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Fertilization and Development of Frozen–Thawed Germinal Vesicle Bovine Oocytes by a One-Step Dilution Method *in Vitro*

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The objective of this study was to evaluate *in vitro* fertilization and cleavage rates of frozen–thawed bovine oocytes at the germinal vesicle (GV) stage. In mouse oocytes, spindle microtubule reorganization after GV breakdown is particularly sensitive to cold and readily damaged by exposure to low temperatures, the damage becoming apparent only at the time of the first mitotic division. The effects of various permeating cryoprotective agents [1.8 *M* ethylene glycol (EG), 1.3 *M* ethylene glycol monomethyl ether (EME), and 1.6 *M* 1,2-propanediol (PROH)] and different concentrations of trehalose (T) and polyvinylpyrrolidone (PVP) on post-thaw developmental capacity were examined. When bovine GV oocytes were frozen slowly in mixtures of 1.8 *M* EG plus 5% PVP and 0.05 *M* T, almost 80% developed to metaphase II; 22.2% degenerated after *in vitro* maturation, and none of those that had been cryopreserved underwent parthenogenetic activation. The total fertilization rate was higher ($P < 0.05$) for oocytes frozen in a mixture of 1.8 *M* EG plus 0.05 *M* T or 0.1 *M* T than in a mixture of 1.8 *M* EG with or without 0.2 *M* T; however, there was no difference in the number of normally fertilized or polyspermic oocytes that had been frozen in various cryoprotective solutions. No significant difference was observed in subsequent development using EG, EME, and PROH for GV oocytes. The addition of 0.05 or 0.1 *M* trehalose to the freezing solution yielded significantly better cleavage and blastocyst rates than the solutions containing 0.2 *M* or no trehalose. For unfrozen controls, GV oocytes yielded significantly higher ($P < 0.01$) cleavage and blastocyst rates compared with frozen–thawed GV oocytes. It was found that 5% PVP had a beneficial effect compared with 10 or 20% concentrations for the development of blastocysts. Transfer of six blastocysts derived from frozen–thawed GV oocytes into three recipient heifers resulted in three pregnancies and the birth of one set of twins and one singleton calf.

Oocytes are extraordinarily large cells. In the germinal vesicle (GV) stage oocyte, chromatin is in a rather decondensed state and a few microtubule organizing centers (MTOCs) are found perinuclearly. At the onset of maturation, cytoplasmic MTOCs are recruited from the cytoplasm. On breakdown of the nuclear envelope and condensation of the chromatin, MTOCs combine with nuclear pericentriolar material (PCM) and polymerize microtubules toward the condensed chromosomes (42). Also, the actin-containing microfilaments are arranged in the perinuclear site (27). The meiotic spindles of *in vitro*-matured bovine oocytes are barrel-shaped, with the diameter of the metaphase plate longer than the pole-to-pole distance. The chromosomes are clustered in a discrete bundle at the metaphase plate. Some microtubules traverse the length of the spindle from pole to pole; others extend from the spindle poles to chromosomes. In mature oocytes, microtubules appear to be restricted largely to the meiotic spindle, with little evidence for foci of pericentriolar material (2). Cooling induces chromosomal abnormalities including disorganized metaphase plates and multipolar spindles in oocytes cooled at all stages of meiosis from germinal vesicle breakdown (GVBD) to metaphase II (23). Glass and Voelkel (11) observed that loss of viability in frozen bovine oocytes was associated with a

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specific step in the cryopreservation process. A limiting factor for achieving efficient cryopreservation of oocytes is direct chilling injury (DCI), which occurs to oocytes during cooling (3). The most striking among the detrimental effects caused by cooling oocytes are on the second meiotic spindle where microtubules are disrupted or disassembled, apparently as a result of tubulin depolymerization (27, 29). Cooling also alters the zona pellucida, resulting in decreased sensitivity to chymotrypsin and reduced fertilization rates, caused by cortical granule exocytosis leading to a premature cortical granule reaction (17). Fertilization and development rates of frozen–thawed bovine ova are lower than those of unfrozen ova. Mature mouse oocytes can be successfully cryopreserved when cooled slowly in 1.5 *M* dimethyl sulfoxide (DMSO) (46). Since this observation was first made, offspring have been produced after transfer of embryos obtained from frozen–thawed rabbit (1, 44), cow (10, 26), and human oocytes (9, 43); however, the overall success of these procedures remains low [with some exceptions (8)], primarily because of the reduced rate of fertilization after freezing and thawing. In the mouse, mature oocytes can be slowly frozen and preserved at −196°C using 1,2-propanediol (PROH) as a cryoprotectant (18). Otoi *et al.* (26) obtained normal offspring from matured bovine oocytes frozen and thawed in PROH following fertilization, culture *in vitro*, and embryo transfer. Three cryoprotectants—ethylene glycol (EG), ethylene glycol monomethyl ether (EME), and PROH—are relatively nontoxic and have been used for bovine embryos, permitting direct rehydration of thawed embryos (40). In a previous report (38), we described the successful cryopreservation of GV bovine oocytes by a combined process of dehydration of the oocytes with trehalose and permeation with PROH before plunging the oocytes into liquid nitrogen. In contrast to the many advanced studies with bovine embryos, however, data regarding unfertilized bovine oocytes are scarce, and the development rates of frozen–thawed oocytes are still low. Moreover, progress in the cryopreservation of GV bovine oocytes has been limited and production of offspring derived from them has not been reported. So more research is needed because storing immature oocytes is an important advance with implications for the cattle breeding industry and for conservation. Along with *in vitro*-matured oocytes (12), if GV stage oocytes also can be cryopreserved successfully, the timing of *in vitro* maturation–*in vitro* fertilization–*in vitro* culture (IVM–IVF–IVC) will be more manageable and genetic resources of various species or strains can be preserved more efficiently. Application of these techniques provides a means of preserving oocytes before maturation and avoids the possible damage that may occur in the spindle of the mature oocyte during freeze–thawing (7). Recently, Leibo and Oda (20) reported high survival of mouse zygotes and embryos cooled rapidly or slowly in EG plus PVP. A gradual dilution of sucrose in several steps seems to be sufficiently effective to mitigate osmotic injury so that the oocytes can develop after IVF (12). This study was conducted to examine the effect of trehalose and PVP on the cryopreservation of bovine oocytes at the GV stage with a single step addition and removal of the cryoprotectants. In addition, we examined the normality of embryos resulting from oocytes frozen in cryoprotectants following maturation, fertilization and culture *in vitro* (IVM–IVF–IVC), and embryo transfer.

MATERIALS AND METHODS

Oocyte Collection

The method for IVM, IVF, and IVC used in these experiments was a modification of the procedure of Boediono *et al.* (5). Ovaries were collected from cows at a local abattoir and were brought to the laboratory in physiological saline [0.89% (w/v) NaCl] at 25 to 30°C within 3 h. The cumulus–oocyte complexes (COCs) in follicular fluid (5–10 per ovary) were aspirated from follicles (2–5 mm in di-

ameter) with a 5-ml syringe with an 18-gauge needle. COCs were collected and washed three times in Dulbecco's phosphate-buffered saline (PBS; Gibco, Grand Island, NY) supplemented with 3 mg ml^{-1} fraction V bovine serum albumin (BSA-V, Sigma Chemical Co., St. Louis MO). All media used for collection and handling of oocytes were kept at 37°C on a warming block.

Freezing and Thawing

COCs were suspended directly in the cryoprotectants for 5 min at room temperature (20°C). Following this exposure, 20 to 30 COCs were loaded into 0.25-ml plastic straws. After loading, the straws were placed in a program freezer (ET-1, Fujihira Co. Ltd, Tokyo, Japan) maintained at 0°C for 2 min. Oocytes were then cooled to −6°C at a rate of 1°C min^{-1}, seeded at −6°C, then held for 10 min, cooled again at a rate of 0.3°C min^{-1} to −30°C, and finally plunged into liquid nitrogen. The straws were held at −196°C for 1 to 6 months. The cryopreserved straws were placed in air for 5 s and then plunged into a 37°C water bath for 10 s for thawing.

In Vitro Maturation

Frozen–thawed GV oocytes were placed in a polystyrene culture dish and then transferred directly into maturation medium containing 25 m*M* Hepes TCM-199 with Earle's salts (Gibco, Grand Island, NY) supplemented with superovulated cow serum (SCS) (22, 39), 0.01 mg ml^{-1} follicle-stimulating hormone (FSH, Denka Pharmaceutical Co., Kawasaki, Japan), and 50 μg ml^{-1} gentamicin (Sigma Chemical Co.). After being washed three times, oocytes surrounded by cumulus cells were kept for 22 h at 38.5°C under 5% CO_2 in air for maturation.

In Vitro Fertilization

Frozen semen was thawed in a water bath (37°C) and washed twice using 2.5 m*M* caffeine in Brackett and Oliphant medium (BO) (6) by centrifugation at 500*g* for 5 min. Then, the semen was resuspended in BO supplemented with 1% bovine serum albumin and 20 μg ml^{-1} heparin (Shimizu Pharmaceutical Co., Ltd., Shimizu, Japan) to yield a sperm concentration of 5×10^6 ml^{-1}. A 100-μl aliquot of sperm suspension was covered with mineral oil. Oocytes that had been cultured for maturation *in vitro* were transferred into sperm microdrops (20–25 oocytes per micro drop) for insemination for 5 h at 38.5°C under 5% CO_2 in air.

In Vitro Cultures

After 5 h of insemination, oocytes with cumulus cells were washed and transferred into culture medium for further development. The culture medium consisted of TCM-199 supplemented with 5% SCS, 5 μg ml^{-1} insulin (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 50 μg ml^{-1} gentamicin. Forty-eight hours after insemination, the cumulus cells surrounding the embryos were removed, and the cumulus cell layer attached to the bottom of the culture dish was used for coculture. The incubation medium was replaced with new medium every 96 h.

Experiment 1

The first experiment was conducted to determine the response to an activating stimulus in GV oocytes previously frozen and then matured *in vitro.* Oocytes were frozen in 1.8 *M* EG + 5% PVP + 0.05 *M* trehalose (T).

Parthenogenetic treatment. The procedures for producing diploid parthenogenetic embryos have been described previously (4). Frozen–thawed oocytes were matured for 32 h at 38.5°C under 5% CO_2 in air. To induce parthenogenetic activation, these matured oocytes were suspended in culture medium containing 7% ethanol for 10 min and then cultured in medium containing 5 mg ml^{-1} cytochalasin D (5 h) to suppress extrusion of the second polar body, thus producing diploid parthenogenotes.

As controls, frozen–thawed GV oocytes were matured for 32 h at 38.5°C under 5%

CO_2 in air. These matured oocytes were then cultured *in vitro* without any fertilization treatment.

Experiment 2

The second experiment was conducted to determine the effects of cryopreservation at the GV stage on subsequent fertilization *in vitro.* COCs were frozen in 1.8 *M* EG, 1.8 *M* EG + 0.05 *M* T, 1.8 *M* EG + 0.1 *M* T, or 1.8 *M* EG + 0.2 *M* T. Frozen–thawed GV oocytes were matured for 22 h at 38.5°C under 5% CO_2 in air. These matured oocytes were then incubated *in vitro* with frozen–thawed sperm. At 15 h after insemination, all oocytes were stained to evaluate fertilization.

Experiment 3

In this experiment, GV oocytes were frozen in (1) 1.8 *M* EG, (2) 1.8 *M* EG + 0.05 *M* T, (3) 1.8 *M* EG + 0.1 *M* T or (4) 1.8 *M* EG + 0.2 *M* T, (5) 1.3 *M* EME, (6) 1.3 *M* EME + 0.05 *M* T, (7) 1.3 *M* EME + 0.1 *M* T or (8) 1.3 *M* EME + 0.2 *M* T, (9) 1.6 *M* PROH, (10) 1.6 *M* PROH + 0.05 *M* T, (11) 1.6 *M* PROH + 0.1 *M* T or (12) 1.6 *M* PROH + 0.2 *M* T. After thawing the oocytes were assessed visually by morphological analysis under the microscope.

Experiment 4

In the fourth experiment, unfrozen oocytes were kept as (1) control (while thawing), and GV oocytes were frozen in (2) 1.8 *M* EG + 0.05 *M* T, (3) 1.8 *M* EG + 0.05 *M* T + 5% PVP, (4) 1.8 *M* EG + 0.05 *M* T + 10% PVP, or (5) 1.8 *M* EG + 0.05 *M* T + 20% PVP. After thawing the oocytes were assessed visually by morphological analysis under the microscope.

Oocyte Staining

After 24 h of maturation and either 15 h of parthenogenetic treatment or 15 h of insemination, the cumulus cells surrounding the embryos were removed by pipetting them in medium containing 150 U ml^{-1} hyaluronidase (Sigma). They were then fixed in Carnoy solution (3 parts ethanol:1 part acetic acid) for 72 h and stained in 1% aceto-orcein to look for the formation of nuclei or pronuclei.

Embryo Evaluation

The proportions of embryos that had developed to the 2-, 4-, and 8-cell stages were recorded 48 h after insemination. Blastocyst development was assessed up to Day 9.

Embryo Transfer

Six morphologically normal blastocysts on Day 8 of the cycle (Day 0 = onset of estrus) after *in vitro* fertilization derived from frozen–thawed GV oocytes were transferred nonsurgically (two embryos per recipient) to the uteri of three heifers (18 months of age). Pregnancy diagnosis was done by ultrasound scan after 60 days.

Statistical Analysis

The data were analyzed by χ^2 test and ANOVA. Probabilities of $P < 0.05$ and $P < 0.01$ were considered to be statistically significant.

RESULTS

Experiment 1

Two groups, each consisting of 27 matured oocytes, were stained to assess the stage of meiosis. The results are shown in Table 1. The proportion of metaphase II formation found in control oocytes was 77.8%, and the proportions of 1PN, 2PN, >2PN, or metaphase II formation found in oocytes subjected to parthenogenetic treatment were 22.2, 44.4, 11.1, and 22.2%.

Experiment 2

The results of this experiment are shown in Table 2. The total fertilization was higher in oocytes cryopreserved in 1.8 *M* EG + 0.05 *M* T or 1.8 *M* EG + 0.1 *M* T treatments than in those frozen in EG either without trehalose or with 0.2 *M* T. Polyspermy occurred in oocytes frozen in 1.8 *M* EG + 0.05 *M* T and in 1.8

TABLE 1
Frozen-Thawed Bovine GV Oocytes (in 1.8 *M* EG + 0.05 *M* T + 5% PVP) Subjected to Parthenogenetic Treatment

Treatment	No. of oocytes assigned	Pronucleus formation			Metaphase II	Degenerated
		1PN	2PN	>2PN		
Control	27	0 (0.0)[a]	0 (0.0)[a]	0 (0.0)[a]	21 (77.8)[a]	6 (22.2)[a]
Ethanol-exposed	27	6 (22.2)[b]	12 (44.4)[b]	3 (11.1)[b]	6 (22.2)[b]	0 (0.0)[b]

EG, ethylene glycol; T, trehalose; PVP, polyvinylpyrrolidone.
[a,b] Values within columns with different superscripts are significantly different ($P < 0.01$).

M EG + 0.1 *M* T, whereas it did not occur in 30 oocytes frozen in 1.8 *M* EG and in another 30 oocytes frozen in 1.8 *M* EG + 0.2 *M* T. The fertilization rate was higher in oocytes cryopreserved in 1.8 *M* EG + 0.05 *M* T or 1.8 *M* EG + 0.1 *M* T than in those frozen in EG either without T or with 0.2 *M* T.

Experiment 3

There were no differences in the proportions of oocytes that cleaved after being frozen in simple solutions of EG, EME, or PROH (Table 3); however, the addition 0.05 or 0.1 *M* trehalose to all three cryoprotectants yielded significantly better rates of cleavage. At best, only a few percent of all of the cryopreserved oocytes developed into blastocysts.

Experiment 4

Addition of PVP to the freezing solution significantly improved the development of cryopreserved oocytes. Table 4 shows the proportions of cryopreserved oocytes that cleaved and developed to the blastocyst stage. The cleavage rate of unfrozen oocytes (control) was significantly higher than that of frozen oocytes. Nevertheless, 5% PVP added to 1.8 *M* EG + 0.05 *M* T had a beneficial effect (with respect to blastocyst production) on the cryopreserved oocytes; after freezing with 10% PVP or 20% PVP, fewer oocytes cleaved. The blastocyst rates, as a percentage of cleaved oocytes, for unfrozen control oocytes and frozen oocytes with 0, 5, 10, or 20% PVP were 45.8, 4.4, 20.0, 9.5, or 0.0%, respectively. Freezing reduced the cleavage and blastocyst rates compared with unfrozen controls. High concentrations of PVP had a negative effect on both cleavage and blastocyst formation, and the best post-thaw development occurred with 5% PVP.

TABLE 2
Fertilization of GV Bovine Oocytes Cryopreserved Using Ethylene Glycol and Trehalose

Group	No. of oocytes	No. (%) of oocytes fertilized		
		Normal	Polyspermic	Total
1.8 *M* EG	30	10 (33.3)[a]	0 (0.0)[a]	10 (33.3)[a]
1.8 *M* EG + 0.05 *M* T	30	12 (40.0)[a]	2 (6.7)[a]	14 (46.7)[b]
1.8 *M* EG + 0.1 *M* T	30	15 (50.0)[a]	1 (3.3)[a]	16 (53.3)[b]
1.8 *M* EG + 0.2 *M* T	30	8 (26.7)[a]	0 (0.0)[a]	8(26.7)[a]

Note. Normal and polyspermic fertilization were identified by the presence of 2PN and >2PN. EG, ethylene glycol; T, trehalose.
[a,b] Values within columns with different superscripts are significantly different ($P < 0.05$).

TABLE 3
Development Capacity of Frozen–Thawed GV Bovine Oocytes Cryopreserved Using Various Cryoprotectants

Cryoprotectant	No. of oocytes assessed	No. cleaved (%)	No. developed to blastocyst (% of embryos cleaved)
1.8 *M* EG	288	72 (25.0)^{bc}	0 (0.0)
1.8 *M* EG + 0.05 *M* T	360	146 (40.6)^{a}	4 (2.7)
1.8 *M* EG + 0.1 *M* T	339	143 (42.1)^{a}	3 (2.1)
1.8 *M* EG + 0.2 *M* T	270	66 (24.4)^{bc}	0 (0.0)
1.3 *M* EME	225	56 (24.9)^{bc}	0 (0.0)
1.3 *M* EME + 0.05 *M* T	324	136 (41.9)^{a}	2 (1.5)
1.3 *M* EME + 0.1 *M* T	252	96 (38.1)^{a}	3 (3.1)
1.3 *M* EME + 0.2 *M* T	186	50 (26.9)^{b}	0 (0.0)
1.6 *M* PROH	342	36 (10.5)^{c}	0 (0.0)
1.6 *M* PROH + 0.05 M *T*	306	120 (39.2)^{a}	2 (1.7)
1.6 *M* PROH + 0.1 *M* T	453	160 (35.3)^{a}	2 (1.3)
1.6 *M* PROH + 0.2 *M* T	378	76 (20.1)^{bc}	0 (0.0)

EG, ethylene glycol; T, trehalose; EME, monomethyl ether; PROH, 1,2-propanediol.
$^{a-c}$ Values within columns with different superscripts are significantly different (ANOVA, Scheffe *F* test $^{ab,bc}P < 0.05$, $^{ac}P < 0.01$.

Development in Vivo of Frozen–Thawed GV Oocytes

Six blastocysts resulting from IVM–IVF–IVC of GV oocytes frozen in EG with 0.05 *M* T and 5% PVP were transferred into three recipients, and all of these resulted in pregnancies diagnosed 60 days after transfer. One recipient delivered a single calf and a second delivered twin calves on March 5th and May 7th, 1995. One pregnancy resulted in abortion by Day 92.

TABLE 4
Development Capacity of Frozen–Thawed GV Bovine Oocytes Cryopreserved Using 1.8 *M* EG + 0.05 *M T* Supplemented with Different Concentration of PVP[A]

Concentration of PVP (%)	No. of oocytes assessed	No. cleaved (%)	No. of blastocysts (% of embryos cleaved)
Unfrozen control	100	83 (83.0)^{a}	38 (45.8)^{a}
0	150	68 (45.3)^{b}	3 (4.4)^{b}
5	170	80 (47.1)^{b}	16 (20.0)^{a}
10	168	42 (25.0)^{c}	4 (9.5)^{b}
20	144	18 (12.5)^{c}	0 (0.0)^{c}

EG, ethylene glycol; T, trehalose; PVP, polyvinylpyrrolidone.
$^{a-c}$ Values within columns with different superscripts are significantly different ($^{ab,bc}P < 0.05$; $^{ac}P < 0.01$).

DISCUSSION

In mouse oocytes, spindle microtubule organization after GVBD is particularly sensitive to cold and is readily damaged by exposure to low temperatures, the damage becoming apparent only at the time of the first mitotic division (33). Parthenogenetic activation occurs after exposure to 1,2-propanediol (41). In the present study, 44% of 27 frozen–thawed GV bovine oocytes developed to the 2PN stage following parthenogenetic treatment; however, without parthenogenetic treatment, most frozen–thawed GV oocytes developed normally to the metaphase II (77.8%). No parthenogenetic activation due to freezing was observed. This suggests that the treatments used in a successful cryopreservation procedure do not cause irreversible damage to the meiotic spindle and parthenogenetic activation of bovine oocytes.

In the second experiment, the total fertilization rates were significantly higher in oocytes cryopreserved in EG plus low concentrations of trehalose (0.05 or 0.1 *M*) than in those frozen in EG alone or in EG plus 0.2 *M* trehalose. We previously reported (38) that the cleavage rates of GV bovine oocytes frozen in 1.6 *M* PROH with 0.1 or 0.2 *M* sucrose by a one-step rehydration procedure were higher than those of oocytes frozen with the same cryoprotectant without sucrose. Rayos *et al.* (28) also reported that sucrose or trehalose in combination with EG was effective in the quick freezing of unfertilized mouse oocytes. It is well known that carbohydrates can be used as an osmotic buffer to maintain osmotic equilibrium between the embryonic cells and the external environment in which the embryo is suspended (30–32), while decreasing the external concentration of cryoprotectant. GV bovine oocytes, however, were more sensitive to a sucrose treatment than *in vitro*-matured oocytes or zygotes (21, 25). Prolonged exposure to sucrose had a deleterious effect on the developmental capacity of GV (immature) or mature bovine oocytes (14). On the other hand, Schellander *et al.* (35) indicated that the use of carbohydrates during cryoprotectant removal is not a key element in improving oocyte cryopreservation.

Hernandez-Ledezma and Wright (13) reported that the use of propanediol, instead of glycerol or DMSO, significantly improved survival and development of cryopreserved mouse oocytes to the two-cell stage. In the present study, we used the permeable cryoprotectants EG, EME, and PROH to freeze GV bovine oocytes (Experiment 3). There were no significant differences in cleavage and blastocyst development rates for oocytes frozen in different cryoprotectants; however, to obtain a higher proportion developing into blastocysts, it was necessary to add a low concentration of a carbohydrate to the freezing solution. Because oocytes and embryos at various stages of development differ both physiologically and morphologically (15, 16, 19), freezing techniques developed for one may not be suitable for the other. Heyman *et al.* (15) reported that very few (6%) bovine oocytes matured *in vitro* after rapid freezing and thawing. Similarly, Fuku *et al.* (10) reported that fewer than 5% of frozen–thawed immature bovine oocytes underwent GVBD and polar body formation. In contrast to these observations, in the present study the total cleavage and development rates to blastocyst were 35.3 to 42.2% and 1.3 to 3.1%, respectively. These findings indicate that cleavage rate was improved, with a small proportion developing to blastocysts in the presence of 0.05 or 0.1 *M* T.

In the fourth experiment, we used PVP to protect GV bovine oocytes against damage caused by freezing and thawing. Although there was no improvement in the cleavage rates, the development into blastocysts was significantly improved in the 5% PVP solution compared with solutions containing 0%, 10%, and 20% PVP. Leibo and Oda (20) reported that when PVP is combined with a relatively low concentration of EG, it apparently enhances the cryoprotective properties of EG solutions, yielding high survival of zygotes and embryos frozen either slowly or rapidly. Such biological macromolecules are often added to cryoprotective agent solutions either for their presumptive cryoprotective effect or as a surfactant (8, 31, 32). Even the use of the supplements PVP and carbohydrates for freezing GV bovine oocytes yielded a cleavage rate significantly lower than that of unfrozen oocytes. This suggests that some membrane damage and extensive disorganization of the ooplasma may have occurred during freezing and thawing because of intracellular ice formation or osmotic stress, which could have interfered in the subsequent fertilization and development of oocytes (34, 36, 47). Failure of fertilization may also be due to the changes in the zona pellucida during freezing and thawing, which inhibits the entry of spermatozoa (17); however, we found that the development of two-cell embryos to the blastocyst stage was similar in oocytes cryopreserved in cryoprotectant supplemented with 5% PVP and unfrozen

control oocytes. Schroeder *et al.* (37) reported that a dramatic change in freezability of mouse oocytes was associated with meiotic maturation *in vivo* or *in vitro*. Moreover, the damaging effects of freeze–thawing were apparent only up to the two-cell stage. Once past this hurdle, the development of oocytes to morulae or blastocysts was not affect further. The findings of Carroll *et al.* (8) support that conclusion. Under conditions where fertilization of oocytes frozen in medium with BSA or PVA was low, substitution of FCS for BSA in the freezing medium restored fertilization to within 15% of control levels. In the present study, when we used the higher concentrations of PVP (10 to 20%), we obtained significantly lower cleavage rates (25.0% and 12.5%) than with a low concentration of PVP (45.3 and 47.1% for 0 and 5%). The reason for this is unclear, but it might be that high concentrations of PVP cause problems in seeding.

Careful attention to temperature control throughout oocyte retrieval, examination, handling, and replacement should help to overcome the problems associated with cooling. Cooling promotes disassembly of microtubules, whereas cryoprotectants promote their uncontrolled assembly. Thus, a delicate balance results in which sufficient cryoprotectant enters to counteract the destabilizing effect of cooling. Manipulating the periods of exposure to cooled cryoprotectant prior to freezing and immediately after thawing should enable an optimal regime for spindle stabilization to be developed (45).

In conclusion, this study suggests that permeating cryoprotective agents in combination with a low concentration of a carbohydrate and PVP can be used effectively in the slow freezing and thawing of GV bovine oocytes. More understanding regarding GVBD and nuclear maturation will be helpful (24).

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