

Inhibins in female and male reproductive physiology: role in gametogenesis, conception, implantation and early pregnancy

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A great deal of new information has arisen in the recent years concerning inhibin physiology and clinical relevance in reproductive medicine. It is now recognized that the two inhibin isoforms, named inhibin A and inhibin B, are produced by the gonads in the course of gamete maturation and in women have a different pattern of secretion throughout the menstrual cycle. Since inhibins are also produced by placenta and fetal membranes, it has been suggested that there is an involvement in physiological adaptation of pregnancy. Evidence from several sources has underlined the clinical usefulness of the measurement of inhibin-related proteins in the diagnosis and follow-up of different fertility disturbances and early pregnancy viability. In the male, inhibin B is produced in the testis, principally by the Sertoli cells. Inhibin B expression and secretion are positively correlated with Sertoli cell function, sperm number, and spermatogenic status and are negatively correlated with FSH. This review covers the most recent advances on the role of inhibins in human reproductive function. Considerable progress in the understanding of inhibin physiology has resulted from selective measurement of the two inhibin molecular forms, named inhibin A and B. Newly recognized alterations of inhibin levels in gynaecological diseases as well as in normal and pathological pregnancy are discussed, with particular emphasis on the potential clinical usefulness of assessing inhibin levels in serum and other biological fluids.

Key words: implantation/inhibin/ovary/pregnancy/testis

Introduction

Inhibins are multifunctional molecules involved in the control of pituitary FSH secretion. A body of observational and experimental evidence from several species, including the human, supports the concept that inhibins are gonadal messengers that exert a physiological negative feedback control on FSH release at the pituitary gland. Apart from their essential role in the selective control of FSH secretion, inhibins are currently recognized as paracrine ovarian and testicular regulators and have multiple paracrine effects in the utero-placental unit, representing a promising marker for male and female infertility, gynaecological and gestational diseases.

Structure and receptor

Inhibins are glycoproteins produced by the granulosa and theca cells of the ovary and by the Sertoli cells of the testis. They are of great importance for the negative feedback control of pituitary

gonadotrophin secretion. There are at least two active molecular forms in circulation, inhibin A and inhibin B, which are heterodimers made by an α subunit and either β_A (inhibin A) or β_B (inhibin B) subunits (Hayes *et al.*, 1998).

Activins (dimers composed of β_A and/or β_B inhibin subunits) act on activin type I and type II receptors which contain an extracellular domain (where the hormone binds), a transmembrane region and a serine/threonine kinase intracellular domain. Although most inhibin effects are associated with its antagonism to activin, there are cells that bind inhibin with much higher affinity than activin due to the existence of specific inhibin-binding molecules (Lebrun and Vale, 1997). One of these molecules was recently discovered to be betaglycan, a membrane-anchored proteoglycan that operates as an inhibin co-receptor. Betaglycan binds inhibin α subunit with high affinity and enhances inhibin binding to activin type II receptor through its β subunit, thus preventing activin access to the type II receptor (Lewis *et al.*, 2000).

Assays for inhibins

Radioimmunoassays were the first assay developed and 'the Monash assay' (McLachlan *et al.*, 1986, 1987; Schneyer *et al.*, 1990) provided a wealth of insight into reproductive physiology. However, the 'Monash' assay, which used a polyclonal antibody raised against 31 kDa bovine inhibin with epitopes for the antibody on the inhibin α subunit, is unable to discriminate between dimeric bioactive inhibin forms and various forms of free α subunit which circulate in 20-fold excess. Several groups prepared monoclonal and polyclonal antibodies with the intention of producing two-site immunoassays to measure specifically the bioactive dimeric forms of inhibin (Illingworth *et al.*, 1991; Baly *et al.*, 1993; Poncelet and Franchimont, 1994). However, due to the high degree of structural conservation of inhibin between species, it is a poor immunogen and the antibodies raised had limited affinity. More recently, a panel of monoclonal antibodies to synthetic peptide immunogens was used to construct ultrasensitive enzyme-linked immunosorbent assays (ELISA) for dimeric inhibin A, inhibin B, and the inhibin precursor pro- α C (Groome *et al.*, 1994, 1995, 1996; Knight and Muttukrishna, 1994; Evans *et al.*, 1997). The inhibin A and inhibin B assays make use of a sample pre-treatment process with hydrogen peroxide (Knight and Muttukrishna, 1994) which oxidizes the methionine residues in the β subunits and significantly increases the sensitivity of the assays. An additional specificity-enhancing step involves heating samples for these assays with a sodium dodecyl sulphate solution, which irreversibly disrupts activin–follistatin complexes and, in the inhibin B assay, removes the effect of heterophil antibodies. A very high specificity and sensitivity is required for measuring concentrations of the various members of the inhibin family in serum where physiological concentrations may be detected at concentrations as low as 5 pg/ml. The Groome ELISA, which are now available commercially (Oxford Bioinnovation, UK or from Diagnostic System Laboratories, DSL, USA), provide precise and replicable results for use in clinical and physiological research.

Pituitary

A decade before the isolation and biochemical characterization of inhibins, their essential biological activity had been demonstrated in testicular extracts that selectively inhibited FSH release (Keogh *et al.*, 1976). Since then, the negative feedback control of pituitary FSH secretion has been the most recognized physiological role of inhibin. The selective inhibition of FSH synthesis and release by circulating inhibins have been demonstrated in several animal species including rodents, other mammals, and non-human primates, and in well-controlled observational and intervention studies in humans.

The physiological role of inhibin has been evaluated by immunoneutralization experiments in which the infusion of anti-inhibin antisera at different phases of the rat estrous cycle caused a robust increase in plasma FSH concentrations, whereas LH remained unchanged (Rivier *et al.*, 1986). Moreover, the administration of inhibin antiserum to female rats (Attardi *et al.*, 1992) or hamsters (Kishi *et al.*, 1996) led to a substantial increase of FSH- β mRNA and protein levels in the pituitary gland,

contrasting with no change in the transcription rate of LH- β or their common α subunit (Attardi *et al.*, 1992).

Another body of evidence for the inhibin effects on gonadotrophin release came from the injection of recombinant human inhibin A into various animal species under different experimental conditions. The administration of recombinant inhibin to female rats prevents the pro-estrous evening FSH surge but does not inhibit ovulation (Rivier *et al.*, 1991a), probably because LH secretion is not affected (Rivier *et al.*, 1991b). Inhibin administration disrupts the FSH pulse frequency, amplitude, and peak levels without changing LH pulsatility, suggesting that inhibin alters mostly the pituitary sensitivity to GnRH, not the hypothalamic GnRH release (Rivier *et al.*, 1991b). Inhibin administration to cycling rhesus monkeys during the early follicular phase produces a rapid inhibition of FSH and thereby delays ovulation (Molskness *et al.*, 1996). Inhibin infusion during the luteal phase does not change LH release, progesterone concentrations nor the duration of the luteal phase, whereas the physiological FSH rise during the luteal–follicular transition is inhibited (Stouffer *et al.*, 1994).

Both observational and experimental evidence in women suggests that inhibins are physiologically important regulators of FSH secretion. The prospective evaluation of women undergoing bilateral oophorectomy showed that serum FSH concentrations begin to increase shortly after surgery in both menstrual cycle phases and, notably, there is a negative correlation between FSH and inhibin A in both phases or between FSH and inhibin B in the follicular phase (Muttukrishna *et al.*, 2002a). At late reproductive years, regularly cycling women with elevated day 3 FSH levels have lower inhibin A and inhibin B levels compared to age-matched controls with normal FSH levels, while activin A, progesterone and estradiol concentrations do not differ (Muttukrishna *et al.*, 2000). In normal cycling, as well as in GnRH deficient women with appropriate GnRH replacement, the suppression of estradiol negative feedback by continuous administration of tamoxifen produces an increase in FSH levels during the luteal–follicular transition, which testifies to the importance of estradiol feedback at this period; nevertheless, the FSH levels remain distant from the post-menopause range, probably because the inhibin feedback is intact. During the late follicular phase, tamoxifen does not induce any significant change in FSH levels, suggesting that at this phase it is the inhibin rather than the estradiol feedback that plays a critical role in the control of FSH secretion (Welt *et al.*, 2003).

Ovary

In the human ovary, inhibin subunit gene expression has been demonstrated in granulosa, theca, and lutein cells with a changing pattern during menstrual cycle (Hayes *et al.*, 1998).

Inhibin peptide localization, however, is essentially restricted to the granulosa layer of antral and pre-ovulatory follicles, as well as granulosa luteal cells of active corpora lutea, being scarce or absent from theca and interstitial cells (Yamamoto *et al.*, 1991, 1992). Inhibin subunits have also been localized in the corpus luteum of pregnancy (Minami *et al.*, 1995). Inhibin α subunit is already expressed during fetal life in antral follicles of sheep ovaries, whereas the β_A subunit is not (Engelhardt *et al.*, 1995).

Although the major importance of inhibins in reproductive function resides on endocrine regulation of pituitary gonadotrophin secretion, some paracrine effects within the ovary have also been described. For example, in granulosa and theca cells isolated from sheep ovaries, inhibin mediation appears to be required for a physiological steroid production in response to gonadotrophin stimulation (Campbell and Baird, 2001).

In the human ovary, inhibin has been shown to increase androgen production by theca cells. Produced by granulosa cells in increasing amounts during follicular development and stored in the antral fluid, inhibin may reach the adjacent thecal layer and positively modulate the LH-induced androgen synthesis. This assumption is supported by observation that recombinant inhibin A enhances LH-stimulated androgen release by cultured human thecal cells (Hillier *et al.*, 1991). Because activin A induces FSH receptors and enhances the proliferation of granulosa cells *in vitro* (Mather *et al.*, 1997), inhibin might also antagonize this activin/FSH effect by competing with activin, characterizing an autocrine regulation of granulosa cell proliferation. However, this is a difficult hypothesis to test *in vitro* because the absence of natural follicular microenvironment would preclude all the physiological conditions (local concentration of inhibins, receptor modulation, other regulatory factors) in which this putative autocrine mechanism may take place.

The paracrine action of inhibins within the ovary is presumably mediated by inhibin/activin receptors in the target cells. Indeed, binding sites for inhibin and activin have been found in human ovary by *in situ* ligand binding (Krummen *et al.*, 1994). Although this methodology does not exclude the influence of soluble activin-binding proteins, like follistatin, the expression of mRNA encoding both type I and type II activin receptors was subsequently shown in human granulosa cells (Eramaa *et al.*, 1995). Furthermore, activin type II receptor has been immunolocalized in ovarian follicles at various developmental stages, since the secondary follicle stage. Inhibin/activin subunits, their receptors, and intracellular signalling proteins from the Smad family have been identified in granulosa and theca cells of small antral follicles as well as granulosa cells of early atretic follicles (Pangas *et al.*, 2002).

Follicular and peritoneal fluid

Both inhibin A and inhibin B are secreted into follicular fluid. An interesting comparison between serum and follicular fluid inhibin levels in matched samples collected from spontaneously cycling women showed that, in the late stage of dominant follicle development, inhibin A is 4-fold more concentrated in follicular fluid than in serum, whereas inhibin B is present in similar concentration in both fluids (Klein *et al.*, 1996). The amount of both inhibins secreted into follicular fluid seems to increase along the process of follicle development, but current data are still conflicting. Based on cross-sectional evaluation of follicular fluid samples collected in different stages of follicle development (4.8–20 mm in diameter) from normal ovulatory women, Magoffin and Jakimiuk (1997) found higher inhibin B levels in follicles of intermediate diameter (13 mm) and higher inhibin B than inhibin A levels in all follicular fluid samples. However, the relatively lower inhibin B concentration in larger follicles seems to result from dilution of the hormone in greater

volumes of follicular fluid rather than a decrease in total hormone release. Similar patterns have been observed in peritoneal fluid of healthy women: inhibin A and inhibin B levels are very high and mimic the cyclic changes of serum inhibin levels (Florio *et al.*, 1998). The amount of both inhibins secreted into follicular fluid seems to increase with follicle development, although their concentrations may slightly decrease in the largest follicles due to dilution in a greater fluid volume (Magoffin and Jakimiuk, 1997). In the sheep, inhibin subunit mRNA are expressed in greater amounts in large than in small follicles, which probably reflects the greater granulosa cell population of large follicles rather than an augmented inhibin production by each individual cell (Campbell *et al.*, 1996; Campbell and Baird, 2001).

Changes of circulating levels during menstrual cycle

Puberty

Associated with the postnatal activation of gonadotrophin secretion, the inhibin B and inhibin A secretion is sustained until the age of 18–24 months. Serum inhibin B levels correlate positively with age several years before the clinical onset of puberty, suggesting increasing follicular activity in late pre-puberty. During female puberty, the inhibin B level increases from Tanner stage 1 to stage 3, suggesting high follicular activity before the development of ovulatory menstrual cycles, but serum inhibin A levels become measurable later in puberty, in agreement with the idea that inhibin A is mainly produced by the corpus luteum (Raivio and Dunkel, 2002).

Menstrual cycle

The major form of inhibin secreted during follicular phase of the menstrual cycle is inhibin B: serum levels rise sharply from early follicular phase of the menstrual cycle, with a peak following the FSH rise and a progressive fall during the remainder of the follicular phase (Figure 1A). Another peak of serum inhibin B is observed 2 days after the midcycle LH peak, followed by rapid decrease and constant low levels during luteal involution (Groome *et al.*, 1996). The circulating levels of inhibin A are low in the early stage of follicular phase and rise from late follicular phase to peak at midluteal phase, after a short indentation coinciding with LH peak. Inhibin B levels change within a range above inhibin A levels during the whole follicular phase of menstrual cycle (Groome *et al.*, 1996; Klein *et al.*, 1996). These different menstrual cycle patterns suggest that the two inhibin forms may have different physiological roles. Whereas inhibin B seems to be the major marker of follicular growth in response to exogenous FSH administration, inhibin A is secreted mostly by the corpus luteum and may be involved in the gradual release of ovarian negative feedback on FSH secretion during the luteal–follicular transition (Welt *et al.*, 1997).

Ageing cycling women have increasing FSH levels during the follicular phase in spite of normal estrogen and LH levels and regular menstrual cycles. This FSH rise occurs some years before menopause and accompanies the decline of ovarian follicular reserve and fertility rate. The selective FSH rise of late reproductive years might be a consequence of declining inhibin secretion by a reduced pool of ovarian follicles. This was

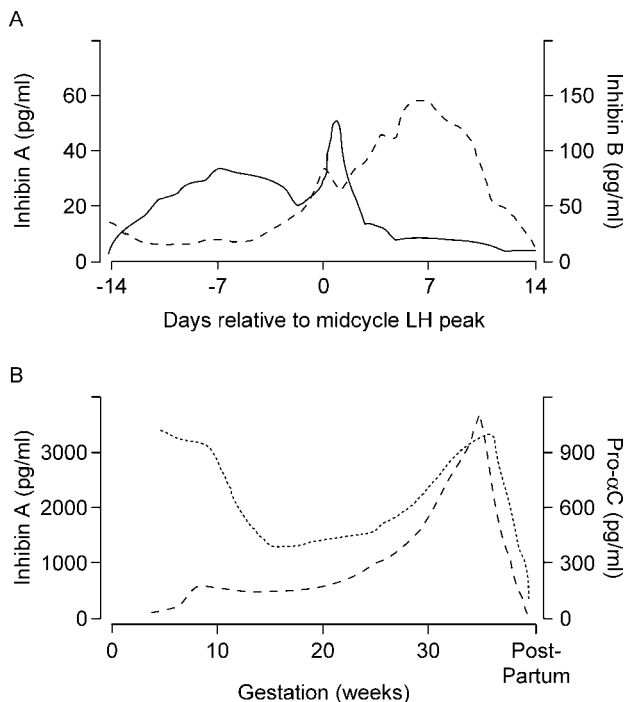


Figure 1. (A) Changes of serum inhibin A and inhibin B levels during the menstrual cycle. Redrawn from Groome *et al.* (1996). (B) Maternal serum inhibin A and pro- α C levels during pregnancy and post-partum. According to Fowler *et al.* (1998).

suggested by early studies performed when assay methods did not discriminate between the two inhibin forms (Lenton *et al.*, 1991). After the development of specific two-site ELISA for both inhibin A and inhibin B, the age-associated changes of ovarian–gonadotrophin interaction have become clearer. It was observed that: (i) ageing women with elevated FSH and normal estradiol levels have markedly lower inhibin B levels during the follicular phase (Klein *et al.*, 1996; Reame *et al.*, 1998); (ii) luteal phase inhibin A levels are also low in ageing cycling women (Danforth *et al.*, 1998); (iii) follicular phase inhibin B levels decrease earlier than inhibin A during the process of ovarian ageing (Burger *et al.*, 1998); (iv) both serum inhibin A and inhibin B levels are nearly undetectable in normal post-menopausal women (Burger *et al.*, 1998; Petraglia *et al.*, 1998).

Changes of inhibin secretion in ovarian disorders

Hypothalamic amenorrhoea

Amenorrhoeic women with deficiency of GnRH secretion due to hypothalamic lesions are an interesting model to study the role of hypothalamic–pituitary axis on inhibin secretion. When treated with exogenous GnRH at regular, physiological pulses, these patients display a normal increase of inhibin B levels during the luteal–follicular transition of induced menstrual cycles. In contrast, abnormally low frequency pulses of GnRH administered during luteal–follicular transition result in sub-physiological levels of FSH and inhibin B (Welt *et al.*, 1997). These findings indicate that the release of inhibin B is dependent on adequate FSH stimulation and may serve to confirm the effectiveness of GnRH replacement therapy on ovarian follicular development.

Conversely, inhibin A and inhibin B levels are not altered in patients with functional hypothalamic amenorrhoea (Petraglia *et al.*, 1998; Casper *et al.*, 2000). These findings indicate that basal inhibin secretion does not require a normal hypothalamus–pituitary function, but may be suddenly suppressed by primary ovarian failure, regardless of senescence.

Polycystic ovarian syndrome

Polycystic ovarian syndrome (PCOS) is characterized by increased inhibin levels due to the persistence of a cohort of small follicles that contribute to the pool of circulating inhibins, but the pulsatile rhythm of inhibin B secretion is blunted (Lockwood *et al.*, 1998a). Treatment of PCOS patients with low doses of FSH may be sufficient to induce the development of a single dominant follicle which grows in parallel with estradiol and inhibin A levels (Anderson *et al.*, 1998a). Overall, these findings indicate that elevated circulating levels of inhibin B lead to the relative deficit of FSH in PCOS, while the disruption of inhibin B pulsatility may be an additional marker of multiple, incomplete follicular growth. Additionally, the paracrine action of inhibins on stimulating androstenedione production by theca cells may reinforce the hyperandrogenism of PCOS (Pigny *et al.*, 1997, 2000).

Premature ovarian failure

Inhibins A and B are dramatically reduced in women with premature ovarian failure (POF) (Petraglia *et al.*, 1998). Serum levels of both inhibins are as low as in normal post-menopausal women matched for time elapsed since last menstrual period, and do not correlate with patient age, length of amenorrhoea, or serum gonadotrophin levels (Petraglia *et al.*, 1998). In addition, circulating levels of inhibin A, inhibin B and pro- α C are reduced after oophorectomy. Women with amenorrhoea induced by GnRH analogue treatment or by antineoplastic chemotherapy still produce inhibin A and pro- α C. This probably reflects a residual ovarian function and hormone synthesis (Cobellis *et al.*, 2002).

The GnRH analogue treatment should be considered in every woman of reproductive age receiving chemotherapy, in addition to assisted reproductive technologies and the investigational attempts of ovarian cryopreservation for future *in vitro* maturation or reimplantation. In addition, inhibin A concentrations may serve as a prognostic factor for predicting the resumption of ovarian function in addition to the levels of FSH, LH and estradiol (Blumenfeld *et al.*, 1999).

The testis

Sites and targets of inhibin

Inhibin subunits are expressed in the human testis from fetal life. At second trimester gestation, both α and β_B subunits are present in Sertoli and Leydig cells, whereas gonocytes express only the β_B subunit and Leydig cells also express the β_A subunit (Rabinovici *et al.*, 1991; Anderson *et al.*, 2002). However, the β_A subunit serves mainly for the synthesis of its homodimer, activin A (Buzzard *et al.*, 2003), whereas inhibin A is produced to a much lesser extent and remains undetectable in

the peripheral serum of adult males (Anawalt *et al.*, 1996; Illingworth *et al.*, 1996).

Sertoli cells are able to produce both α and β_B inhibin subunits before puberty, as suggested by immunolocalization and by the finding of normal serum inhibin B levels in pre-pubertal boys with Sertoli cell-only syndrome. After puberty, only α subunit continues to be expressed by Sertoli cells whereas the β_B subunit is predominantly detected in maturing germ cells and with less intensity in Leydig cells (Andersson *et al.*, 1998). Coherently, the acute administration of FSH to healthy adult men does not induce any significant change in serum inhibin B, while pro- α C levels do increase (Kinniburgh and Anderson, 2001). This suggests that Sertoli cells produce predominantly α subunit in response to FSH stimulation, whereas β_B subunit and dimeric inhibin B require a more complex process to be released.

Testicular targets of inhibins

The receptors, co-receptors and intracellular signalling molecules thus far implicated in the inhibin mechanism of action are all expressed in the testis. Type II activin receptor and Smad proteins have been localized in Sertoli, Leydig, and germ cells (de Kretser *et al.*, 2001; Hu *et al.*, 2003), whereas the inhibin co-receptors betaglycan and inhibin-binding protein seem to be restricted to Leydig cells (Bernard *et al.*, 2002). However, the physiological role of paracrine/autocrine inhibin effects within the testis has not been clarified. While the α subunit knockout mouse model suggests that this protein protects against the development of testicular tumours, there is no evidence for a physiological role of paracrine/autocrine inhibin signalling on spermatogenesis or steroidogenesis (reviewed by de Kretser *et al.*, 2001).

Physiology

Mutual regulation between inhibin and gonadotrophins in men

Physiological inhibin production by the adult testis requires a normal population of Sertoli cells, FSH stimulation, and spermatogenesis to be present. The two later factors are not absolutely necessary for a basal inhibin B release, which is seen in some forms of hypogonadism, impaired spermatogenesis (Foresta *et al.*, 1999), and in men submitted to pharmacological gonadotrophin suppression by testosterone treatment (reviewed by Anderson, 2001). Spermatid vein blood sampling has revealed a pulsatile pattern of immunoreactive inhibin release and the pulses coincided with those of testosterone, suggesting that both hormones may respond to the same pulses of gonadotrophins (Winters, 1990). However, experimental evidence in non-human primates and clinical observations show unequivocally that the gonadotrophic effect of the pituitary gland on testicular inhibin secretion comes from FSH action on Sertoli cells (Ramaswamy and Plant, 2001). Not only is the contribution of Leydig cells to the circulating inhibin negligible (Andersson *et al.*, 1998), but the LH effect on inhibin release also appears to be inhibitory rather than stimulatory (Ramaswamy and Plant, 2001).

Serum inhibin B concentrations are substantially decreased or undetectable in men with either hypo- or hypergonadotrophic hypogonadism (Anawalt *et al.*, 1996). Among infertile men with

elevated FSH levels, the FSH concentration is inversely correlated with inhibin B but not with pro- α C, suggesting that the physiologically important hormone that exerts tonic negative feedback upon FSH secretion in men is inhibin B (Illingworth *et al.*, 1996).

Inhibin B as a marker of spermatogenesis

The functional relationship between spermatogenesis and serum inhibin B concentrations is nicely demonstrated by the study of patients with variable degrees of spermatogenesis impairment due to a single genetic disorder, the presence of microdeletions in the AZFc region of the Y chromosome (Frydelund-Larsen *et al.*, 2002). In this selected population, serum inhibin B concentrations are absolutely normal in the individuals with bilateral spermatocytic arrest but undetectable in patients with a predominant Sertoli cell-only histological pattern (Frydelund-Larsen *et al.*, 2002).

Among the germ cells, spermatids appear to play a critical role in the mechanism of testicular inhibin B secretion in adulthood. Men with obstructive azoospermia and spermatidic arrest show normal inhibin B concentrations, whereas those with Sertoli cell-only syndrome or spermatogenesis arrest at pre-spermatid phases have considerably lower, often undetectable, inhibin B concentrations (Andersson *et al.*, 1998; Foresta *et al.*, 1999; Frydelund-Larsen *et al.*, 2002). Testicular irradiation for treatment of carcinoma *in situ* leads to a Sertoli cell-only pattern with undetectable inhibin B levels in men with previously normal inhibin B concentrations (Petersen *et al.*, 1999). Nevertheless, a minority of individuals with Sertoli cell-only syndrome are still capable of producing some inhibin B, the source of which has not been determined (Foresta *et al.*, 1999).

Sertoli cells also release inhibin B from its apical side into the seminiferous tubules, thus contributing to the seminal plasma inhibin B content. Seminal plasma inhibin B concentrations are substantially reduced in azoospermic men regardless of obstructive, non-obstructive or post-vasectomy azoospermia (Anderson *et al.*, 1998b; Gareu *et al.*, 2002), and correlate directly with sperm count (Anderson *et al.*, 1998b). Seminal plasma inhibin B is inversely related with serum FSH but their correlation is not strong, probably because the regulation of peripheral serum inhibin B (which exerts negative feedback on FSH secretion) differs from that of seminal plasma inhibin B (Anderson *et al.*, 1998b; Gareu *et al.*, 2002).

Time-course from fetal to adult life and senescence

Inhibins A and B become detectable in male fetal serum at 14–16 weeks of gestation (Muttukrishna *et al.*, 2004). In male fetuses, inhibin B concentrations at mid-trimester correlate directly with testosterone (Muttukrishna *et al.*, 2004) and inversely with FSH (Debieve *et al.*, 2000), suggesting that the pituitary–testicular axis, and in particular the mutual regulation of inhibin B and FSH release, is already operative during fetal life.

Testicular cells collected from newborns produce more inhibin B *in vitro* than cells obtained from older children (Berenshtein *et al.*, 2000). In the peripheral serum, inhibin B concentrations are detectable from birth (Florio *et al.*, 1999). In neonates, serum inhibin B concentrations are higher in males than in females, probably reflecting a testicular production, but there

appears to be no correlation with FSH levels at this stage of development (De Schepper *et al.*, 2000). There is a rapid post-natal increment which parallels the rise of FSH, then a peak at ~3–4 months and a rapid decrease to low but detectable concentrations during the following years until the pubertal rebound (Chada *et al.*, 2003). The most pronounced increase in serum inhibin B levels during pubertal transition occurs between Tanner stages G1 and G2, coinciding with the LH and testosterone surges (Raivio *et al.*, 1998; Chada *et al.*, 2003). The decline in testicular function in older men is accompanied by a slight reduction in inhibin B concentrations, the mechanism of which has not been established (Mahmoud *et al.*, 2000; Anderson, 2001).

Pathophysiology

Pubertal delay and hypogonadism

In boys with constitutional delay of puberty, as well as men with hypogonadotropic hypogonadism, serum inhibin B concentrations are low and dissociated from FSH. Upon GnRH replacement, the testis begins to respond to gonadotrophin stimulation by releasing inhibin B, which then reaches the physiological concentration range and establishes the negative feedback control of FSH release, as evidenced by an inverse correlation between inhibin B and FSH levels (Seminara *et al.*, 1996; Raivio *et al.*, 2000).

Cryptorchidism

Inhibin B can be used as a sensitive marker of testicular function in boys with various testicular disorders (Kubini *et al.*, 2000). Basal inhibin B strongly correlates with the hCG-induced testosterone increment and helps in the differential diagnosis between anorchia, abdominal testis with or without testicular damage, gonadal dysgenesis, and androgen insensitivity (Kubini *et al.*, 2000). There is a direct correlation between inhibin B levels and the number of spermatogonia in testicular biopsies (Longui *et al.*, 1998). During early childhood, it is the basal inhibin B concentration that better detects testicular function, whereas in older children some increment of inhibin B following hCG injection may also be observed (Raivio and Dunkel, 1999).

Adults with cryptorchidism and subfertility have lower inhibin B levels than fertile men or even subfertile men without cryptorchidism (Gouveia Brazao *et al.*, 2003).

Germ cell depletion by cancer therapy

Inhibin B is a reliable marker of spermatogenesis soon after puberty in cancer survivors who had received chemotherapy and/or radiation therapy in the past. These treatments cause selective depletion of germ cells without affecting Leydig cell function or steroidogenesis. Consequently, at puberty these boys have low inhibin B levels which correlate inversely with FSH and testicular size but not with testosterone or LH (Lahteenmaki *et al.*, 1999; Cicognani *et al.*, 2000). In adult men, the decrease of inhibin B and the consequent rise in FSH concentrations have been documented a few months after testicular irradiation for the treatment of carcinoma *in situ* (Petersen *et al.*, 1999). Interestingly, these patients continue to produce free α subunits that can

be detected by total inhibin assays (Tsatsoulis *et al.*, 1990), while functional, dimeric inhibin B is suppressed.

Clinical usefulness in reproductive medicine

Monitoring of ovulation induction cycles

During controlled ovarian stimulation for assisted reproduction treatment, inhibin B can be used in the same way as estradiol to monitor the follicular growth. In a cohort of women undergoing ovulation induction for assisted reproduction treatment, a peak of inhibin B has been characterized at midfollicular phase, preceding the pre-ovulatory estradiol peak, that correlated with the number of oocytes retrieved (Dokras *et al.*, 2000) and fertilized (Fawzy *et al.*, 2002). Higher serum inhibin A and inhibin B levels on the day of hCG administration to trigger ovulation are associated with a greater probability of pregnancy in assisted reproduction treatment cycles, but these markers do not add substantial predictive power to classical parameters such as age and number of oocytes retrieved (Hall *et al.*, 1999; Engel *et al.*, 2003).

Both inhibin A and inhibin B are produced in greater amounts during IVF cycles complicated by ovarian hyperstimulation syndrome (Enskog *et al.*, 2000). Higher inhibin levels are already present during follicular growth and before the onset of the syndrome, but the usefulness of inhibin determinations to predict this complication and guide cycle cancellation has not been established.

Evaluation of ovarian reserve and prediction of response to ovulation induction

Women with abnormally high FSH response to clomiphene citrate challenge also have lower serum inhibin B levels during the follicular phase of the menstrual cycle, which explains the exaggerated basal and/or post-test FSH levels (Hofmann *et al.*, 1998). This occurs because normal ovulatory women in their late reproductive years have lower follicular phase inhibin B levels compared to young women, which reflects their reduced pool of growing follicles (Klein *et al.*, 1996). In addition to the number of follicles, inhibin B possibly reflects their quality as well. Thus, luteinized granulosa cells collected from women with elevated FSH levels have a reduced inhibin production rate *in vitro* (Seifer *et al.*, 1996).

As the physiological principle of the clomiphene citrate test is to unmask an exaggerated FSH release due to insufficient inhibin feedback, the recent improvement of inhibin assays has inspired the use of direct inhibin measurements to assess ovarian reserve instead of, or in addition to, FSH. As expected, low inhibin B levels at early follicular phase of spontaneous cycles predict an increased risk of poor response to controlled ovulation induction. Importantly, inhibin B offered significant prognostic information even after data correction for confounding variables such as the FSH concentration (Balasch *et al.*, 1996; Seifer *et al.*, 1997; Hall *et al.*, 1999; Tinkanen *et al.*, 1999; Ficicioglu *et al.*, 2003). Whether this information is likely to influence the clinical decision as to justify the inclusion of inhibin B measurement in the work-up of assisted reproduction treatment patients is still uncertain. The latest clinical guideline released by the National Collaborating Centre for Women's and Children's Health (2004)

states that the role of inhibin B for this purpose needs further evaluation.

Inhibin B has also been evaluated as an additional marker to predict the response to ovulation induction in women whose main infertility factor was ovulatory dysfunction but, in such cases, it does not appear to be of clinical relevance (Imani *et al.*, 2000, 2002). This is not surprising because anovulatory women who fail to respond to ovulation induction might have this resistance explained by a number of alternative mechanisms, apart from a diminished ovarian reserve.

Prediction of sperm retrieval in men with non-obstructive azoospermia

Serum inhibin B concentrations may be useful to predict the chances of successful sperm recovery among men with non-obstructive azoospermia submitted to testicular sperm extraction (TESE) (Ballesca *et al.*, 2000; Bailly *et al.*, 2003). There appears to be an association between lower inhibin B levels and unsuccessful sperm retrieval by TESE (Ballesca *et al.*, 2000; Brugo-Olmedo *et al.*, 2001; Bailly *et al.*, 2003). This outcome cannot be predicted by FSH, probably because multiple factors other than spermatogenesis influence its peripheral concentration, whereas inhibin B reflects more faithfully the sperm production (Ballesca *et al.*, 2000; Bailly *et al.*, 2003). Despite this evidence, the precise accuracy of inhibin B test to predict the outcome of TESE–ICSI is still unknown. Whereas some studies suggested that inhibin B is a sensitive and specific marker of sperm recovery (Ballesca *et al.*, 2000; Brugo-Olmedo *et al.*, 2001), others have found it less effective (von Eckardstein *et al.*, 1999; Vernaev *et al.*, 2002). Only one study has stratified the population according to the different histopathologic patterns encompassed by the common denomination of non-obstructive azoospermia, and the sensitivity of inhibin B was much better if the pattern was maturation arrest compared to germ cell aplasia (Vernaev *et al.*, 2002). There is no evidence that combining inhibin B with another serum marker (e.g. FSH) will be advantageous over inhibin B alone to predict the outcome of TESE. Finally, it should be remembered that undetectable serum inhibin B does not exclude the possibility of successful pregnancy and delivery using TESE and ICSI (Guthausen *et al.*, 2002). In contrast to serum measurements, seminal plasma inhibin B does not distinguish patients with positive or negative outcome of TESE (Garem *et al.*, 2002).

Early pregnancy

Physiology

Inhibins are also involved in the control of the fetomaternal communication required to maintain pregnancy. Human placenta, decidua, and fetal membranes are the major sites of production and secretion of inhibin A and inhibin B in maternal serum, amniotic fluid, and cord blood. During the first trimester of pregnancy, the fetoplacental unit is the main source of circulating inhibin A and inhibin B. In fact, inhibin subunit mRNA (α , β_A , β_B) were localized in placental trophoblast (Petraglia *et al.*, 1987, 1991). Inhibin A is secreted into the maternal circulation (Petraglia *et al.*, 1993; Muttukrishna *et al.*, 1995, 1997), and coelomic fluid (Riley *et al.*, 1996; Wallace *et al.*, 1997a;

Luisi *et al.*, 1998). From 6 to 12 weeks of gestation, a precursor of inhibin A, pro- α C peptide, is mainly secreted by the corpus luteum (Muttukrishna *et al.*, 1997; Lockwood *et al.*, 1998b). During early pregnancy, pro- α C peptide levels increase to reach high concentrations at 5 weeks, and then fall after week 9 in coincidence with a small rise in inhibin A and with the peak in hCG (Fowler *et al.*, 1998). Therefore, the profile of first trimester circulating maternal pro- α C is markedly different from inhibin A, with an excess of pro- α C over inhibin A, indicating that a shift occurs in the tissue source and/or processing pattern of the α subunit precursor as pregnancy advances (Figure 1B). It is likely that these events reflect the switch from corpus luteum to placental support for the pregnancy. Furthermore, pro- α C concentrations in week 4 of pregnancy are higher in singleton pregnancy resulting from IVF treatment cycles (i.e. with multiple corpora lutea) than in spontaneous singleton pregnancies (i.e. with single corpus luteum), but extremely low in pregnancies arising from frozen–thawed embryo (i.e. established without corpus luteum), suggesting that the corpus luteum is the main source of the α monomer during early pregnancy (Lockwood *et al.*, 1997).

Contrasting findings have been reported by Birdsall *et al.* (1997) as inhibin A concentrations in the first trimester of pregnancy are not significantly different in women with or without corpora lutea, and multiple donor oocyte pregnancies were found to have higher concentrations of inhibin A compared with singleton donor oocyte pregnancies, supporting a placental source (Birdsall *et al.*, 1997). It has also been reported that while maternal circulating concentrations of inhibin A after the removal of fetoplacental unit gradually decreased within the first hour and decreased to even lower concentrations within the next 3 hours, maternal pro- α C levels decreased only within the first hour and then remained unaltered with levels similar to the luteal phase (Muttukrishna *et al.*, 1997), indicating a significant contribution by a viable corpus luteum. Taken together, these findings suggest that in early pregnancy the fetoplacental unit is the major source of inhibin A; and that pro- α C is mainly secreted by the corpus luteum.

During the first trimester of pregnancy, maternal serum levels of inhibin A are higher than in non-pregnancy, while inhibin B (Petraglia, 1997) does not significantly differ from levels detected during the menstrual cycle. At this gestational period, coelomic fluid inhibin B levels are higher than in maternal serum and amniotic fluid (Luisi *et al.*, 1998). In amniotic fluid, inhibin A is not detectable (Riley *et al.*, 1996).

With respect to the possible clinical implications, the measurement of inhibin-related proteins in early pregnancy could be useful in the diagnosis of impending abortion, hydatidiform mole and Down's syndrome.

Pathologies

Impending abortion

Early diagnosis of a complicated or poor pregnancy outcome, particularly in patients undergoing assisted reproduction treatment, could aid their counselling and management, and the role for the inhibin family as markers of early pregnancy viability has been investigated in this respect. Initially low levels and a very rapid decline of inhibin A in non-viable clinical pregnancies

with embryonic failure has been reported; and non-viable clinical pregnancies have very low levels of inhibin A and the initially low levels and very rapid decline in inhibin A in pregnancies with embryonic failure suggest a role for this glycoprotein as a monitor of early pregnancy viability (Lockwood *et al.*, 1997, 1998a). Furthermore, in IVF pregnancies following inadvertent periconceptual exposure to GnRH analogue, the levels of pro- α C are found to be higher in successful pregnancies than in early pregnancy failure, being indicative of lytic damage, and herald pregnancy failure despite luteal supplementation with progesterone (Lockwood *et al.*, 1998b).

However, all those studies have evaluated the early pregnancy outcome in women who became pregnant after assisted reproduction treatment. Recently, inhibin A concentrations were measured in maternal circulation of healthy spontaneously pregnant women progressing to deliver a healthy term singleton baby, in patients with incomplete miscarriage [carrying a non-viable (absence of heart beat activity) embryo or an anembryonic gestational sac *in utero*] and with complete miscarriage (an empty uterus with a history of passage of products of conception), in order to ascertain whether their measurement, in comparison to hCG, might provide a rapid and useful marker of early pregnancy viable placentation (Luisi *et al.*, 2003).

Patients with complete miscarriage had the lowest hCG, and inhibin A levels, that were lower in complete than in incomplete abortion (Figure 2). Thus, in the presence of complete miscarriage, a condition associated with trophoblast dismissal, maternal serum levels of hCG and inhibin A are the lowest, indicating the presence of a failed trophoblast. In fact, in patients who underwent voluntary pregnancy interruption, maternal inhibin A concentrations quickly decreased to low levels after the removal of the placenta (Muttukrishna *et al.*, 1997), reinforcing the concept that the fetoplacental unit significantly contributes to the maternal levels. Thus, when evaluated in a larger population, according to different gestational ages (Luisi *et al.*, 2003), or longitudinally in the same patients (Muttukrishna *et al.*, 1997) inhibin A and hCG levels were significantly lower in miscarriage than in healthy patients.

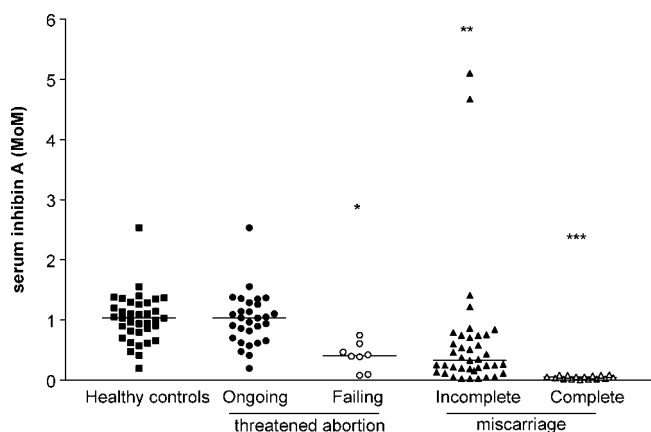


Figure 2. Maternal serum inhibin A levels in healthy pregnant women (control), patients with threatened abortion with ongoing and failing pregnancy, incomplete and complete miscarriage. Individual values are plotted (expressed as mean of mean) and horizontal bars represent the group medians. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$ versus healthy controls and threatened abortion with ongoing pregnancy.

The measurement of inhibin A during the first trimester of pregnancy could be useful in the diagnosis of trophoblast dysfunctions, and therefore be helpful in the management of early pregnancy problems, to predict the first trimester pregnancy outcome in patients with early pregnancy vaginal bleeding due to threatened abortion. In fact, patients with early pregnancy bleeding and an intrauterine sac with fetal cardiac activity (threatened abortion) progressing till term have higher inhibin A than those with threatened abortion but whose pregnancy failed later (Florio *et al.*, 2004). This finding may add significant prognostic information for predicting the pregnancy outcome. In fact, using the best thresholds indicated by the receiver operating characteristic (ROC) analysis, only inhibin A at the threshold 0.553 MoM achieved the best accuracy for prediction of failing pregnancy, showing a sensitivity of 90.6% and a specificity of 99.5% (Florio *et al.*, 2004) (Figure 2).

Inhibin A concentrations were also evaluated in women with a history of unexplained recurrent miscarriages: levels were lower in those patients that had a subsequent miscarriage compared with those who had a live birth (Muttukrishna *et al.*, 2002b).

Taken together, all these findings would support the utility of serum inhibin A measurement in the prediction of poor outcome of pregnancy, or perhaps even as a marker of placental dysfunction and damage both in the presence and before the onset of the clinical symptoms of recurrent miscarriage.

Hydatidiform mole

Partial hydatidiform mole is a triploid gestational trophoblastic tumour, occurring in about three per 1000 pregnancies (Seckl *et al.*, 2000). Inhibin subunits are localized in trophoblastic tumours (McCluggage *et al.*, 1998; Pelkey *et al.*, 1999; Shih and Kurman, 1999; McCluggage, 2001) and the detection by immunohistochemistry of the inhibin subunits has been recently proposed as a useful immunohistochemical marker of trophoblastic neoplasia to be included in the diagnostic antibody panel in association with β -hCG (Pelkey *et al.*, 1999; McCluggage, 2001). In addition, serum immunoreactive inhibin levels were found to be high in women with hydatidiform mole (Yohkaichiya *et al.*, 1989; Badonnel *et al.*, 1994), and suggested as a reliable tumour marker. However, that assay was never available on a large scale, it was of poor reliability and did not distinguish specifically between the various forms of inhibin-related proteins.

Inhibin A levels are consistently elevated in molar pregnancy without any considerable overlap with normal pregnancy values at the same stage of pregnancy (Florio *et al.*, 2002), suggesting a role for inhibin A measurement in diagnosing molar pregnancies (Figure 3). In addition, inhibin A levels after molar evacuation decline significantly to values similar to those measured in non-pregnant women, whereas hCG levels, despite decreasing, remain far higher than in non-pregnant women, suggesting that inhibin A is more sensitive than hCG in identifying patients with spontaneous remission after molar evacuation.

Down's syndrome

Inhibin α subunit is overexpressed in second trimester placental tissue of pregnancy affected by fetal Down's syndrome

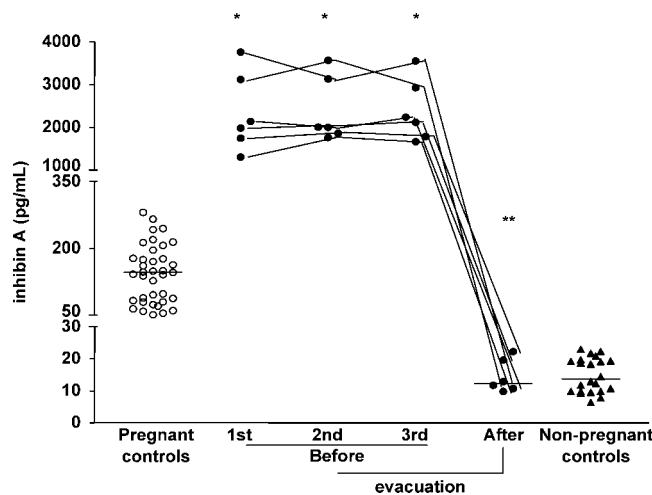


Figure 3. Maternal serum inhibin A levels in healthy pregnant women (○), partial hydatidiform mole (●) before and after evacuation, and in non-pregnant controls (△) during the follicular phase of the menstrual cycle. Medians are indicated by horizontal bars. * $P < 0.001$ versus molar pregnancy before evacuation; ** $P < 0.001$ versus values before evacuation.

(Lambert-Messerlian *et al.*, 1998), suggesting that increased α subunit expression is one of the mechanisms leading to increased levels of inhibin A in serum. Indeed, during the second trimester of gestation, inhibin A levels were significantly elevated in maternal serum of women carrying a Down's syndrome pregnancy (Van Lith *et al.*, 1992; Spencer *et al.*, 1993; Cuckle *et al.*, 1994), even in the presence of unchanged inhibin B (Wallace *et al.*, 1998a,b) and activin A levels (Lambert-Messerlian *et al.*, 1996, 1998), at least between 16 and 19 weeks of gestation. Amniotic fluid inhibin A levels are abnormally reduced in this syndrome, contrarily to what happens with maternal serum levels, indicating that the two compartments receive inhibin A from different sources: whereas the fetal membranes are the main source of inhibin A for the amniotic cavity, the placenta is responsible for most of that in the maternal circulation (Wallace *et al.*, 1997b, 1999).

The finding of high maternal serum inhibin A levels in association with Down's syndrome may be used as a prenatal screening marker. Although not sensitive enough to be used as a single marker (Wenstrom *et al.*, 1999), inhibin A may be usefully introduced into a multiple marker screening test for Down's syndrome. The Serum Urine and Ultrasound Screening Study (SURUSS) conducted on 47 507 women recruited between September 1996 and April 2000 who attended 25 maternity centres (24 in the UK and one in Austria) has provided the largest data set reported on women seen in both the first and second trimesters of pregnancy without intervention in the first trimester (Wald *et al.*, 2003, 2004). The Quadruple test, an early second trimester (14–20 completed weeks) test based on the measurement of α -fetoprotein, unconjugated estriol (uE3), free β -hCG (or total hCG) and inhibin A together with maternal age, is now widely used in the USA and UK, and it shows a 6.2% false-positive rate for an 85% detection rate, and the corresponding odds of being affected given a positive result were 1:32 (Wald *et al.*, 2003, 2004). The integration of nuchal translucency measurement and pregnancy-associated plasma protein A (PAPP-A) in the first trimester with the Quadruple test in the second trimester

seems to be the most effective screening test, with an estimated 85% detection rate for a 0.9% false-positive rate, that increases to 1.3% without measuring inhibin A. The use of serum markers only (PAPP-A in the first trimester and the Quadruple test in the second trimester) has an 85% detection rate for a 3.9% false-positive rate: however, the false-positive rate for the test without inhibin A measurement is 6.0% (Wald *et al.*, 2003, 2004).

Conclusion

Based on the findings of the present review, it can be concluded that:

- Inhibin A and inhibin B are made at different times in human males and females and from several different cell types. They have a demonstrable role in suppressing FSH production and there is some evidence for local paracrine effects in the gonads.
- Ovary and testis are the main inhibin A- and inhibin B-producing organs from puberty.
- Inhibin A is secreted mainly by the corpus luteum, and is involved in the negative feedback control of FSH secretion during luteal–follicular transition.
- Inhibin A concentrations may serve as a prognostic factor for predicting the resumption of ovarian function.
- Inhibin B is secreted by antral follicles in response to FSH, and is the major marker of follicular growth. Thus, inhibin B level is a marker of ovarian function, which may become a useful tool to identify the potential ovarian responsiveness to ovulation induction.
- Inhibin B is a marker of spermatogenesis, and the decline in testicular function in older men is accompanied by a slight reduction in inhibin B concentrations, the mechanism of which has not been established.
- During pregnancy, the placenta becomes the predominant secretory organ for inhibin A, which is also produced by the corpus luteum.
- The measurement of inhibin A has clinical value in women at early pregnancy with impending abortion or hydatiform mole.
- The increase of serum inhibin A in Down's syndrome pregnancies may have a clinical value, because it improves the prenatal detection of the disease.

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