Influence of protein supplements on the secretion of leukaemia inhibitory factor by mitomycin-pretreated Vero cells: possible application to the *in vitro* production of bovine blastocysts with high cryotolerance

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A co-culture system for bovine embryos using mitomycin-treated Vero cells and serum-supplemented modified synthetic oviduct fluid (mSOF) supports the development of *in vitro* maturation and fertilization-derived oocytes to hatched blastocysts. In this system, it has been suggested that one contribution made by the co-culture cells to embryo development is production of the cytokine leukaemia inhibitory factor (LIF). However, there are concerns about exposure of early embryos to serum due to its incompatibility with embryo cryosurvival. In this study, the influence of two protein supplements (synthetic serum substitute (SSS), a lipid-free human serum-derived product) and oestrous cow serum (ECS) on Vero cell LIF secretion was compared, with the aim of designing a co-culture system that is supportive of bovine embryo cryopreservation. Vero cells cultured for 72 h in medium 199 + 5% fetal bovine serum (FBS) (recommended maintenance medium for this cell line) secreted detectable amounts of LIF (13.1 ± 0.9 pg LIF per 10^5 cells). Culture in mSOF, the medium routinely used in this laboratory for embryo culture, also supported LIF secretion in Vero cells. However, the amount of LIF was tenfold higher (24.7 ± 6.2 pg LIF per 10^5 cells; $P < 0.05$) when mSOF was supplemented with 10% (v/v) ECS compared with supplementation with 2% (v/v) SSS. Results of a second series of experiments in which supplementation with each protein was normalized to 10% revealed similar differences in LIF secretion, indicating that LIF secretion was affected by the type, not the amount, of protein. Time course analysis revealed stepwise increases ($P < 0.05$) in cumulative LIF secretion with every 24 h of culture in mSOF + either SSS or ECS. In terms of embryo development and post-cryopreservation viability, medium supplementation with 2% (v/v) SSS alone versus the two-step system of 2% (v/v) SSS (days 1–4) + 10% (v/v) ECS (days 4–10) had no influence ($P > 0.05$) on the ability of bovine blastocysts to hatch, with or without intervening cryostorage. However, the rate of blastocyst formation (expressed as the percentage of cleaved embryos) was only 27% in the presence of 2% (v/v) SSS, and increased almost twofold ($P < 0.05$) when ECS was added beginning on day 4 of co-culture. In summary, Vero cell LIF secretion was increased markedly by ECS. A two-step system of medium supplementation, in which embryos are exposed to ECS beginning on day 4 of *in vitro* development combined high rates of blastocyst formation with cryotolerance. This effect may be a result of limiting embryo exposure to serum-derived lipid until after the eight-cell stage and providing an increase in LIF during the critical developmental stages of compaction and cavitation.

Introduction

Carnegie *et al.* (1997) reported a co-culture protocol for bovine embryos using mitomycin-pretreated Vero cells that

consistently provided rates of blastocyst and hatched blastocyst formation from *in vitro* maturation and fertilization (IVM/IVF)-derived oocytes of > 40%. Vero cells are a line of Green monkey kidney epithelial cells that improved the *in vitro* development of human embryos (Menezo *et al.*, 1990) and resulted in more pregnancies after embryo transfer (Menezo *et al.*, 1992). Furthermore, co-

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culture of bovine embryos with non-proliferating, rather than proliferating, Vero cell monolayers resulted in higher rates of development to hatched blastocysts (Carnegie *et al.*, 1997). This was probably due, at least in part, to the fact that these cells remained at a constant cell density throughout the 7–10 day co-culture period and were not competing with the developing embryos as vigorously as would proliferating cells for nutrients present in the culture medium. It was also suggested that the co-culture cells may improve *in vitro* embryonic development as a result of production of the cytokine leukaemia inhibitory factor (LIF) (Carnegie *et al.*, 1997).

LIF is present in the mouse uterus at embryo implantation (Stewart *et al.*, 1992) and both promotes the development to hatched blastocysts of murine eight-cell embryos and supports trophoblastic outgrowth (Lavrinos *et al.*, 1995). Vogiagis *et al.* (1997) reported the presence of this cytokine in ovine endometrium of early pregnancy as well as in day 17 trophoblastic tissue. Furthermore, LIF increased the viability of cultured ovine morulae and blastocysts (Fry *et al.*, 1992), promoted the development of bovine embryos during serum-free culture (Fukui and Matsuyama, 1994) and increased the cryotolerance of bovine blastocysts (Han *et al.*, 1995). LIF is secreted by mitomycin-pretreated Vero cells (Carnegie *et al.*, 1997).

On the basis of the observed beneficial effects of LIF on embryo viability and cryotolerance, the aim of this study was to develop a co-culture protocol that was maximally supportive of Vero cell LIF production during blastocyst formation. The overall aim was to produce embryos capable of surviving the stresses of cryopreservation and subsequent thawing and embryo transfer. The influence of two different protein supplements on the secretion of LIF by proliferation-inactivated Vero cells was examined. In addition to standard fetal bovine serum (FBS) used for Vero cell culture (ATCC recommendations), the protein supplements compared were synthetic serum substitute (SSS) and oestrous cow serum (ECS). SSS is a human serum-derived product (84% human serum albumin and 16% human globulins) that contains no lipid fraction and increases the rate of human embryo growth *in vitro* (Weathersbee *et al.*, 1995), supports the establishment of pregnancy (Desai *et al.*, 1996; Dugan *et al.*, 1997) and is compatible with human embryo cryopreservation (T. B. Pool, personal communication). ECS is the standard protein supplement used for embryo culture in this laboratory and supports high rates of hatched blastocyst formation during embryo co-culture with Vero cells (Carnegie *et al.*, 1997).

The time course of LIF secretion during culture with each of the protein supplements was investigated to determine changes in the concentration of LIF to which embryos might be exposed during co-culture. In addition, the rates of blastocyst formation and post-thaw *in vitro* hatching of embryos obtained using Vero cell co-culture in the presence of SSS and ECS alone or in combination were compared. Finally, pregnancy rates were compared after the transfer of IVM/IVF-derived fresh versus frozen-thawed blastocysts into synchronized recipients using blastocysts from the co-culture protocol determined to be optimal on the basis of *in vitro* criteria.

Materials and Methods

Maintenance of Vero cells

Routine culture of Vero cells (American Type Culture Collection, Rockville, MD) was in medium 199 (M199) (GIBCO Laboratories, Mississauga, ON, Canada) supplemented with FBS (5%; GIBCO), gentamicin (100 mg ml⁻¹; GIBCO) and fungizone (0.63 mg ml⁻¹; GIBCO). Immediately after receipt of the cells, the cell stock was expanded in culture and numerous vials of cells at the second passage were prepared in M199 + 15% (v/v) dimethylsulfoxide and 20% FBS and stored in liquid nitrogen. Each vial of cells was used for a maximum of 20 passages (10 weeks). The cells were grown to confluence and subcultured twice a week using a solution of 0.25% (w/v) trypsin and 5 × 10⁻⁴ mol EDTA l⁻¹ (trypsin/EDTA) prepared in Dulbecco's modified (Ca²⁺/Mg²⁺-free) PBS (D-PBS). In all experiments, the proliferation of confluent monolayers of Vero cells was inactivated by pretreatment (2 h) with mitomycin C (10 mg ml⁻¹ in M199; Sigma, St Louis, MO). Hence, Vero cells used for assessment of LIF secretion or for co-culture with embryos were mitomycin-pretreated non-proliferating cell monolayers.

Measurement of LIF secretion using ELISA

Mitomycin-pretreated Vero cells were established in monolayer culture in M199 + 5% FBS. After overnight culture, the cells were washed and cultured for an additional 72 h under the following conditions. In the first set of experiments, cells were cultured in M199 + 5% FBS (Vero cell maintenance medium) or in modified synthetic oviduct fluid (mSOF) (Takahashi and First, 1992, 1993) + either 2% (v/v) SSS (Immucor Canada Inc., Edmonton, AL) or 10% (v/v) ECS (the two media preparations used for embryo culture in our laboratory). In the second set of experiments, the concentrations of all three serum sources was normalized to 10%. For the second and third sets of experiments, the Vero cell plating density was halved to 7.5 × 10⁴ cells per well, which is used routinely in this laboratory for co-culture with embryos and provided sufficient cells for the measurement of secreted LIF. In the third set, the time-dependent (24–72 h) secretion of LIF was compared in mSOF + either 2% (v/v) SSS or 10% (v/v) ECS. For all assays, the culture supernatants were collected and stored at -20°C before measurement of LIF concentrations using a human LIF ELISA kit (R&D Systems, Minneapolis, MN). Absorbance was determined at 450 and 570 nm using a MicroElisa MR580 plate reader. This assay had a sensitivity of 2 pg LIF ml⁻¹ and a mean intra-assay coefficient of variation of 9.1%. Each experiment was repeated three times (duplicate wells for each treatment group) and a single LIF assay was used to evaluate the three replicates. Duplicate wells were used to measure the concentration of LIF in each sample; LIF was not detected in any media preparations that had not been cultured with Vero cells.

Production of bovine zygotes by IVM/IVF

For all culture procedures involving embryos, M199 was

supplemented with 5% FBS (GIBCO) and 100 µg gentamicin ml⁻¹ (GIBCO). Cumulus-oocyte complexes (COCs) were collected by needle aspiration from follicles of 2–6 mm in diameter on abattoir-derived ovaries. After maturation (22–24 h in M199 containing 10% (v/v) heat-inactivated ECS, 100 µg gentamicin ml⁻¹, 4 µg FSH ml⁻¹ (Folltropin; Vetrepharm Canada Inc., London, ON) and 6 µg LH ml⁻¹ (Lutropin; Vetrepharm) as described by Hasler *et al.* (1995)), COCs with well-expanded cumulus complexes were inseminated with frozen-thawed spermatozoa (2.5×10^5 ml⁻¹) prepared by Percoll discontinuous gradient centrifugation (Hasler *et al.*, 1995). Semen collected from a single bull was used for all experiments; the insemination medium was Tyrode's medium (TALP) + 6 mg BSA ml⁻¹, 2 µg heparin ml⁻¹, 20 µmol penicillamine l⁻¹, 10 µmol hypotaurine l⁻¹ and 2 µmol epinephrine l⁻¹ (Miller *et al.*, 1994). After 18 h, the cumulus cells were removed from the presumptive zygotes by intermittent gentle vortexing over 20 s. The pooled zygotes were then randomly allocated (duplicate wells) to appropriate co-culture treatment groups.

Embryo co-culture and scoring of development

After mitomycin pretreatment, washed Vero cells were collected using trypsin/EDTA, resuspended (1.5×10^5 cells ml⁻¹) in M199 + 5% FBS, allocated (0.5 ml per well) to Nunc four-well culture dishes, and cultured (48 h at 39°C in 5% CO₂ in air) to establish cell monolayers for embryo co-culture. On day 1 of co-culture, all Vero cell monolayers were washed with mSOF containing minimal essential medium amino acids and basal medium Eagle amino acids (GIBCO), glucose (1.39 mmol l⁻¹), glutamine (1 mmol l⁻¹) and 2% (v/v) SSS. The monolayers were equilibrated with this medium (0.5 ml per well) for 2 h before addition of the zygotes.

All embryos were cultured (duplicate wells) for 7 days; 40% of the medium was renewed on day 4 and day 6. At day 7 of co-culture, the rate of development to blastocysts was scored as a percentage of the number of cleaved embryos at day 2. The blastocysts were then divided into two groups. Co-culture of one group was continued to allow measurement of hatching rates by day 10, whereas the other group was cryopreserved in liquid nitrogen and subsequently thawed for *in vitro* assessment of embryo viability. In the first set of experiments, the influence of 7 days of co-culture in the absence of serum or serum derivatives, or in the presence of 2% (v/v) SSS or 10% (v/v) ECS on post-thaw development to hatched blastocysts was investigated. In the second set of experiments, the effect of 2% (v/v) SSS or 2% (v/v) SSS plus 10% (v/v) ECS beginning on day 4 of culture on the *in vitro* rate of blastocyst formation and post-thaw hatching was determined.

Embryo cryopreservation and assessment of post-thaw developmental potential

Embryos at the early blastocyst stage were pre-equilibrated (5–10 min) in 1.5 mol ethylene glycol l⁻¹ + 0.2 mol sucrose l⁻¹ (Emcare freezing medium; ImmunoChemical Products Ltd,

Auckland), loaded into straws and immediately immersed in cold ethanol (–6°C) in a BioCool II programmable freezer (Caltec Scientific, Toronto, ON). The straws were manually seeded and cooled (0.6°C min⁻¹) to –35°C before they were plunged into liquid nitrogen.

For thawing, straws were held for 5 s in air and then for 15 s in a 37°C water bath (Semple *et al.*, 1995). The embryos were expelled directly into 2 ml of M199 + 10% (v/v) ECS (to allow immediate dilution of the cryoprotectant). After additional transfer through two changes of M199 + 10% (v/v) ECS (removal of cryoprotectant plus embryo rehydration), the embryos were co-cultured (39°C and 5% CO₂ in air) with Vero cells for 3 days in M199 + 100 µg gentamicin ml⁻¹ and 10% (v/v) ECS to allow hatching. Development to hatched blastocysts by day 10 with or without freeze-thaw was scored as a percentage of either the number of cleaved embryos (day 2) or blastocysts (day 7), depending on the comparisons to be made. In additional experiments, individual embryos produced by Vero cell co-culture (mSOF + 2% (v/v) SSS (day 1 to day 4) and mSOF + 2% (v/v) SSS and 10% (v/v) ECS (day 4 to day 7)) were either transferred immediately ($n = 19$) as early blastocysts to synchronized recipients or transferred directly ($n = 36$) after freeze-thaw, as described earlier. Pregnancies were initially assessed (day 26) and subsequently confirmed (day 45) by ultrasound and the sex of each fetus (first experiment only) was determined by ultrasound at day 60 of gestation.

Statistical analysis

With the exception of the pregnancy and fetal sexing data, all data were evaluated using analysis of variance (ANOVA), and significance of difference between individual means was determined using Duncan's multiple-range test. For those experiments in which variances were dissimilar between treatment groups, the data were transformed to natural logarithms before statistical evaluation. Data from pregnancy rates and fetal sex determination were analysed using the chi-squared test.

Results

Secretion of LIF by Vero co-culture cells

Mitomycin-pretreated Vero cells secreted LIF under all of the culture conditions examined. However the amount of secretion was influenced by composition of the medium (Fig. 1). M199 + 5% FBS is the established maintenance medium for this cell line; culture for 72 h in this medium was associated with a mean LIF secretion of 13.1 ± 0.9 pg per 10^5 cells. mSOF is the medium used routinely in this laboratory for the support of *in vitro* embryo development. Cells cultured in mSOF + 2% (v/v) SSS secreted 2.3 ± 0.9 pg LIF per 10^5 cells during 72 h of culture, whereas secretion in cells maintained in mSOF + 10% (v/v) ECS was tenfold higher (24.7 ± 6.2 pg per 10^5 cells; $P < 0.05$) (Fig. 1). Experiments in which both proteins were used at 10% confirmed that the observed difference in LIF secretion was the result of the

Fig. 1. Influence of medium composition on the secretion of leukaemia inhibitory factor (LIF) by Vero cells during 72 h of culture. □, Medium 199 + 5% fetal bovine serum (FBS); ▨, modified synthetic oviduct fluid (mSOF) + 2% (v/v) synthetic serum substitute (SSS); ■, mSOF + 10% (v/v) oestrous cow serum (ECS). For each experiment duplicate wells were plated at 1.5×10^5 cells per well. Each bar is the mean $\pm$ SEM of three experiments. Statistically significant differences between values ($P < 0.05$) are indicated by different letters.

Fig. 2. Influence of type of protein supplement on the secretion of leukaemia inhibitory factor (LIF) by Vero cells (7.5×10^4 cells per well) during 72 h of culture in modified synthetic oviduct fluid (mSOF). □, Medium 199 + 10% fetal bovine serum (FBS) (control); ▨, modified synthetic oviduct fluid (mSOF) + 10% (v/v) synthetic serum substitute (SSS); ■, mSOF + 10% (v/v) oestrous cow serum (ECS). Each bar is the mean $\pm$ SEM of three experiments (duplicate wells per experiment). Letters indicate values that are significantly different ($P < 0.01$).

different types of protein, rather than different amounts. The pattern of secretion in response to medium composition was identical to that for the earlier experiments (Fig. 2 versus Fig. 1). The concentration of LIF in mSOF + 10% (v/v) ECS (93.5 ± 16.3 pg LIF per 10^5 cells) was sevenfold higher (13.6 ± 3.1 pg per 10^5 cells; $P < 0.01$) than that in mSOF + 10% (v/v) SSS, and threefold higher (28.4 ± 1.2 pg per 10^5 cells; $P < 0.01$) than that in maintenance medium (M199 + 10% FBS; Fig. 2).

Time course analysis revealed a stepwise increase ($P < 0.05$) in cumulative LIF secretion by Vero cells during 72 h of culture in mSOF + either 2% (v/v) SSS or 10% (v/v) ECS; the amount of LIF increased at least twofold after every 24 h of

Fig. 3. Time course of leukaemia inhibitory factor (LIF) secretion by Vero cells (7.5×10^4 cells per well) cultured in modified synthetic oviduct fluid (mSOF) + either 2% (v/v) synthetic serum substitute (SSS) or 10% (v/v) oestrous cow serum (ECS). □, 24 h; ▨, 48 h; ■, 72 h. Data are expressed as a percentage of secretion at 24 h and are the means $\pm$ SEM of three experiments (duplicate wells). Medium LIF concentrations at 24 h were 2.78 ± 0.74 and 4.39 ± 1.54 pg per 10^5 cells, respectively, for the mSOF + SSS and mSOF + ECS treatment groups. Statistically significant differences between values are indicated by different letters (a,b: $P < 0.05$; c: $P < 0.01$).

Table 1. Influence of protein supplements on the cryotolerance of bovine blastocysts produced by co-culture with Vero cells

Treatment group	Percentage of hatched blastocysts*			
	10% ECS	No serum	2% SSS	10% SSS
Fresh	82.3 $\pm$ 3.6 ^a	79.7 $\pm$ 6.2 ^a	86.6 $\pm$ 1.7 ^a	66.9 $\pm$ 6.9 ^b
Frozen	2.2 $\pm$ 2.2 ^d	42.6 $\pm$ 4.9 ^c	62.3 $\pm$ 4.7 ^b	63.4 $\pm$ 3.4 ^b

ECS, oestrous cow serum; SSS, synthetic serum substitute. Values are mean $\pm$ SEM of three experiments involving a total of 361 day 7 blastocysts. Blastocysts were randomly divided into two groups and cultured for three additional days (with or without intervening cryopreservation in liquid nitrogen) to hatched blastocysts.

*Expressed as a percentage of cleaved embryos (day 2 of co-culture).
^{a-d}Means with different superscripts are significantly different ($P < 0.01$).

culture (Fig. 3). LIF secretion was consistently higher ($P < 0.05$) during culture in mSOF + ECS compared with mSOF + SSS. Although the amount of LIF increased steadily during Vero cell culture in mSOF + SSS, the concentration at 48 and 72 h of culture was 27% ($P < 0.05$) and 22% ($P < 0.01$), respectively, of that measured in Vero cell-conditioned ECS-supplemented mSOF (Fig. 3).

Influence of co-culture conditions on embryo viability with or without cryopreservation

Co-culture of bovine zygotes in the presence of 10% (v/v) ECS (day 1 to day 7) produced blastocysts with very poor cryotolerance (post-thaw development to hatched blastocysts $2.2 \pm 2.2\%$ versus $82.3 \pm 3.6\%$ for fresh blastocysts, $P < 0.01$; Table 1). This rate was increased almost 20-fold ($P < 0.01$) by

Table 2. Influence of protein supplementation during bovine embryo co-culture on the rate of hatched blastocyst production (blastocyst formation $\times$ post-thaw hatching)

Treatment group	Percentage of blastocysts (day 7)*	Percentage of hatched blastocysts (after thawing)	Percentage embryo production (percentage blastocysts $\times$ percentage hatched)
2% SSS throughout	27 $\pm$ 4 ^a	61 $\pm$ 16 ^a	18 $\pm$ 7 ^a
2% SSS + 10% ECS (day 4)	47 $\pm$ 2 ^b	64 $\pm$ 11 ^a	30 $\pm$ 5 ^a

ECS, oestrous cow serum; SSS, synthetic serum substitute.

Values are mean $\pm$ SEM of three experiments involving a total of 575 presumptive zygotes from which 433 cleaved embryos were obtained by day 2 of co-culture.

Half of the blastocysts obtained on day 7 of co-culture (103) underwent cryopreservation and the remainder continued in co-culture as controls.

*Expressed as percentage of cleaved embryos (day 2 of co-culture).

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

Table 3. Pregnancy rates after transfer of fresh versus frozen bovine blastocysts*

Treatment group	Number transferred [†]		Percentage pregnancies [‡]		Mean percentage pregnancies
	Trial 1	Trial 2	Trial 1	Trial 2	
Fresh	9	10	56	40	48
Frozen	20	16	70	50	60

*Vero cell co-culture in modified synthetic oviduct fluid (mSOF) + synthetic serum substitute (SSS) (day 1 to day 4) and mSOF + SSS and oestrous cow serum (ECS) (day 4 to day 7).

[†]A single embryo was transferred to each of 29 (Trial 1) and 26 (Trial 2) recipients.

[‡]Pregnancy determined at day 26 and confirmed at day 45 by ultrasound.

removal of the serum from the co-culture medium and was increased further to at least 60% ($P < 0.01$) by replacing ECS with SSS (2 or 10%). The post-thaw rate of hatched blastocyst formation of $> 60\%$ was maintained when embryos were co-cultured with 2% (v/v) SSS from day 1 to day 4 and exposed to 10% (v/v) ECS beginning on the afternoon of day 4 (Table 2).

This two-step medium supplementation protocol was selected because it was observed that the initial rates of blastocyst formation *in vitro* were influenced markedly by the regimen used for protein supplementation of the co-culture medium. Only 27% of embryos co-cultured in medium containing 2% (v/v) SSS as the sole protein supplement throughout the 7 day culture period had formed blastocysts by day 7 (Table 2). Similar rates were obtained in the presence of 10% (v/v) SSS (J. A. Carnegie, J. J. Morgan, N. McDiarmid and R. Durnford, unpublished). However, this rate increased almost twofold ($P < 0.05$) when additional protein supplementation of the medium with 10% (v/v) ECS was initiated on day 4 of co-culture (Table 2). In the two-step protein supplementation regimen, similar post-thaw survival rates were obtained compared with co-culture in SSS (61–64%; Table 1) and high initial rates of blastocyst formation were obtained when supplementation with ECS was initiated on day 4 (47 $\pm$ 2%). Thus a trend toward higher embryo production (30 $\pm$ 5% versus 18 $\pm$ 7% (SSS alone)) was observed with the two-step regimen.

Pregnancy rates after transfer of cryopreserved bovine embryos

Two trials involving transfer of cryopreserved blastocysts produced by IVM/IVF and the preferred Vero cell co-culture

protocol of mSOF + 2% (v/v) SSS (day 1 to day 4) followed by mSOF + SSS and 10% (v/v) ECS (day 4 to day 7; ECS-group embryos) yielded a mean pregnancy rate of 60% as assessed by ultrasound (initially at day 26 and confirmed at day 45; Table 3). This percentage of *in vivo* embryo viability was at least as high ($P > 0.05$) as that obtained using fresh embryos (48%; Table 3). In the first group of embryos ($n = 29$), fetal sex determination at day 60 revealed an equal distribution ($P > 0.05$) of males (56 and 46%) and females (44 and 54%) for the fresh and frozen transfers, respectively.

Discussion

The *in vitro* production of embryos is a valuable tool for the dairy and beef industries when these embryos can establish viable pregnancies and produce healthy calves after cryostorage. Unfortunately, *in vitro*-derived bovine embryos are more easily damaged by cryopreservation than those developed *in vivo* (Leibo and Loskutoff, 1993; Massip *et al.*, 1995). It was proposed that a potential contributor to the reduced freezing tolerance was exposure to serum-derived lipid during the 7 days required to yield blastocysts (Shamsuddin *et al.*, 1994; Shamsuddin and Rodriguez-Martinez, 1994; Dobrinsky, 1996). Indeed, as has been reported by others (Shamsuddin *et al.*, 1994; Shamsuddin and Rodriguez-Martinez, 1994; Semple *et al.*, 1995), in the present study, bovine embryos produced under serum-free culture conditions had higher post-thaw survival and hatching rates than those produced in serum supplemented media. Bovine embryos produced by culture in the presence of serum have a lower buoyant density than those developing *in vivo*, a feature that has been attributed to a

higher intracellular lipid content (Leibo and Loskutoff, 1993). Furthermore, the removal of lipid from pig and bovine embryos before freezing reduced embryo chilling sensitivity and increased post-thaw survival rates (Nagashima *et al.*, 1994, 1995; Leibo *et al.*, 1995; Diez *et al.*, 1996). However, the absence of serum has also been associated with reduced rates of *in vitro* bovine embryo development to blastocysts (Shamsuddin and Rodriguez-Martinez, 1994; Semple *et al.*, 1995) as well as a lower number of cells per blastocyst (Dinnyes *et al.*, 1996). The aim of the present study was to modify an established Vero cell co-culture protocol (Carnegie *et al.*, 1997) to combine a high tolerance of cryopreservation with reasonable *in vitro* rates of formation of viable blastocysts.

SSS, a 6% human protein solution that was specifically designed as a supplement for the culture of human embryos, increased the *in vitro* rates of development of both mouse and human embryos (Weathersbee *et al.*, 1995; Desai *et al.*, 1996). It has been suggested that the beneficial effect of SSS may be due, at least in part, to the polyhydroxyl domains of the globulins that bind water and convey weak gel-like properties to the microenvironment surrounding the developing embryos (Weathersbee *et al.*, 1995). This saline solution of HSA plus primarily α - and β -globulins contains no lipid component (Weathersbee *et al.*, 1995) and has been associated with high post-thaw survival of human embryos (T. Pool, personal communication). Although, in the present study, embryos cultured for 7 days in mSOF supplemented with SSS alone showed rates of post-thaw viability in excess of 60%, the overall rates of cryopreservable embryo production were decreased by inadequate support of the morula-blastocyst transition by SSS at either 2% (this study) or 10% (J. A. Carnegie, unpublished). However, although embryos exposed to ECS throughout their development from zygotes to blastocysts showed minimal cryotolerance, the addition of ECS to SSS-supplemented mSOF beginning on day 4 of co-culture not only restored the blastocyst formation rate (to almost 50%) but also produced blastocysts that could be frozen successfully.

In addition to limiting exposure to serum-derived lipid until most embryos had progressed well beyond the four-cell stage, this two-step protocol for the production of cryopreservable blastocysts may also derive its success from the influence of the different culture supplements on the amount of LIF secretion by the Vero cell feeder layer. LIF is secreted by both human and murine uterine endometrial tissue at blastocyst hatching and implantation (Bhatt *et al.*, 1991; Shen and Leder, 1992; Charnock-Jones *et al.*, 1994; Kojima *et al.*, 1994; Cullinan *et al.*, 1996). Furthermore, the presence of LIF receptor mRNA in human blastocysts has been demonstrated by RT-PCR (Charnock-Jones *et al.*, 1994) and a role for LIF in stimulating blastocyst implantation was proposed on the basis of a measured stimulatory influence of this cytokine on the differentiation of purified human cytotrophoblasts toward an anchoring trophoblastic cell phenotype that expresses fibronectin (Nachtigall *et al.*, 1996). In addition to promoting the viability of ovine embryos (Fry *et al.*, 1992), the transitory production of LIF in mice has been implicated in the support of primitive endoderm formation in the embryo, while at the same time temporarily inhibiting

further differentiation of primitive ectoderm and mesoderm (Shen and Leder, 1992). Exposure of mouse eight-cell embryos to LIF during culture increased the proportion of embryos capable of completing hatching and was also associated with more vigorous trophoblastic outgrowth *in vitro* (Lavranos *et al.*, 1995). Finally, the addition of LIF to cultured bovine embryos stimulated development more effectively when commenced at the stage of morula formation, rather than earlier (Fukui and Matsuyama, 1994; Carnegie *et al.*, 1997), and the presence of LIF during the final 2–3 days of culture before freezing significantly increased post-thaw hatching rates for IVF-derived bovine blastocysts (Han *et al.*, 1995).

Support for the contention of an embryotrophic role for feeder cell-derived LIF comes from experiments using buffalo rat liver cells and STO cells (murine embryonic fibroblastic cell line), two cell lines frequently used for the derivation and maintenance of embryonic cell lines because of their ability to secrete LIF (for example, Robertson, 1987; Smith and Hooper, 1987; Gough *et al.*, 1989). Buffalo rat liver cells supported the *in vitro* development of bovine IVM/IVF-derived bovine oocytes to hatched blastocysts (Rehman *et al.*, 1994), whereas STO cells performed a comparable role for ovine embryos (Rexroad and Powell, 1993). Previous studies have shown that there is greater sequence homology between human and ovine sources of LIF than between ovine and murine sources of this cytokine (Fry, 1992). Hence Vero cells may be a more appropriate feeder layer for bovine embryos developing *in vitro* by providing a primate rather than a rodent source of LIF.

Carnegie *et al.* (1997) reported the secretion of LIF by mitomycin-pretreated Vero cells cultured in M199 + 5% FBS, the recommended maintenance medium for this cell line, and this medium was included as a positive control in the present study. Vero cell monolayers cultured in mSOF + 2% (v/v) SSS also secreted measurable amounts of this cytokine in this study. However, the approximately tenfold greater secretion of LIF by Vero monolayers cultured in ECS-supplemented medium indicates that developing bovine embryos cultured according to the two-step protocol would be exposed to considerably higher concentrations of the cytokine during the latter period of co-culture when they are undergoing cavitation to form blastocysts and when, *in vivo*, they would normally be entering the uterus. These differences in LIF secretory activity were not a reflection of the different amount of protein supplementation of the mSOF (2% (v/v) SSS versus 10% (v/v) ECS), but appeared to be the result of the influence of protein supplement composition on the Vero cells. ECS in particular was associated with a high LIF secretion by Vero cell monolayers. In agreement with an earlier report from this laboratory (Carnegie *et al.*, 1997), Menck *et al.* (1997) also reported Vero-cell support of *in vitro* bovine embryo development that was equivalent to or better than that obtained using co-culture with bovine oviductal epithelial cells. Their co-culture protocol used Menezo B₂ medium supplemented with 10% FBS (day 1 to day 7) during co-culture with proliferating Vero cells and did not involve renewal of the medium throughout the co-culture period. Hence, although direct comparison cannot be made due to the numerous differences in protocol, it should

be noted that the rate of blastocyst formation in the presence of Vero cells was closer to that obtained with 2% (v/v) SSS alone throughout co-culture, compared with the two-step system involving introduction of ECS at day 4. In mice, the two peaks in uterine LIF secretion, one at ovulation and the second at day 3.5 of gestation, occur at times of increased circulating concentrations of oestradiol and it has been suggested that oestradiol may regulate uterine secretion of LIF (Shen and Leder, 1992). Hence, it is possible that a similar effect of oestrogen, a steroid present in blood at high concentration at oestrus, is responsible for the marked stimulatory effect of ECS on Vero cell LIF secretion. Although it remains to be determined whether Vero cells bind oestrogen, the expression of oestrogen receptor α mRNA has been demonstrated in the rat kidney (Kuiper *et al.*, 1997).

The ultimate test of an embryo culture and cryo-preservation protocol is its ability to support the establishment of viable pregnancies that yield live young after thawing and embryo transfer. The pregnancy rates obtained using frozen embryos in this study are comparable with those reported in the literature (for example, Farin and Farin, 1995; Massip *et al.*, 1996) and indicate that the co-culture protocol used enables embryos of sufficient quality to withstand the stresses imposed by exposure to cryoprotectants and low temperature.

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