Effect of chilling on the development of in vitro produced bovine embryos at various cleavage stages

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Purpose: Bovine embryos and zygotes are known to be sensitive to “temperature shock” when cooled to temperatures near 0°C. The effect of chilling on in vitro derived embryos at various cleavage stages was investigated.

Methods: Cumulus-oocyte-complexes (COCs) were matured in IVM medium with serum. Presumptive zygotes were cultured in serum free in vitro culture (IVC) medium. Embryos were used as chilled or control samples at the 2-cell, 4-cell, 8-cell, morula, and blastocyst stages. Embryos in 0.2 mL PBS in plastic straws were cooled rapidly in ethanol baths at 0°C for 30 min. Embryo viability was assessed by in vitro development.

Results: The percentage of control embryos that hatched as blastocysts increased the later stage at which they were selected. Relative proportion of embryos increased from 28% to 48% to 68% when chilled at the 8-cell, morula or blastocyst stages.

Conclusions: IVF-produced embryos are differentially susceptible to cooling injury. Cell counts made of those blastocysts formed from chilled embryos indicated subtle effects of chilling.

KEY WORDS: Bovine; cell number; chilling; IVF embryos; viability.

INTRODUCTION

In the livestock industry, effective exploitation of genetically valuable embryos relies on the development of a suitable technique for the low temperature storage of embryos. Because of the inherent practical value of embryo freezing as well as its intellectually provocative nature, the cryobiology of mammalian embryos has been studied intensively (1). In recent years, the capability to produce in vitro cattle em-

bryos reliably from ovarian oocytes has led to the availability of all stages of early embryos from 2-cell to morulae (2), whereas previously, they could only be recovered surgically. Availability of such early cleavage stage embryos has led to attempts to manipulate and freeze them. The damage of many types of cells when exposed to temperatures close to 0°C in the absence of freezing has been referred to as “temperature shock” (3). Later it was referred to as “direct chilling injury” or “cold shock” (4). Recently, the term “chilling sensitivity” has been applied to the effects of low temperatures on embryos of several non-mammalian species (5,6).

The practical value of a technique to chill cattle embryos without damaging them would lie in its facilitating their movement from one geographical region to another. Most freeze thaw procedures for embryos result in the loss of some cells. Therefore, the option of chilling embryos to 0–5°C or storing them at ambient temperature without injury might be a

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viability alternative (2,7). Since mouse embryos at all cleavage stages are not damaged by chilling, it has been possible to store them for many days at 0°C without freezing (8).

The abnormal development of embryos when maintained in non-nutrient media (e.g., PBS) for any extended period at room temperature or higher can be prevented by chilling them (those beyond a certain stage of development) to 0–4°C (9). In most instances, early in vitro derived blastocysts suspended in PBS fail to survive rapid or slow cooling to 0°C (10).

Work at Cambridge during the 1970s showed a clear relationship between an embryo's quality and developmental stage and its ability to survive chilling and freezing (11,12). In cattle, embryos collected before compaction of the blastomeres were sensitive to cooling below 7.5°C. Early bovine blastocysts were apparently resistant to cooling damage whereas pre-blastocyst stages were not (13). Hence, it appears as if the extent and nature of cryoinjury may be dependent on the developmental stage of the embryo and its species (14).

Cooling compact morulae from 30 to 0°C had little or no adverse effect on in vivo embryos, whereas none of the in vitro derived morulae cooled below 15°C survived. This led to the conclusion that fundamental cellular differences may exist between these two categories. The sensitivity cannot be inherent in that embryonic stage, but rather results from the manner in which the embryo itself was derived and produced (15).

With that in view, the present study was undertaken to examine the effect of chilling on the viability of different developmental stages of in vitro produced bovine embryos, and to determine the cell count in blastocysts that developed from in vitro embryos that had been chilled at different developmental stages.

MATERIALS AND METHODS

Chemicals and Reagents

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Source and Collection of Oocytes

Bovine ovaries were obtained from a local abattoir and transported to the laboratory within 3 h

after collection in phosphate-buffered saline (PBS) at 30–34°C. The collection of the cumulus-oocyte-complexes (COCs) was performed in a warm hood (32–36°C) by making cross hatched incisions on the ovarian cortex with sterile razor blades and rinsing the surface with warm 10 mM HEPES buffered Ham's F10 medium supplemented with sodium bicarbonate (1.2 g/L), penicillin (50,000 IU/L), streptomycin (50,000 µg/L), heparin (2,000 IU/L), and 2% steer serum (SS; Cansera Inc., Rexdale, Ontario, Canada).

In Vitro Maturation

COCs having three or more complete layers of cumulus cells and fully grown oocytes with homogenous cytoplasm were rinsed three times in in vitro maturation medium (IVM) and selected for IVM. Groups of 20 COCs were transferred into individual 75 µL droplets of M199 (Earle's salts; Canadian life Technologies, Burlington, Ontario) supplemented with 25 mM HEPES, sodium bicarbonate (2.2 g/L), sodium pyruvate (110 mg/L), penicillin (3,000 IU/L), streptomycin (3,000 µg/L), and 10% SS and cultured for 22–24 h at 38.5°C in humidified 5% CO₂ in air.

In Vitro Fertilization

COCs at 22–24 h of maturation were vortexed for 20 s in 5 mL of HEPES-TALP medium to remove partially their cumulus cells, and were placed into IVF drops (20 oocytes per 75 µL IVF drop) after being rinsed three times with HEPES-TALP supplemented with 2% bovine serum albumin (BSA, Fraction V) and an additional three times with IVF-TALP medium.

In this study, multiple ejaculates of semen from a single bull whose spermatozoa had been frozen in egg yolk-citrate buffer and glycerol solutions were used. Frozen thawed bull semen were washed two times with 10 mL HEPES-TALP medium by centrifugation at 200×g for 10 min, and then resuspended in 0.6–0.8 mL of HEPES-TALP medium. The sperm suspension was gently layered over a wet glass wool column, and allowed to filter by gravity. Then, 10 µL of the filtered sperm suspension were added to each IVF drop to yield a final sperm concentration of $\sim 2 \times 10^6$ sperm/mL and cocultured with bovine oviductal epithelial cells (BOECs) explants in 5% CO₂ in air at 38.5°C. BOECs were prepared by the method of Rieger *et al.* (16).

In Vitro Culture

After 20–24 h of culture with sperm, presumptive zygotes were rinsed with serum-free M199 medium containing 0.1 mg/mL polyvinyl alcohol (PVA, MW 30,000–70,000; Sigma), placed into serum-free IVC drops containing BOEC (20 zygotes per 50 μ L IVC medium) and cultured at 38.5°C in 5% CO₂ in air. Cleavage of zygotes was determined at 48 hpi (Day 2). On Days 3 and 6, the cultures were “fed” with 25 μ L of fresh serum-free IVC medium.

Experimental Procedures

Experiment 1. The effect of chilling on the in vitro viability of in vitro produced bovine embryos at different developmental stages was examined. Depending on the embryo stage, 5–7 replicate experiments were performed. In each replicate, batches of embryos at the 2-cell stage harvested at 30–34 hpi, at the 4-cell stage at 42–46 hpi, at the 8-cell stage at 48–52 hpi, at the morula stage at 142–146 hpi, and at the blastocyst stage at 166–170 hpi were obtained from a single oocyte collection. For each stage, embryos were randomly assigned to two equal groups (control or chilled) without any intentional selection and allocated to a cooling experiment. All procedures of embryo handling were carried out at 30–32°C and monitored. Embryos were first transferred into PBS solution: Dulbecco's Phosphate Buffered Saline (D-PBS) containing 1,000 mg/L of D-glucose, 36 mg/L Na pyruvate, and 5 mg/L phenol red, supplemented with 0.1 g/L of CaCl₂, penicillin (3,000 IU/L), streptomycin (3,000 μ g/L), and 10% SS with an osmolality of 270–290 mOsm and pH adjusted to 7.3–7.4. One group of embryos was loaded into ~0.2 mL D-PBS contained in plastic straws (#A201; IMV, l'Aigle, France) and heat-sealed. Cooling was done by placing the straws directly into a well-stirred ethanol bath in a programmable freezer (BioCool III FTS systems, Stone Ridge, NY) at 0°C for 30 min. During this time, embryos in the control group were maintained in D-PBS at 38.5°C in 5% CO₂ in air. At the end of the cooling period, the straws were removed from 0°C and warmed for 5 s in air followed by 15 s in a 35°C water bath. In both the control and treated groups, the embryos were immediately transferred into equilibrated fresh IVC medium and rinsed three times and placed into 50 μ L droplets of serum-free IVC medium containing the same BOEC explants used to produce embryos. Viability of the embryos after cooling was assessed by in vitro culture. Cleavage

and blastocyst development were evaluated at ~24 h intervals up to Day 8 postinsemination. Blastocysts were cultured for an additional 2 days to record the number that hatched on Day 9 and 10. The hatching rate was calculated as the percentage of all blastocysts present on Day 8 that hatched completely from their zonae on each day. The results of this experiment formed the basis for the second experiment.

Experiment 2. The purpose of this experiment was to assess whether the number of cells of which blastocysts were comprised was influenced by the developmental stage at which embryos were chilled. A minimum of five replicates was carried out for each developmental stage. The cooling procedure was the same as that described for Experiment 1. Expanded blastocysts developed on Day 7 or 8 from 8-cell and morula-stage embryos that had been chilled at ~48 h or ~142 hpi, respectively; hatched blastocysts developed on Day 8 or 9 from blastocysts that had been chilled at ~166 hpi. Expanded or hatched blastocysts were collected and processed for cell counts. Embryos were placed in 1% sodium citrate solution for 4 min at room temperature, transferred onto clean, grease-free glass slides, and 10 μ L of a 1:1 mixture of freshly prepared acetic acid and methanol was dropped onto them; cells were spread by gently blowing on the slide under a lamp until it dried. The slides were fixed overnight in acetic acid and methanol mixture (1:3, v/v) and stained with 4% Giemsa stain for 4 min, air dried, and the total number of cells was counted under a phase contrast microscope.

Statistical Analysis

Comparison of data among the survival of different developmental stages of embryos after chilling was made by one-way ANOVA after arcsine transformation. The mean (SEM) of cell numbers of blastocysts was compared within and between the control and chilled embryos by using Student's *t*-test. Differences were considered significant when $P < 0.05$.

RESULTS

The effect of chilling to 0°C for 30 min on the morphology of different developmental stages of in vitro derived bovine embryos was evaluated immediately after chilling and at ~12 h intervals thereafter until the embryos degenerated or developed into blastocysts. Immediately after cooling, all the embryos appeared to be morphologically normal.

Table I. Effect of Chilling on Survival of Different Developmental Stages of In Vitro Produced Bovine Embryos

Embryo stage	Group	Embryos used	Number of embryos that developed into (%)		Normalized survival (%)
			Expanded blastocyst	Hatching blastocyst	
2-Cell	Control	77	25 (32.5) ^a	22 (28.6) ^a	100
	Chilled	77	2 (2.6) ^b	0 (0) ^b	0
4-Cell	Control	59	23 (39.0) ^{a,e}	19 (32.2) ^a	100
	Chilled	59	0 (0) ^b	0 (0) ^b	0
8-Cell	Control	54	27 (50.0) ^c	18 (33.3) ^a	100
	Chilled	54	8 (14.8) ^d	5 (9.3) ^b	27.9
Morula	Control	73	31 (42.5) ^e	25 (34.2) ^a	100
	Chilled	73	16 (21.9) ^d	12 (16.4) ^c	48.0
Blastocyst	Control	71	—	59 (83.1) ^d	100
	Chilled	71	—	40 (56.3) ^e	67.7

Note. Values with different letters within a column are significantly different ($P < 0.05$).

However, some of the embryos exhibited a darkened “sun-burnt” appearance of the cytoplasm within ~12 h after chilling. Although the embryos appeared structurally unaltered, the blastomeres appeared asymmetrical with a hazy outline and degeneration was evident within 36 h after chilling. None of the embryos that exhibited such morphological changes developed further. Embryos that survived chilling appeared to have even granulation, a well-defined distinct outline with no blastomere extrusion, and looked like the control embryos.

The effect of chilling to 0°C for 30 min on the capacity of different developmental stages of in vitro produced bovine embryos to develop in culture is shown in Table I. The development of blastocysts from 2-cell, 4-cell, 8-cell, and morula-stage embryos in the control group ranged from ~30 to 50%. In the chilled group, only two of 77 chilled 2-cell embryos formed a blastocyst, and none of 59 chilled 4-cell embryos developed. In contrast, 15% of chilled 8-cell embryos developed into blastocysts and 22% of chilled morulae developed. The majority of the blastocysts that developed from control 2-cell and 4-cell embryos also hatched in culture, although only two-thirds of the control 8-cell embryos did so. None of the chilled 2-cell or 4-cell embryos hatched, whereas 9, 16, and 56% of the embryos chilled at the 8-cell, morula, and blastocyst stages did so. Considered as a proportion of the blastocysts that had formed by Day 7 or 8, there was no significant difference in the hatching rate between blastocysts derived from control and chilled 8-cell embryos, whereas there was a significant difference between blastocysts of control and chilled groups at the morula and blastocyst stages. The effect of embryo stage on the

likelihood of development was even clearer when blastocyst development from chilled embryos was normalized to that of the controls at the same developmental stage. Compared to their controls, about 1/4, 1/2, and 2/3 of 8-cell, morulae and blastocysts developed into hatched blastocysts.

The mean cell numbers of different developmental stages of in vitro derived bovine embryos after chilling are presented in Table II. In the control group, the mean cell numbers of Day-7 to -8 blastocysts derived from 8-cell and morula-stage embryos were 145.0 ± 8.0 and 142.5 ± 6.5 , respectively. Significant differences ($P < 0.05$) in the mean number of cells were found in the Day 7 blastocyst formed from 8-cell (94.0) and morula chilled embryos (101.8 ± 8.2). Similarly the mean number of cells was statistically different ($P < 0.05$) between Day 8 blastocysts derived from 8-cell whereas no significant difference was observed in morula stage of control and chilled group. In the blastocyst stage there was no significant difference ($P > 0.05$) in cell numbers among the hatched blastocysts on Days 8 and 9 of the control and chilled groups.

DISCUSSION

In vitro embryo production (IVP) techniques are attractive because of the possibilities for the low-cost mass production of bovine embryos for transfer, embryo diagnosis, somatic cell and embryo cloning and for the production of transgenic cows, as well as for basic research on the mechanisms of fertilization and embryogenesis (17). As is true for all techniques of assisted reproduction, most efficient utilization of in vitro derived embryos requires that they be

Table II. Comparison of Cell Number of Different Developmental Stages of In Vitro Produced Bovine Embryos After Chilling

Stage	Group	Embryos used	Blastocyst cell count (Mean $\pm$ SEM)		
			Day 7	Day 8	Day 9
8-cell	Control	22	145.0 $\pm$ 8.0 ^a	—	—
		25	—	160.8 $\pm$ 7.0 ^a	—
	Chilled	1	94	—	—
		14	—	119.4 $\pm$ 3.3 ^b	—
Morula	Control	27	142.5 $\pm$ 6.5 ^a	—	—
		29	—	156.9 $\pm$ 7.8 ^a	—
	Chilled	23	101.8 $\pm$ 8.1 ^b	—	—
		20	—	125.3 $\pm$ 4.6 ^{ab}	—
blastocyst	Control	14	—	175.5 $\pm$ 2.8 ^a	—
		14	—	—	191.8 $\pm$ 2.2 ^a
	Chilled	10	—	170.6 $\pm$ 1.9 ^a	—
		8	—	—	188.1 $\pm$ 3.3 ^a

^{a,b}Number of cells with different letters within the same column is significantly different ($P < 0.05$).

cryopreserved for transfer at later times in different locations. Consequently, there is growing interest in the cryopreservation of all cleavage stages of embryos of the large domestic species. This has led to the rediscovery of the phenomenon of chilling injury, namely, the damage and death of cells caused simply by exposing them to temperatures close to 0°C (15).

In this study, the morphological evaluation of different developmental stages of in vitro derived bovine embryos immediately after being cooled to 0°C for 30 min revealed no differences in the morphology between control and chilled embryos. However, within ~12 h of chilling, some embryos acquired a dark appearance in culture. The present observations are consistent with those of Looney *et al.* (18) who found that embryos appeared to be morphologically normal when evaluated immediately after cooling, but underwent lysis of cellular membranes after 3–7 h in culture at 38°C.

Bovine embryos at the 8- to 16-cell stage appear morphologically similar to embryos of other mammalian species at comparable stages of development. Primitive junctional complexes began to form between some blastomeres of 8-cell embryos. (14). Vesicular inclusions have been reported in preimplantation embryos of many mammalian species and were a prominent feature of the Day 5 bovine embryos. Inclusions that are present from the 1-cell stage and decline in number with development may be a form of stored material that is utilized during cleavage (19).

Oocytes and early embryos of cattle, sheep, and swine contain large numbers of spherical inclusions variously referred to as yolk, globules, lipid droplets, and cytoplasmic vesicles (20). Standard cryopreser-

vation methods yielding high rates for in vivo derived bovine embryos often result in low survival rates for in vitro derived embryos (10,21). It is interesting that the cryotolerance of bovine IVM/IVF embryos produced in a serum supplemented medium could be increased following removal of cytoplasmic lipid droplets (LD) by centrifugation, suggesting that the cytoplasmic LD may affect the sensitivity of embryos to chilling and freezing (22).

Chilling sensitivity depends on the embryo stage and seems to follow a general pattern. For a given developmental stage, survival declines with increasing length of exposure to a given low temperature. At low temperature, the rate of the survival decline increases (5,6). The survival of 8- to 16-cell bovine embryos is severely reduced after cooling below 10°C (23). Martino *et al.* (24) reported that indirect chilling injury is independent of cooling rate and is usually manifested after extended periods of exposure to low temperatures.

Our results show that few, if any, of 2-cell and 4-cell embryos survived chilling, but that resistance to chilling increased with advancing stage from 8-cell to morula to blastocyst. These findings suggest that the different developmental stages of in vitro produced bovine embryos have varying degrees of sensitivity to chilling. Bovine embryos cultured in serum-supplemented medium contain numerous cytoplasmic lipid droplets and immature mitochondria compared to those cultured in serum-free medium. The accumulation of cytoplasmic lipids in embryos developed in serum containing medium may be a result of incorporation of lipoproteins from the serum and may result in impaired function of mitochondria, thus having a negative effect on the

sensitivity of embryos to chilling and freezing (25). Failure of the development of 2- and 4-cell stage embryos might be due to denaturation of lipids and or with cellular components associated with the lipid in the cytoplasm (26). Our data are also consistent with the view that sensitivity of embryos to cooling is associated with the presence of a large number of vesicular inclusions within the embryonic cells, since bovine embryos also have a large number of these inclusions in the early cleavage stages and the absence of these vesicles at the blastocyst stage is coincident with a loss of sensitivity to cooling (11).

The number of cells present in the preimplantation embryos may be a good indicator of their viability, although morphological criteria alone are not sufficient indications (27). Wurth *et al.* (28) concluded that the presence of mitotic activity and the number of blastomeres in relation to their age form valuable parameters to assess embryo quality and can be used for evaluation of in vitro production system of bovine embryos.

In our study, the mean cell numbers recorded in blastocysts that developed on Days 7 and 8 from chilled 8-cell embryos and morulae were lower than that of their corresponding controls. But there was no significant difference in the cell number of Days 8 and 9 blastocysts between chilled and control groups. The reduction in the mean cell number of the blastocysts derived from 8 cell and morula stage embryos of chilled group may be attributed to death of few blastomeres or a decrease in their ability to divide at normal frequency which could have resulted after extended period (30 min) of exposure to 0°C, but not sufficient enough to compromise further developmental potency. Hence these embryos over-time in culture resulted in blastocysts with low cell numbers.

An embryo might progress to the 8-cell stage and for some unknown reason two or three blastomeres die while the remainder continue to develop. This blastomere death can happen at any stage of development. But the important thing is that as long as some blastomeres have remained healthy the embryo can result in pregnancy (29). However, it seems from the present data that the reduced cell number in the blastocysts derived from the 8-cell and the morula-stage chilled embryos had no deleterious effect on hatching as evidenced by the comparable hatching rates obtained in chilled and control groups. Hence it could be speculated that such embryos, if transferred, could develop to term. However, further studies in similar aspects which may throw insight

to this subject and would add benefit to the current trends in cryobiology and in turn to the bovine embryo transfer industry, are needed for careful assessment after transfer of chilled embryos to recipient cows.

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