TS 2000).
5. Brady labels (PTL-19-427).
6. 100 x 15 mm Petri dish (Falcon # 351029).
7. Center-well organ culture dish (Falcon 353037).
8. Styrofoam container.
9. Visotubes 10 mm (IMV 5561).
10. Cryo canes aluminium (ThermoScientific 5015-0001).

29.2.2 Reagents

1. Serum Substitute Supplement (SSS) (Irvine)
2. Wash solution as a part of the Thaw kit (Irvine)
3. Vit-Kit-Freeze (Irvine Scientific #90133DSOC).
4. Vit-Kit-Thaw (Irvine Scientific # 90137DSOC).

29.2.3 Equipment

1. Dissecting stereo microscope (Olympus SZX-12, Bausch Lomb or Leica) with warming stage.
2. Laminar Flow hood (Origio).
3. Inverted Microscope (Olympus IX-71).
4. Infrared 1.48 μm diode laser (Hamilton Thorne-Lykos laser (Hamilton Thorne Research, Beverly, MA).

29.2.4 Methods

29.2.4.1 Vitrification Procedure

Vitrification of blastocysts is undertaken utilizing a “closed system” (HSV: High Security Vitrification Kit; CryoBio System, L’Aigle, France; FDA 510(k) clearance for cleavage stage embryos and blastocysts) after a two-step loading with cryoprotectant agents at 24°C. The composition for the “freeze” and “warm” kit are summarized in Table 29.1. Each single step is described in detail below.

1. Aseptic techniques are required at all stages. For equilibration and vitrification procedures ensure the benchwarmer is at room temperature (~24°C).
2. Take reagents from the refrigerator and allow them to warm to room temperature.
3. Move blastocysts to freeze into a separate well. To perform assisted collapsing (AC), bring this dish to the inverted microscope and with the embryo positioned with the laser objective (LYKOS, Hamilton Thorne) use a single pulse (100 percent power, 500 μs pulse length) to hit the blastocysts between the junction of two trophectoderm cells to collapse the embryo. Place the dish with the blastocyst back into the incubator for 5–10 min.

Table 29.1 Composition of vitrification and warming solutions (Irvine vit-kit “freeze” and “warm”)

	Composition		
	Ethylene glycol (%)	Dimethyl sulfoxide (%)	Sucrose (M)
Equilibration solution (ES)	7.5	7.5	0
Vitrification solution (VS)	15	15	0.5
Warming solution (TS)	0	0	1.0
Diluent solution (DS)	0	0	0.5
Wash solution (WS)	0	0	0

M-199 + 20% SSS [Wash solution–WS]

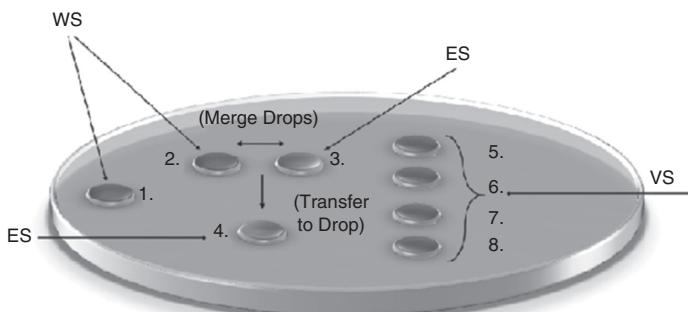


Figure 29.1 Setup for the vitrification procedure on a plain 100 mm Petri dish lid surface. (ES = Equilibration solution; VS = Vitrification solution; WS = Washing solution)

4. Label a Petri dish with the patient’s name under the lid as follows: Washing solution (WS), Equilibration solution (ES), and Vitrification solution (VS). Prepare 2 x 50 μ L of WS, 2 x 50 μ L of ES and 4 x 50 μ L of VS (see Figure 29.1).
5. Brady label should include the patient’s last name, first name, accession #, Medical Personal Identification# (MPI), date plus number, and type of embryos; apply the label to the open end of the empty outer straw.
6. Each sample that is vitrified will be done in a separate hood and verified by a second embryologist before proceeding. Vitrify good, expanded/hatching blastocysts on day 5/6/7.
7. Remove embryos from culture dishes using a stripper tip into the WS (drop 1), gently aspirating to remove any residual culture medium and oil.
8. Pipette from WS (drop 1) to the other drop of WS (drop 2) and immediately merge it with the 1st drop of ES (drop 3). Set the timer for 5 min.
9. After 5 min, transfer the embryos to the remaining drop of ES (drop 4). Set the timer for 3 min. Place the embryos on the top of the drop and let them settle to the bottom of the dish.

10. Fill styrofoam container with LN2.
11. Then load the blastocysts in a VS back loaded stripper tip, and rinse through the 4 droplets of VS (drop 5–8); between each droplet, clean the tip.
12. Placement into the VS and loading of the HSV should take about 1 minute, so that the total incubation time in VS is about 45 sec. After 45 sec, using a stripper tip gently load the blastocyst(s) in a small volume (less than 0.5 µL) to the tip of the HSV.
13. Visually confirm placement of embryos on the HSV.
14. Load the HSV stick into the empty outer straw, the side with the embryos first. Use the blue handle to make sure the stick is in as far as it goes. Then, using the heat sealer, seal the open end of the stick and plunge the whole straw with a stirring movement into the LN2. Finally, place the straw in a 5-mL liquid nitrogen prefilled tube (Visotube Rond, IMV, France) and connect it to aluminum cane for further storage.
15. Store the cane in a nitrogen tank.
16. Make sure to record cane location on the freezing worksheet and cryo inventory log.
17. Complete all paperwork and recheck that all vial locations are logged into the Embryo Inventory.

29.2.4.2 Warming Procedure

It is important to mention that regardless of the day of cryopreservation of the embryo (whether day 5, 6 or 7); at thawing, blastocysts should be treated as if they had been cryopreserved on the fifth day of development. To remove the cryoprotectants, blastocysts need to be warmed and diluted in a three-step process. Each single step is described in detail below.

1. Take reagents from the refrigerator and allow them to warm to room temperature. After the first step being done at 37°C, the following steps for removing the cryoprotectants are done at 24°C.
2. Place a single 1 x 200 µL drop of TS on a 100-mm Petri dish and place it on a warming plate to warm it up to 37°C (Figure 29.2).
3. Label a Petri dish (Nunc) with the patient's name under the lid as follows: TS, DS, and WS. Then prepare on a separate 100-mm dish lid 1 x 50 µL of TS, 3 x 50 µL of DS and 4 x 50 µL of WS.
4. Before warming, use a Stripper tip with 275 µm end hole for removing the blastocysts from the HSV tip.
5. Fill styrofoam container with LN2.
6. Confirm location and identification with a second embryologist before warming any HSV kit. Warm one kit at a time.
7. Each sample that is warmed is done in a separate hood and verified by a second embryologist before proceeding.
8. With the HSV kit submerged in LN2, open the kit by cutting the outer straw and removing the inner straw.
9. Then the carrier with the blastocysts can be removed from the LN2 and submerge the HSV kit containing the blastocysts directly in the pre-warmed 37°C drop containing 200 µL of 1.0 M sucrose (TS), which should be as close as possible to the LN2 styrofoam container. As soon as the HSV kit contents liquefy (within 1sec), try to locate the blastocyst(s) before removing it (them) with a stripper tip. After locating all the blastocysts, remove them from the HSV tip and place them in a fresh drop of TS at 24°C

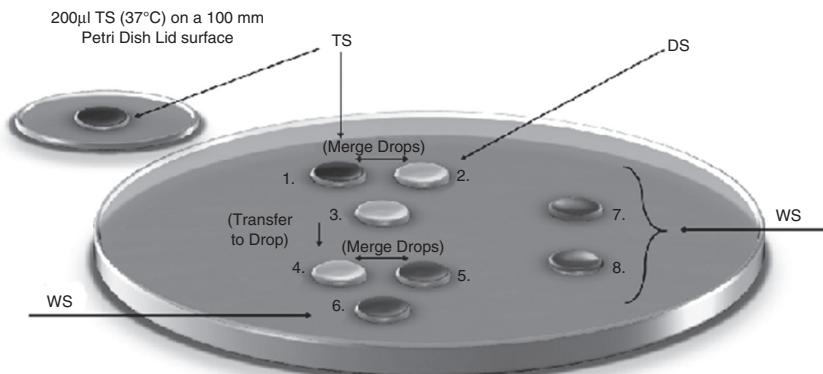


Figure 29.2 Setup for the warming procedure on a plain 100 mm Petri dish bottom surface. (TS = Thawing Solution; DS = Diluent solution; WS = Washing solution)

(drop 1) at room temperature, then connect immediately with the 1st droplet of DS (drop 2). Wait for 3 min. When switching the embryos between different concentrations of warming solutions, be sure to fill up the pipette with the next lower concentration of warming solution before picking up the cells for moving into the next dilution.

10. Then connect with the 2nd droplet of DS (drop 3) for 2 min.
11. Then transfer blastocysts to 0.5 M sucrose (drop 4) and connect with 1st of WS (drop 5) for 3 min.
12. After 3 min connect with 2nd drop of WS (drop 6) for 2 min. Finally rinse through a series of two additional drops of WS for 1 min (drop 7 and 8).
13. Perform assisted hatching on the warming dish before placing the blastocysts into a culture dish with (SAGE+20%SSS); (SAGE Blastocyst Medium, Trumbull, CT, USA), and put them back in the incubator for subsequent culture until transfer.
14. Record the survival and appearance of all blastocysts. Update log with warming data, and notify the physician of result.

29.3 Successful Application of Blastocyst Vitrification

Between 2004 and 2016 the Fertility Centers of Illinois “IVF Laboratory River North” (Chicago) has vitrified 33,476 blastocysts from 8289 patients (Table 29.2). The majority of blastocysts were vitrified on day 5 (55.0 percent), 43.5 percent on day 6, with a minority on day 7 (1.5 percent). After 13 years of vitrifying blastocysts using an open (Cryotop) as well as closed (HSV) system, and 7754 FETs with an average number of 1.7 embryos transferred, the perinatal outcomes are as follows: number of babies from 1914 deliveries until January 2015 equals 2336 (1203 girls and 1133 boys) (Table 29.3). No abnormalities were recorded.

Table 29.4 is summarizing data on gestational age and live birth weight from 1363 children born. On the whole, 926 babies were from frozen-vitrified day-5 and day-6 transfers, whereas 437 babies were born from fresh elective single embryo transfers (eSET). Furthermore, comparing the 437 newborns from the eSET group to the 926 newborns from frozen-vitrified group; (VBT), there was a significant difference in mean age of

Table 29.2 Retrospective data from 8289 patients (average age 34.6±6.0) with blastocyst vitrification between 2004 and 2016

	Day of Development			Total
	Day 5	Day 6	Day 7	
Number of blastocysts vitrified	18,495 (55.0%)	14,505 (43.5%)	476 (1.5%)	33,476 (100%)

Table 29.3 Perinatal outcome of vitrified blastocysts after 7754 transfers between 2004 and 2016 (babies delivered until January 2015)

	Day 5 + Day 6	Day of Development		
		Day 5	Day 6	Day 7
Deliveries (total)	1914	1264	641	9
Babies born (total)	2336	1555	771	10
Female	1203	813	386	4
Male	1133	742	385	6
Singletons	1502 (78.5)	979 (77.5)	515 (80.0)	8 (89.0)
Twins	402 (21.0)	279 (22.0)	122 (19.0)	1 (11.0)
Triplets	10 (0.5)	6 (0.5)	4 (1.0)	-

Note: Percentages are indicated between parentheses.

the patient ($p<0.001$). In addition, looking at gestational age (GA) and live birth weight (LBW) of newborns derived from vitrified day-5 blastocysts ($n = 561$) and day-6 blastocysts ($n = 365$) following data were observed: T-test results indicate no significant difference in gestational age ($p = 0.71$) nor birth weight ($p=0.124$) for the frozen transfers based on day of development. Obviously, gestational age is highly correlated with birth weight, but there is no significant difference in gestational age between eSET and VBT. However, Table 29.4 shows that one-way ANOVA of LBW by both groups indicate a statistically significant difference between the means of eSET vs VBT ($p = 0.013$) with heavier babies in the VBT group. This observation was also published earlier by the Pinborg group [28]. As shown in Table 29.4, Chi-square statistic indicates no difference in the gender distribution between fresh and frozen-vitrified transfers ($\chi^2; p = 0.3454$ respectively). Running a T-test on gender weight combining the gender from fresh with frozen-vitrified transfers indicates a significant difference in average weight by gender ($p<0.001$; Table 29.5). Males were on average 157.2 grams heavier than females. Table 29.5 shows the weight distribution for babies born in both groups. Overall, 110 babies weighed less than 2500 grams (*considered as low birth weight*), 159 babies born from both groups weighed between 4000 and 4500 grams

Table 29.4 Live birth weight (LBW) and gestational age (GA) of babies ($n=1363$) delivered after fresh elective single blastocyst transfers (eSBT; $n=437$) compared to those delivered from a vitrified blastocyst transfer (VBT; $n=926$) and related to day of development after VBT (day 5 vs day 6)

	eSBT – Day 5	VBT – Day 5 + Day 6	Day of development	
			VBT – Day 5	VBT – Day 6
Average age	31.1 ± 3.1^c	34.7 ± 4.9^c	34.7 ± 5.0	34.8 ± 4.8
Deliveries	437	926	561	365
Female	238 ^a	479 ^a	288	191
Male	199 ^a	447 ^a	273	174
GA	38.1 ± 2.5^a	37.9 ± 2.7^a	38.0 ± 2.6^a	37.9 ± 2.9^a
LBW (g)	3294.9 ± 609.4^b	3390.1 ± 710.6^b	3419.1 ± 712.6^a	3345.5 ± 705.8^a

Note: T-test (Satterthwaite-Unequal variance) & χ^2 test: ^a $P>0.05$; ^b $P=0.013$; ^c $P<0.001$

Table 29.5 Distribution (%) of live birth weight (LBW) of babies ($n=1363$) delivered after fresh elective single blastocyst transfers (eSBT; $n=437$) compared to those delivered from vitrified blastocyst transfers (VBT; $n=926$) and gestational age (GA) and gender-specific live birth weight (LBW) between fresh and frozen embryo transfers combined

	eSBT – Day 5 (n) [%]	VBT – Day 5 + Day 6 (n) [%]	Total (n) [%]
LBW (g) < 2500	34 [7.8]	76 [8.2]	110
LBW (g) ≥ 4000	38 [8.7]	154 [16.6]	192
LBW (g) ≥ 4000 and ≤ 4500	32 [7.3]	127 [13.7]	159
LBW (g) > 4500	6 [1.4]	27 [2.9]	33
Gender	Girls from eSET and VBT		Boys from eSET and VBT
N	717		646
GA (weeks)	37.9 ± 2.6^a		38.0 ± 2.6^a
LBW (g)	3284.1 ± 663.8^b		3441.3 ± 691.0^b

Note: Percentages are indicated between brackets. T-test & χ^2 test: ^a $P>0.05$; ^b $P<0.001$

(considered as large birth weight) and 33 babies weighed more than 4500 grams (considered as macrosomic birth weight).

The outcome with regards to day of development and age of the patient including donor-recipient cycles, between 2004 and October 2016 are summarized in Tables 29.6

and 29.7. In good prognosis patients under 35 years old when transferring day-5 blastocysts, an ongoing pregnancy and implantation of 49.2 and 45.1 percent was noted (Table 29.6). In contrast, transferring day-6 blastocysts in patients younger than 35 years of age, an ongoing pregnancy and implantation of 36.0 and 32.4 percent was recorded (Table 29.7).

In October 2007 the Fertility Centers of Illinois “IVF Laboratory River North” moved forward from the use of an open carrier system (Cryotop – embryos are in direct contact with liquid nitrogen) to a closed system (embryos are sealed before contact with liquid nitrogen). Using a closed carrier (High Security Vitrification Kit, HSV) for aseptic vitrification, the following data from day-5, day-6 and day-7 blastocysts were observed and are summarized in Table 29.8: (a) clinical pregnancy rate (cPR): 54.8% vs 42.5% vs 18.2%; (b) ongoing pregnancy (oPR): 46.3% vs 33.3% vs 15.9%; and (c) implantation rate (IR): 44.0% vs 31.9% vs 12.5% (Table 29.8). As shown in Table 29.8, oPR, cPR, and IR occurring in the day-5 blastocyst group were significantly higher than transferring day-6 or even day-7 blastocysts.

Between 2007 and October 2016 the Fertility Centers of Illinois “IVF Laboratory River North” (Chicago) performed 2765 vitrified blastocyst transfers (VBT) without collapsing prior to vitrification (group A), and in 2765 VBT (group B) where artificial collapse was performed prior to vitrification. When the vitrified-warmed blastocysts were divided into day-5 and day-6 groups, the following data were observed (Table 29.9): in 1622 VBT transferring day-5 blastocysts from group A (mean age of 35.5 ± 5.0), the IR, cPR, and oPR were 38.4%, 48.9%, and 40.0%, compared to 49.0%, 59.7%, and 51.5% of day-5 blastocysts from group B ($n = 1930$; mean age of 35.2 ± 4.8). As shown in Table 29.9, implantation, cPR

Table 29.6 Retrospective outcome data (2004–10/2016) at the Fertility Centers of Illinois, Chicago from vitrified day-5 blastocysts in regard to the patients’ age

	Patient’s age (y)				
	<35	35–37	38–40	>40	Donor
Average age	31.3 ± 2.4	35.9 ± 0.8	38.9 ± 0.8	42.6 ± 1.9	43.5 ± 4.8
Cycles	1978	895	587	307	355
Transfers	1977	894	585	306	355
Blastocysts survived (%)	98.4	98.7	98.8	98.7	99.0
Blastocysts transferred (mean)	1.6	1.6	1.7	1.7	1.7
Positive pregnancy/VET (%)	64.9	62.9	60.5	59.8	62.8
Clinical pregnancy/VET (%)	56.0	52.0	49.0	48.0	53.0
Ongoing/delivered pregnancies (%)	49.2	44.0	39.0	37.0	41.0
Implantations	1442	585	355	173	240
Implantation rate (%)	45.1	42.0	36.5	34.3	40.5

Note: VET = vitrified embryo transfer.

Table 29.7 Retrospective outcome data (2004–10/2016) at the *Fertility Centers of Illinois, Chicago* from vitrified day-6 blastocysts in regard to the patients' age

	Patient's age (y)				
	<35	35–37	38–40	>40	Donor
Average age	31.2 ± 2.3	36.0 ± 0.8	38.9 ± 0.8	42.6 ± 1.9	43.0 ± 4.7
Cycles	1039	579	451	316	178
Transfers	1032	574	448	314	177
Blastocysts survived (%)	97.7	98.7	98.2	97.0	99.7
Blastocysts transferred (mean)	1.7	1.6	1.7	1.6	1.7
Positive pregnancy/VET (%)	52.6	48.4	48.0	45.5	46.3
Clinical pregnancy/VET (%)	44.0	41.0	40.0	35.0	38.0
Ongoing/delivered pregnancies (%)	36.0	33.0	31.0	24.0	27.0
Implantations	579	291	224	124	79
Implantation rate (%)	32.4	30.6	29.5	24.4	26.6

Note: VET = vitrified embryo transfer.

Table 29.8 A comparison of retrospective data of vitrified day-5, day-6 and day-7 blastocysts using aseptic vitrification technology between October 2007 and October 2016 at *Fertility Centers of Illinois, Chicago*

	Day 5 + Day 6 + Day 7	Day 5	Day 6	Day 7
Patient's age (y)	35.7 ± 4.9	35.3 ± 4.9	36.2 ± 4.8	36.0 ± 4.1
Transfers	5575	3552	1979	44
Blastocysts warmed	9102	5679	3349	74
Blastocysts survived	9011 (99.0)	5635 (99.2)	3304 (98.7)	72 (97.3)
Blastocysts transferred	8856	5546	3238	72
Blastocysts transferred (mean)	1.6	1.6	1.6	1.6
Implantations	3483 (39.3)	2440 (44.0) ^a	1034 (31.9) ^a	9 (12.5) ^a
Positive pregnancy/VET	3334 (59.8)	2297 (64.7) ^b	1023 (51.7) ^b	14 (31.8) ^b
Clinical pregnancy/VET	2795 (50.1)	1947 (54.8) ^c	840 (42.5) ^c	8 (18.2) ^c
Ongoing/delivered pregnancies	2309 (41.4)	1643 (46.3) ^d	659 (33.3) ^d	7 (15.9) ^d
Live births (n) until 01/2015	1726	1200	520	6

Note: Percentages are indicated between parentheses. χ^2 ; ^a $P>0.05$; ^b $P<0.001$; ^{ab,c,d} $P<0.001$; VET = vitrified embryo transfer.

Table 29.9 A comparison of retrospective data of vitrified day-5 and day-6 blastocysts without artificial collapsing (group A) and with artificial collapsing (group B) using aseptic vitrification technology between 2007 and October 2016 at *Fertility Centers of Illinois, Chicago*

	Technique			
	Group A		Group B	
	Day 5	Day 6	Day 5	Day 6
Patient's age (y)	35.5 ± 5.0	36.1 ± 4.8	35.2 ± 4.8	36.4 ± 4.7
Transfers	1622	1143	1930	835
Blastocysts warmed	2735	2004	2943	1343
Blastocysts survived	2709 (99.0)	1972 (98.4)	2925 (99.4)	1330 (99.0)
Blastocysts transferred	2632	1930	2913	1306
Blastocysts transferred (mean)	1.6	1.7	1.5	1.6
Implantations	1012 (38.4) ^a	514 (26.6) ^{aa}	1426 (49.0) ^a	519 (39.7) ^{aa}
Positive pregnancy/VET	926 (57.1) ^b	497 (43.5) ^{bb}	1370 (71.0) ^b	525 (62.9) ^{bb}
Clinical pregnancy/VET	793 (48.9) ^c	416 (36.4) ^{cc}	1153 (59.7) ^c	423 (50.7) ^{cc}
Ongoing/delivered pregnancies	648 (40.0) ^d	312 (27.3) ^{dd}	994 (51.5) ^d	347 (41.6) ^{dd}

Note: Percentages are indicated between parentheses. χ²; Day 5: ^aP<0.01; ^{b,c,d}P<0.001; Day 6: ^{aa}P<0.01; ^{bb,cc,dd}P<0.001; VET = vitrified embryo transfer.

and oPR occurring from the day 5 blastocysts in group B were significantly higher than from the day-5 blastocyst in group A.

If we compare day 6 in group A ($n=1143$; mean age of 36.1 ± 4.8) with day 6 outcome in group B ($n=835$; mean age of 36.4 ± 4.7), the following data in terms of implantation, cPR and oPR were observed: 26.6%, 36.4%, 27.3% vs 39.7%, 50.7%, and 41.6%, respectively (Table 29.9). As shown in Table 29.9, implantation, cPR, and oPR occurring in the day-6 blastocysts of group B were significantly higher than transferring day-6 blastocysts from group A.

29.4 Conclusions and Future Directions

Vitrification is a cryopreservation method with many advantages, and an ever increasingly consistent clinical track record. A standardized vitrification protocol applicable to all stages of the preimplantation embryo may not be realistic because of: (a) different surface-to-volume ratios; (b) the pattern of the movement of water and cell-permeating cryoprotectants is rather more stage-specific; (c) differing cooling rate requirements between oocytes, zygotes, cleavage stage embryos and blastocysts; and (d) variable chill-sensitivity between these different developmental stages. Currently however, the most widely used protocol applied to any embryo stage is the two-step equilibration in an equimolar combination of

the cryoprotectants ethylene glycol and DMSO. With the increased use in human assisted reproduction will come evolution of the vitrification process, as it is fine-tuned to clinical needs, thus pushing forward its development to higher levels of clinical efficiency, utilization and universal acceptance.

29.5 Practical Implications Vitrifying at the Blastocyst Stage

Our data have shown that freezing at the blastocyst stage provides excellent survival, implantation, and ongoing pregnancy rates. To achieve these outcomes, consider these points: (a) Without a successful blastocyst vitrification storage program, extended culture should never be attempted; (b) The blastocyst is composed of more cells and is therefore better able to compensate for cryoinjury; (c) The cells are smaller, which makes cryoprotectant penetration faster. On average, fewer embryos per patient are cryostored, but each one has a greater potential for implantation when thawed.

Juergen Liebermann wants to thank the physicians at the Fertility Centers of Illinois (FCI) and the embryologists at the FCI IVF Laboratory River North (Jill Matthews, BS, Sara Sanchez, BS, Rebecca Brohammer, BS; Fanjing Meng, PhD; Alina Diamond, BS; Janna Schwab, MS; Ivana Elzy, BS; Jordan Machlin, BS; and Laura Baranyi, BS) for their invaluable contributions and support in pushing vitrification to become our standard protocol for cryopreservation of human oocytes and blastocysts within our program since 2004.

References

1. Whittingham DG, Leibo SP, Mazur P. Survival of mouse embryos, frozen to -196°C and -289°C . *Science* 1972;178:411–14.
2. Fahy GM, MacFarlane, DR, Angell, CA et al. Vitrification as an approach to cryopreservation. *Cryobiology* 1984;21:407–26.
3. Fahy GM. Vitrification: a new approach to organ cryopreservation. In: *Transplantation: Approaches to Graft Rejection*, pp. 305–35, 1986 Ed. Merryman HT, Alan R Liss, New York.
4. Seki S, Mazur P. The dominance of warming rate over cooling rate in the survival of mouse oocytes subjected to a vitrification procedure. *Cryobiology* 2009;59:75–82.
5. Mazur P, Seki S. Survival of mouse oocytes after being cooled in a vitrification solution to -196°C at 95° to $70,000\text{ }^{\circ}\text{C/min}$ and warmed at 610° to $118,000\text{ }^{\circ}\text{C/min}$: A new paradigm for cryopreservation by vitrification. *Cryobiology* 2011;62:1–7.
6. Rall WF and Fahy GM. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature* 1985;313:573–75.
7. Kuleshova L, Gianaroli L, Magli C et al. Birth following vitrification of a small number of human oocytes: case report. *Hum Reprod* 1999;14:3077–79.
8. Yoon TK, Chung HM, Lim JM et al. Pregnancy and delivery of healthy infants developed from vitrified oocytes in a stimulated in vitro fertilization-embryo transfer program. *Fertil Steril* 2000;74:180–81.
9. Walker DL, Tummon IS, Hammitt DG et al. Vitrification versus programmable rate freezing of late stage murine embryos: a randomized comparison prior to application in clinical IVF. *Reprod Biomed Online* 2004;8:558–68.
10. Trapphoff T. Vitrification of oocytes: Imprinting and disturbance in spindle formation and chromosome segregation. In: *Vitrification in Assisted Reproduction* (2nd. Edition), Chapter 12, p. 105116, 2015 Ed. Michael J Tucker, Juergen Liebermann, Informa Healthcare, London, UK.
11. Takahashi K, Mukaida T, Goto T et al. Perinatal outcome of blastocyst transfer with vitrification using cryoloop: a 4-year follow-up study. *Fertil Steril* 2005;84:88–92.

12. Liebermann J, Tucker MJ. Comparison of vitrification versus conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. *Fertil Steril* 2006;86:20–26.
13. Liebermann J. Vitrification of human blastocysts: an update. *Reprod Biomed Online* 2009;19: Suppl 4: 105–14.
14. Liebermann J, Nawroth F, Isachenko V et al. Potential importance of vitrification in reproductive medicine. *Biol Reprod* 2002;67:1671–80.
15. Liebermann J, Dietl J, Vanderzwalmen P et al. Recent developments in human oocyte, embryo and blastocyst vitrification: where are we now? *Reprod Biomed Online* 2003;7:623–33.
16. Vanderzwalmen P, Bertin G, Debauche Ch et al. Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification. *Hum Reprod* 2002;17:744–51.
17. Mukaida T, Oka C, Goto T, Takahashi K. Artificial shrinkage of blastocoeluses using either a microneedle or a laser pulse prior to the cooling steps of vitrification improves survival rate and pregnancy outcome of vitrified human blastocysts. *Hum Reprod* 2006;21:3246–52.
18. Edashige K, Kasai M. The movement of water and cryoprotectants in mammalian oocytes and embryos: Membrane permeability and aquaporines. In: *Vitrification in Assisted Reproduction* (2nd. Edition), Chapter 5, pp. 47–54, 2015 Ed. Michael J Tucker, Juergen Liebermann, Informa Healthcare, London, UK.
19. Son WY, Yoon SH, Yoon HJ, Lee SM, Lim JH. Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. *Hum Reprod* 2003;18:137–39.
20. Hiraoka K, Hiraoka K, Kinutani M, Kinutani K. Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. *Hum Reprod* 2004;19:2884–88.
21. Mukaida T, Oka C, Goto T, Takahashi K. Artificial shrinkage of blastocoeluses using either a micro-needle or a laser pulse prior to the cooling steps of vitrification improves survival rate and pregnancy outcome of vitrified human blastocysts. *Hum Reprod* 2006;21:3246–52.
22. Iwayama H, Hochi S, Yamashita M. In vitro and in vivo viability of human blastocysts collapsed by laser pulse or osmotic shocks prior to vitrification. *J Assist Reprod Genet* 2011;28:355–61.
23. Hur YS, Park JH, Ryu EK et al. Lim JH. Effect of artificial shrinkage on clinical outcome in fresh blastocyst transfer cycle. *Clin Exp Repro Med* 2011;38:87–92.
24. Liebermann J, Conaghan J. ()Artificial collapse prior blastocyst vitrification: Improvement of clinical outcome. *The Journal of Clinical Embryology* 2013;16(1).
25. Kazemi P, Dashtizad M, Shamsara M et al. Effect of blastocoel fluid reduction before vitrification on gene expression in mouse blastocysts. *Molecular Reproduction and Development* 2016;83: 735–42.
26. Larman MG, Sheehan CB, Gardner DK. Calcium-free vitrification reduces cryoprotectant-induced zona pellucida hardening and increases fertilization rates in mouse oocytes. *Reproduction* 2006;131:53–61.
27. Conaghan J, Vaccari S. Development and hatching of human blastocysts after vitrification and warming. In: *Vitrification in Assisted Reproduction* (2nd. Edition), Chapter 20, pp. 175–84, 2015 Ed. Michael J Tucker, Juergen Liebermann, Informa Healthcare, London, UK.
28. Pinborg A, Henningsen AA, Loft A et al. Large baby syndrome in singleton born after frozen embryo transfers (FET): Is it due the maternal factors or the cryotechnique? *Hum Reprod* 2014;29:618–27.

On The Strategy of “Freezing Only” Embryos

Bruce S. Shapiro and Forest C. Garner

In the United States, there has been a recent and dramatic shift toward increased use of “freeze-all” cycles, in which all embryos or oocytes are cryopreserved for use in subsequent frozen-thawed embryo transfer (FET) cycles (Figure 30.1) [1]. There has also been a corresponding parallel increase in the number of transfers of frozen-thawed embryos, while the number of fresh transfers has declined. This suggests that freeze-all cycles are steadily replacing fresh transfers as a primary treatment option.

The shift toward greater use of embryo cryopreservation accompanied a synergistic increase in success rates with frozen-thawed embryos. For the first time, in 2014 the national average implantation rates in the United States were greater with frozen-thawed embryo transfers than with fresh embryo transfers in every autologous age group (Figure 30.2) [1]. The risk ratio for implantation in frozen-thawed embryo transfers to that in fresh embryo transfers increased steadily with increasing age, from 1.06 in patients <35 years of age to 5.61 in patients >44 years of age (Figure 30.3) [1]. The cause of this steady increase cannot be discerned from national summary statistics. One potential explanation is relatively greater use of embryo cryopreservation in older patients using preimplantation genetic screening. Another is greater endometrial impact of controlled ovarian stimulation (COS) in older patients.

The reported benefits of the freeze-all strategy have been summarized previously [2–4]. The benefits that are mentioned most often include greater endometrial receptivity [5–7] and certain reduced maternal and perinatal risks [8–11], when compared to fresh autologous embryo transfer. Another benefit is that cryopreservation allows sufficient time for preimplantation genetic screening (PGS) results to become available before embryo transfer [12, 13].

The greater endometrial receptivity in cycles using FET seems to arise from the absence of effects of COS on endometrial development and maturation [14]. Much of the impaired endometrial receptivity following ovarian stimulation seems to be in the form of an advanced receptive phase, inducing an asynchrony between endometrial and embryonic development so that slowly developing embryos are less likely to implant than their faster counterparts [15–17]. This effect seems to be exacerbated in cycles with premature progesterone elevation [18–19]. Older patients appear to have increased risk of asynchronous transfers [20]. Although commonly believed or suspected previously, the asynchrony effect does not appear to be a direct effect of elevated estradiol alone [21].

Despite well-documented negative effects of ovarian stimulation on the endometrium, it may not be inherently obvious that freezing all embryos would increase success rates. Freezing allows the transfer of embryos in a subsequent cycle unimpaired by the effects of COS. However, there are also risks associated with embryo freezing that can cause cycle

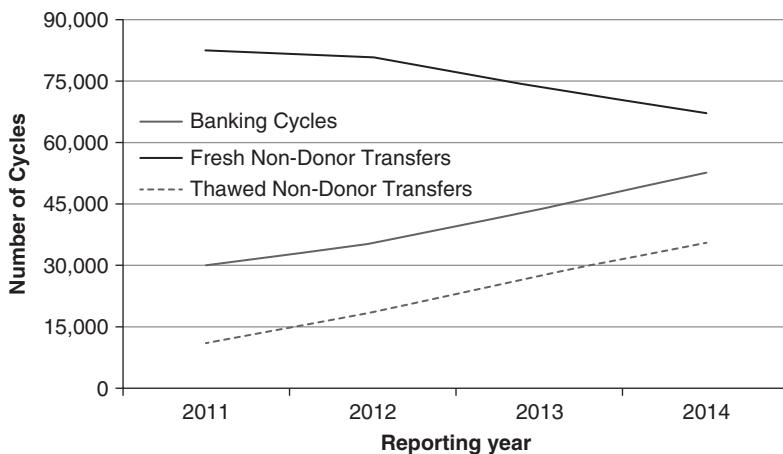


Figure 30.1
Trends in frequencies of banking cycles (red line), fresh autologous embryo transfers (green line), and transfers of frozen-thawed embryos (purple line). Results are calculated from the United States Centers for Disease Control and Prevention annual report for reporting year 2014.

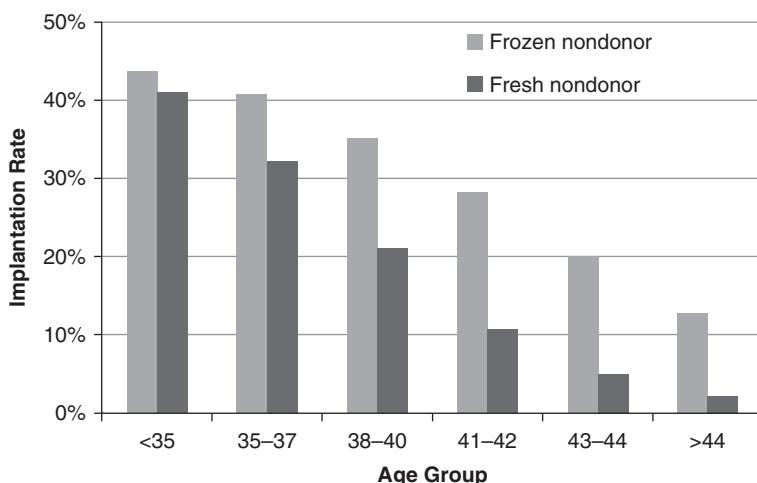


Figure 30.2
Implantation rates for each autologous age group, broken out by whether the embryos were fresh (red bars) or frozen-thawed (blue bars). Data are from the United States Centers for Disease Control and Prevention annual report for reporting year 2014

failure. These include embryonic cryo-damage, which can result in immediate embryo non-survival or latent damage with eventual failure to continue post-thaw development [19]. Therefore, whether the patient is benefitted depends upon the balance between the offsetting effects of endometrial impairment following COS in fresh transfer and embryonic cryo-damage following freeze-all in FET cycles. However, these factors may be locally influenced by COS protocols, freezing techniques, patient populations, and other factors that are specific to each center and laboratory.

To date, there have been five published randomized trials from four research teams comparing fresh and frozen-thawed embryo transfers. All five of these studies reported greater success rates with frozen-thawed embryo transfers than with fresh transfers, and in three of those five studies that difference was statistically significant. However, those five studies differed in inclusion criteria, COS methods, freezing methods, embryonic stage of

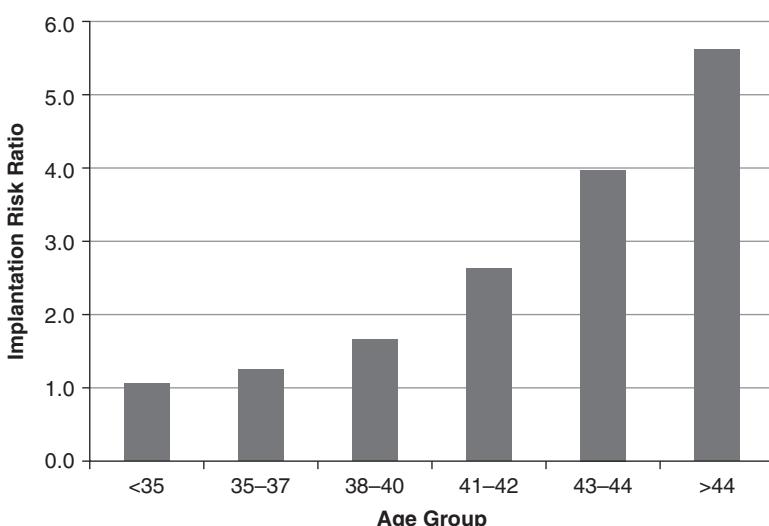


Figure 30.3
Ratios of implantation rates for frozen-thawed and fresh transfers. The risk ratio increased from 1.06 in patients <35 years of age to 5.61 in patients >44 years of age. Results are calculated from the United States Centers for Disease Control and Prevention annual report for reporting year 2014

cryopreservation, embryonic stage of embryo transfer, and outcome measures. However, one of the studies that reported a significant difference was subsequently withdrawn by the publisher over concerns regarding methodology.

Two trials by one center [5, 6], one in 137 randomized normal responders and the other in 122 randomized high responders, compared transfers of blastocysts derived from conventionally frozen bipronuclear oocytes to fresh blastocyst transfers. Post-thaw extended culture was chosen to try to ensure that the transferred blastocysts had clearly resumed post-thaw development. Resumed development is a higher standard than mere post-thaw survival, as many apparently surviving embryos on immediate post-thaw inspection fail to continue development to the blastocyst stage [19]. The implantation rates with that method were 65–71 percent in those two randomized trials, indicating the method was reasonably successful at eliminating cryo-damaged embryos from consideration for transfer. The initial analyses were per protocol (per transfer), not intention to treat, because the study question regarded endometrial receptivity. The main outcome measure in both studies was clinical pregnancy rate (based on fetal heart motion) per transfer. The clinical pregnancy rates per transfer in the high responder study were 79.6 percent in the freeze-all group and 65.4 percent in the fresh transfer group ($P = 0.11$). The clinical pregnancy rates per transfer in the normal responder study were 84.0 percent with freeze-all and 54.7 percent with fresh transfer ($P = 0.01$). The latter study concluded there was evidence of impaired endometrial receptivity following ovarian stimulation in normal responders.

The live birth results for those two studies were published subsequently for the purpose of comparing birthweights [11]. The live birth rates per transfer for the two studies, combined, were 74.7 percent in the freeze-all groups and 57.1 percent in the fresh transfer groups ($P = 0.01$). The live birth rates per randomized patient were 56.9 percent and 45.5 percent, respectively ($P = 0.11$). The relative risk of live birth was 1.24 (95% CI 0.96 to 1.60).

More recently, Chen and colleagues [7] reported a randomized trial in 1508 PCO patients. Their methods included cleavage-stage vitrification and cleavage-stage transfer

of two embryos, followed by intention to treat analysis of live births. The live birth rates per randomized patient were 49.3 percent in the freeze-all arm and 42.0 percent in the fresh transfer arm ($P<0.01$). The relative risk of live birth was 1.17.

As this is written, the most recent randomized trial is currently available only in abstract form. This study by Vuong et al. [22] in 782 non-PCO patients employed vitrification of cleavage-stage embryos and cleavage stage embryo transfer. The main outcome measure was ongoing pregnancy. The ongoing pregnancy rates per randomized patient were 36.3 percent in the freeze-all arm and 34.5 percent in the fresh transfer arm ($P=0.65$). The relative risk of ongoing pregnancy was 1.05.

The relative risk ranged from 1.05 to 1.24 in favor of freeze-all, and the weighted average of the relative risks is approximately 1.14. Some of the variation in observed relative risks may have been caused by differences in methodology, study populations, or outcome measures. Among these studies, the greatest success rates and the greatest relative risk in favor of FET were found with blastocyst transfer, specifically with blastocysts selected after thaw as the morphologically best of each thawed complete cohort [11]. Indeed, the failure to provide equivalent embryo selection methods to fresh and frozen arms of a trial must induce some degree of bias. Unfortunately, it is common practice to thaw embryos without preference, and to transfer thawed embryos without confirming resumed post-thaw development.

A meta-analysis of these results is needed. A previous meta-analysis [23] should now be repeated because one of the included studies was subsequently withdrawn and because the two largest trials to date were completed after that meta-analysis was performed.

FET has been associated with certain reduced maternal and perinatal risks. The reported reduced maternal risks include late-onset ovarian hyperstimulation syndrome (OHSS), ectopic pregnancy, antepartum hemorrhage, placenta previa, and placental abruption [8, 24, 25]. The reduced perinatal risks include low birthweight, preterm delivery, very preterm delivery, small for gestational age, and perinatal mortality [8]. However, there are also increased risks associated with FET, including macrosomia and caesarian section delivery [8]. The reported birthweight differences in numerous registry studies have varied from 50 to 250 g [11], while randomized trials have reported birthweight differences of 162–166 g [7, 11], with the infants from frozen-thawed embryo transfers being consistently heavier than those from fresh embryos. The remarkable consistency of reported birthweight differences in those two randomized trials [7,11] is interesting because of the varied methodologies in those trials. One study used conventional slow freezing of bipronuclear oocytes followed by post-thaw culture to the blastocyst stage while the other study used vitrification of cleavage-stage embryos. This suggests the birthweight difference is independent of the freezing method employed.

The specific cause of this birthweight difference, whether uterine or embryonic, cannot be discerned directly from comparisons of autologous fresh and frozen-thawed embryo transfers because those comparisons are confounded by the two simultaneous methodological differences in embryo cryopreservation and uterine COS exposure. However, the root cause has been better examined by studies of super-ovulated rats [26] and by study of oocyte donation cycles [10]. It seems the hypothesis of a direct effect of embryo cryopreservation as the cause of the greater birthweights, perhaps through some epigenetic mechanism, is contradicted by the lack of birthweight differences in fresh and frozen-thawed embryo transfers in oocyte donation cycles [10]. Therefore, the evidence suggests the observed

birthweight differences are due to uterine COS exposure reducing birthweights in fresh autologous embryo transfers, presumably through altered placentation.

The observed birthweight differences between fresh and FET cycles may require a distinct control group from which to judge assisted reproduction cycle outcomes. However, obtaining an appropriate control group is not trivial, and comparisons between births following assisted reproduction cycles and those in the general population will have numerous confounding differences, such as the intendedness of pregnancies. The factors related to intendedness, such as differences in stress levels, contraceptive use, nutrition, tobacco use, contraindicated prescription drug use, drug/alcohol abuse, and prenatal care, might result in increased prevalence of prematurity and low birthweight in unintended pregnancies. Indeed, a meta-analysis found unintended pregnancies had increased risk for low birthweight (odds ratio 1.36) and pre-term birth (OR 1.31) when compared to intended pregnancies [27]. All assisted reproduction pregnancies are obviously intended. Therefore, a comparison between birthweight effects following assisted reproduction cycles and those from spontaneous conceptions will use a control group that is biased toward low mean birthweights if the latter includes unintended pregnancies. Of course, age, infertility diagnosis, economic status, the number delivered, vanished twins, and other factors must also be addressed.

COS protocols developed for achieving pregnancy and live birth in fresh transfer cycles evolved over several decades to maximize success rates of fresh transfers. This implies the COS protocol must obtain viable oocytes while minimizing the impact of COS on endometrial receptivity. These cross purposes resulted in a compromise that may have been unrecognized by those developing COS protocols. For example, the criteria for timing of the ovulatory trigger, typically when two or three lead follicles reached 17–18mm mean diameter, were derived from an attempt to obtain viable oocytes while also controlling the unrecognized risk that larger follicles would produce excessive pre-ovulatory progesterone, impairing endometrial receptivity [28]. However, this early trigger ignores cohort heterogeneity and therefore might limit the number of mature follicles and thus result in many follicles being smaller than ideal for oocyte maturity and fertilization [29].

Most recent studies of freeze-all cycles, including the randomized trials described earlier, used COS protocols that were identical to those used in fresh transfer cycles, and were not specifically optimized for the freeze-all paradigm. A COS protocol optimized for a freeze-all cycle would focus on obtaining viable embryos while controlling costs, with no constraints regarding any impact on endometrial receptivity. Such a protocol would not necessarily be restricted to starting in the early follicular phase [30]. It might use inexpensive oral progestogens to suppress hypothalamic GnRH instead of relatively expensive injected GnRH agonists or GnRH antagonists [31]. While random-start stimulation and such use of progestogens are incompatible with endometrial receptivity for a fresh embryo transfer, this is not a concern for freeze-all cycles. Furthermore, the trigger point might be extended so that more follicles of ideal size are obtained [29], without regard for premature progesterone elevation.

OHSS incidence has declined over the last decade, largely through the use of GnRH agonist for the ovulatory trigger and increased use of freeze-all cycles. The agonist trigger typically results in rapid, complete, and irreversible luteolysis [32], which terminates follicular production of the excess VEGF that causes OHSS. Clinically significant OHSS incidence following agonist trigger is greatly reduced [33], but a small number of individual

cases have been reported. However, the use of the agonist trigger followed by fresh autologous embryo transfer has been associated with reduced success rates in some studies [34] so that the use of freeze-all may be indicated following agonist trigger. The use of freeze-all cycles eliminates the potential for late-onset OHSS from the rising serum hCG levels associated with early pregnancy [35].

Centers should be able to achieve excellent success rates by transferring fresh embryos under ideal conditions, specifically when blastocysts develop rapidly, pre-ovulatory progesterone levels are low, and OHSS risk is low, while otherwise freezing all embryos. However, if a COS protocol optimized for the freeze-all cycle is routinely employed, fresh autologous transfers may be precluded.

The relative cost-effectiveness of the freeze-all strategy versus fresh transfer requires detailed analyses. The first published comparisons were encouraging for the freeze-all paradigm [36, 37]. Further research should also consider costs associated with maternal complications (e.g. OHSS) and perinatal outcomes (e.g. low birthweight). Further benefit should be realized as the increasing implantation rates realized through superior endometrial receptivity allow and encourage increased use of elective single embryo transfer, eliminating most of the elevated risk of multiple pregnancies currently associated with assisted reproduction. The implantation rate with transfer of vitrified and warmed blastocysts is reportedly as high as 70 percent in a group of patients 18–40 years of age with at least normal ovarian reserve, even without PGS [38]. This is adequate to support elective single embryo transfer in that group.

The freeze-all protocol also allows time for the PGS results to become available, and for planning around those results. The combination of genetic screening, blastocyst vitrification and single blastocyst transfer is the current leading edge of IVF practice, resulting in high success rates with low risks of multiple pregnancy, OHSS, and adverse perinatal outcome [12, 13].

References

1. Centers for Disease Control and Prevention, American Society for Reproductive Medicine, Society for Assisted Reproductive Technology. 2014 Assisted Reproductive Technology Fertility Clinic Success Rates Report. Atlanta (GA): US Dept. of Health and Human Services; 2016.
2. Maheshwari A, Bhattacharya S. Elective frozen replacement cycles for all: ready for prime time? *Hum Reprod.* 2013 January;28(1):6–9.
3. Roque M. Freeze-all policy: is it time for that? *J Assist Reprod Genet.* 2015 February;32(2): 171–76.
4. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C. Clinical rationale for cryopreservation of entire embryo cohorts in lieu of fresh transfer. *Fertil Steril.* 2014 July;102(1):3–9.
5. Shapiro BS, Daneshmand ST, Garner FC et al. Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: a prospective randomized trial comparing fresh and frozen-thawed embryo transfer in normal responders. *Fertil Steril.* 2011 August;96 (2): 344–48.
6. Shapiro BS, Daneshmand ST, Garner FC et al. Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: a prospective randomized trial comparing fresh and frozen-thawed embryo transfers in high responders. *Fertil Steril.* 2011 August;96(2): 516–18.
7. Chen ZJ, Shi Y, Sun Y et al. Fresh versus frozen embryos for infertility in the polycystic ovary syndrome. *N Engl J Med.* 2016 August 11;375(6): 523–33.
8. Maheshwari A, Pandey S, Shetty A, Hamilton M, Bhattacharya S. Obstetric and

- perinatal outcomes in singleton pregnancies resulting from the transfer of frozen thawed versus fresh embryos generated through in vitro fertilization treatment: a systematic review and meta-analysis. *Fertil Steril.* 2012 August;98(2): 368–77.
9. Wennerholm UB, Henningsen AK, Romundstad LB et al. Perinatal outcomes of children born after frozen-thawed embryo transfer: a Nordic cohort study from the CoNARTaS group. *Hum Reprod.* 2013 September;28(9): 2545–53.
 10. Kalra SK, Ratcliffe SJ, Coutifaris C, Molinaro T, Barnhart KT. Ovarian stimulation and low birth weight in newborns conceived through in vitro fertilization. *Obstet Gynecol.* 2011 October;118(4): 863–71.
 11. Shapiro BS, Daneshmand ST, Bedient CE, Garner FC. Comparison of birth weights in patients randomly assigned to fresh or frozen-thawed embryo transfer. *Fertil Steril.* 2016 August;106(2): 317–21.
 12. Rodriguez-Purata J, Lee J, Whitehouse M et al. Reproductive outcome is optimized by genomic embryo screening, vitrification, and subsequent transfer into a prepared synchronous endometrium. *J Assist Reprod Genet.* 2016 March;33(3): 401–12.
 13. Grifo J, Kofinas J, Schoolcraft WB. The practice of in vitro fertilization according to the published literature. *Fertil Steril.* 2014 September;102(3): 658–59.
 14. Kolibianakis E, Bourgain C, Albano C et al. Effect of ovarian stimulation with recombinant follicle-stimulating hormone, gonadotropin releasing hormone antagonists, and human chorionic gonadotropin on endometrial maturation on the day of oocyte pick-up. *Fertil Steril.* 2002 November;78(5): 1025–29.
 15. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Ross R. Contrasting patterns in in vitro fertilization pregnancy rates among fresh autologous, fresh oocyte donor, and cryopreserved cycles with the use of day 5 or day 6 blastocysts may reflect differences in embryo-endometrium synchrony. *Fertil Steril.* 2008 January;89(1):20–26.
 16. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Thomas S. Large blastocyst diameter, early blastulation, and low preovulatory serum progesterone are dominant predictors of clinical pregnancy in fresh autologous cycles. *Fertil Steril.* 2008 August;90(2): 302–09.
 17. Shapiro BS, Daneshmand ST, Restrepo H et al. Matched-cohort comparison of single-embryo transfers in fresh and frozen-thawed embryo transfer cycles. *Fertil Steril.* 2013 February;99(2): 389–92.
 18. Venetis CA, Kolibianakis EM, Bosdou JK, Tarlatzis BC. Progesterone elevation and probability of pregnancy after IVF: a systematic review and meta-analysis of over 60 000 cycles. *Hum Reprod Update.* 2013 September–October;19(5): 433–57.
 19. Shapiro BS, Daneshmand ST, Garner FC et al. Embryo cryopreservation rescues cycles with premature luteinization. *Fertil Steril.* 2010 February;93(2): 636–41.
 20. Shapiro BS, Daneshmand ST, Desai J et al. The risk of embryo-endometrium asynchrony increases with maternal age after ovarian stimulation and IVF. *Reprod Biomed Online.* 2016 July;33(1):50–55.
 21. Levi AJ, Drews MR, Bergh PA, Miller BT, Scott RT Jr. Controlled ovarian hyperstimulation does not adversely affect endometrial receptivity in in vitro fertilization cycles. *Fertil Steril.* 2001 October;76(4): 670–74.
 22. Vuong LT, Dang VQ, Ho TM et al. Freeze-all versus fresh embryo transfer in IVF/ICSI, a randomised controlled trial (NCT02471573). *Fertil Steril.* 2018 January;106(3):e376.
 23. Roque M, Lattes K, Serra S et al. Fresh embryo transfer versus frozen embryo transfer in in vitro fertilization cycles: a systematic review and meta-analysis. *Fertil Steril.* 2013 January;99(1): 156–62.
 24. Perkins KM, Boulet SL, Kissin DM, Jamieson DJ; National ART Surveillance (NASS) Group. Risk of ectopic pregnancy associated with assisted reproductive technology in the United States,

- 2001–2011. *Obstet Gynecol.* 2015 January;125(1):70–78.
25. Shapiro BS, Daneshmand ST, De Leon L et al. Frozen-thawed embryo transfer is associated with a significantly reduced incidence of ectopic pregnancy. *Fertil Steril.* 2012 December;98(6): 1490–94.
26. Mainigi MA, Olalere D, Burd I et al. Peri-implantation hormonal milieu: elucidating mechanisms of abnormal placentation and fetal growth. *Biol Reprod.* 2014 February 13;90(2):26.
27. Shah PS, Balkhair T, Ohlsson A et al. Intention to become pregnant and low birth weight and preterm birth: a systematic review. *Matern Child Health J.* 2011 February;15(2): 205–16.
28. Kolibianakis EM, Albano C, Camus M et al. Prolongation of the follicular phase in in vitro fertilization results in a lower ongoing pregnancy rate in cycles stimulated with recombinant follicle-stimulating hormone and gonadotropin-releasing hormone antagonists. *Fertil Steril.* 2004 July;82(1): 102–07.
29. Rosen MP, Shen S, Dobson AT et al. A quantitative assessment of follicle size on oocyte developmental competence. *Fertil Steril.* 2008 September;90(3): 684–90.
30. Cakmak H, Katz A, Cedars MI, Rosen MP. Effective method for emergency fertility preservation: random-start controlled ovarian stimulation. *Fertil Steril.* 2013 December;100(6): 1673–80.
31. Kuang Y, Chen Q, Fu Y et al. Medroxyprogesterone acetate is an effective oral alternative for preventing premature luteinizing hormone surges in women undergoing controlled ovarian hyperstimulation for in vitro fertilization. *Fertil Steril.* 2015 July;104(1):62–70.
32. Kol S. Luteolysis induced by a gonadotropin-releasing hormone agonist is the key to prevention of ovarian hyperstimulation syndrome. *Fertil Steril.* 2004 January;81(1):1–5.
33. Engmann L, Benadiva C, Humaidan P. GnRH agonist trigger for the induction of oocyte maturation in GnRH antagonist IVF cycles: a SWOT analysis. *Reprod Biomed Online.* 2016 March;32(3): 274–85.
34. Youssef MA, Van der Veen F, Al-Inany HG et al. Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist-assisted reproductive technology. *Cochrane Database Syst Rev.* 2014 October 31;(10):CD008046.
35. Devroey P, Polyzos NP, Blockeel C. An OHSS-Free clinic by segmentation of IVF treatment. *Hum Reprod.* 2011 October;26(10): 2593–97.
36. Roque M, Valle M, Guimarães F, Sampaio M, Geber S. Cost-effectiveness of the freeze-all policy. *JBRA Assist Reprod.* 2015 August 1;19(3): 125–30.
37. Papaleo E, Pagliardini L, Vanni VS et al. A direct healthcare cost analysis of the cryopreserved versus fresh transfer policy at the blastocyst stage. *Reproductive Biomedicine Online.* In press.
38. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C. Freeze-all at the blastocyst or bipronuclear stage: a randomized clinical trial. *Fertil Steril.* 2015;104: 1138–44.

Fertility Preservation

Eggs, Embryos and Ovarian Tissue, and the Use of GnRH Analogues to Protect Fertility

Richard A. Anderson

The improvement in survival rates from many malignant diseases has been one of the great successes of modern medicine. This has led to the emergence of the field of 'late effects', recognizing the adverse effects of the treatments involved and the wide range of health-related problems that may result from cancer treatment. For young men and women, one important concern is the potential loss of fertility, and this applies also to children treated before puberty. The effects of chemotherapy on the ovary have been recognized for several decades, going back to original studies looking at the histology of ovaries of girls who had died due to chemotherapy compared to from accidental causes, showing depletion of growing follicles associated with treatment [1]. Contemporaneous studies also showed the long-term effect of chemotherapy on the ovary, resulting in the increased risk of premature ovarian insufficiency (POI) [2]. The high risk associated with alkylating agent therapy was already recognized in those early studies.

The basis for the adverse effects of chemotherapy and radiotherapy on female fertility is largely related to the established finding that the stock of primordial follicles that constitutes the ovarian reserve is formed in fetal life. Accelerated depletion can result in a range of reproductive problems characterized in the literature as acute ovarian failure, infertility and subsequently POI, with the latter often described as chemotherapy-associated amenorrhoea to allow for the possibility of late recovery of ovarian activity. The risk of these is often related to diagnosis, but in fact it is, of course, the treatment that is the main determinant of risk rather than the diagnosis in itself. This is very elegantly shown, for example, in the treatment of adult Hodgkin Lymphoma where the hazard ratio for POI (defined as menopause before the age of 40 years) is unchanged by the ABVD chemotherapy regimen, but increased to 20 with alkylating agent therapy, to 23 with pelvic radiotherapy and to 35 with combination of alkylating agent chemotherapy and pelvic radiotherapy [3]. Similarly a large analysis of parenthood in female survivors of childhood Hodgkin Lymphoma in Germany showed that the overall chance of parenthood was the same in Hodgkin Lymphoma survivors as in the general population, except at older ages [4]. However, specific therapies that were associated with reduced chance of parenthood included radiotherapy to the pelvis and the highest doses of alkylating agents, although procarbazine up to $11,400 \text{ mg/m}^2$ and cyclophosphamide up to $6,000 \text{ mg/m}^2$ had non-significant or only minor effects. Radiotherapy is very injurious to oocytes, and thus radiotherapy under the diaphragm or, even more so, to the pelvis is associated with significant risk of ovarian failure [5].

Assessment of the risks the patient is facing is therefore central to an informed discussion regarding the potential need for fertility preservation. We have proposed a framework for this, dividing issues into those intrinsic to the patient and those extrinsic to her [6]. The latter are focused on the nature of the predicted treatment, that is, whether it is assessed as being of high, medium or low risk to her fertility, but of course there may be ongoing uncertainty, particularly when there are results from, for example, pathology and staging tests that are yet to be completed. There are also issues regarding the options that are in practice available to her, in the time available, and also dependent on local provision and indeed funding.

Intrinsic factors include the health of the patient, issues regarding consent, particularly where children are involved, the declining ovarian reserve with age and indeed whether the direct assessment of it may or may not be valuable. While many adults with cancer do not have particular health issues that would impact on the risks of undertaking fertility preservation, it is increasingly recognized that markers of the ovarian reserve, including antral follicle count and anti-Müllerian hormone (AMH), are reduced in women with malignant disease [7] or indeed other serious illnesses and that this is then reflected in a lower oocyte yield after ovarian stimulation [8]. There may, however, be more specific issues regarding, for example, a coagulopathy or bleeding disorder or indeed the location of the tumour if it is within the pelvis. Issues regarding consent are of particular importance where that is by proxy, and can be, at least in part, mitigated by fertility preservation procedures being undertaken when another procedure is also required, such as insertion of a central line. This aspect is further complicated by the currently experimental status of ovarian tissue cryopreservation, which is the procedure appropriate to prepubertal girls [9]. Age is a critical concern, impacting both the risks of the proposed treatment to subsequent fertility and the likelihood of successful fertility preservation. The risk to fertility has been most widely explored in relation to post-chemotherapy amenorrhea which, in the context of breast cancer, rises from approximately 10–15 percent to over 70 percent risk with age under 35 to over 40, respectively [10]. This effect has also been clearly demonstrated in survivors of childhood cancer, with a widening gap in the cumulative incidence of first pregnancy when that occurs after age 30 compared to overall [11]. Intriguingly, however, a greater prevalence of early menopause was reported by younger women than older women, following recovery of menses after cancer treatments [12]. The interaction between age and chemotherapy-related loss of reproductive lifespan requires further investigation, especially where the ovarian reserve is further lost by removal of ovarian tissue for fertility preservation. While unilateral oophorectomy has remarkably little effect on reproductive lifespan [13], indicating the ability of the remaining ovary to compensate and reduce the rate of follicle growth activation, the impact of subsequent chemotherapy has not been closely investigated.

Declining fertility with age has contributions from both follicle number and oocyte quality, with substantial research endeavours particularly focusing on the quantitative aspect as a predictive measure (as the qualitative aspect is much harder to determine). Thus, the question is whether individuals with a higher ovarian reserve, as detected by AMH or antral follicle count (AFC), are less likely to have chemotherapy-induced amenorrhea and/or infertility after treatment than those with a lower ovarian reserve. Several studies have now demonstrated that this is indeed the case, particularly in relation

to breast cancer where an interaction with age and body mass index has also been demonstrated [14,15,16].

The available options for women are cryopreservation of egg oocytes, embryos or ovarian tissue. These options have been extensively reviewed elsewhere [9,17]; thus, only the key features will be mentioned here. The cryopreservation by vitrification of oocytes has been a major development in the field, allowing effective fertility preservation for adult women without committing their oocytes to fertilization by a male. While widely practiced, most of the outcomes presented in the literature refer to healthy young women as oocyte donors, or women undergoing elective oocyte banking [18,19]. These clearly show the potential of this approach, but it is unclear whether oocytes taken from women for fertility preservation for malignant disease or other serious illnesses have the same potential or show some degree of compromise. Embryo cryopreservation is, of course, very long established and an appropriate option for women in a stable relationship but such women may still prefer to have oocyte cryopreservation or indeed divide the oocytes between being fertilized and not. Ovarian tissue is widely, although not universally, regarded as still experimental, with a growing number of babies born as a result of this. The literature, however, still consists of case series, and while these are increasingly large [20], there remains substantial potential for publication bias. A recent report on 95 ovarian tissue replacements in 74 women with an average age of 30 years at cryopreservation and 34 years at transplantation reported a pregnancy rate of 27.5 percent, with successful delivery in 22.5 percent [21]. Slightly more than half of the pregnancies in this and other similar case series have been as a result of natural conception, which is perhaps a key advantage of this approach to fertility preservation. A further key approach is the potential for hormonal function, and indeed in the above series 62.5 percent of women with well-defined POI before ovarian transplantation showed evidence of ovarian activity one year after transplantation. In addition to this slightly limited success rate in terms of successful pregnancies (especially given the young age of the women at tissue storage), a key issue with this approach is the potential for reintroduction of malignant cells within the ovarian tissue. While this is a very substantial risk in women with leukaemia, the emerging evidence suggests this risk is very limited in women with many solid malignancies where there is no evidence of metastatic disease [22]. For some conditions, there are specific molecular markers which may be used with very high sensitivity and specificity, but where this is not available, conventional histological analysis needs to be relied on and none of these methods can test the pieces of tissue that are actually being replaced. While there have been case reports of women having recurrence of malignancies following ovarian tissue transplantation, at present there are none where that recurrence of disease has been linked to the tissue replacement.

31.1 The Possibility of Ovarian Protection Using GnRH Analogues

Prevention is always better than cure; thus there is a long-standing and continuing interest in protecting the ovary against chemotherapy. The use of GnRH analogues to suppress the reproductive axis has long been investigated as a way of potentially protecting the ovary. This may have originally stemmed from the fallacious view that the prepubertal gonad is protected, whereas in fact it is probably largely protected because of the high ovarian reserve at that time, rather than the endocrinological hypogonadotropic status. The first randomized trial to investigate this was reported in 1987, and included 30 men and 18

women with Hodgkin disease who were randomized to chemotherapy with or without coadministration of buserelin [23]. This early trial did not show any benefit, but was clearly underpowered. This was followed by a number of uncontrolled observational reports proposing a benefit, but more recently there have been larger RCTs investigating the concept. A large trial using triptorelin in women with early breast cancer showed a reduced risk of early menopause to 8.9 percent in those treated with chemotherapy plus triptorelin, compared to 25 percent in the chemotherapy alone group [24]. This trial included some 280 women. Subsequently, the largely US-based POEMS study showed very similar results using goserelin, with the prevalence of ovarian failure being 8 percent versus 22 percent, giving an odds ratio of 0.30, 95 percent confidence interval 0.09–0.97 [25]. There was also a suggestion in that study that there were more pregnancies achieved in the goserelin group than the controls, but both this and other studies have reported pregnancy outcomes have not had this as a primary outcome and therefore this result must be taken with considerable caution. Several meta-analyses have recently been published, and generally support the hypothesis that GnRH agonist therapy has a beneficial effect on the risk of POI in breast cancer [26]. Importantly, there seems to be no adverse effect on survival, as this is a concern with women with hormone-sensitive tumours: this has been addressed in three studies, which show no effect (HR 1.00, 95% CI 0.49–2.04) [26]. The degree of ovarian function protected by GnRH agonist therapy has not been investigated in any of the above studies to any great extent, but it has been reported in the UK-based OPTION study where AMH measurements were available on a sub-group of women taking part in the trial. Thus, whereas overall a significant reduction in the prevalence of POI one year after chemotherapy was detected in women treated with goserelin, there was no significant difference in AMH concentrations between the two groups at that time, with very marked reductions in AMH in both groups [27]. Additionally, that study showed that there was no evidence of a protective effect in women over the age of 40. All the trials thus far have focused on POI as the primary outcome; thus, trials addressing more relevant clinical endpoints such as fertility and the clinical consequences of early oestrogen deficiency have not been performed. The data from the OPTION study suggest that any preservation of ovarian function is likely to be very modest although it is indeed the case that in some, particularly younger, women this may be sufficient to allow subsequent conception.

Most of the data in this field concern women with breast cancer but women with other cancers have also been investigated. A recent trial reporting long-term outcomes in women with lymphoma treated with GnRH agonist during chemotherapy did not show any effect on POI risk [28]. It is unclear as to whether this reflects the ages of the patients included, or the different risks associated with the specific therapies in that study. It is likely that if the effect of chemotherapy on risk of POI is modest then likewise any beneficial effect of GnRH agonist may be difficult to show, and similarly if the effect of chemotherapy is very high (or varied regimens are studied, as is more the case with lymphoma than breast cancer) then again a beneficial effect will be difficult to show. Thus, while there seems to be no specific reason why GnRH agonist use with a certain diagnosis may have benefit conferred whereas it does not with other diagnoses, it is perhaps more likely to reflect the associated chemotherapy protocols. It is, however, the case that GnRH agonist administration may have additional beneficial effects, for example, in suppression of menstruation during chemotherapy and particularly avoidance of heavy bleeding due to thrombocytopenia. At present, therefore, while the data do support its use in younger women with breast

cancer its beneficial effect may be limited, and there may be increased side effects such as hot flashes from oestrogen deficiency during treatment which should be weighed in discussion with the patient.

31.2 Conclusions

The use of oocyte and embryo fertility cryopreservation is growing rapidly but there remains little evidence of how many women return to use them, and thus specific demonstration of success rates for cancer patients is needed. Ovarian tissue cryopreservation remains experimental, especially when applied to girls, but with growing evidence for its potential to restore hormone function as well as fertility. There remain concerns as to how best to deal with the problem of malignant cell contamination in leukaemia especially. The use of GnRH analogues to protect the ovary against chemotherapy is now supported by high-quality evidence of a reduction in risk of POI, but whether this translates into opportunities for pregnancy is not clear, and the added reproductive lifespan may be limited.

References

1. Himelstein-Braw R, Peters H and Faber M. Morphological study of the ovaries of leukaemic children. *Br J Cancer* 1978; **38**:82–87.
2. Chapman RM, Sutcliffe SB and Malpas JS. Cytotoxic-induced ovarian failure in women with Hodgkin's disease. I. Hormone function. *JAMA* 1979; **242**:1877–81.
3. Swerdlow AJ, Cooke R, Bates A et al. Risk of premature menopause after treatment for Hodgkin's lymphoma. *J Natl Cancer Inst* 2014;106.
4. Bramswig JH, Riepenhausen M and Schellong G. Parenthood in adult female survivors treated for Hodgkin's lymphoma during childhood and adolescence: a prospective, longitudinal study. *Lancet Oncol* 2015; **16**:667–75.
5. Wallace WH, Thomson AB, Saran F and Kelsey TW. Predicting age of ovarian failure after radiation to a field that includes the ovaries. *Int J Radiat Oncol Biol Phys* 2005; **62**:738–44.
6. Wallace WH, Critchley HO and Anderson RA. Optimizing reproductive outcome in children and young people with cancer. *J Clin Oncol* 2012; **30**:3–5.
7. Lawrence B, Fehm T, von Wolff M et al. Reduced pretreatment ovarian reserve in premenopausal female patients with Hodgkin lymphoma or non-Hodgkin lymphoma—evaluation by using antimüllerian hormone and retrieved oocytes. *Fertil Steril* 2012; **98**:141–44.
8. Friedler S, Koc O, Gidoni Y, Raziel A and Ron-El R. Ovarian response to stimulation for fertility preservation in women with malignant disease: a systematic review and meta-analysis. *Fertil Steril* 2012; **97**:125–33.
9. Anderson RA, Mitchell RT, Kelsey TW et al. Cancer treatment and gonadal function: experimental and established strategies for fertility preservation in children and young adults. *Lancet Diabetes Endocrinol* 2015; **3**:556–67.
10. Petrek JA, Naughton MJ, Case LD et al. Incidence, time course, and determinants of menstrual bleeding after breast cancer treatment: a prospective study. *J Clin Oncol* 2006; **24**:1045–51.
11. Chow EJ, Stratton KL, Leisenring WM et al. Pregnancy after chemotherapy in male and female survivors of childhood cancer treated between 1970 and 1999: a report from the Childhood Cancer Survivor Study cohort. *Lancet Oncol* 2016; **17**:567–76.
12. Letourneau JM, Ebbel EE, Katz PP et al. Acute ovarian failure underestimates age-specific reproductive impairment for young women undergoing chemotherapy for cancer. *Cancer* 2012; **118**:1933–39.

13. Brook JD, Gosden RG and Chandley AC. Maternal ageing and aneuploid embryos—evidence from the mouse that biological and not chronological age is the important influence. *Hum Genet* 1984; **66**:41–45.
14. Anderson RA, Adamson D, Yellowlees A et al. Administration of a GnRH agonist during chemotherapy during chemotherapy for breast cancer reduces ovarian toxicity in women aged under 40 years. *ESHRE Meeting, Helsinki* 2016:P488.
15. Anderson RA, Rosendahl M, Kelsey TW and Cameron DA. Pretreatment anti-Mullerian hormone predicts for loss of ovarian function after chemotherapy for early breast cancer. *Eur J Cancer* 2013; **49**:3404–11.
16. Su HC, Haunschmid C, Chung K et al. Prechemotherapy antimüllerian hormone, age, and body size predict timing of return of ovarian function in young breast cancer patients. *Cancer* 2014; **120**:3691–98.
17. De Vos M, Smitz J and Woodruff TK. Fertility preservation in women with cancer. *Lancet* 2014; **384**:1302–10.
18. Cobo A, Garcia-Velasco JA, Coello A et al. Oocyte vitrification as an efficient option for elective fertility preservation. *Fertil Steril* 2016; **105**:755–64 e758.
19. Doyle JO, Richter KS, Lim J et al. Successful elective and medically indicated oocyte vitrification and warming for autologous in vitro fertilization, with predicted birth probabilities for fertility preservation according to number of cryopreserved oocytes and age at retrieval. *Fertil Steril* 2016; **105**:459–66 e452.
20. Donne J, Dolmans MM, Pellicer A et al. Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation. *Fertil Steril* 2013; **99**:1503–13.
21. Van der Ven H, Liebenthron J, Beckmann M et al. Ninety-five orthotopic transplantsations in 74 women of ovarian tissue after cytotoxic treatment in a fertility preservation network: tissue activity, pregnancy and delivery rates. *Hum Reprod* 2016; **31**:2031–41.
22. Dolmans MM, Luyckx V, Donne J, Andersen CY and Greve T. Risk of transferring malignant cells with transplanted frozen-thawed ovarian tissue. *Fertil Steril* 2013; **99**:1514–22.
23. Waxman JH, Ahmed R, Smith D et al. Failure to preserve fertility in patients with Hodgkin's disease. *Cancer Chemother Pharmacol* 1987; **19**:159–62.
24. Del Mastro L, Boni L, Michelotti A et al. Effect of the gonadotropin-releasing hormone analogue triptorelin on the occurrence of chemotherapy-induced early menopause in premenopausal women with breast cancer: a randomized trial. *JAMA* 2011; **306**:269–76.
25. Moore HC, Unger JM, Phillips KA et al. Goserelin for ovarian protection during breast-cancer adjuvant chemotherapy. *N Engl J Med* 2015; **372**:923–32.
26. Lambertini M, Ceppi M, Poggio F et al. Ovarian suppression using luteinizing hormone-releasing hormone agonists during chemotherapy to preserve ovarian function and fertility of breast cancer patients: a meta-analysis of randomized studies. *Ann Oncol* 2015; **26**:2408–19.
27. Leonard R, Adamson D, Bertelli G et al. GnRH agonist for protection against ovarian toxicity during chemotherapy for early breast cancer: the Anglo Celtic Group OPTION trial. *Ann Oncol* 2017; **28**:1811–16.
28. Demeestere I, Brice P, Peccatori FA et al. No evidence for the benefit of gonadotropin-releasing hormone agonist in preserving ovarian function and fertility in lymphoma survivors treated with chemotherapy: final long-term report of a prospective randomized trial. *J Clin Oncol* 2016.

Stimulating the Poor Responder

Yadava B. Jeve and Harish M. Bhandari

32.1 Introduction

Poor ovarian response (POR) to controlled ovarian stimulation poses a significant clinical challenge. The prevalence of POR has been estimated to range from 9 to 24 percent. However, the true incidence is unknown as until recently there was no consensus on the definition of POR, resulting in variations in the cohort of patients included in the reporting trials. The European Society of Human Reproduction and Embryology (ESHRE), working group on poor ovarian response definition, reached a consensus on the ‘minimal criteria’, also called the ‘Bologna criteria’, needed to define POR. Two of the following three features must be present to define a patient as a poor responder: (i) advanced maternal age (≥ 40 years) or any other risk factor for POR; (ii) a previous characterized POR cycle (≤ 3 oocytes with a conventional stimulation protocol); (iii) an abnormal ovarian reserve test (antral follicle count $< 5-7$ follicles or anti-Müllerian hormone (AMH) $< 0.5-1.1$ ng/ml) [1]. The working group also proposed that in the absence of advanced maternal age or abnormal ovarian reserve tests two episodes of poor ovarian response after maximal stimulation are sufficient to define POR.

Maternal age is a key determinant of successful IVF outcome. Evidence has shown convincingly that poor ovarian response is a first sign of ovarian ageing and that ovarian ageing could be independent of chronological age. Though prognosis for a poor responder is poor, with fewer than 10 percent recorded live birth rate per treatment cycle, the live birth rate does not appear to be homogenous and younger women with poor ovarian response achieve better pregnancy results [2].

Management of poor response to ovarian stimulation is a widely researched subject in assisted conception. Though various strategies have been used in this group of women to improve reproductive outcome, the overall success rate remains low. The options range from changes to standard ovarian stimulation protocols to no or minimal ovarian stimulation to use of novel adjuvants. This chapter discusses various strategies used to stimulate poor responders to achieve an optimal ovarian response and improved reproductive outcome.

32.2 Different Strategies for Ovarian Stimulation in Women with Poor Ovarian Response

Pituitary Down-Regulation

Ovarian stimulation in a gonadotropin-releasing hormone agonist (GnRHa) protocol to prevent premature LH surge has been the standard in assisted conception cycles. In a long

GnRHa protocol, the GnRHa is started either in the mid-luteal phase or in the early follicular phase of the preceding cycle and is continued until the final follicular maturation trigger. The gonadotropin administration is delayed until pituitary desensitization has been achieved, which is determined on the basis of no evidence of ovarian follicular activity on a transvaginal pelvic ultrasound scan with or without serum oestrogen levels. GnRH receptors are present in the human ovary, and the long GnRHa down-regulation in poor responders appears to have a direct, deleterious effect on the ovary, which is not ideal for women with limited cohort of follicles.

Pituitary down-regulation in a long GnRH agonist cycle can also cause over-suppression leading to prolonged cycle length and an increased treatment cost. Therefore, in recent years, pituitary down-regulation with GnRH antagonist which causes chemical hypophysectomy and microdose flare-up GnRH agonist (commenced in the early follicular phase in conjunction with gonadotropin ovarian stimulation) have become more acceptable options over long GnRHa protocol. According to the results of a recent worldwide, web-based survey [3] of clinicians from 272 IVF units from 45 countries, GnRH antagonist protocol (52%) was the most preferred protocol followed by short GnRH agonist flare-up (20%) and microdose protocols (15%).

32.2.1 GnRH Antagonist Protocols

GnRH antagonists cause immediate, rapid gonadotropin suppression by competitively blocking GnRH receptors in the anterior pituitary gland, thereby preventing endogenous premature release of LH. The GnRH-antagonist down-regulation does not cause inhibition of early folliculogenesis, which is a critical point for a poor responder woman with a limited cohort of antral follicles. Antagonists could be used as daily dose or single depot dose injections and both approaches appear to be equally effective. In a fixed protocol, the GnRH antagonist is started on day 6 of the stimulation, whereas in a 'flexible' protocol, the pituitary down-regulation is when the lead follicle reaches an average of 12–14 mm in diameter until the day of final maturation trigger. It has also been shown that the use of GnRH antagonist for down-regulation reduces the amount of gonadotropin used, the length of stimulation, the number of cancelled cycles due to poor response, the overall cost normally associated with long GnRH agonist protocol and an increase in the number of oocytes retrieved with satisfactory pregnancy rates [4]. Meta-analysis of 17 randomized controlled trials ($n = 1696$) compared pituitary down-regulation with GnRH agonist versus GnRH antagonist and reported no significant difference in the number of oocytes retrieved (mean difference 0.09, 95% CI -0.53 to 0.36) and no difference in clinical pregnancy rates (OR 1.24 95% CI 0.88 to 1.73) [5].

Combined protocol (agonist–antagonist) described in limited number of studies included start of treatment with GnRH agonist down-regulation followed by ovarian stimulation with high dose (average 450 IU) gonadotropin. When the dominant follicle is ≥ 12 mm and the E2 level ≤ 400 , stop the GnRH agonist and start the GnRH antagonist. The advantage of this approach is that GnRH agonist initially provides endogenous gonadotropin secretion (flare effect) and with the addition of exogenous gonadotropins, the ovarian response is increased for a short time. Premature LH peak could be prevented by starting the GnRH antagonist at the right time. The study results failed to achieve any statistical significance when compared with standard protocol [6].

Letrazole in GnRH antagonist protocol is a cost-effective and patient-friendly protocol, but it has not been shown to improve clinical pregnancy rate and live birth rates [7].

Although use of GnRH antagonist for poor ovarian response is one of the popular approaches, it is not supported by robust data. The only potential benefit then is of acceptability of a shorter course when the outcome is likely to be poor.

32.2.2 Modifications to GnRH Agonist Protocols

32.2.2.1 GnRH Agonist Flare Protocol and Microdose Flare Protocol

Ovarian suppression with long GnRH agonist protocol results in excessive dampening of the ovarian response to hormonal stimulation and can cause refractoriness to gonadotropin stimulation, particularly in women with POR.

GnRH agonist administered early in the menstrual cycle (flare) causes an endogenous release of gonadotropins. GnRH agonist flare has been used in conjunction with gonadotropin ovarian stimulation for poor responders. It is believed that it enhances the effect of exogenous gonadotropins and in theory eliminates excessive ovarian suppression. The fundamental principle of this approach is to administer the minimal dose of GnRHa necessary to induce gonadotropin release while minimizing premature ovulation. Pretreatment with an oral contraceptive or progestin has also been suggested to minimize the incidence of corpus luteum rescue and undesired rise in follicular phase progesterone and androgen. Flare protocol includes pituitary down-regulation with GnRHa, starting on day 2 at 1 mg/day for 3 days, followed by 250 µg/day and gonadotropins for ovarian stimulation from day 2. Microdose flare protocol includes pituitary down-regulation with GnRHa, 20–50 µg administered twice daily and gonadotropins for ovarian stimulation from day 2.

Evidence suggests no significant difference in the outcome among various GnRH agonist flare protocols [8]. Cochrane review suggests no significant difference in the clinical pregnancy rate per woman comparing GnRH antagonist versus GnRHa flare protocol [9]. Although flare protocol strategy seems logical, its benefit in the management of the poor ovarian response has not been consistently demonstrated.

32.2.2.2 GnRH Agonist Stop Protocols

Continuation of GnRHa even at low doses during ovarian stimulation is thought to have deleterious effect in a patient with diminished ovarian reserve and it has been found that GnRHa cessation following down-regulation can be effective in the prevention of premature rise in LH. In GnRHa stop protocol, GnRHa administration is stopped prior to initiation of gonadotropin stimulation but after down-regulation has been achieved. It was suggested that stop protocol may improve ovarian responsiveness based on a hypothetical effect on the ovary, directly via GnRH receptor or by indirectly regulating the vascular network within the ovary. However, there is no good evidence to suggest that this approach offers any advantage over the conventional, long down-regulation protocol except in the requirement of less gonadotropin for stimulation.

32.2.3 Gonadotropin Modifications

A simple approach to improve ovarian response in POR women could be to increase the dose of the gonadotropin administration. However, the same has not been proven in studies

which suggest that POR women who failed to conceive on a daily gonadotropin dose of 450 IU did not benefit from increasing the dose to 600 IU [10]. Increasing the daily dose of gonadotropins higher than 450 IU or a total dose of 3000 IU per treatment cycle has questionable clinical advantage, but results in higher treatment cost.

LH is crucial for the development of follicular growth and oocyte maturation. It helps to maintain adequate concentrations of intra-ovarian androgens and promotes steroidogenesis with follicular growth. It has been proposed that the addition of LH to ovarian stimulation protocol may benefit from a better ovarian response in women with POR (see Chapter 14). Eight randomized trials ($n = 847$) studied the role of LH supplementation, and the meta-analysis of these studies found no significant difference in clinical pregnancy rates (OR 1.32 95% CI 0.93 to 1.87) [5]. When recombinant FSH plus recombinant LH (r-FSH/r-LH) were compared with r-FSH monotherapy, the reproductive outcome was comparable [11]. Human chorionic gonadotropin (hCG) is a functional analogue of LH and it was found that uHCG supplementation produced better clinical outcomes compared with r-LH in women with poor ovarian response [12].

Corifollitropin alfa is a novel recombinant follicle-stimulating hormone (r-FSH) analogue, with longer half-life and slower absorption rate compared to r-FSH. Its use for poor ovarian response has not shown any benefit [13].

32.2.4 Use of Adjuvants

32.2.4.1 Growth Hormone (GH) Supplementation (see Chapter 15)

Growth hormone acts on the ovaries during early as well as late follicular phase to stimulate the proliferation and differentiation of granulosa cells. GH also increases the production of oestradiol in the ovaries by enhancing aromatase and 3-β-hydrogenase activity. GH can stimulate the growth and function of granulose cells by increasing intra-ovarian production of insulin-like growth factor (IGF) and in several animal models of in vitro maturation; it was shown that exogenous GH increased follicular IGF-1 and IGF-2 and oocyte competence. A recent meta-analysis showed GH supplementation increases clinical pregnancy rates (RR 1.65, 95% CI 1.23–2.22) and live birth rates (RR 1.73, 95% CI 1.25–2.40) with significantly less cancelled cycles [14]. There is wide range of GH dose and schedule described in trials. Suggested GH dose is 0.1 IU/kg/day with gonadotropins. GH supplementation could be used in both GnRH antagonist protocol and GnRH agonist flare protocols. GH supplementation offers promising research direction for poor ovarian response and further good-quality studies would be required before recommending GH for routine clinical use.

32.2.4.2 Androgens (see Chapter 11)

DHEA Supplementation

Dehydroepiandrosterone (DHEA) is being increasingly used for POR. It is the precursor of oestradiol (E2) and testosterone (T) and originates from the adrenal zona reticularis and from ovarian theca cells. Its conversion may not be symmetrical favouring testosterone over the oestradiol and may depend on the steroidogenic enzymes present in peripheral target tissues. It is not clear whether DHEA also exerts its actions through direct interaction with the androgen receptor or after conversion to more potent androgens such as testosterone. DHEA augments the follicular sensitivity towards FSH stimulation and increases ovarian

IGF-1, which is known to have a positive effect on the follicular development and oocyte quality. The beneficial effect of DHEA may also be due to rescue of small antral follicles from atresia. Increasing intra-follicular androgens results in an increased AMH expression by the granulosa cells and inhibin-B production. With DHEA supplementation, virilizing effects such as acne, deepening of voice and facial hair growth are reported to be minimal. The doses of DHEA range from 25 mg to 30 mg three times a day for three months before the IVF cycle. Although observational studies showed improved clinical pregnancy rates, the difference was not significant when the data were restricted to randomized trials [15]. Well-designed multicentre randomized controlled trials are required to provide more insight on the effectiveness of DHEA.

Testosterone Supplementation

Androgen receptors are abundant in granulosa cells of healthy pre-antral and antral follicles and their expression is upregulated by androgen administration. Androgen-excess stimulates early stage of follicular growth and increases the number of pre-antral and antral follicles; it also augments FSH receptor expression in granulosa cells. It results in enhanced responsiveness of ovaries to FSH, and reduction in granulosa cell apoptosis. Initial studies suggested that transdermal testosterone pretreatment increased clinical pregnancy and live birth rates in poor responders undergoing ovarian stimulation for IVF. The commonest protocol used in these trials was 10 mg of transdermal testosterone (1 gram gel) for 21 days before starting ovarian stimulation cycle and the treatment was required for three to four weeks to result in increased antral follicle count (AFC) and ovarian stromal blood flow. The meta-analysis (3 RCTs; n = 225) showed significantly improved live birth rates (OR 2.18, 95% CI 1.01 to 4.68), but the number of oocytes retrieved was not statistically significant (Mean Difference 0.94, 95% CI 0.24 to 1.64) [5]. However, when the results of a recent randomized trial [16] are added to this meta-analysis there appears to be no difference in the outcome (OR 1.96, 95% CI 0.96 to 4.01). Therefore, based on the current evidence, routine use of testosterone pretreatment could not be recommended to improve pregnancy outcome in poor responders.

32.2.5 Natural Cycle and Mild Stimulation Protocols

Natural cycle IVF and mild stimulation protocols have been proposed as an alternative to conventional stimulation protocols for poor responders as being more cost-effective and acceptable. Natural cycle IVF appears to have a very poor prognosis for poor responders and does not appear to provide substantial benefits. Mild stimulation protocols are reported to reduce the mean number of days of stimulation, the total amount of gonadotropins used and the mean number of oocytes retrieved. Mild protocols include use of clomiphene citrate or letrozole with FSH. A typical protocol includes clomiphene citrate 50–100 mg/d from day 2 to 6 of the menstrual cycle with FSH 150 IU/d introduced from day 5 of the cycle and GnRH-antagonist (0.25 mg/d) from the 8th day of the cycle until the day of final maturation trigger. Although there is a wide variation in the protocols used in mild stimulation, the dose of FSH is restricted to a maximum of 150 IU. Evidence from meta-analysis suggests that mild protocols with clomiphene citrate has equal pregnancy outcome for women with POR as compared with that of conventional protocol [17]. Letrazole in combination with 150 IU/d gonadotropins has also been found to be as effective in stimulation with higher doses of

gonadotropins [18]. A recent multicentre randomized trial showed that in women with poor ovarian reserve (defined as FSH>10, AFC<5, previous poor response), mild ovarian stimulation strategy (ovarian stimulation with 150 IU/d of gonadotropin and GnRH antagonist down-regulation) showed similar ongoing pregnancy rates to a conventional ovarian stimulation strategy [19]. Although acceptable results have been reported, further large prospective randomized studies are needed to better evaluate the efficacy of mild stimulation protocols compared with conventional stimulation approaches.

32.3 Conclusion

Despite two decades and various attempts, it has not been possible to find an optimal ovarian stimulation protocol for poor responders, which remains a major clinical challenge. With the current insufficient evidence, no single treatment can be recommended over another which can significantly improve reproductive outcome for women with POR. Traditional long protocols with high doses of gonadotropins are not ideal as it has been demonstrated that similar success rates could be achieved with less intensive approach. Limited evidence supports pituitary down-regulation using GnRH antagonists to avoid severe pituitary suppression and smaller doses of gonadotropins as possible treatment options. Various adjuvants such as DHEA, GH and testosterone have shown some promising initial results, but it is early for them to be recommended for routine clinical use.

References

1. Ferraretti AP, La Marca A, Fauser BC, et.al. ESHRE consensus on the definition of 'poor response' to ovarian stimulation for in vitro fertilization: the Bologna criteria. *Human Reproduction* (Oxford, England). 2011;26(7): 1616–24.
2. Bozdag G, Polat M, Yarali I, Yarali H. Live birth rates in various subgroups of poor ovarian responders fulfilling the Bologna criteria. *Reproductive Biomedicine Online*. 2017; S1472–6483(17)30141–4. doi:10.1016/j.rbmo.2017.03.009.
3. Patrizio P, Vaiarelli A, Levi Setti PE et al. How to define, diagnose and treat poor responders? Responses from a worldwide survey of IVF clinics. *Reproductive Biomedicine Online*. 2015;30: 581–92.
4. Boza A, Cakar E, Boza B, et.al. Microdose flare-up gonadotropin-releasing hormone (GnRH) agonist versus GnRH antagonist protocols in poor ovarian responders undergoing intracytoplasmic sperm injection. *Journal of Reproduction & Infertility*. 2016;17: 163–68.
5. Jeve, YB and Bhandari HM. Effective treatment protocol for poor ovarian response: A systematic review and meta-analysis. *J Hum Reprod Sci*. 2016;9:70–81.
6. Turgay Çelik Gİ, Sütçü HK, Akpak YK, Akar ME. A flexible multidose GnRH antagonist versus a microdose flare-up GnRH agonist combined with a flexible multidose GnRH antagonist protocol in poor responders to IVF. *BioMed Research International*. 2015;2015:970163.
7. Yang R, Li H, Li R, Liu P, Qiao J. A comparison among different methods of letrozole combined with gonadotropin in an antagonist protocol and high-dose gonadotropin ovarian stimulation antagonist protocol in poor ovarian responders undergoing in vitro fertilization. *Archives of Gynecology and Obstetrics*. 2016;294: 1091–97.
8. Surrey ES. Management of the poor responder: the role of GnRH agonists and antagonists. *Journal of Assisted Reproduction and Genetics*. 2007;24: 613–19.
9. Pandian Z, McTavish AR, Aucott L, Hamilton MP, Bhattacharya S. Interventions for 'poor responders' to controlled ovarian hyper stimulation (COH) in in-vitro fertilisation (IVF). Cochrane Database of Systematic Reviews (Online). 2010(1):CD004379.

10. Lefebvre J, Antaki R, Kadoch I-J et al. 450 IU versus 600 IU gonadotropin for controlled ovarian stimulation in poor responders: a randomized controlled trial. *Fertility and Sterility*. 2017;104: 1419–25.
11. Humaidan P, Chin W, Rogoff D et al. Efficacy and safety of follitropin alfa/ lutropin alfa in ART: a randomized controlled trial in poor ovarian responders. *Human Reproduction* (Oxford, England). 2017;32: 544–55.
12. Mak SM, Wong WY, Chung HS et al. Effect of mid-follicular phase recombinant LH versus urinary HCG supplementation in poor ovarian responders undergoing IVF – a prospective double-blinded randomized study. *Reproductive Biomedicine Online*. 2017;34: 258–66.
13. Selman H, Rinaldi L. Effectiveness of corifollitropin alfa used for ovarian stimulation of poor responder patients. *International Journal of Women's Health*. 2016;8: 609–15.
14. Li XL, Wang L, Lv F et al. The influence of different growth hormone addition protocols to poor ovarian responders on clinical outcomes in controlled ovary stimulation cycles: A systematic review and meta-analysis. *Medicine*. 2017;96:e6443.
15. Qin JC, Fan L, Qin AP. The effect of dehydroepiandrosterone (DHEA) supplementation on women with diminished ovarian reserve (DOR) in IVF cycle: Evidence from a meta-analysis. *Journal of Gynecology Obstetrics and Human Reproduction*. 2017;46:1–7.
16. Bosdou JK, Venetis CA, Dafopoulos K et al. Transdermal testosterone pretreatment in poor responders undergoing ICSI: a randomized clinical trial. *Human Reproduction* (Oxford, England). 2016;31: 977–85.
17. Song D, Shi Y, Zhong Y et al. Efficiency of mild ovarian stimulation with clomiphene on poor ovarian responders during IVF /ICSI procedures: a meta-analysis. *European Journal of Obstetrics and Gynecology and Reproductive Biology*. 2016;204:36–43.
18. Bastu E, Buyru F, Ozsurmeli M et al. A randomized, single-blind, prospective trial comparing three different gonadotropin doses with or without addition of letrozole during ovulation stimulation in patients with poor ovarian response. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*. 2016;203:30–34.
19. Youssef MA, van Wely M, Al-Inany H et al. A mild ovarian stimulation strategy in women with poor ovarian reserve undergoing IVF: a multicenter randomized non-inferiority trial. *Human Reproduction* (Oxford, England). 2017;32: 112–18.