

EFFECTS OF HYALURONIC ACID IN CULTURE AND CYTOCHALASIN B TREATMENT BEFORE FREEZING ON SURVIVAL OF CRYOPRESERVED BOVINE EMBRYOS PRODUCED IN VITRO

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SUMMARY

One limitation to the widespread use of in vitro–produced embryos in cattle is their poor survival following cryopreservation. Two approaches for enhancing survival of in vitro–produced bovine embryos following cryopreservation were evaluated: culture in the presence of hyaluronic acid and alterations in the cytoskeleton through cytochalasin B treatment. The experiment was a 2×2 factorial design to test main effects of hyaluronic acid added to culture at day 5 after insemination (+ or –) and cryopreservation treatment (control or cytochalasin B). Embryos used for cryopreservation were blastocysts and expanded blastocysts harvested on day 7 after insemination. Cytochalasin B increased the percent of embryos that re-expanded ($P < 0.0001$) and that hatched following thawing ($P < 0.05$). The hatching percent was 29.6% for embryos treated with cytochalasin B versus 9.1% for control embryos. There was no significant effect of hyaluronic acid on survival although there was a tendency for embryos cultured with hyaluronic acid to have higher percent hatching if not treated with cytochalasin B (12.7% for hyaluronic acid versus 4.5% for control; hyaluronic acid \times cytochalasin B interaction; $P = 0.09$). In conclusion, cytochalasin B treatment before freezing improved cryosurvival of bovine embryos produced in vitro. Such a treatment could be incorporated into methods for cryopreservation of bovine embryos provided post-transfer survival is adequate. In contrast, culture with hyaluronic acid was of minimal benefit—the increased cryosurvival in the absence of cytochalasin B was not sufficient to allow an adequate number of embryos to survive.

Key words: cytochalasin B; hyaluronic acid; cryopreservation; embryo; in vitro fertilization.

INTRODUCTION

In vitro production of embryos is an important tool for improving genetic merit and fertility of cattle and is an indispensable component of other technologies such as somatic cell cloning and transgenesis (Hansen and Block, 2004). One limitation to the widespread use of in vitro–produced embryos in the cattle industry is the poor survivability of cryopreservation by in vitro–produced embryos. In vitro survival rates following thawing (Pollard and Leibo, 1993; Guyader-Joly et al., 1999; Enright et al., 2000; Khurana and Niemann, 2000a; Diez et al., 2001) and pregnancy rates following thawing and transfer (Hasler et al., 1995; Agca et al., 1998; Ambrose et al., 1999; Al-Katanani et al., 2002) are consistently lower for embryos produced in vitro when compared to embryos produced in vivo by superovulation.

The poor survival of the in vitro–produced embryo is associated with culture-induced changes in ultrastructure (Rizos et al., 2002), gene expression (Bertolini et al., 2002; Lazzari et al., 2002; Lonergan et al., 2003), and metabolism (Krisner et al., 1999; Khurana and Niemann, 2000b) that make it distinct from the embryo produced in vivo. Among the metabolic changes are an increase in lipid content (Abe et al., 1999; Rizos et al., 2002), a condition that

has been linked to poor freezability. Mechanical delipidation (Tomimaga et al., 2000; Diez et al., 2001) and addition of inhibitors of fatty acid synthesis (De La Torre-Sanchez et al., 2005) can improve survival following cryopreservation.

In the current study, two approaches for enhancing survival of bovine embryos following cryopreservation were evaluated. The first was to culture embryos in the presence of hyaluronic acid. This un sulphated glycosaminoglycan is present in follicular, oviductal, and uterine fluids in several species, including cattle (Lee and Ax, 1984). Receptors for hyaluronic acid (CD44) have been reported on the bovine oocyte, cumulus cell, and preimplantation-stage embryo (Valcarcel et al., 1999). Addition of hyaluronic acid to culture medium has been reported to increase blastocyst re-expansion and hatching after freezing (Stojkovic et al., 2002; Lane et al., 2003). The second approach was to determine whether altering the cytoskeleton before cryopreservation would enhance embryo survival. The rationale for this treatment is that cryoinjuries such as intracellular ice formation and osmotic shock induce irreversible disruption in microtubules and microfilaments (Kuwayama et al., 1994; Fair et al., 2001) and that temporary depolymerization of actin microfilaments before cryopreservation could reduce cytoskeletal damage and plasma membrane fracture caused by alterations in cytoskeletal architecture (Dobrinsky, 1996). Addition of cytochalasin B to cause actin depolymerization had no effect on survival of

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TABLE 1

EFFECT OF HYALURONIC ACID ADDED AT DAY 5 AFTER INSEMINATION ON PRODUCTION OF BLASTOCYSTS AT DAY 7 AND 8 AFTER INSEMINATION.^{a,b}

Culture medium	Number of oocytes	Percent cleaved	Blastocysts/oocyte (%) ^c	Blastocysts/ cleaved embryo (%) ^c	Percent of total blastocysts that were collected at day 7
Control	1935	76.0 ± 0.9	36.0 ± 1.2*	47.2 ± 1.3**	68.8 ± 2.4
Hyaluronic acid	3087	77.7 ± 0.9	31.5 ± 1.2	40.7 ± 1.3	62.2 ± 2.4

^a n = 18 replicates^b Means within a column that differ significantly are indicated by *(*P* < 0.05) and **(*P* < 0.01)^c Includes blastocysts collected at day 7 and those collected at day 8.

eight-cell embryos in the mouse (Prather and First, 1986) but enhanced survival of expanded and hatched blastocysts without affecting survival of morula and early blastocysts in the pig (Dobrin-sky et al., 2000).

MATERIALS AND METHODS

Embryo production. Procedures, reagents, and media formulation for oocyte maturation, fertilization, and embryo culture were as previously described (Roth and Hansen, 2005) with some modifications. Briefly, cumulus oocyte complexes (COCs) were harvested from ovaries of a variety of breeds collected at a local abattoir located at a travel distance of approximately 1.5 h from the laboratory. The COCs were matured in tissue culture medium-199 with Earle's salts supplemented with 10% (v/v) steer serum, 2 µg/ml estradiol 17-β, 20 µg/ml follicle stimulating hormone, 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin, and an additional 1 mM glutamine for approximately 22 h at 38.5° C in an atmosphere of 5% (v/v) CO₂ in humidified air. Insemination with a cocktail of Percoll-purified spermatozoa from three different bulls was performed in vitro fertilization-Tyrode's albumin lactate pyruvate (TALP) solution. At 8–12 h postinsemination (hpi), putative zygotes were denuded of cumulus cells by suspension in *N*-2-hydroxyethylpiperazine-*N*-2-ethane-sulfonic acid (HEPES)-TALP medium containing 1000 units/ml hyaluronidase type IV (Sigma Chemical Co., St Louis, MO) and vortexing in a microcentrifuge tube for 5 min. Presumptive zygotes were then placed in groups of ~30 in 50-µl microdrops of a modified synthetic oviductal fluid (SOF) prepared as described by Fisher-Brown et al. (2002). Embryos were cultured at 38.5° C in a humidified atmosphere of 5% (v/v) CO₂, 5% O₂, and with the balance N₂. Blastocysts were collected for cryopreservation on day 7 after insemination.

Experimental design and embryo manipulation. The experiment was a 2 × 2 factorial design to test main effects of hyaluronic acid during culture (+ or -) and cytochalasin B before cryopreservation (+ or -). Data on development were obtained from 18 replicates using 5022 oocytes; data on cryopreservation were obtained from 7 replicates using a total of 197 blastocysts.

Following insemination and transfer to fresh microdrops, embryos cultured without hyaluronic acid were cultured in SOF for 7 d beginning after insemination. Embryos treated with hyaluronic acid were cultured in SOF until day 5, when all embryos were transferred to a fresh microdrop of SOF containing 6 mg/ml hyaluronic acid from *Streptococcus zooepidemicus* (Sigma).

Blastocysts and expanded blastocysts were harvested on the morning of day 7 after insemination and washed twice in holding medium consisting of HEPES-TALP (Parrish et al., 1986) containing 10% (v/v) fetal calf serum (FCS). Embryos treated with cytochalasin B were incubated for 10 min at 38.5° C in air while in HEPES-TALP containing 10% (v/v) FCS and 7.5 µg/ml cytochalasin B (Sigma) in a 1.5-ml microcentrifuge tube (Tominaga et al., 2000). Cytochalasin B was initially dissolved in dimethyl sulfoxide at a concentration of 5 mg/ml and was then added to HEPES-TALP to achieve a final concentration of 7.5 µg/ml. Control embryos were incubated similarly in HEPES-TALP containing 10% (v/v) FCS.

Cryopreservation. Procedures for freezing were modified from those reported elsewhere (Hasler et al., 1995; Enright et al., 2000). In brief, blastocysts were transferred in groups of 10 to a fresh 100-µl microdrop of HEPES-TALP containing 10% FCS at 38.5° C for the time it took to harvest all

embryos (~ 10 min). Next, embryos in groups of 5–8 per treatment (hyaluronic acid or control) were randomly selected to receive cytochalasin B treatment before freezing or not as described above. Afterwards, each group of 5–8 embryos was placed in a 50-µl microdrop of 10% (v/v) glycerol in Dulbecco's phosphate-buffered saline (DPBS) containing 0.4% (w/v) bovine serum albumin (freezing medium) in a grid plate over a slide warmer at 30° C. Within 10 min, embryos were loaded in a 50-µl volume into 0.25-ml plastic straws (Agtech, Manhattan, KS). Up to 8 embryos were loaded in each straw. Two columns of 50-µl freezing medium separated by air bubbles were always placed above and below the column of embryos. Straws were transferred to a freezing chamber (Cryologic Model CL5500, Mulgrave, Victoria, Australia) for 2 min at -5° C and ice crystals were induced by touching the straw where the top column of medium resided with a cotton plug that had been immersed in liquid nitrogen. After an additional 3 min at -5° C, embryos were cooled to -32° C at a rate of -0.6° C/min. After 2 min at -32° C, straws were directly immersed in liquid N₂ and stored until thawing (4 days–1 wk later).

Thawing and determination of survival. Straws containing embryos were thawed by warming for 10 s in air at room temperature and 20 s in a 32° C water bath. All subsequent steps before culture were performed with media prewarmed to ~ 30° C and with dishes placed on a slide-warmer set at 30° C. Embryos were then expelled into an empty petri dish and immediately transferred to a fresh 60 µl-drop of DPBS containing 6.6% (v/v) glycerol and 0.3 M sucrose in an grid dish. After 5 min, embryos were sequentially transferred to DPBS containing 3.3% (v/v) glycerol and 0.3 M sucrose for 5 min and DPBS + 0.3 M sucrose for 5 min. Embryos were then washed three times in HEPES-TALP + 10% (v/v) FCS and placed into culture in groups of 5–8 in 25-µl microdrops of SOF containing 10% (v/v) FCS. Culture was at 38.5° C in a humidified atmosphere of 5% (v/v) CO₂, 5% O₂, and 90% N₂. Re-expansion was determined at 48 h after thawing and hatching at 72 h.

Statistical analysis. Data on the proportion of oocytes that cleaved and the proportion of embryos that developed to the blastocyst stage on day 7 and day 8 were determined for each replicate. Treatment effects were determined by least-squares analysis of variance using the GLM procedure of SAS (SAS for Windows 90, Cary, NC). The model included the main effects of replicate and treatment. Data for the proportion of frozen/thawed embryos that re-expanded and the proportion that hatched by 72 h of culture were analyzed using the CATMOD procedure of SAS. The initial model included all main effects and two-way interactions. After removing nonsignificant effects, the final model included replicate, hyaluronic acid, preparation before freezing (none, cytochalasin B), and the interaction of hyaluronic acid and preparation before freezing.

RESULTS

Effect of hyaluronic acid on embryonic development. As shown in Table 1, addition of hyaluronic acid at day 5 after insemination caused a slight reduction in the yield of blastocysts on day 7 and day 8 after insemination regardless of whether data were expressed as the proportion of oocytes developing to the blastocyst stage (*P* < 0.05) or the proportion of cleaved embryos developing to the blastocyst stage (*P* < 0.01). Of the blastocysts that were recovered, 62–68% were recovered at day 7 and the balance at day 8. There

TABLE 2

EFFECT OF CULTURE IN HYALURONIC ACID AND TREATMENT WITH CYTOCHALASIN B ON SURVIVAL AFTER CRYOPRESERVATION.^a

Culture medium	Cytochalasin treatment	Re-expansion by 72 hr ^b	Hatching by 72 hr
Control	Control	8/44 (18.2%)	2/44 (4.5%)
Control	Cytochalasin B	22/43 (51.2%)	17/43 (39.5%)
Hyaluronic acid	Control	16/55 (29.0%)	7/55 (12.7%)
Hyaluronic acid	Cytochalasin B	26/55 (47.3%)	12/55 (21.8%)

^a Data are the fraction of embryos, and in parentheses, percent. Number of replicates was seven.

^b Effect of cytochalasin B ($P < .0001$).

^c Effect of cytochalasin B ($P < 0.05$), hyaluronic acid ($P < 0.10$), and the cytochalasin B \times hyaluronic acid interaction ($P = 0.09$).

was no effect of hyaluronic acid on the proportion of blastocysts collected at day 7 (Table 1).

Survival after cryopreservation. Overall, cytochalasin B increased the percent of embryos that re-expanded following thawing ($P < 0.0001$) and that hatched following thawing ($P < 0.05$) (Table 2). Overall re-expansion rates were 49.0% (48/98) for embryos treated with cytochalasin B and 24.2% (24/99) for embryos not subjected to cytochalasin B. Hatching rates were 29.6% (29/98) for embryos treated with cytochalasin B and 9.1% (9/99) for embryos not subjected to cytochalasin B.

Although there was no significant effect of hyaluronic acid on cryosurvival, there was a tendency ($P = 0.09$) for a hyaluronic acid \times cytochalasin B interaction affecting percent of blastocysts that hatched following thawing. This interaction reflects the fact that hyaluronic acid increased the percentage of hatching for embryos not subjected to cytochalasin B treatment and decreased the percentage of hatching for embryos subjected to cytochalasin B.

DISCUSSION

Of the two treatments evaluated for enhancing cryosurvival of in vitro-produced bovine embryos, cytochalasin B treatment was the most effective as determined by an improvement in both embryo re-expansion and hatching. The rationale for this treatment is to reduce cellular injury caused by disruption in microtubules and microfilaments (Kuwayama et al., 1994; Fair et al., 2001) and to increase flexibility of the plasma membrane to allow it to tolerate forces associated with freezing that lead to membrane damage. In other studies, addition of cytochalasin B had no effect on survival of eight-cell embryos in the mouse (Prather and First, 1986), enhanced survival of expanded and hatched pig blastocysts without affecting survival of morula and early blastocysts (Dobrinsky et al., 2000), and improved survival of in vivo derived bovine blastocysts subjected to vitrification (Dobrinsky et al., 1995).

For embryos not exposed to cytochalasin B, there was a tendency for those cultured in hyaluronic acid to have a higher re-expansion rate and hatching rate than embryos cultured without hyaluronic acid. Both Stojkovic et al. (2002) and Lane et al. (2003) reported improved survival rates to freezing when embryos were cultured in hyaluronic acid; such a beneficial effect has not always been observed (Furnus et al., 1998s). Surprisingly, embryos cultured in hyaluronic acid were less likely to survive freezing than were control

embryos when the cytochalasin B treatment was applied. Perhaps physiological changes induced by hyaluronic acid cause the embryo to be less able to adjust to the cellular actions of cytochalasin B. Those changes are potentially numerous because hyaluronic acid acts to affect cell function through several means, including signaling through cell surface receptors, modifying the biophysical properties of extracellular and pericellular matrices by attracting water, and interacting physically with a variety of ions and other molecules (Laurent, 1987; Ruoslahti and Yamaguchi, 1991; Hardingham and Fosang, 1992; Yasuda et al., 2002; Toole et al., 2005). One possible mechanism by which hyaluronic acid could increase embryo survival to freezing is by increasing the total number of cells in the embryo (Stojkovic et al., 2002; Jang et al., 2003; Kim et al., 2005).

One unexpected finding was the reduction in the percentage of hyaluronic acid-treated embryos that became blastocysts. In other studies, hyaluronic acid either had no effect (Stojkovic et al., 2002; Lane et al., 2003) or caused an increase in blastocyst yield (Furnus et al., 1998; Jang et al., 2003). Differences in origin and concentration of hyaluronic acid could explain some of this difference between studies. Hyaluronic acid can be isolated from different sources (e.g., bacteria, rooster comb, and umbilical cord) and preparations can differ in protein, endotoxin, and nucleotide content (Shiedlin et al., 2004). Stojkovic et al. (2002) reported that preliminary results indicated that embryo development in vitro was dependent upon the origin of the commercially available hyaluronic acid. Also, however, embryos cultured with hyaluronic acid experienced a change in culture medium at day 5 whereas control embryos did not. Such a difference could have obscured beneficial effects of hyaluronic acid although another paper indicates no effect of changing culture medium at 72 hpi on blastocyst yield in cattle (Ikeda et al., 2000).

The percent of embryos that underwent hatching after freezing in glycerol and thawing has varied from 0% (Enright et al., 2000) to 22% (Diez et al., 2001; Nedambale et al., 2004), 32% (Guyader-Joly et al., 1999), and 69% (Hasler et al., 1997). The best survival achieved in this study was for embryos cultured without hyaluronic acid and treated with cytochalasin B. In this group, 51.2% of cryopreserved embryos were capable of re-expansion and 39.5% hatched. It is likely that the percentage of hatched embryos can be further improved by modifying postthaw culture conditions. Massip et al. (1993) found hatching rates for frozen/thawed, in vitro-produced embryos were 41% when culture was performed in the presence of bovine oviductal epithelial cells, whereas hatching rate using other culture conditions not involving coculture was 0–6%. Nonetheless, one would not expect optimal pregnancy rates to be achieved following direct transfer of embryos frozen in glycerol even with the inclusion of cytochalasin B treatment. Rather, it is suggested that pregnancy rates following transfer of embryos cryopreserved using slow-freezing procedures can be optimized by selecting embryos for transfer based on development in culture after thawing.

In contrast to the poor survival of in vitro-produced embryos frozen using conventional slow-freezing techniques, several experiments indicate that cryosurvival can be enhanced by using vitrification (Vajta, 2000). It remains to be tested whether survival of embryos produced in vitro after vitrification can be improved by cytochalasin B treatment. There was a beneficial effect of cytochalasin B treatment on cryosurvival of embryos derived in vivo following vitrification (Dobrinsky et al., 1995).

In conclusion, cytochalasin B treatment before freezing improved cryosurvival of bovine embryos produced in vitro and subjected to slow-freezing in glycerol. Such a treatment could be incorporated into methods for cryopreservation of bovine embryos provided post-transfer survival is adequate. In contrast, culture with hyaluronic acid was of minimal benefit—the increased cryosurvival in the absence of cytochalasin B was not sufficient to allow an adequate number of embryos to survive.

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