Available online at www.sciencedirect.com
SCIENCE DIRECT®
Theriogenology 60 (2003) 407–419
Theriogenology

Use of G1.2/G2.2 media for commercial bovine embryo culture: equivalent development and pregnancy rates compared to co-culture

Michelle Lane[a,*], David K. Gardner[a], Marilyn J. Hasler[b], John F. Hasler[c]

[a]*Research and Development Department, Colorado Center for Reproductive Medicine, 799 East Hampden Avenue, Suite 520, Englewood, CO 80110, USA*
[b]*Franklin and Marshall College, Lancaster, PA, USA*
[c]*Em Tran Inc., Elizabethtown, PA, USA*

Received 30 May 2002; accepted 10 September 2002

Abstract

The expanded application of commercial bovine IVM, IVF, and IVC systems is dependent on the ability to produce embryos in culture that are capable of producing normal pregnancies. Because serum containing culture systems can induce neonatal and fetal problems there exists a definite need for a serum-free culture system that produces viable blastocysts. This study demonstrated that the physiological sequential media system G1.2/G2.2 could produce bovine blastocysts at rates equivalent to co-culture. Additionally, these blastocysts had equivalent or increased cell numbers and inner cell mass development. Blastocysts produced in the G1.2/G2.2 culture system produced pregnancies following both fresh transfer and cryopreservation at equivalent rates to co-culture. Finally, this study demonstrated that the media system G1.2/G2.2 could be used in a commercial OPU transfer program without any loss in the numbers of blastocysts produced or the numbers of pregnancies resulting following transfer from either fresh or cryopreserved blastocysts.

Keywords: Defined media; Embryo transfer; IVF; Viability

* Corresponding author. Tel.: +1-303-788-4868; fax: +1-303-788-4438.
E-mail address: mlane@colocrm.com (M. Lane).

0093-691X/03/$ – see front matter
doi:10.1016/S0093-691X(03)00030-X

1. Introduction

If bovine IVM, IVF, and IVC systems are to be commercially successful, it is essential to produce a high rate of viable embryos that result in normal pregnancies. Traditionally, the commercial development of bovine embryos derived from IVM, IVF and IVC has employed co-culture of somatic cell lines such as buffalo rat liver cells (BRL) or vero cells in complex tissue culture media supplemented with serum for embryo culture [1–3]. While these culture systems have been shown to support high rates of blastocyst development, the commercial applications of these techniques have been hampered by problems associated with resultant pregnancies. The use of co-culture with somatic cells and serum for embryo development is associated with high rates of fetal loss, pregnancy complications and high rates of neonatal loss associated with abnormally large calves [4,5]. There are an increasing number of studies that indicate that these abnormalities can be attributed to the use of serum [6,7]. It has been reported that co-culture systems can still give rise to good rates of blastocyst development if the serum is restricted to the period after the first 72 h of culture. However, this did not reduce pregnancy complications [8]. Inclusion of serum in the culture medium has direct detrimental effects on the embryo itself resulting in perturbations in metabolism [9], disruptions in the ultrastructure of the mitochondria, and development of large lipid vesicles that displace the normal ultrastructure [5,6,8,10,11]. Therefore, if the application of this technology is to continue, it is essential to use serum-free culture systems that support high rates of development in culture, enable subsequent cryopreservation of the blastocysts and result in the establishment of normal pregnancies in a commercial application.

One such serum-free culture system is the sequential media G1.2 and G2.2 system [12]. These media were formulated specifically to prevent intracellular stress to the embryo thereby maintaining embryo viability [12]. Additionally, these media take into account the changing carbohydrate and amino acid requirements of the embryo. As a result these media are able to support high rates of blastocyst development in culture of embryos from many species [12].

The aim of this study was to assess the ability of G1.2/G2.2 sequential media to support bovine embryo development and pregnancy rates in a commercial embryo transfer program.

2. Materials and methods

2.1. Oocyte recovery and in vitro maturation

Ovaries were obtained from a slaughterhouse and transported to the lab in a plastic bag at 26–30 °C. Upon arrival in the laboratory, ovaries were rinsed with tap water at 28 °C and then washed with 1% Nolvasan solution (Aveco Co. Inc., Fort Dodge, IA, USA) and 1% 7X (ICN Biochemicals Inc., Costa Mesa, CA, USA) in 28 °C tap water. Ovaries were then rinsed very well with tap water and maintained at 23–27 °C until aspiration. Cumulus–oocytes complexes (COC) were aspirated from 2 to 10 mm follicles using a

19-gauge needle attached to a Pioneer Pro-Pump (Pioneer Medical, Madison, CT, USA) [13].

For aspiration directly from donor cows, oocytes were collected by transvaginal ultrasound-guided aspiration from client owned cows, primarily Holsteins, with an Aloka 500 ultrasound monitor (Corometrics Medical Systems, Wallingford, CT, USA) and a 5 MHz sector linear scanning transducer housed together in a vaginal probe with a stainless steel guide containing a 17-gauge single lumen needle. Aspiration pressure was controlled by a foot pedal-operated Pioneer Pro-Pump set at 50 mmHg. The follicular aspirate was rinsed with PBS through a Em-Con filter (Immuno systems, Spring Valley, WI, USA).

Following aspiration, oocytes from slaughterhouse ovaries that had three or more compact layers of cumulus were used for maturation regardless of cytoplasmic appearance. For oocytes collected from donor cows all oocytes with one or more layers of cumulus were used. Oocytes were washed five times in a modified Tyrode's medium (TALP; Bio Whittaker, Walkersville, MD, USA) and placed into 0.5 ml of TCM-199 with Earle's salts (Sigma, St. Louis, MO, USA) supplemented with 2.2 g/l sodium bicarbonate (Gibco BRL, Grand Island, NY, USA), 10% heat-inactivated fetal calf serum (FCS; Hyclone Laboratories, Logan, UT, USA), 4 μg FSH and 6 μg LH (Sioux Biochemicals, Sioux Center, IA, USA). All culture procedures for IVM, IVF, and IVC were performed in four-well plates (Nuclon, Roskilde, Denmark). Oocytes were placed into maturation medium within 5–9 h of ovary collection or within 30 min of aspiration. Oocytes were matured at 39 °C in a humidified atmosphere of 5% CO_2 in air for 21–24 h.

2.2. *In vitro fertilization*

Following IVM, oocytes were rinsed twice in TALP and placed in 0.5 ml of Fert-TALP [14]. Frozen semen was thawed in a 35 °C water bath and layered on a discontinuous gradient of Percoll (Sigma) in a 15 ml centrifuge tube [13]. Following centrifugation the sperm pellet was re-suspended in Sperm-Talp [15]. Depending on the bull used (as requested by client), spermatozoa were added to the fertilization drops at concentrations ranging from 0.1 to 0.5×10^6/ml. At the time the sperm was added to the wells, 20 μmol/l penicillamine, 10 μmol/l hypotaurine, 1 μmol/l epinephrine, and 2 μg/ml heparin were added. Gametes were co-incubated for 18 h at 39 °C in a humidified atmosphere of 5% CO_2 in air. Following in vitro fertilization oocytes were removed, washed twice in TALP and then vortexed for 2 min to remove cumulus cells.

2.3. *In vitro culture*

For co-culture, putative zygotes were cultured in 0.5 ml Menezo's B2 (Laboratoire C.C.D., Paris, France) with 10% FCS (Hyclone) on a monolayer of BRL cells (American Type Culture Collection, Rockville, MD, USA). The BRL cells were plated at a concentration of approximately 200,000, 100,000 or 50,000 cells at 24, 48, or 72 h before use. Embryos were cultured at 39 °C in a humidified atmosphere of 5% CO_2 in air. After

72 h of in vitro culture, embryos were transferred to a fresh co-culture well and development continued until Day 7.

For culture in sequential media, putative zygotes were washed twice and placed in 500 μl of medium G1.2 (Table 1). Embryos were cultured at 39 °C in a humidified atmosphere of 5% CO_2:5% O_2:90% N_2 in modular incubator chambers. After 72 h of culture, embryos were washed vigorously two to three times in medium G2.2 (Table 1) and cultured for a further 72 h in 500 μl of medium G2.2.

Table 1
Composition of media G1.2/G2.2

Component	G1.2 (mM)	G2.2 (mM)
NaCl	90.08	90.08
KCl	5.5	5.5
NaH_2PO_4	0.25	0.25
$MgSO_4$	1.0	1.0
$NaHCO_3$	25.0	25.0
$CaCl_2$	1.8	1.8
Glucose	0.5	3.15
Lactate	10.5	5.87
Pyruvate	0.32	0.1
EDTA	0.01	–
Taurine	0.1	–
Alanyl-glutamine	0.5	1.0
Alanine	0.1	0.1
Arginine	–	0.6
Asparagine	0.1	0.1
Aspartate	0.1	0.1
Cystine	–	0.1
Glutamate	0.1	0.1
Glycine	0.1	0.1
Histidine	–	0.2
Isoleucine	–	0.4
Leucine	–	0.4
Lysine	–	0.4
Methionine	–	0.1
Phenylalanine	–	0.2
Proline	0.1	0.1
Serine	0.1	0.1
Threonine	–	0.4
Tryptophan	–	0.5
Tryosine	–	0.2
Valine	–	0.4
Ca pantothenate	–	0.0042
Choline chloride	–	0.0072
Folic acid	–	0.0023
Inositol	–	0.010
Niacinamide	–	0.0082
Pyridoxal	–	0.0049
Riboflavin	–	0.0003
Thiamine	–	0.003

2.4. Assessment of embryo development

In vitro developed embryos were assessed after 144 h of culture (168 h after insemination) and classified as early blastocysts, mid-blastocysts, expanded blastocysts, and hatching blastocysts as described by Hasler et al. [4].

2.5. Differential labeling of ICM and trophectoderm cells

Numbers of ICM and TE cells in blastocysts were determined using the differential staining procedure as described by Van Soom et al. [16].

2.6. Freezing procedure

Blastocysts were cryopreserved using a slow-freezing protocol with ethylene glycol as the cryoprotectant. Blastocysts were equilibrated for 10–15 min at ambient temperature in 1.5 M ethylene glycol and 0.1 M sucrose in 0.25 ml straws. Straws were loaded into a programmable freezer at −6 °C and seeded after approximately 1 min. After being held at the seeding temperature for 15 min, straws were cooled at −0.5 °C per min to −32 °C and then plunged into liquid nitrogen.

Blastocysts were thawed for 10 s in air and then placed in a 30 °C water bath until the ice melted. Following thawing, straws were then immediately loaded into a transfer gun.

2.7. Embryo biopsy

Blastocysts were removed from culture and washed through four rinses of TALP medium without protein supplementation. The embryos were viewed at 100× magnification using an inverted microscope and a trophectoderm biopsy consisting of approximately 10 cells was removed with an ophthalmic blade (A. B. Technology, Inc., Pullman, WA, USA) attached to a micromanipulator. The sex of the blastocyst was then determined by polymerase chain reaction [17,18].

2.8. Transfer procedure

Fresh blastocysts for transfer were rinsed in PBS and loaded into 0.25 ml straws. Following thawing, frozen embryos were transferred directly from the straw in which they were frozen. Single embryos were transferred nonsurgically to the uterine horn ipsilateral to the corpus luteum of Holstein heifers that had been observed in estrus between 2 days before and the same day as the IVF of the embryos [4]. Pregnancy was determined by palpation per rectum between 55 and 60 days of gestation.

2.9. Statistical analysis

Differences in embryo development in culture were assessed using linear-logistic regression where the error distribution was assumed to be binomial. The null hypothesis of no treatment effect against a treatment effect was tested using the log-likelihood ratio

statistic. The day of experiment was fitted as a factor. Cell numbers were assessed by Student's *t*-test. A value of $P < 0.05$ was considered to be significantly different.

3. Experimental design

3.1. Experiment 1

3.1.1. Comparison of bovine embryo development in co-culture and sequential G1.2/G2.2 culture systems from slaughterhouse oocytes

Oocytes were matured and fertilized under standard conditions and randomly allocated to culture in either BRL co-culture system or media G1.2/G2.2 at 5% CO_2:5% O_2:90% N_2. Development to the blastocyst stage, blastocyst cell number, allocation to the ICM and survival following cryopreservation were assessed after 144 h of culture.

3.2. Experiment 2

3.2.1. Effect of oxygen tension on embryo development in sequential media G1.2/G2.2

Oocytes were matured and fertilized in standard conditions and then randomly allocated to culture in G1.2/G2.2 at either 5% CO_2 in air or 5% CO_2:5% O_2:90% N_2. Development to the blastocyst stage was assessed after 144 h of culture.

3.3. Experiment 3

3.3.1. Effect of G1.2/G2.2 media age on embryo development

Oocytes were matured and fertilized under standard conditions and cultured in media G1.2/G2.2 that was freshly prepared (<2 weeks old) and media that was stored at 4 °C for 15 weeks from preparation. This experiment was performed for two independent batches of media.

3.4. Experiment 4

3.4.1. Assessment of viability of blastocysts grown in sequential media G1.2/G2.2

Oocytes were collected from slaugtherhouse ovaries and matured and fertilized under standard conditions before culture in media G1.2/G2.2 at 5% CO_2:5% O_2:90% N_2. Blastocysts on Day 7 were either transferred to recipients or cryopreserved for subsequent transfer.

3.5. Experiment 5

3.5.1. Use of sequential media G1.2/G2.2 in a commercial IVF program

Oocytes were aspirated from cows and matured and fertilized under standard conditions. Embryos were then cultured in media G1.2/G2.2 to the blastocyst stage in 5% CO_2:5% O_2:90% N_2. On Day 7, blastocysts were biopsied for sex determination and either transferred or cryopreserved for subsequent transfer.

4. Results

4.1. Experiment 1

Development to the blastocyst and expanded blastocyst stages was equivalent for zygotes cultured in either the BRL co-culture system or G1.2/G2.2 sequential media system in 5% CO_2:5% O_2:90% N_2 (Table 2). There was also no difference in the cell numbers of the early blastocysts or mid blastocysts between the two culture systems (Table 3). However, expanded blastocysts grown in sequential media G1.2/G2.2 had significantly higher total cell numbers and ICM cell numbers compared to expanded blastocysts grown in BRL co-culture (Table 3, Fig. 1).

Additionally, there was no difference in the ability of blastocysts to re-expand and hatch following cryopreservation when they were grown in the BRL co-culture system compared to sequential media G1.2/G2.2 (Table 4).

4.2. Experiment 2

Blastocyst development on Day 7 was significantly increased by culturing embryos at a reduced oxygen concentration of 5% compared to the atmospheric oxygen concentration of

Table 2
Comparison of development of embryos cultured in BRL co-culture or G1.2/G2.2 after 144 h of culture[1]

Culture system	Oocytes (*n*)	Oocytes fertilized (*n*)	Blastocyst development/oocyte % (*n*)	Blastocyst development/cleaved % (*n*)
BRL co-culture	1388	937	26.7 (370)	39.4 (370)
G1.2/G2.2	1557	1059	21.4 (323)	31.4 (323)

[1] There were no significant differences between the two culture treatments in blastocyst development following 144 h of culture.

Table 3
Cell numbers of blastocysts grown in different culture treatments for 144 h

Culture system	Early blastocyst	Blastocyst	Expanded blastocyst
BRL co-culture	115 ± 8^a (20)	124 ± 10^a (21)	169 ± 9^a (22)
G1.2/G2.2	112 ± 8^a (27)	156 ± 15^a (9)	207 ± 13^b (10)

Different letters (a, b) within a column are significantly different ($P < 0.05$). Number of embryos are represented in parenthesis. Values are mean ± S.E.M.

Table 4
Effect of culture in sequential media G1.2/G2.2 on survival following cryopreservation[1]

Culture system	*N*	Expanded blastocysts at 24 h *n* (%)	Hatching blastocysts at 96 h *n* (%)
BRL co-culture	180	146 (81.1%)	96 (53.3%)
G1.2/G2.2	189	133 (70.3%)	91 (48.1%)

[1] There was no difference in the ability of blastocysts to survive cryopreservation between the two culture systems.

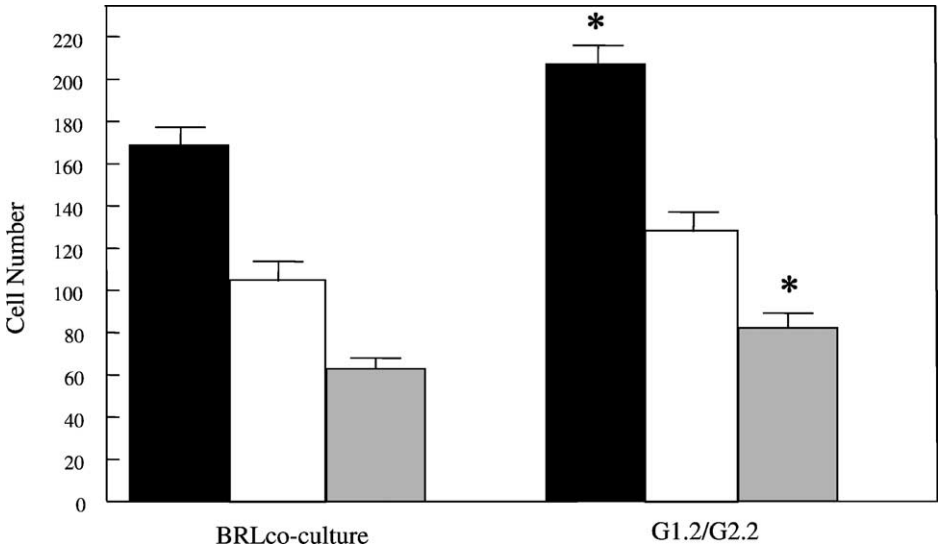

Fig. 1. Distribution of ICM and TE cells in expanded blastocysts grown for 144 h. Solid bars indicate blastocyst cell number. Open bars indicate trophectoderm cell number. Shaded bars indicate ICM cell number. (∗) Significantly different from BRL co-culture ($P < 0.05$).

20% (Table 5). All subsequent experiments were therefore performed using the gas phase of 5% CO_2:5% O_2:90% N_2.

4.3. Experiment 3

Development to the blastocyst stage on Day 7 was not affected by the age range (up to 15 weeks) of the two lots of media used in this study (Fig. 2).

4.4. Experiment 4

All blastocysts were grown in sequential media G1.2/G2.2 system and on Day 7 of development (144 h of culture) blastocysts were transferred. For grade 1 blastocysts a pregnancy rate of 56.4% was obtained following the transfer of a single blastocyst to a recipient heifer. This rate dropped to 41% for grade 2 blastocysts resulting in an overall

Table 5
Effect of oxygen concentration on blastocyst development in media G1.2/G2.2

Oxygen concentration (%)	Oocytes (*n*)	Cleavage rate % (*n*)	Blastocyst development/oocyte % (*n*)	Blastocyst development/cleaved % (*n*)
20	676	59.6 (403)	7.6 (49)	12.2 (49)
5	641	68.7 (440)	21.0** (142)	32.3** (142)

Blastocyst development was assessed after 144 h of culture. Number of embryos are represented in parenthesis.
** Significantly different from 20% oxygen concentration ($P < 0.01$).

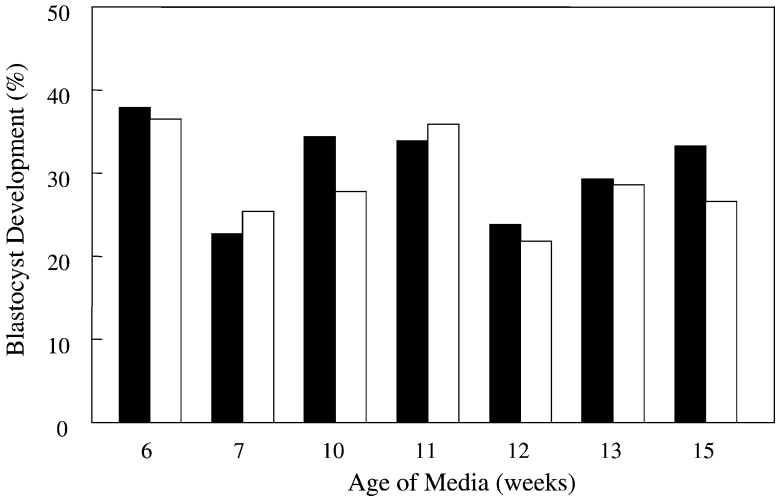

Fig. 2. Effect of medium age on blastocyst development (144 h of culture). Solid bars represent fresh media (<2 weeks from preparation). Open bars represent stored media. There was no significant difference in the rate of blastocyst development between fresh (<2 weeks from preparation) and stored media.

pregnancy rate of 51.3% (Table 6). This pregnancy rate was not reduced when blastocysts were cryopreserved before transfer (Table 7).

4.5. Experiment 5

Equivalent rates of blastocyst development were achieved from OPU oocytes whether they were grown in a sequential media G1.2/G2.2 system or a BRL co-culture system.

Table 6
Pregnancy rate obtained following transfer of fresh blastocysts produced in media G1.2/G2.2

Grade of blastocyst	Blastocysts transferred (*n*)	Pregnancies (*n*)	Pregnancy rate (%)
1	156	88	56.4
2	78	32	41.0
Overall	234	120	51.3

Blastocysts were cultured in media G1.2/G2.2 for 144 h before transfer.

Table 7
Pregnancy rate following transfer of cryopreserved blastocysts grown in media G1.2/G2.2

Culture system	Blastocysts transferred (*n*)	Pregnancies (*n*)	Pregnancy rate (%)
BRL co-culture	67	28	42.0
G1.2/G2.2	40	19	47.5

Blastocysts were cultured for 144 h before cryopreservation. There was no difference in pregnancy rate between the two culture systems.

Table 8
Sex distribution of blastocysts developed in either BRL co-culture or media G1.2/G2.2 derived from OPU oocytes

Culture system	Oocytes (*n*)	Blastocyst development/oocyte % (*n*)	Female:male ratio	Percentage of male embryos (%)
BRL co-culture	3250	19.6 (638)	443:523	54.1[+]
G1.2/G2.2	4417	13.7 (603)	101:90	47.1

Blastocysts were assessed after 144 h of culture. Number of embryos are represented in parenthesis.
[+] Different from a 50:50 ratio ($P < 0.08$).

Table 9
Pregnancy rates of blastocysts derived from OPU oocytes[1]

Culture system	Biopsied blastocysts transferred (*n*)	Pregnancies following transfer of biopsied blastocysts (*n*)	Pregnancy rate following transfer of biopsied blastocysts (%)
BRL co-culture	95	43	45.0
G1.2/G2.2	92	38	41.3

[1] There was no difference in pregnancy rate on Day 60 between the two culture systems.

Blastocysts that were developed in the G1.2/G2.2 system and biopsied for sexing resulted in a sex ratio not different from 50:50. However, blastocysts developed in the BRL co-culture system had a sex ratio favoring males ($P = 0.08$; Table 8). Pregnancy rates of these biopsied blastocysts were equivalent for the two culture systems (Table 9).

5. Discussion

The data presented in this manuscript demonstrate that it is possible to obtain equivalent rates of blastocyst development in a commercial IVM, IVF, and IVC production system using a physiologically based sequential serum-free culture system (G1.2/G2.2) to those obtained using a BRL co-culture system. Most importantly, however, the blastocysts developed in a serum-free system had equivalent rates of cryosurvival and gave rise to equivalent rates of pregnancies after transfer. These data demonstrate that it is possible to use a serum-free culture system to develop bovine embryos without sacrificing embryo quality and most importantly viability.

Problems with pregnancies following IVM, IVF, and IVC have primarily been attributed to the presence of serum in the culture system. These problems have included heavier birth weights (commonly referred to as the large lamb or calf syndrome), extended gestation periods, higher rates of abortion and increased rates of perinatal mortality [5,8,19]. Studies investigating culture of embryos with or without serum have determined that many of these problems with pregnancy and parturition are eliminated when serum is replaced in the medium with purified preparations of BSA [7]. Restricting the use of serum to the second 48 h of culture did not reduce abnormalities associated with pregnancies in a very large field trial involving several thousand transfers [8]. Furthermore, studies of the fetuses have

determined that the growth coefficients for fetal liver and heart were increased in fetuses resulting from blastocysts that were grown in the presence of co-culture or serum [20], while fetuses arising from blastocysts grown in the absence of serum were the same as in vivo developed controls.

Therefore, the need for a culture system that does not employ serum is essential for the extended application of IVM, IVF, and IVC procedures in a commercial setting. The problems associated with pregnancies following in vitro production procedures have resulted in a decreased demand for IVM, IVF, and IVC and it has been primarily restricted to use with infertile donors [8]. Therefore, it is essential that a robust system be developed that can routinely produce viable embryos in the absence of serum.

It has been reported that in order to produce high rates of viable blastocysts serum is an essential medium component for development of the embryo to the post-compaction stage [21]. However, the data presented in this study demonstrate that if the culture media used are physiologically based, then serum is not required for the development of viable blastocysts. Therefore, the use of co-culture and serum supplementation is not necessary. Data presented in this study show that in a comparison of over 5000 oocytes, embryo development to the blastocyst stage in sequential media system G1.2/G2.2 is equivalent to that observed in co-culture. Additionally, the cell number of the blastocysts is equivalent and, for expanded blastocysts, the cell numbers are significantly higher in G1.2/G2.2 blastocysts compared to co-culture. An analysis of the ability of the blastocysts to re-expand and hatch following cryopreservation also revealed that the blastocysts grown in G1.2/G2.2 had equivalent rates of cryosurvival. However, most importantly, the pregnancy rates of blastocysts grown in G1.2/G2.2 and transferred either fresh or following cryopreservation are also equivalent to co-culture. This observation is in agreement with a smaller transfer study of OPU oocytes, in which pregnancy rates following transfer of blastocysts grown in G1.2/G2.2 were equivalent to co-culture and significantly higher than those for a one-step culture system (BARC-1) [22].

It has previously been reported that the number of ICM cells in blastocysts that are developed in co-culture systems are less than those observed for blastocysts that are developed in vivo [16,23]. However, in this study the number of ICM cells in the expanded blastocysts developed in G1.2/G2.2 sequential media was significantly higher than that observed for co-culture. This indicates that the blastocysts derived from this culture system better supports differentiation of the ICM compared to co-culture systems.

The high rates of blastocyst development for the physiologically based culture system G1.2/G2.2 were dependent on the use of a physiological oxygen environment of 5%. This observation is in agreement with studies in numerous species that have demonstrated that blastocyst development is improved by reducing the oxygen concentration [24–26]. This requirement for low oxygen concentration is not necessary for co-culture systems. However, one of the reported benefits of co-culture is the reduction of oxygen tension in the cultures [27].

In addition to use of the G1.2/G2.2 system for slaughterhouse oocytes, the media system was used in the commercial IVM, IVF, and IVC programs at Em Tran. Comparison between G1.2/G2.2 and co-culture revealed that there were no differences in the ability to produce blastocysts of transferable quality between the two systems. Additionally, the blastocysts produced in the G1.2/G2.2 system were successfully biopsied for sex

determination and then transferred giving rise to pregnancies at the same rates as those grown in BRL co-culture. Therefore, blastocysts developed in G1.2/G2.2 appear to be as robust as co-culture blastocysts.

While the number of sampled blastocysts in the G1.2/G2.2 group is small (191) compared to the co-culture group (966) there appears to be a shift in the sex ratio of the blastocysts from favoring males in the co-culture group to favoring females in the G1.2/G2.2 group. An increase in the number of males produced in co-culture systems has previously been reported [28–30]. It has been suggested that this sex ratio difference is attributable to the fact that male embryos develop faster and that female embryos are more susceptible to the culture environment [29,31]. Therefore, it appears that physiological sequential media, which are formulated to reflect the carbohydrate levels of the reproductive tract and reduce cellular stress on the embryo [32] result in equivalent numbers of female and male embryos developing on Day 7. The significance of this finding is currently under further investigation.

In conclusion, a physiologically based serum-free sequential culture system can be used in a commercial embryo production program without any reductions in the number and quality of blastocysts produced or in the number of pregnancies that are established after either fresh blastocyst transfer or transfer following cryopreservation.

Acknowledgements

We thank the staff of Em Tran Inc. for helping to make this study possible and Vitrolife AB for funding.

References

[1] Gordon I. Laboratory production of cattle embryos. Wallingford, UK: CAB International; 1994.
[2] Hasler JF. The current status of oocyte recovery, in vitro embryo production, and embryo transfer in domestic animals, with an emphasis on the bovine. J Anim Sci 1998;76:52–74.
[3] Gardner DK. Embryo development and culture techniques. In: Clark JR, editor. Animal breeding: technology for the 21st century. London: Harwood Academic Publishers; 1998. p. 13–46.
[4] Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, et al. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology 1995;43:141–52.
[5] Farin PW, Crosier AE, Farin CE. Influence of in vitro systems on embryo survival and fetal development in cattle. Theriogenology 2001;55:151–70.
[6] Walker SK, Heard TM, Seamark RF. In vitro culture of sheep embryos without co-culture: successes and perspectives. Theriogenology 1992;37:111–26.
[7] Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. Biol Reprod 1995;53:1385–91.
[8] Hasler JF. In vitro production of cattle embryos: problems with pregnancies and parturition. Hum Reprod 2000;15:47–58.
[9] Gardner DK, Lane M, Spitzer A, Batt PA. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. Biol Reprod 1994;50:390–400.
[10] Dorland M, Gardner DK, Trounson A. Serum in synthetic oviduct fluid causes mitochondrial degeneration in ovine embryos. J Reprod Fertil 1994;13:70 [abstract].

[11] Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE. Ultrastructural morphometry of bovine compact morulae produced in vivo or in vitro. Biol Reprod 2000;62:1459–65.
[12] Gardner DK. Mammalian embryo culture in the absence of serum or somatic cell support. Cell Biol Int 1994;18:1163–79.
[13] Hasler JF. In vitro culture of bovine embryos in Menezo's B2 medium with or without coculture and serum: the normalcy of pregnancies and calves resulting from transferred embryos. Anim Reprod Sci 2000;60/61:81–91.
[14] Bavister BD, Yanagimachi R. The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatozoa in vitro. Biol Reprod 1977;16:228–37.
[15] Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988;38:1171–80.
[16] Van Soom A, Boerjan M, Ysebaert MT, de Kruif A. Cell allocation to the inner cell mass and the trophectoderm in bovine embryos cultured in two different media. Mol Reprod Dev 1996;45:171–82.
[17] Peippo J, Bredbacka P. Sex-related growth rate differences in mouse preimplantation embryos in vivo and in vitro. Mol Reprod Dev 1995;40:56–61.
[18] Bredbacka P. Recent developments in embryo sexing and its field application. Reprod Nutr Dev 1998; 38:605–13.
[19] Holm P, Walker SK, Seamark RF. Embryo viability, duration of gestation and birth weight in sheep after transfer of in vitro matured and in vitro fertilized zygotes cultured in vitro or in vivo. J Reprod Fertil 1996;107:175–81.
[20] Sinclair KD, McEvoy TG, Maxfield EK, Maltin CA, Young LE, Wilmut I, et al. Aberrant fetal growth and development after in vitro culture of sheep zygotes. J Reprod Fertil 1999;116:177–86.
[21] Pinyopummintr T, Bavister BD. In vitro-matured/in vitro-fertilized bovine oocytes can develop into morulae/blastocysts in chemically defined, protein-free culture media. Biol Reprod 1991;45:736–42.
[22] Long CR, Pryor JH, Wells K, Lane M, Gardner DK, Looney CR. In vitro development and subsequent pregnancy rates of in vitro-produced embryos in various culture media. Theriogenology 2000;53:99 [abstract].
[23] Iwasaki S, Yoshiba N, Ushijima H, Watanabe S, Nakahara T. Morphology and proportion of inner cell mass of bovine blastocysts fertilized in vitro and in vivo. J Reprod Fertil 1990;90:279–84.
[24] Quinn P, Harlow GM. The effect of oxygen on the development of preimplantation mouse embryos in vitro. J Exp Zool 1978;206:73–80.
[25] Batt PA, Gardner DK, Cameron AW. Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. Reprod Fertil Dev 1991;3:601–7.
[26] Thompson JG, Simpson AC, Pugh PA, Donnelly PE, Tervit HR. Effect of oxygen concentration on in vitro development of preimplantation sheep and cattle embryos. J Reprod Fertil 1990;89:573–8.
[27] Edwards LJ, Batt PA, Gandolfi F, Gardner DK. Modifications made to culture medium by bovine oviduct epithelial cells: changes to carbohydrates stimulate bovine embryo development. Mol Reprod Dev 1997;46:146–54.
[28] Avery B, Back A, Schmidt T. Differential cleavage rates and sex determination in bovine embryos. Theriogenology 1989;32:139–47.
[29] King WA, Picard L, Bousquet D, Goff AK. Sex-dependent loss of bisected bovine morulae after culture and freezing. J Reprod Fertil 1992;96:453–9.
[30] Xu KP, Yadav BR, King WA, Betteridge KJ. Sex-related differences in developmental rates of bovine embryos produced and cultured in vitro. Mol Reprod Dev 1992;31:249–52.
[31] Gutierrez-Adan A, Granados J, Pintado B, De La Fuente J. Influence of glucose on the sex ratio of bovine IVM/IVF embryos cultured in vitro. Reprod Fertil Dev 2001;13:361–5.
[32] Gardner DK, Lane M. Development of viable mammalian embryos in vitro: evolution of sequential media. In: Cibelli J, Lanza R, Campbell K, West MD, editors. Principles of cloning. San Diego: Academic Press; 2002. p. 187–213.