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A serum-free culture system for efficient *in vitro* production of bovine blastocysts with improved viability after freezing and thawing

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Abstract

The aim of this study was to evaluate whether two completely serum-free media (IVMD101 and IVD101) could improve the yield and quality of bovine blastocysts from *in vitro* matured and fertilized oocytes. The media were evaluated in the presence (IVMD101) or absence (IVD101) of bovine cumulus/granulosa cell (BCGC) cocultures. The proportion of embryos developing to the blastocyst stage in IVMD101 medium with BCGC cocultures (36.5%) and IVD101 medium without BCGC cocultures (37.1%) was significantly higher than in serum-supplemented medium (TCM199 + 5% calf serum) with BCGC cocultures (25.1%). Furthermore, the mean cell numbers per blastocyst on Day 7 developed in IVMD101 medium (179.5 cells) and IVD101 medium (177.1 cells) were greater than in the serum-supplemented medium (145.7 cells). The survival rates of blastocysts derived in IVMD101 medium (73.3%) and IVD101 medium (60.0%) based on hatching after 72 h of post-thaw culture were superior to that of blastocysts derived in the serum-supplemented medium (48.1%). Under microscopic observation, bovine blastocysts derived in the serum-supplemented medium showed abundant lipid droplets, largely into the trophectoderm cells. This morphological difference may partly explain the sensitivity of serum-derived embryos after freezing and thawing. In conclusion, these new serum-free culture media are useful, not only to study the mechanisms of early embryogenesis, but also for mass production of good quality embryos for embryo transfer, cloning and transgenesis.

Abbreviations: bFGF, basic fibroblast growth factor; BCGC, bovine cumulus/granulosa cell; CS, calf serum; COCs, cumulus-oocytec omplexes; D-PBS, Dulbecco's phosphate buffered saline; FORs, free oxygen radicals; GSH, glutathione; IVC, *in vitro* embryo culture; IVF, *in vitro* fertilization; IVM, *in vitro* maturation; OCM, oocyte collection medium; TGF-α, transforming growth factor-α; TGF-β_1, transforming growth factor-β_1; TIMP-1, tissue inhibitor of metalloproteinase-1.

Introduction

The techniques for producing bovine embryos by *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) have shown remarkable progress in recent years. Basic research on early embryonic development as well as on the applications for low cost, mass production of bovine calves by embryo transfer, cloning by nuclear transfer, and gene manipulation for the production of transgenic cows has developed at a rapid pace.

Serum has been commonly used for *in vitro* maturation of bovine oocytes and *in vitro* embryo culture (IVC). Serum contains many kinds of substances such as amino acids, carbohydrates, lipids, inorganic salts, vitamins, hormones and growth factors that may stimulate and/or inhibit oocyte maturation and embryonic development. Bovine embryos become arrested in development at the 8- to 16-cell stages when they were cultured in media supplemented only with serum (Wright and Bondioli, 1981). Coculture systems with a variety of somatic cells have been established which

allow successful development of early embryos beyond the 8- to 16-cell stage to the blastocyst stage (Eyestone and First, 1989; Fukui, 1989; Rexroad, 1989). These results suggest that embryo-somatic cell interactions play an important role in early mammalian embryogenesis. However, the presence of undefined components such as serum in the culture system made it difficult to determine the precise role of growth factors and/or secretory products from either somatic cells or embryos which were capable of promoting early embryonic development. Furthermore, it was more complicate to prepare somatic cells for the efficient production of bovine embryos *in vitro*.

Successful cryopreservation of *in vitro*-derived embryos and subsequent pregnancies of these embryos after thawing has been reported (Fukuda et al., 1990). However, survival rates of frozen *in vitro*-derived embryos, as measured either by post-thaw development in culture or by pregnancies following embryo transfer, have been lower than those reported for *in vivo*-derived counterparts (Massip et al., 1995). Survival of frozen-thawed *in vitro*-derived embryos has also been reported to be affected by the culture condition in which the embryos were produced (Semple et al., 1995).

The objectives of the present study were to examine whether an effective serum-free media, in the presence or absence of bovine cumulus/granulosa cell (BCGC) coculture, could be developed which would yield a superior proportion of blastocysts with a higher survival rate after freezing and thawing, compared to conventional serum-supplemented medium.

Materials and methods

Culture Media

Medium was prepared to volume with water purified by reverse osmosis and filtration through a Milli-Q system (Japan Millipore Co., Tokyo, Japan). All the media were sterilized by filtering through a 0.22 μm membrane filter (Gelman Science, Ann Arbor, MI). Media were stored at 4 °C and fresh media were prepared every 2 weeks. The osmolarity and pH of all media were 275–290 mOsmoles and 7.2–7.3, respectively. The osmolarity of the media was adjusted by changing the concentration of NaCl.

Serum-supplemented medium for maturation of oocytes and embryo culture was tissue culture medium 199 (TCM199) with Earle's salts buffered with 25 mM HEPES (GIBCO Laboratories, Grand Island, NY) containing 5% heat-inactivated calf serum (CS; GIBCO). The formulations of complete serum-free media (IVMD101 and IVD101) are shown in Table 1. The basal medium of IVMD101 and IVD101 was prepared from specially ordered TCM199 medium (DM199; Kyokuto Pharmaceutical Co., Tokyo, Japan) which excludes glucose, Tween-80 and para-aminobenzoic acid from the original components of TCM199. Both serum-free media contained Earle's salts buffered with 25 mM HEPES (Sigma Chemical Co. St. Louis, MO). The concentration of glucose in IVD101 medium was reduced to 2.22 mM compared to 5.56 mM in TCM199 and IVMD101 media. The concentration of reduced glutathione (GSH) in IVD101 medium was increased to 200 mM compared to 0.2 μM in TCM199 and IVMD101 media.

Complete IVMD101 medium was composed of DM199 basal medium supplemented with 5.56 mM glucose, 0.91 mM pyruvate, 5 mM taurine, 5 nM Se, 5 μg/ml bovine insulin, 10 ng/ml human recombinant transforming growth factor-α (TGF-α), 10 μg/ml bovine apotransferrin and 10 μg/ml gentamycin. Complete IVD101 medium was composed of

Table 1. Composition of complete serum-free media for *in vitro* maturation (IVM) and *in vitro* embryo culture (IVC)

Components	IVMD101[a]	IVD101[a]
D-glucose[b]	5.56 mM	2.22 mM
Sodium pyruvate[c]	0.91 mM	0.27 mM
Sodium lactate[d]	–	2.48 mM
L-cysteine[e]	–	0.05 mM
GSH[c]	–	200 μM
Taurine[d]	5 mM	5 mM
Selenium[c]	5 nM	5 nM
Insulin[d]	5 μg/ml	–
TGF-α[f]	10 ng/ml	–
Apotransferrin[c]	10 μg/ml	10 μg/ml
b FGF[f]	–	10 ng/ml
TGF-β_1[f]	–	1 ng/ml
TIMP-1[g]	–	0.5 μg/ml
Aprotinin[d]	–	0.5 μg/ml
Gentamycin sulfate[d]	10 μg/ml	10 μg/ml

[a] Basal medium of IVMD101 and IVD101 complete serum-free media is DM199 described in the Section 'Materials and methods'.
Each component was purchased from:
[b] Nakarai Tesque, Inc., Kyoto, Japan; [c] GIBCO;
[d] Sigma;
[e] Funakoshi, Inc., Tokyo, Japan;
[f] Morinaga Institute of Biological Science, Yokohama, Japan.
[g] Bovine TIMP-1 was purified from conditioned medium of bovine cumulus/granulosa cells described previously (Satoh et al., 1994).

DM199 basal medium supplemented with 2.22 mM glucose, 0.27 mM pyruvate, 2.48 mM lactate, 0.05 mM cysteine, 200 μM GSH, 10 ng/ml human recombinant basic fibroblast growth factor (bFGF), 1 ng/ml human recombinant transforming growth factor-β_1 (TGF-β_1), 0.5 μg/ml bovine tissue inhibitor of metalloproteinase-1 (TIMP-1), 0.5 μg/ml bovine aprotinin. The IVD101 medium contained the same concentrations of taurine, Se, apotransferrin and gentamycin as in IVMD101 medium, but no insulin or TGF-α.

Oocyte collection

Oocyte collection was carried out as reported previously (Kobayashi et al., 1992). Briefly, ovaries of Japanese Black and Holstein cows and heifers were obtained at a local abattoir and transported to the laboratory in sterile 0.9% NaCl at 15–20 °C within 2 h. The ovaries were pooled regardless of stages of the estrous cycle of donors. The ovaries were washed several times with oocyte collection medium (OCM; Research Institute for the Functional Peptides, Yamagata, Japan) which consisted of Dulbecco's phosphate buffered saline (D-PBS) supplemented with 5.56 mM glucose, 1.25 mM sodium pyruvate, 15 μg/ml sodium heparin (Sigma), 8 μg/ml phenol red and 10 μg/ml gentamycin. Oocytes were aspirated from 2- to 6-mm diameter follicles and washed twice in the OCM. Oocytes with 3–5 layers of compact cumulus cells and evenly granulated cytoplasm were used in the following experiments.

Sperm preparation and in vitro fertilization (IVF)

IVF100 medium (Research Institute for the Functional Peptides, Yamagata, Japan) was used for the treatment of spermatozoa and fertilization of oocytes. The IVF100 medium consisted of a modified BO medium (Brackett and Oliphant, 1975) supplemented with 1.25 mM sodium pyruvate, 0.5 mM cysteine, 5 mg/ml BSA (Kokusai Shiyaku, Kobe, Japan), 7.5 μg/ml sodium heparin, 5 mM caffeine (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 10 μg/ml gentamycin. A frozen stock of semen obtained from a Japanese Black bull was rapidly thawed at 38 °C, diluted with the fertilization medium (IVF100) and washed twice by centrifugation at 2200 rpm for 5 min. The final sperm pellet was resuspended in the same medium to give a concentration of 1.0×10^7 spermatozoa/ml. For IVF, a 50 μl aliquot of spermatozoa suspension was combined with a 50 μl droplet of IVF100 medium. After *in vitro* maturation, cumulus oocyte complexes (COCs) were transferred in a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 6 h.

Preparation of bovine cumulus/granulosa cell (BCGC) coculture

BCGCs used for coculture were prepared by gentle pipetting of the COCs in either serum-free medium (IVMD101) or serum-supplemented medium (TCM199 + 5% CS). BCGCs were then cultured in 6-well Repro C-1 culture plates (Research Institute for the Functional Peptides, Yamagata, Japan) containing either 230 μl of the same serum-free medium or serum-supplemented medium. Media were changed every 3–4 days, and confluent BCGC monolayers were obtained 8–9 days after seeding, which were subsequently used for the *in vitro* embryo culture (IVC) and embryo freezing and thawing experiments.

Experimental design

Experiment 1

The effect of either serum-free or serum-supplemented media in the presence or absence of BCGC was determined on *in vitro* development of bovine embryos. For serum-free cultures, the COCs were washed in pre-equilibrated complete serum-free maturation medium (IVMD101). Then 25–30 COCs were aliquoted into 6-well Repro C-1 culture plates in 230 μl of the maturation medium and overlaid with 100 μl of paraffin liquid (Merck, Darmstadt, Germany). For serum-supplemented cultures, the COCs were handled in the same manner, except that TCM199 containing 5% CS (TCM199 + 5% CS) was used. The COCs were matured in either serum-free or serum-supplemented medium in a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 24 h.

After the IVF described in Materials and Methods, 25–30 inseminated oocytes were placed in 6-well Repro C-1 culture plates in 230 μl of either IVMD101 medium or TCM199 + 5% CS medium, overlaid with 100 μl of paraffin liquid and incubated for 24 h. These embryos were then denuded by careful pipetting with capillary pipettes to completely remove the BCGC. 25–30 denuded embryos in 230 μl of media were transferred to the previously prepared confluent BCGC cultures which had been grown in serum-free or serum-supplemented media. The cocultures were

incubated in a humidified atmosphere of 5% CO_2 in air at 38.5 °C. The same number of denuded embryos were transferred to 6-well Repro C-1 plates in 230 μl serum-free medium (IVD101) without BCGC coculture and incubated in a humidified atmosphere of 5% CO_2/5% O_2/90% N_2 at 38.5 °C. Serum-free embryo cultures were incubated for up to 9 days without medium change, while those in serum-supplemented media were changed every 2 days. Cultured embryos were observed daily under a microscope. The efficacy of the culture systems was evaluated by the proportion of embryos that cleaved on Day 2 (2 – cell stage), by the proportion of 8-cell stage embryos on Day 2–3, and the proportion of blastocysts on Day 7–9 (day of insemination = Day 0).

Experiment 2

On day 7 of culture, blastocysts which were developed *in vitro* in Experiment 1 were examined for the number of cells by the modified methods of Lee and Fukui (1996). Briefly, the embryos derived in the three different media were treated with 0.5% (v/v) sodium citrate solution for 5 min, and fixed in a cold solution of methanol:acetic acid:distilled water (3:2:1) for 5 min. The fixed embryos were mounted on slides with a small volume of the fixative, dried overnight and stained with 5% (v/v) Giemsa's solution (Merck) for 10 min. The cell number was determined by counting stained nuclei under a phase-contrast microscope.

Experiment 3

Blastocyst and expanded blastocyst stage embryos obtained on Day 7 from the three different media in Experiment 1 were used. Embryo freezing was carried out as previously reported (Takagi et al., 1994). Briefly, the embryos were suspended in D-PBS supplemented with 1.8 M ethylene glycol (Amresco, Solon, OH), 5.56 mM glucose, 1.25 mM sodium pyruvate, 5 mg/ml BSA and 10 μg/ml gentamycin. After a 10 min equilibration period, the embryos were loaded into 0.25 ml plastic straws, with a maximum of 10 embryos per straw, placed directly into a 0 °C alcohol bath chamber, and held for 2 min. Then, the embryos were cooled from 0 °C to –6 °C at 1 °C/min and seeded at –6 °C. After seeding, the straws were held for 15 min at –6 °C, and cooled to –30 °C at a rate of 0.3 °C/min. Finally, the straws were plunged into and stored in liquid nitrogen. After storage in liquid nitrogen for 2–3 weeks, frozen embryos were immediately thawed by placing the straws in a 35 °C water bath and the contents drained into a sterile Petri dish. The embryos were then placed into the culture medium for rehydration and washed 3 times. Embryos were cultured on feeder layers of BCGC in TCM199 supplemented with 5% CS in 5% CO_2 in air at 38.5 °C. Embryos were evaluated microscopically at 24 h intervals up to 72 h. Survival rates were assessed by these parameters: (1) re-appearance of the blastocoele after 24 h culture and (2) the ability to hatch *in vitro* after 72 h in culture.

Experiment 4

Morphological observation using transmission electron microscopy of Day 7 blastocysts in either serum-free medium (IVD101) without BCGC coculture or serum-containing medium with BCGC coculture (TCM199 + 5% CS) was carried out. A small number (3–4 from each medium treatment) of blastocysts were processed for transmission electron microscopy by fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4) at 0–4 °C for 1–3 h. After the fixation, the blastocysts were washed in the PB and then post-fixed with 1% osmium tetroxide in 0.1 M PB at 0–4 °C for 1 h. Subsequently, the blastocysts were individually embedded in 1% agar prior to the epoxy resin embedding processes. All samples were dehydrated in ascending concentrations of ethanol solutions (50–100%), substituted in propylene oxide, and embedded in epoxy resin (Taab Laboratories Equipment Ltd., Berkshire, UK). Ultrathin sections were cut with an ultramicrotome (Reichert Ultracuts, Leica, Heerbrugg, Switzerland), stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEM – 1210, Jeol, Tokyo, Japan).

Statistical analysis

Statistical analyses were carried out by one-way analysis of variance (ANOVA, Abacus Concepts, StatView, Berkeley, CA) and Fisher's protected least significant difference test. The percentage data were subjected to arcsine transformation before the statistical analysis.

Results

Experiment 1

The effect of media (serum-free vs. serum-supplemented) with or without BCGC cocultures on *in vitro*

Table 2. Development of *in vitro* matured and fertilized bovine embryos in three different media

Culture medium	BCGC coculture	No. of inseminated oocytes	No. of replicates	No. and (%)[a] of oocytes developed to		
				≧2-cell	≧ 8-cell	Blastocyst
Serum-free medium						
IVMD101	+	208	7	184 (88.5%)[b]	142 (68.3%)[b]	76 (36.5%)[b]
IVD101	–	197	7	182 (92.4%)[b]	151 (76.6%)[c]	73 (37.1%)[b]
Serum-supplemented medium						
TCM199 + 5% CS	+	233	8	180 (80.7%)[c]	133 (59.6%)[d]	56 (25.1%)[c]

[a] Percentage of the number of inseminated oocytes cultured.
[b−−d] Value with different superscripts within each column differ significantly ($p < 0.005$).

development of bovine embryos was determined (Table 2). The percentage of zygotes that developed to the 2-cell, 8-cell and blastocyst stages when cultured in serum-free IVMD101 with BCGC coculture (36.5%) or IVD101 medium without BCGC coculture (37.1%) was significantly higher ($p<0.05$) than those in serum-supplemented medium (25.1%) cocultured with BCGC.

Experiment 2

The cell numbers of blastocysts on day 7 cultured in the three different media in Experiment 1 were measured (Table 3). The cell numbers per blastocyst were highly variable in all culture conditions. The mean cell numbers in serum-free media (IVMD101 with BCGC coculture and IVD101 without BCGC coculture; 179.5±45.7 and 177.1±59.7, respectively) were higher ($p<0.01$) than those in serum-supplemented medium (TCM199 + 5% CS; 145.7±47.3).

Experiment 3

Blastocyst and expanded blastocyst stage embryos obtained from the three different media on Day 7 were frozen and the post-thaw viability of these embryos was evaluated (Table 4). After 24 h incubation, the survival of blastocysts developed in IVMD101 serum-free medium with BCGC coculture (93.3%) was significantly better ($p<0.05$) than those developed in IVD101 serum-free medium without BCGC coculture (80.0%) and serum-supplemented medium with BCGC coculture (74.1%). After 72 h incubation, hatching rates of blastocysts developed in IVMD101 (73.3%) and IVD101 (60.0%) were significantly higher ($p<0.05$) than those developed in the serum-supplemented medium (48.1%).

Experiment 4

Morphological observation of blastocysts by differential interference contrast microscopy revealed that blastocysts cultured in serum-supplemented medium in the presence of BCGC cocultures had a dark, granular morphology with the inclusion of abundant lipid-like vesicles, while blastocysts cultured in the serum-free media (IVMD101 and IVD101) were more translucent and had less lipid-like droplets (data not shown). Transmission electron micrographs of blastocysts derived in the same serum-supplemented medium showed many large lipid droplets in the trophectodermal cells inside of the blastocysts compared to those cultured in serum-free medium (IVD101) (Figure 1).

Discussion

Bovine *in vitro* matured and fertilized oocytes are routinely cultured up to the blastocyst stage in serum-supplemented media with or without somatic cell cocultures. Although serum is commonly added to culture medium to promote blastocyst formation of bovine embryos, serum and serum fractions are known to have inhibitory activity at specific stages of embryo development (Pinyopummintr and Bavister, 1991; Lim et al., 1994). Since serum contains various unknown components, deletion of serum from the culture medium was essential for analyzing the roles of specific factors on embryo development. The results of

Table 3. Mean cell numbers of bovine blastocysts cultured in three different media

Culture medium	BCGC coculture	No. of blastocysts examined	Mean±S.D. cells per blastocyst	Range (min–max)
Serum-free medium				
IVMD101	+	37	179.5±45.7	(89–271)[a]
IVD101	–	38	177.1±59.7	(86–281)[a]
Serum-supplemented medium				
TCM199 + 5% CS	+	36	145.7±47.3	(77–284)[b]

[a−−b] Values with different supercripts within each column differ significantly ($p < 0.01$).

Table 4. Post-thaw survival of bovine blastocysts developed in three different media

Culture medium	BCGC coculture	No. of blasyocysts thawed	Blastocysts after 24 h n (%)	Hatched blastocysts after 72 h n (%)
Serum-free medium				
IVMD101	+	60	56 (93.3)[a]	44 (73.3)[a]
IVD101	–	70	56 (80.0)[b]	42 (60.0)[a,b]
Serum-supplemented medium				
TCM199 + 5% CS	+	54	40 (74.1)[b]	26 (48.1)[b]

[a−−b] Values with different superscripts within each column differ significantly ($p < 0.05$).

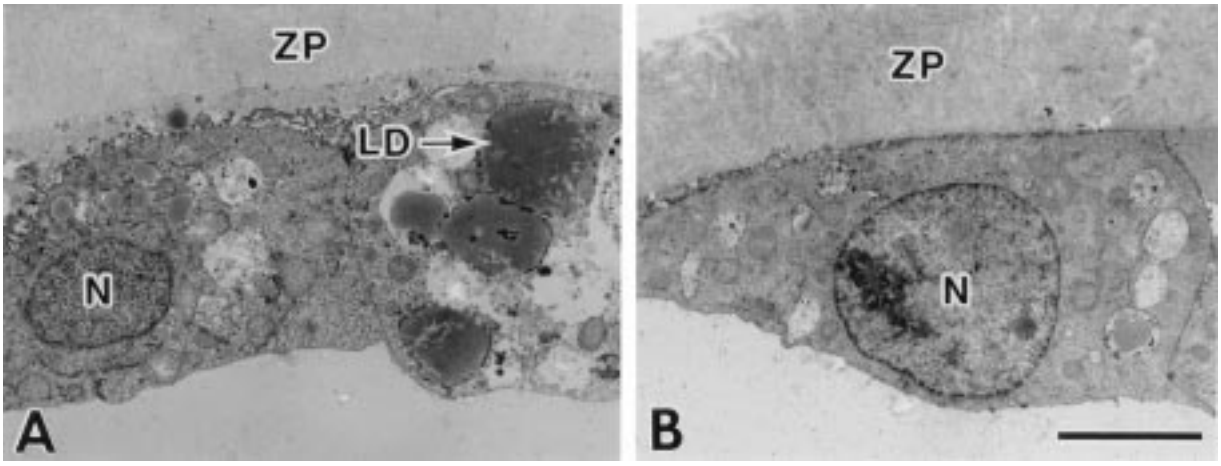

Figure 1. Electron micrographs of trophoblast cells from blastocysts developed in serum-supplemented medium (A) and in serum-free medium (B). (A) Many large lipid droplets (LD) were present in the trophoblast cells. N, nucleus; ZP, zona pellucida. Bar = 5 μm.

this study indicated that the improved serum-free media (IVMD101 in the presence of BCGC and IVD101 in the absence of BCGC) could be successfully used for increased production of bovine blastocysts *in vitro*. The major characteristics of IVMD101 medium used were the supplementation with growth factors

(TGF-α, insulin) and antioxidant-related components (taurine, Se, apotransferrin). IVD101 medium contained a low concentration of glucose (2.22 mM), lactate, growth factors (bFGF, TGF-β_1, TIMP-1) and antioxidant-related factors (increased concentrations of GSH, taurine, Se, apotransferrin, cysteine) (Table 1). IVMD101 medium was used in conjunction with the somatic cell cocultures of BCGC under atmospheric oxygen conditions (20% O_2) while IVD101 medium was designed for embryo growth in the absence of somatic cell cocultures under low oxygen conditions (5% O_2).

The proportions of embryos developed to the blastocyst stage cultured in either IVMD101 or IVD101 serum-free media were higher than those in serum-containing medium even using BCGC cocultures (Table 2). Furthermore, the mean cell numbers per blastocyst obtained in the same serum-free media were superior to those of blastocysts developed in serum-supplemented medium (Table 3). Consistent with our previous findings (Kobayashi et al., 1994b), a high glucose concentration (5.5 mM) in the defined IVMD101 medium resulted in a high yield of blastocysts under a high oxygen atmosphere (20% O_2) when in coculture with BCGC. In contrast, a low concentration of glucose (2.2 mM) in IVD101 was highly effective for production of blastocysts under a low oxygen culture (5% O_2) in the absence of BCGC cocultures. These results suggest that the presence of somatic cells may provide the optimal requirements of glucose in the culture medium and oxygen in the atmosphere for embryo development *in vitro*.

It is possible that the cellular metabolism of somatic cells, involved in cell survival and proliferation, utilizes a considerable amount of glucose and oxygen resulting in conditions which provide for better development of the embryos. In fact, the presence of high glucose concentration in early pre-implantation embryos up to the 8-cell stage can be deleterious to further embryo development and lactate, a metabolite of glucose produced by somatic cells, appears to be a useful energy substrate for *in vitro* development of bovine embryos (Pinyopummintr and Bavister, 1991; Takahashi and First, 1992).

In the absence of somatic cell cocultures, it appears that atmospheric O_2 (20%) is deleterious to embryo development, while a low oxygen concentration (5% O_2) supports the increased production of blastocysts *in vitro* (Fukui et al., 1991; Kobayashi et al., 1994b). These data suggest that one function of somatic cell coculture systems may be reducing oxygen tension to less toxic levels. Free oxygen radicals (FORs) have recently been implicated as major causes of embryonic arrest and cell death (Johnson and Nasr-Esfahani, 1994). Several mechanisms may be involved in defending against the potential deleterious effects of FORs, including iron-binding proteins, free radical scavengers, and antioxidant enzyme activities (Nasr-Esfahani et al., 1990; Nasr-Esfahani and Johnson, 1992). Serum-free IVMD101 medium contains taurine, Se, apotransferrin and IVD101 medium contains a large amount of GSH (200 μM) and cysteine in addition to the same components of IVMD101 medium described above. Free transition metal ions can activate superoxides to produce the FORs which are highly toxic for embryo development (Nasr-Esfahani et al., 1990; Johnson and Nasr-Esfahani, 1994). Iron-carrier proteins such as apotransferrin could act as iron chelators resulting in the promotion of development of mouse embryos *in vitro* through the two-cell block (Nasr-Esfahani and Johnson, 1992). Taurine is considered to act as a potent anti-oxidant to prevent the accumulation of superoxides and the generation of the FORs as well as an osmolyte, protecting embryos against osmotic shock (Bavister, 1995). Luvoni et al. (1996) have recently reported that the addition of reduced GSH in the defined medium could improve bovine embryo development *in vitro*. GSH plays an important role as a cellular antioxidant interacting with superoxide and hydroxyl radicals. As the substrate in the reduction of hydrogen peroxide catalyzed by glutathione peroxidase, GSH detoxifies intracellular peroxides. An extracellular role of GSH in the prevention of lipid peroxidation of cellular membranes has been also reported (Thomas et al., 1988). Selenium was shown to prevent oxidative damage to mammalian cells by regulating glutathione peroxidase activity (Stadtamn, 1974). It has also been found to have a stimulatory effect on growth of many other somatic cell lines under serum-free culture conditions (Barnes and Sato, 1980).

Growth factors may play an important role in modulating embryonic and fetal growth and development (for review, see Schultz and Heyner, 1993). Transcripts of a variety of growth factors and their receptors have been found to be expressed in mammalian pre-implantation embryos at different stages of development (Adamson, 1993). Several growth factors have been shown to affect mammalian embryo development through increased protein and RNA synthesis, resulting in increased cell number and enhanced rates of blastocyst formation (Herrler et al., 1992; Larson

et al., 1992; Adamson, 1993). In accordance with our present results, Larson et al. (1992) reported that bFGF and TGF-β_1 synergistically stimulated bovine embryo development beyond the block stage. Addition of TGF-α or EGF to the IVM medium was found to stimulate cumulus expansion of the COCs which improved the fertilization rate and developmental potential in bovine oocytes (Kobayashi et al., 1994a). The yield and viability of *in vitro*-derived embryos were promoted by the use of somatic cell cocultures (Goto et al., 1994; Freeman et al., 1995). The BCGCs used in our study produced active embryotrophic factors (Kobayashi et al., 1992), and TIMP-1 isolated from BCGC conditioned medium was found to stimulate bovine embryo development (Satoh et al., 1994).

A comparative study of *in vivo* and *in vitro* produced embryos revealed remarkable differences in their morphology and metabolism (Massip et al., 1995). Although the development of bovine IVM/IVF embryos up to blastocyst stage is now possible under various culture conditions, there are large differences in the quality of the blastocysts with regard to morphology, cell numbers, and frozen embryo viability (Shamsuddin et al., 1993; Massip et al., 1995). The present data indicate that the post-thaw viability of bovine blastocysts derived in serum-free media was significantly better than those developed in the serum medium (Table 4). Morphological observation using transmission electron microscopy has clearly shown the abundant lipid droplets in trophectodermal cells of blastocysts cultured in serum-supplemented medium (Figure 1). Thompson et al. (1995) recently reported that ovine embryos cultured in serum-containing medium (SOF + human serum) were morphologically different from those cultured in serum-free medium (SOF + amino acids + BSA), having abundant lipid droplets. Leibo and Loskutoff (1993) reported that the *in vitro*-derived embryos at the compact morula stage had a much lower buoyant density, which might result from a higher ratio of lipid to protein. They also found that displacement of intracellular lipids by centrifugation significantly improved the survival of *in vitro* produced embryos, suggesting that those lipids were partially responsible for the sensitivity to chilling and freezing (Leibo et al., 1995). The sensitivity of *in vitro*-derived embryos to cryopreservation can also be influenced by different culture conditions (Greve et al., 1993; Leibo and Loskutoff, 1993).

In summary, we developed serum-free culture systems for *in vitro* development of bovine embryos with or without somatic cell cocultures. These serum-free culture systems can offer several advantages over the culture in serum-containing medium, including increased rates of blastocysts formation, cell numbers, and embryo viability after freezing and thawing.

In particular, IVD101 medium without somatic cell cocultures is useful, not only to obtain a large number of good quality embryos for practical uses such as embryo transfer, cloning and transgenesis but also to study the mechanisms of early embryogenesis.

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