Viability of one-cell bovine embryos cultured *in vitro*: comparison of cell-free culture with co-culture

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In this study, the quality (number of cells) and pregnancy rates of bovine blastocysts produced by *in vitro* maturation/*in vitro* fertilization (IVM/IVF) following cultivation in either cell-free culture or co-culture were compared. Bovine one-cell IVM/IVF embryos obtained 6 h after insemination were stripped of cumulus cells and assigned to either cell-free culture or co-culture with granulosa cell monolayers for 9 days (Expt 1) or 10 days (Expts 2 and 3). In Expt 3, day-7 (day 0 = day of insemination) blastocysts, day-8 expanded blastocysts and day-9 hatched blastocysts were air-dried, fixed and stained to determine the number of cells. Expanded blastocysts obtained in Expt 1 were cryo-preserved using propylene glycol as a cryoprotectant and were used later for embryo transfer. There were no significant differences between cell-free culture and co-culture in the percentage of one-cell embryos that developed to 2- to 16-cells (66.7% versus 72.4% for Expt 1, 71.0% versus 78.2% for Expt 2). However, significantly more ($P < 0.05$) of the one-cell embryos co-cultured with granulosa cell monolayers developed to morula, blastocyst and expanded blastocyst stages compared with those in cell-free culture (35.0 versus 27.1%, 25.1 versus 14.2%, 15.6 versus 5.4% for Expt 1; 37.6 versus 24.0%, 25.7 versus 11.0%, 16.8 versus 3.0% for Expt 2). Only embryos co-cultured with granulosa cell monolayers hatched (Expt 2). Embryos co-cultured with granulosa cell monolayers also had higher ($P < 0.01$) numbers of cells (92 ± 42 versus 56 ± 21 for blastocysts, 149 ± 53 versus 81 ± 29 for expanded blastocysts). Pregnancy rates tended to be higher for embryos produced by co-culture compared with those from cell-free culture (40.0 versus 27.3%, respectively). The results suggest that embryos produced in cell-free culture were of poorer quality than those produced in co-culture; however, some of them were developmentally competent as confirmed by the births of three calves after transfer to eleven recipient cows.

Introduction

The biochemical requirements for normal, *in vitro* development of early preimplantation embryos of domestic animals have not been defined (Rexroad, 1989). Somatic cell co-culture is one method for overcoming the *in vitro* developmental block of cultured mammalian embryos. However, the ideal culture system should exclude co-culture with somatic cells or supplementation with serum because neither of these systems are chemically defined. The somatic cells used for co-culture may produce unknown, embryo growth promoting factor(s) and/or delete embryo-toxic factor(s) from the culture medium (Eyestone and First, 1989), making it difficult to determine the exact requirements for embryo development, and preventing a clear understanding of embryo metabolism, independent of other cell types. A successful embryo culture system using a medium free of somatic cells is needed for the *in vitro* study of

embryo development. Such a system for *in vitro* matured/*in vitro* fertilized (IVM/IVF) bovine embryos has been developed by several workers (Fukui *et al.*, 1991; Rosenkrans and First, 1991; Pinyopummintr and Bavister, 1991; Takahashi and First, 1992); however, the viability of blastocysts produced by their system has not been examined following embryo transfer. Larson *et al.* (1992) and Goto *et al.* (1992) succeeded in obtaining calves following the transfer of IVM/IVF bovine embryos developed in a cell-free culture system. However, the latter study showed that the viability, as assessed by embryo transfer, of IVM/IVF bovine embryos produced in the cell-free system was lower than that of embryos produced in the co-culture system.

The objective of this study was to compare the quality and pregnancy rate of blastocysts produced by either cell-free culture or co-culture systems directly. Blastocyst quality was assessed *in vitro* by the number of cells per blastocyst and development to hatched blastocyst and *in vivo* viability was determined after transfer to recipient cows.

Materials and Methods

In vitro maturation and fertilization of ovarian oocytes

The method for the production of IVM/IVF zygotes was a modification of the procedure of Goto *et al.* (1988). Briefly, cumulus-oocyte complexes were aspirated from small (1–7 mm in diameter) antral follicles on bovine ovaries obtained from an abattoir. They were washed three times with Whittingham's modified Dulbecco's phosphate-buffered saline (mPBS; Whittingham, 1971) supplemented with 1% newborn calf serum (CS; Gibco, New York), followed by one wash with maturation medium. The maturation medium was 12.5 mmol Hepes TCM 199 l^{-1} (Gibco) supplemented with 5% calf serum. Only oocytes with an intact, non-expanded cumulus mass and evenly granulated cytoplasm (60–90 cumulus-oocyte complexes per 2.5 ml medium) were then cultured for the 22 h maturation.

In vitro fertilization was performed using frozen semen from a single ejaculate from one bull. The frozen-thawed semen (0.5 ml) was diluted with BO medium (Brackett and Oliphant, 1975) without BSA but with caffeine (5 mmol l^{-1}) and heparin (60 μg ml^{-1}). The spermatozoa were washed with this medium (8 ml) twice, by centrifugation at 700 g for 7 min. The spermatozoa were then resuspended to a concentration of 5×10^6 cells ml^{-1} and were preincubated for 2 h in BO medium supplemented with 2.5 mmol caffeine l^{-1} , 30 μg heparin ml^{-1} and 5 mg BSA ml^{-1} . *In vitro* fertilization was conducted by co-incubating spermatozoa (5×10^6 cells ml^{-1}) and cumulus-oocyte complexes (15–20 per 110 μl drop) for 6 h.

Preparation of granulosa cell monolayers

Granulosa cell monolayers were prepared from granulosa cells aspirated from bovine follicles using established methods (Goto *et al.*, 1990). Briefly, follicular fluid containing granulosa cells was centrifuged (180 g for 5 min). The pellet of granulosa cells was washed by centrifugation at 180 g for 5 min in 5 ml of mPBS supplemented with 1% calf serum. The pellet was suspended in 1 ml of an enzyme solution containing 1000 U protease ml^{-1} (Dispase; Sanko Junyaku, Tokyo). Cells were incubated for 20–40 min at 38.7°C. The cells were then vigorously pipetted to permit the separation of cells, followed by three washings (180 g for 5 min) in mPBS. After the final washing, 5 ml of maturation medium (12.5 mmol Hepes TCM 199 + 5% calf serum) was added to the cells, and the cell suspension was passed through a wire mesh to remove large cell clumps. Aliquots of cell suspension (2.5 ml, 10^5 cells ml^{-1} of culture medium) were placed in each culture dish (35 M/M; Termo, Tokyo) so that 80–100% confluence would be attained within 48 h. The percentage of live cells was over 80% at the beginning of culture.

In vitro culture of one-cell embryos

One-cell embryos were stripped of cumulus cells after co-incubation with spermatozoa by vortexing in TCM 199 supplemented with 0.1% hyaluronidase. The embryos were

then washed three times with development medium (12.5 mmol Hepes TCM 199 + 1% calf serum + 1 mmol sodium pyruvate l^{-1}). The cumulus-free one-cell embryos (30–45 embryos per 2.5 ml culture medium) were randomly assigned to either cell-free culture or co-culture with granulosa cell monolayers for 9 days (Expt 1) or 10 days (Expts 2 and 3). Developmental stages of embryos were examined every 24 h. The development medium was replaced with new medium every 48 h. All of the culture media used were supplemented with antibiotics (100 IU penicillin ml^{-1} and 100 μg streptomycin ml^{-1}). All cultures were maintained at 38.7°C in a humidified atmosphere of 5% CO_2 in air.

Evaluation of number of cells per embryo

In Expt 3, day 7 (day 0 = day of insemination) blastocysts, day 8 expanded blastocysts and day-9 hatched blastocysts were air-dried and the number of cells counted (Tokumaru *et al.*, 1989). Briefly, the embryos were treated with a hypotonic solution (0.5% sodium citrate) and then fixed with acetic acid:methanol (1:3). After staining with 5% Giemsa solution, the total number of cells were counted under a microscope.

Cryopreservation and embryo transfer

Expanded blastocysts from Expt 1 obtained 8 days after insemination from either cell-free culture or co-culture were equilibrated in a three-step procedure with 0.5 mol propylene glycol l^{-1} for 5 min, 1.0 mol propylene glycol l^{-1} for 5 min and 1.6 mol propylene glycol l^{-1} for 10–15 min in a base medium of mPBS supplemented with 20% calf serum. Embryos in the 1.6 mol propylene glycol l^{-1} were loaded into straws (one embryo per straw) and were separated by two air bubbles from the medium (0.2 mol sucrose l^{-1} with 20% calf serum in mPBS) filling the remainder of the straw. Straws were placed directly into a cooling chamber (ET-1, Fujihira, Tokyo) kept at 0°C and cooled to $-6^\circ C$ at $-1^\circ C$ min^{-1} . After holding for 10 min at this temperature, including time for seeding, the straws were cooled to $-30^\circ C$ at $-0.3^\circ C$ min^{-1} . The straws were held for 10 min at this temperature before being plunged into liquid nitrogen. Straws were thawed at 30°C in water and the embryos were directly transferred into recipient cows (7–8 days after standing oestrus; one embryo per recipient).

Pregnancy diagnosis

Pregnancy diagnosis was made by ultrasound 40–42 days after embryo transfer.

Statistical analysis

Chi-square test was used to examine differences in developmental rates between the two culture groups. The total number of cells in blastocysts was subjected to logarithmic transformation before being subjected to analysis of variance. Mean differences were determined by Student's *t* test. Pregnancy

Table 1. *In vitro* development of bovine one-cell embryos cultured for 9 days in cell-free culture or co-culture systems

Culture system	Number of one-cell embryos*	Number (%) of embryos developed to:			
		2- to 16-cell (day 2–3)	Morula (day 4–6)	Blastocyst (day 6–8)	Expanded blastocyst (day 7–9)
Cell-free	240	160 (66.7) ^a	64 (27.1) ^a	34 (14.2) ^a	13 (5.4) ^a
Co-culture	243	176 (72.4) ^a	85 (35.0) ^b	61 (25.1) ^b	38 (15.6) ^b

*Total from six replicates.

^{ab}Means within a column with different superscripts are significantly different ($P < 0.05$).**Table 2.** *In vitro* development of bovine one-cell embryos cultured for 10 days in cell-free culture or co-culture systems

Culture system	Number of one-cell embryos*	Number (%) of embryos developed to:				
		2- to 16-cell (day 2–3)	Morula (day 4–6)	Blastocyst (day 6–8)	Expanded blastocyst (day 7–9)	Hatched blastocyst (day 8–10)
Cell-free	100	71 (71.0) ^a	24 (24.0) ^a	11 (11.0) ^a	3 (3.0) ^a	0 (0) ^a
Co-culture	101	79 (78.2) ^a	38 (37.6) ^b	26 (25.7) ^b	17 (16.8) ^b	11 (10.9) ^b

*Total from three replicates.

^{ab}Means within a column with different superscripts are significantly different ($P < 0.05$).**Table 3.** Number of cells of IVM/IVF* blastocysts produced in cell-free culture or co-culture systems

Culture system	Stage of embryo		
	Blastocyst (day 7)	Expanded blastocyst (day 8)	Hatched blastocyst (day 9)
Cell-free	56 ± 21 ^a (30)	81 ± 29 ^a (27)	None (None)
Co-culture	92 ± 42 ^b (45)	149 ± 53 ^b (75)	189 ± 60 (35)

Values are means ± SD. Number of embryos examined in parentheses.

^{ab}Means within a column with different superscripts are significantly different ($P < 0.01$).*IVM: *in vitro* maturation; IVF: *in vitro* fertilization.

rates were compared using a χ^2 test incorporating Yates' continuity correction.

Results

There was no significant difference in the percentage of one-cell embryos that developed to the 2- to 16-cell stage in cell-free culture and co-culture (66.7 and 72.4%, respectively; Table 1). However, one-cell embryos co-cultured with granulosa cell monolayers showed significantly higher development ($P < 0.05$) to morula, blastocyst and expanded blastocyst stages than did those of cell-free culture (35.0 versus 27.1%, 25.1 versus 14.2%, 15.6 versus 5.4%, respectively). Similar results were obtained in Expt 2 (Table 2). Only embryos co-cultured with granulosa cell monolayers hatched from their zonae pellucidae (Table 2).

The mean number of cells in blastocysts and expanded blastocysts derived from cell-free culture (56 and 81, respectively) was lower (Table 3, $P < 0.01$) than those obtained from co-culture (92 and 149, respectively). The mean number of cells in hatched blastocysts (co-culture only) was 189.

Pregnancy rate after the transfer of frozen-thawed IVM/IVF/IVC embryos was 27.3 and 40.0% for cell-free culture and co-culture, respectively (Table 4). However, the difference was not significant ($P > 0.05$) owing to the limited number of embryo transfers.

Three calves were born to eleven recipient cows that received embryos produced in cell-free culture, whereas five calves were born to fifteen recipient cows that received embryos produced in co-culture. One pregnancy in the co-culture group was lost at four months gestation.

Table 4. Effect of culture system on pregnancy rate of frozen-thawed IVM/IVF* bovine embryos

Culture system	Number of embryos transferred	Number of pregnancies	Pregnancy rate (%)**	Number of calves born
Cell-free	11	3	27.3	3
Co-culture	15	6	40.0	5 ^a

*IVM: *in vitro* maturation; IVF: *in vitro* fertilization.

**No significant difference ($P > 0.05$) between culture systems.

^aOne pregnancy was lost at 4 months gestation.

Discussion

This study demonstrates that a cell-free culture system containing calf serum can be used to culture IVM/IVF bovine embryos to the blastocyst stage. However, developmental rate and quality of blastocysts produced in cell-free culture were poorer than those obtained following co-culture. The contribution(s) made by somatic cells to the *in vitro* development of bovine embryos may fall into one of four categories: (1) production of factors that stimulate embryo development (Gandolfi and Moor, 1987; Heyman *et al.*, 1987; Eyestone and First, 1989; Moor *et al.*, 1990); (2) removal of inhibitory compounds present in the culture environment (Carney *et al.*, 1990; Bavister *et al.*, 1992); (3) reduction of oxygen concentration (Tervit *et al.*, 1972; Wright *et al.*, 1976; Thompson *et al.*, 1990; Fukui *et al.*, 1991; Voelkel and Hu, 1992); or (4) physical contact between the embryo and the somatic cells (Kuzan and Wright, 1982; Allen and Wright, 1984). In the present study it was not determined which of these mechanisms was responsible for the increased blastocyst quality.

Poor quality embryos produced in cell-free culture have also been reported by Takahashi and First (1992), who used a semi-defined medium containing BSA, and by Larson *et al.* (1992) who used a chemically defined medium containing growth factors. However, a direct comparison of embryo quality and pregnancy rate between cell-free culture and co-culture was not made in these studies. The results of the present study indicate that embryos produced in cell-free culture were of poorer quality (fewer cells per blastocyst and poorer development beyond morula stage) and possessed poorer *in vivo* developmental capability (lower pregnancy rate) compared with those produced in co-culture. Moreover, from visual assessment, the development of embryos in cell-free culture was about 0.5–1 day behind that of co-cultured embryos (data not shown). This is a further indication of the poor quality of embryos produced in cell-free culture, and supports the work of Goto *et al.* (1992), who reported that the number of cells per bovine blastocyst decreased as the day of blastocoel formation (after IVF) was delayed. A similar observation was reported for rabbit embryos (Fischer and Schumacher, 1991).

Obviously, development of a new culture medium that supports *in vitro* embryonic development comparable to *in vivo* is needed. Recent observations (Rieger and Guay, 1988; Butler and Williams, 1991; Tiffin *et al.*, 1991; Rieger *et al.*, 1992) on the metabolism of ovine and bovine embryos may help in designing a better culture medium for supporting embryo development *in vitro* comparable to that observed *in vivo*.

These results demonstrate that embryos produced in cell-free culture were of poorer quality than those produced in co-culture. However, some of these embryos are developmentally competent, as confirmed by the birth of three calves after transfer of frozen-thawed blastocysts to recipient cows. Additional improvements in the culture of IVM/IVF embryos should enable the use of this technology, in conjunction with ultrasound-guided transvaginal oocyte retrieval (Pieterse *et al.*, 1991), as part of bovine genetic improvement programmes.

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