Enhanced Cryosurvival of Bovine Blastocysts Produced In Vitro in Serum-Free Medium

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Purpose: Culture systems affect the development of IVP embryos and consequently their cryosurvival potential. The viability of postthawed bovine IVP embryos developed from IVM/IVC medium in the presence or absence of serum was compared.

Methods: Cumulus-oocyte complexes were matured in IVM medium supplemented with or without serum. Some oocytes were evaluated for nuclear maturation status and others were inseminated with semen. Presumptive zygotes were cultured in IVC medium supplemented with or without serum for 9 days. Blastocysts were cryopreserved with 1.5 M ethylene glycol in PBS.

Results: No difference was observed in the nuclear maturation status and cleavage rates in both groups, but significantly ($P < 0.05$) higher in blastocyst rates in the serum-supplemented group. After freezing, survival of blastocysts was higher in the serum-free group. At 36 h culture after thawing, blastocysts developed without serum had significantly ($P < 0.05$) higher cell number than those cultured with serum.

Conclusions: We conclude that serum-free culture system enhances the viability of frozen-thawed bovine embryos.

KEY WORDS: Bovine; cryopreservation; culture; embryo; serum.

INTRODUCTION

Bovine in vitro embryo production techniques have been increasingly refined, to the point that blastocyst development is successful in various media formulations and under different culture conditions (1,2). Bovine embryos are routinely cultured in serum-supplemented media with or without somatic cell cocultures. The supplementation of bovine embryo culture medium with serum, especially fetal calf serum (FCS), is practiced widely, despite the undefined and variable nature of serum composition. Serum has been shown to have a biphasic effect on development of bovine embryos (3), whereas some batches

of bovine serum albumin (BSA) stimulate and others inhibit embryo development (4). Since the first calf born from a frozen embryo (5), there has been considerable progress in cryopreservation of in vivo-produced embryos. The presence of serum during in vitro culture (IVC) might have an influence on cryoresistance of the resulting embryos. In recent years, major efforts have been directed at improving survival rates of in vitro produced (IVP) embryos after cryopreservation, and the greatest portion of this effort has been concentrated on the bovine species (6,7). Under semidefined conditions in M199 supplemented with BSA, insulin, transferrin, and selenium, higher survivability of frozen-thawed blastocysts was observed compared to those produced in M199 supplemented with estrous cow serum (ECS) alone or cocultured with bovine oviductal epithelial cells (BOEC) (8). Although the development of bovine IVM-IVF embryos up to the blastocyst stage is now possible under a wide range of culture conditions, there are

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differences in the quality of the blastocysts in terms of their morphology, cell-numbers, and viability after cryopreservation (6,9,10). Furthermore, standard cryopreservation methods yielding high survival rates for in vivo-produced embryos resulted in significantly lower survival rates for IVP embryos (6,10), indicating that the conditions prevailing during development of embryos have significant effects on the ability of embryos to survive after freezing.

It has therefore been suggested that improving the culture conditions (11) can minimize the sensitivity of IVP embryos to cryopreservation. Substantial differences exist in the quality of blastocysts developed under various culture conditions after cryopreservation (2,9,12). Recently, Yamashita *et al.* (13) have reported that complete serum-free media could improve the yield and quality of bovine blastocysts derived from IVM/IVF oocytes. IVP embryos are generally less compact, zona pellucida sensitive to enzymatic digestion, and usually darker and more buoyant, presumably because of an increased lipid content (14). Elimination of serum during culture of presumptive bovine zygotes has been shown to improve the cryoresistance of blastocysts (15–17). In farm animals, the maintenance of genetic diversity relies on successful cryopreservation of germ cells and embryos for use in the embryo transfer industry (18).

Therefore, the objectives of the present study were to evaluate the in vitro development of blastocysts and the viability of postthawed bovine embryos produced under different culture conditions with or without serum in IVM/IVC medium.

MATERIALS AND METHODS

Media

Chemicals were purchased from the Sigma Chemical Company (St. Louis, MO), and media from GIBCO (Canadian Life Technologies, Burlington, ON) unless otherwise specified. The medium used for maturation of cumulus-oocyte complexes (COC) was M199 containing Earle's salts, 25 mM Hepes, 10 μ g/mL FSH, 10 μ g/mL LH, 1 μ g/mL estradiol-17 β , 2.5 mM Na pyruvate, 1 mM L-glutamine, 1.0% penicillin-streptomycin (10,000 IU and 10,000 μ L/mL, respectively; Pen-Strep; GIBCO), and with or without 10% fetal calf serum (FCS). The medium (IVC medium) used for culture of embryos was M199 containing Earle's salts, 2.5 mM Na pyruvate, 1 mM L-glutamine, 0.5% Pen-Strep and with or without 10% FCS. Tyrode's albumin lactate pyruvate

medium containing 2% (w/v) bovine serum albumin (BSA; Fraction V, Sigma) supplemented with 10 mM Hepes (HEPES-TALP) or without (IVF-TALP) was used for sperm preparation. Serum-free media contained 0.1% polyvinyl alcohol (PVA) in order to facilitate handling of embryos. For all media, the pH was adjusted to 7.4 and the osmolality to 280 mOsm/kg.

Oocyte Preparation

Ovaries obtained from a local slaughterhouse were sliced to collect COCs in Ham's F10 medium. Sets of 15 COCs were matured in 50 μ L droplets of IVM medium supplemented with or without 10% FCS under paraffin oil (Yakuri, Japan) at 39°C in a humidified atmosphere of 5% CO₂ in air. After 22-h culture, samples of oocytes were mechanically denuded of cumulus cells by vortexing in 3% sodium citrate solution and were assessed for their meiotic status by staining. For this, oocytes were fixed with aceto-methanol (acetic acid:methanol, 1:3, v/v) for 24 h, stained with 1% aceto-orcein. Nuclear status was examined under a phase-contrast microscope at $\times 400$ magnification.

Embryo Production

For sperm preparation, frozen semen straws were thawed and intact sperm was isolated by Percoll-density gradient method as described by Rosenkrans *et al.* (10). In brief, 100% Percoll (Pharmacia, Uppsala, Sweden) solution was mixed with 10 $\times$ salt solution (NaCl, 2.889 g; KCl, 0.238 g; KH₂PO₄, 0.116 g; CaCl₂, 0.112 g; Hepes 0.163 g; 50 mL of milli-Q water) to form 90% Percoll solution. A 45% Percoll solution was prepared from this by addition of an equal volume of HEPES-TALP. The gradient was formed by pipetting 1.5 mL of 90% Percoll solution into a 15 mL conical tube that was overlaid with 1.5 mL of 45% Percoll solution. Frozen-thawed bull semen was placed onto the top of the 45% gradient and centrifuged at 850 $\times$ g at room temperature for 15 min. After removing the supernatant, the pellet was washed twice with 10 mL of HEPES-TALP by centrifugation at 300 $\times$ g for 10 min, and resuspended in IVF-TALP.

For IVF, expanded cumulus cells were partially removed from oocytes after 22 h IVM by vortexing for 10 s in HEPES-TALP. Sets of 15 oocytes were then inseminated with spermatozoa that had been prepared by Percoll density-gradient at a final concentration of 2×10^6 spermatozoa/mL in 50 μ L of IVF-TALP. At 16 h postinsemination (hpi), these sets of 15 presumptive zygotes were cultured in 50 μ L of IVC medium

in the presence or absence of 10% FCS. At 48 and 120 hpi, the cultures were “fed” with 25 μ L of fresh IVC medium to each drop and were maintained for 192 hpi to assess the blastocyst development rate and cell number among treatment groups.

Embryo Freezing

At 192 hpi, embryos developed to blastocyst stage were frozen as described by Semple *et al.* (11). Briefly, embryos were equilibrated in Dulbecco's phosphate buffered saline (D-PBS) supplemented with 10% FCS, 1.0% Pen-Strep, and 1.5 M ethylene glycol. The embryos were loaded individually into 0.25 mL plastic straws. The straws were transferred into a freezer control (BIOGENICS CL863, USA) equilibrated for 10 min at 7°C during which time seeding was initiated. Freezing was accomplished by a cooling rate of $-0.6^{\circ}\text{C}/\text{min}$ from -7°C to -35°C , and then straws were plunged into liquid nitrogen for storage. Frozen straws were subsequently warmed for 5 s in air, followed by 15 s at 37°C . The thawed embryos were diluted directly into D-PBS and further cultured in IVC medium for 36 h.

Differential Staining

Differential staining was used to count cells and determine the ratio of ICM to total cell number according to the methods of Papaioannou and Ebert (19). Briefly, embryo zona pellucidae were digested by incubation with 0.5% pronase solution for 5 min and incubated with 10% rabbit antiovine-spleen serum in D-PBS for 30 min at 39°C . After washing three times with D-PBS + 10% BSA, embryos were incubated in D-PBS supplemented with 10% guinea-pig serum (for complement), 10 $\mu\text{g}/\text{mL}$ propidium iodide, and 10 $\mu\text{g}/\text{mL}$ bisbenzimidazole (Hoechst 33342) for 30 min at 39°C . The cells were counted on a Zeiss fluorescence microscope fitted with an excitation filter of 488 nm and a barrier filter of 543 nm; the nuclei of ICM cells were seen as blue and those of trophoblast cells as pink.

Experimental Design

The study comprised three experiments. In Experiment 1, the nuclear maturation of oocytes cultured in IVM medium in the presence or absence of serum was assessed (Table I). In Experiment 2, the rates of cleavage and blastocyst development were compared among different IVM/IVC medium with or without serum (Table II). On Day-8 of culture, the embryos

Table I. Nuclear Status of Bovine Oocytes Matured in M199 Supplemented With or Without Serum for 22 h^a

Serum ^b	Oocytes (n)	Nuclear status (%) ^c			
		MI	AI-TI	MII	Degenerated
+	82	11 (13.4)	2 (2.4)	65 (79.2)	4 (4.8)
–	85	9 (10.5)	8 (9.4)	61 (71.7)	9 (10.5)

^a Results of four replicates.

^b +, supplemented with 10% FCS; –, supplemented without 10% FCS.

^c MI, Metaphase I; AI-TI, Anaphase I-Telophase I; MII, Metaphase II.

developed to blastocyst stage were assessed for their total cell number and the ratio of ICM cells (Table III). In Experiment 3, Day-8 blastocysts produced by culture of different IVM/IVC medium with or without serum were evaluated for survival after freezing and thawing (Table IV). Following culture for 36 h, total cell number and the ratio of ICM cells were determined (Table V).

Statistical Analysis

Difference between treatments were analysed using one-way analysis of variance (ANOVA) after arcsine transformation of the proportional data of viability and ICM cell numbers. Comparison of means among treatments was performed using the Turkey-Kramer multiple comparisons test. Differences were considered significant when $P < 0.05$.

RESULTS

Experiment 1

A total of 167 oocytes were assessed for their nuclear status after 22 h of IVM. Table I shows the

Table II. Development of IVP Bovine Embryos Cultured in M199 Supplemented With or Without Serum^a

Serum ^b		Oocytes (n)	Development (%)	
IVM	IVC		Cleavage	Blastocysts ^c
+	+	374	257 (68.7)	55 (21.4) ^a
+	–	362	215 (59.3)	27 (12.5) ^b
–	+	376	247 (65.6)	49 (19.8) ^a
–	–	362	225 (62.1)	21 (9.3) ^b

^a Results of six replicates.

^b +, supplemented with 10% FCS; –, supplemented without 10% FCS.

^c Values with different superscripts (a, b) in the same column were significantly different ($P < 0.05$).

Table III. Total Cell Number and ICM Ratio of Day-8 Blastocysts Cultured in M199 Supplemented With or Without Serum

Serum ^a		Embryos (n)	Cell number (Mean $\pm$ SEM) ^b			Percent of ICM
IVM	IVC		Total	ICM	TE	
+	+	14	120 $\pm$ 6	19	101	15.8
+	–	15	105 $\pm$ 7	24	81	22.8
–	+	10	118 $\pm$ 5	20	98	16.9
–	–	12	103 $\pm$ 4	22	81	21.3

^a +, supplemented with 10% FCS; –, supplemented without 10% FCS.

^b ICM, inner cell mass; TE, trophoblasts.

results of this experiment. In both culture groups in the presence or absence of serum, the percentages of Metaphase II were 79 and 72%, respectively, not significantly different ($P < 0.05$).

Experiment 2

A total of 1474 oocytes over six replicates, with each replicate composed of 362–376 oocytes were assessed for in vitro development under different culture condition with or without serum (Table II). No difference in cleavage rate (59–69%) was seen with or without serum supplement. Serum supplementation in IVM did not affect subsequent blastocyst development; whereas, a significantly higher ($P < 0.05$) development rate for blastocyst was observed in the serum-supplemented IVC group compared to that of the serum-free group (21 and 20% vs. 13 and 9%).

Table III shows total cell number and ICM ratio of Day-8 blastocysts produced by different IVM/IVC conditions. Serum supplementation during IVM did not affect the total cell number of the embryos. However, blastocysts obtained from serum-supplemented IVC groups had more cells (120 $\pm$ 6, 118 $\pm$ 5) than

Table IV. Survivability of Frozen-Thawed Blastocysts Cultured in M199 Supplemented With or Without Serum

Serum ^a		Embryos ^b (n)	No. of (%) blastocysts hatched ^c
IVM	IVC		
+	+	49	25 (51.0) ^a
+	–	25	19 (76.0) ^b
–	+	32	18 (56.3) ^a
–	–	32	25 (78.1) ^b

^a +, supplemented with 10% FCS; –, supplemented without 10% FCS.

^b Cultured for more 24 h post thawing of Day-8 blastocysts.

^c Values with different superscripts (a, b) in the same column were significantly different ($P < 0.05$).

Table V. Total Cell Number and ICM Ratio Following 36-h Culture of Frozen-Thawed Day-8 Blastocysts Cultured in M199 Supplemented With or Without Serum

Serum ^a		Embryos ^b (n)	No. of cells (Mean $\pm$ SEM) ^c			Percent of ICM
IVM	IVC		Total ^d	ICM	TE	
+	+	16	159 $\pm$ 4 ^a	24	135	15.0
+	–	15	177 $\pm$ 7 ^b	38	139	21.4
–	+	15	151 $\pm$ 2 ^a	25	126	16.5
–	–	15	180 $\pm$ 5 ^b	39	141	21.0

^a +, supplemented with 10% FCS; –, supplemented without 10% FCS.

^b Cultured for more 36 h post thawing of Day-8 blastocysts.

^c ICM, inner cell mass; TE, trophoblasts.

^d Values with different superscripts (a, b) in the same column were significantly different ($P < 0.05$).

those from serum-free groups (105 $\pm$ 7, 103 $\pm$ 4). No difference in the ratio of ICM was seen between serum-free and serum-supplemented groups.

Experiment 3

The survivability of frozen-thawed blastocysts produced in vitro by different culture conditions is presented in Table IV. Each group consists of 25–49 embryos. After freezing of blastocysts cultured with or without serum, a significantly ($P < 0.05$) higher survival as assessed by the subsequent hatching rate was observed in the serum-free IVC groups (76, 78% vs. 51, 56%).

The total cell number and the ratio of ICM to trophoblast in frozen-thawed Day-8 blastocysts obtained from different culture conditions are presented in Table V. Following 36 h of culture after thawing, the blastocysts developed from serum-free IVC had significantly ($P < 0.05$) higher cell number (180 $\pm$ 5, 177 $\pm$ 7) than those cultured with serum (159 $\pm$ 4, 151 $\pm$ 2). However, no difference in the ratio of ICM was seen between with and without serum supplement.

DISCUSSION

To facilitate studies on early embryo development of domestic animals and to enhance the efficiency of in vitro embryo production procedures, it is highly desirable to optimize culture conditions in the absence of serum. Our study confirmed that the supplementation or elimination of serum from maturation medium did not significantly ($P < 0.05$) affect nuclear maturation rates (79% vs. 72%) under our laboratory conditions. This data demonstrate that serum

supplementation is not essential during IVM for nuclei maturation. Zhang and Sirard (20) have reported that nuclear and cytoplasmic maturation can be influenced by culture conditions including protein supplementation and hormones. However, in the present study the proportion of oocytes completing nuclear maturation in the presence of serum was not different from that of serum-free group (Table I). This confirms the suggestion that protein-supplementation especially in the form of serum is not necessary during IVM of bovine oocytes.

According to results of the second experiment, no difference in cleavage rate (59~69%) was observed with or without serum supplementation. However, significantly higher ($P < 0.05$) blastocyst development rates were observed in serum supplemented IVC groups compared to the serum-free group (21% vs. 13%). Our study suggests that the cleavage rate was not affected by protein supplementation, confirming the earlier reports of Saeki *et al.* (21). Eckert *et al.* (22) also reported similar results of cleavage (59% vs. 54%) and blastocyst rates (25% vs. 8%, $P < 0.05$). On the other hand, Yamashita *et al.* (23) reported that the efficiency of blastocyst formation and mean numbers of blastocysts in serum-free medium supplemented with growth factors were much greater compared to serum-containing medium. These results indicate the potential value of serum-free culture but suggest factors in serum might accelerate the proliferation of blastomeres and blastocyst development.

Culture conditions, in particular the presence of serum, has been implicated in abnormal embryonic, fetal growth, and development as well as reduced calving rates (24,25). One hypothesis is that the *in vitro* environment, possibly leading to perturbation of differentiation and organogenesis (26) affects transcription of one or several developmentally important genes.

In this study, around 77% of the frozen-thawed blastocysts showed increased survivability following serum-free culture. Semple *et al.* (11) have shown that freezing of Day-7 bovine blastocysts resulted in mean survival rates (reexpansion within 24 h) of 81 and 71%, with subsequent hatching by 72 h in 59 and 52% for the serum-free and serum groups, respectively. A subsequent study (15) showed 96 and 42% hatching of Day 7 blastocysts and Day 6 morulae respectively, when cryopreserved after serum-free coculture. These results suggest a role for serum lipids in the stage-specific sensitivity to cryopreservation, which has been previously reported for *in vitro* produced embryos (27). Dorland *et al.* (28) claimed that the presence of fetal calf serum had adverse effects

on the structure of mitochondria and the accumulation of lipid droplets was caused by impaired function of mitochondria, since intracellular lipid is normally metabolized by mitochondria. Moreover it has been suggested that embryos with high lipid content were more sensitive to cryopreservation procedures (6,29). Hence, mechanical delipidation of bovine embryos led to an increase in survival rate after freezing and thawing (14,30,31). Recently, Yamashita *et al.* (13) reported that survival rates of blastocysts derived from serum-free medium were superior at 48 and 72 h postthaw to that of blastocysts grown in serum supplemented medium, which contained numerous lipid droplets in the cytoplasm. The presence of serum lipids during culture might influence cryoresistance of resulting embryos. Our findings and others suggest that cryopreservation of *in vitro* produced bovine embryos appear to be improved by a serum-free culture system.

The number of inner cell mass (ICM) cells and trophoblast (TE) cells may be a valuable indicator of viability and quality of mammalian blastocysts (19). The results reported reveal total cell numbers and number of TE cells of expanded and hatched blastocysts cultured with or without serum was similar. However, by 36 h postthaw, the total cell number and number of TE cells of blastocysts previously cultured in serum-free medium were significantly greater than of those cultured in serum-containing medium.

In conclusion, the present study shows that the use of serum-free medium did not improve the number of blastocysts produced, but did improve the morphology and cell number of resultant embryos and resulted in increased tolerance to cryopreservation. In light of our findings, it would be appropriate to carry out transfer studies using fresh and cryopreserved embryos to determine if our culture results are consistent with pregnancy outcome.

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