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The Influence of Polyvinylpyrrolidone on Freezing of Bovine IVF Blastocysts Following Biopsy

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A study was conducted to develop a better freezing protocol for *in vitro* developed biopsied bovine blastocysts. Biopsied blastocysts were exposed to 1.8 *M* ethylene glycol (EG) + 0.05 *M* trehalose (T) and different concentration (5, 10, and 20%) of polyvinylpyrrolidone (PVP). Exposure to the solutions alone did not affect their *in vitro* development (Experiment 1). Experiments 2, 3, and 4 tested the viability of biopsied blastocysts cryopreserved in 1.8 *M* EG + different concentrations of T (0, 0.05, 0.1, and 0.3 *M*), 1.8 *M* EG + different concentrations of PVP (0, 5, 10, and 20%), and 1.8 *M* EG + 0.05 *M* T + different concentrations of PVP (0, 5, 10, and 20%), respectively. The proportion of biopsied blastocysts that reexpanded following cryopreservation in 1.8 *M* EG + 0.05 *M* T (38.5%) and 1.8 *M* EG + 0.1 *M* T (36.1%) was significantly ($P < 0.05$) higher than the proportion that reexpanded in 1.8 *M* EG + 0.3 *M* T (13.9%) (Experiment 2). The viability and the percentage of embryos that developed to >250 μm in diameter in the 5, 10, and 20% PVP groups (77.8 and 50.0%, 78.1 and 43.8%, 76.9 and 65.4%, respectively) were significantly higher than those that developed cryopreserved without PVP (55.1 and 20.7%) (Experiment 3). Optimum development of *in vitro* culture of frozen–thawed biopsied blastocysts was obtained using 1.8 *M* EG + 0.05 *M* T and 20% PVP. Analysis of blastocysts >250 μm in diameter showed that the number of ICM cells of biopsied blastocysts cryopreserved in 1.8 *M* EG + 0.05 *M* T with or without PVP was not different from the number of unfrozen biopsied blastocysts. These results indicate that PVP has some beneficial effect on freezing of biopsied bovine blastocysts.

At present, the most practical and efficient livestock embryo-sexing method is the one utilizing the polymerase chain reaction (PCR) using Y-specific primers (14). In the context of bovine embryo sexing, PCR has been used to amplify male-specific DNA from embryo biopsies with a successful amplification product indicating a male biopsy (embryo) (2, 7, 14, 18, 19, 25). Since microsurgical techniques are required to obtain blastomeres from the embryo and around 4 h is required to obtain the test results by PCR, it would be desirable to keep these biopsied embryos at −196°C before transfer. However, results to date indicate that the viability of the biopsied or bisected bovine embryo is reduced after cryopreservation (22). In particular, bisection of frozen–thawed bovine embryos resulted in decreased survival (13, 24). The use of bovine embryo bisection in conjunction with sexing has been reported (3, 19), but there is little information about the viability of frozen–thawed IVF bovine embryos without a zona pellucida following biopsy. Embryos frozen in ethylene glycol (EG) or propylene glycol (PG) can be rehydrated directly in holding medium without stepwise dilution of the cryoprotectant (21, 26). However, even for such permeable cryoprotectants as EG and PG it may be necessary to include a low concentration of sugar in the holding medium to protect the embryos from osmotic shock. Recently, Leibo and Oda (10) reported that polyvinylpyrrolidone (PVP) was effective for mouse embryo freezing. The present study was aimed at developing a freezing protocol for *in vitro* developed biopsied blastocysts without a zona pellucida and evaluating the viability of these biopsied blastocysts following freezing in different cryoprotectant solutions.

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MATERIALS AND METHODS

In Vitro Blastocyst Production

Ovaries were obtained from a local slaughterhouse. Oocytes with a compact cumulus were cultured for 20 to 22 h (38.5°C, 5% CO_2 in air) in medium-199 (TCM-199 with Earle's salts, L-glutamine, 2.200 mg/ml sodium bicarbonate,

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and 25 m*M* Hepes; Gibco, U.S.A.) supplemented with 0.01 mg/ml follicle-stimulating hormone (FHS; Denka, Japan). The medium was also supplemented with 5% superovulated cow serum (SCS) collected on Day 7 from superovulated donors that produced good quality embryos (11, 20). Frozen–thawed spermatozoa were centrifuged twice in Brackett and Oliphant medium (1) containing 2.5 m*M* caffeine and 20 μg/ml heparin and were then incubated with mature oocytes for 5 h at 38.5°C and 5% CO_2 in air. Cleaved embryos were cultured in culture medium containing TCM-199 supplemented with 5% SCS and 5 μg/ml insulin (Wako Ltd., Japan) (4, 6).

Blastocyst Biopsy

The biopsy medium used was PBS (Gibco) (37°C) containing 5% PVP (MW 40,000; Denka, Kawasaki, Japan). Grade 1 *in vitro* produced blastocysts (Day 8 embryos) were placed in this medium for 10 to 15 min prior to microsurgery. Blastocysts were biopsied, removing around 1/10 of the whole embryo, using the scratched bottom technique (12). Cells were removed from the trophectoderm. The zona pellucida was removed from the biopsied blastocysts and they were transferred to a cumulus cell layer in a culture dish containing TCM-199 supplemented with 5% SCS, 5 μg/ml insulin under paraffin oil and incubated (38.5°C, 5% CO_2 in air) for 3 h prior to freezing.

Experiment 1

This experiment was conducted to determine the effects of exposure of the biopsied blastocysts to different cryoprotectant solutions followed by removal of the cryoprotectants. Blastocysts were exposed to three cryoprotectant solutions: A, 1.8 *M* EG + 5% PVP + 0.05 *M* trehalose (T); B, 1.8 *M* EG + 10% PVP + 0.05 *M* T; and C, 1.8 *M* EG + 20% PVP + 0.05 *M* T for 10 min at 25°C. After exposure, the embryos in <5 μl cryoprotectant solution were transferred directly to 2.5 ml of culture medium to achieve direct rehydration and washed three times. The embryos were then transferred to culture wells and cultured on feeder layers of bovine cumulus cells in culture medium. The proportion that developed to fully expanded blastocysts and expanded blastocysts up to 250 μm diameter during 48 h culture was recorded.

Freezing of Biopsied Blastocysts

Cryoprotectant solutions were prepared in modified PBS (m-PBS), PBS supplemented with 3 mg/ml bovine serum albumin (BSA, fraction V; Sigma, U.S.A.). Biopsied blastocysts were exposed at room temperature (25°C) to the cryoprotectants, added in a one-step manner, in Experiments 2, 3, and 4.

Experiment 2

Groups 1 (1.8 *M* EG (control), 2 (1.8 *M* EG + 0.05 *M* T), 3 (1.8 *M* EG + 0.1 *M* T), and 4 (1.8 *M* EG + 0.3 *M* T) were evaluated.

Experiment 3

Groups 1 (1.8 *M* EG (control), 2 (1.8 *M* EG + 5% PVP) 3 (1.8 *M* EG + 10% PVP), and 4 (1.8 *M* EG + 20% PVP) were evaluated.

Experiment 4

Groups 1 (1.8 *M* EG + 0.05 *M* T (control), 2 (1.8 *M* EG + 0.05 *M* T + 5% PVP), 3 (1.8 *M* EG + 0.05 *M* T + 10% PVP), and 4 (1.8 *M* EG + 0.05 *M* T + 20% PVP) were evaluated.

In all the above experiments, biopsied blastocysts were cultured for 3 h and then exposed to the cryoprotectants for 5 min at room temperature. Following this exposure, 5 to 10 embryos were loaded into 0.25-ml plastic straws. After loading, the straws were placed in a programmable freezer (ET-1, Fujihira, Japan) maintained at 0°C. Blastocysts were then cooled to −7°C at a rate of 1°C/min, seeded at −7°C, cooled again at a rate of 0.3°C/min to −30°C, and then plunged into liquid nitrogen.

Biopsied and nonbiopsied blastocysts were frozen using 1.8 *M* EG + 0.05 *M* T + 20% PVP with the same procedure as mentioned above. The viabilities after thawing were compared.

In Vitro Viability Assessments

After a 7- to 14-day storage period in liquid nitrogen, the cryopreserved straws were placed

in air for 5 s and plunged into a 30°C water bath for thawing. After thawing, cryoprotectants were removed by the one-step procedure as described in Experiment 1. Upon cryoprotectant removal, embryos were transferred to a cumulus layer as described in Experiment 1 and cultured. Embryos were evaluated microscopically at 12 and 48 h. Embryos were classified after freezing into two groups: those which reexpanded to less than and those which expanded to more than 250 μm in diameter after 48 h in culture.

Determination of ICM and Trophectoderm Cells

After 48 h of culture, only the viable embryos which developed to 250 μm in diameter were stained. Immunosurgery and differential staining techniques as described in earlier reports (8, 23) were used to determine ICM and trophectoderm cell numbers. The blastocysts were incubated in TCM-199 supplemented with 20% rabbit anti-bovine lymphocyte antiserum for 30–35 min at 38.5°C. Subsequently, the embryos were washed three times with TCM-199 supplemented with guinea pig complement and 5% SCS. Propidium iodide (Sigma) and bisbenzimide (Hoechst 33342, Sigma) were added at a final concentration of 10 μg/ml to the complement solution. Finally, the embryos were washed in PBS with 3 mg/ml BSA and mounted on a glass slide. ICM cells were separated from the trophectoderm by placing a coverslip and applying gentle pressure. Glass slides were then examined under the fluorescence microscope (filter 330–380 nm) for ICM cells. This resulted in vital nuclei fluorescing blue (bisbenzimide-positive) and nonvital nuclei fluorescing pink (propidium iodide). Color photographs of all ICM cells were taken and the number of viable ICM cells was counted. The total numbers of ICM and trophectoderm cells were also noted following hypotonic treatment and Giemsa staining.

Statistical Analysis

The viability of blastocysts at different times after culture was analyzed by the χ^2 test. The mean total ICM cells number and percentage of live ICM cells of unfrozen and postthawed biopsied blastocysts were analyzed by analysis of variance (ANOVA). Differences at a probability value (P) of 0.05 or less were considered significant.

RESULTS

Table 1 shows the percentages of control embryos and embryos exposed to the various cryoprotectants without freezing that reexpanded and those that reached 250 μm in diameter after culture on a cumulus cell monolayer for 48 h. There was no significant difference in viability among treatment groups at any time point. Table 2 shows the survival rate and the number of blastocysts that developed to 250 μm in diameter following cryopreservation in EG with different concentrations of T. The viability and the number that expanded up to 250 μm in culture following cryopreservation in 1.8 *M* EG + 0.05 *M* T (74.4 and 38.5%) and 1.8 *M* EG + 0.1 *M* T (69.4 and 36.1%) were significantly higher than those cryopreserved in 1.8 *M* EG + 0.3 *M* T (41.7 and 13.9%). The survival rate and the number that developed up to 250 μm following cryopreservation in 1.8 *M* EG and different concentrations of PVP are shown in Table 3. The viability and the percentage of embryos that developed to 250 μm in diameter in the 5, 10, and 20% PVP groups (77.8 and 50.0%, 78.1 and 43.8%, 76.9 and 65.4%, respectively) were sig-

TABLE 1
Development of Solution Control Blastocysts Cultured on Cumulus Cell Monolayers

Medium[a]	No. of embryos	Indication of embryo viability at 48 h (%)		
		Not expanded	<250 μm[b]	>250 μm[c]
Control	25	2 (8.0)	6 (24.0)	17 (68.0)
A	23	3 (13.0)	5 (21.7)	15 (65.2)
B	26	1 (3.8)	7 (26.9)	18 (69.2)
C	22	3 (13.6)	4 (18.2)	15 (68.2)

[a] Treatment A, 1.8 *M* EG + 5% PVP + 0.05 *M* T; B, 1.8 *M* EG + 10% PVP + 0.05 *M* T; C, 1.8 *M* EG + 20% PVP + 0.05 *M* T (EG, ethylene glycol; PVP, polyvinylpyrrolidone; T, trehalose).
[b] Expanding to <250 μm in diameter.
[c] Expanding to >250 μm in diameter.

TABLE 2

Development of Biopsied Blastocysts Following Cryopreservation in 1.8 *M* Ethylene Glycol Supplemented with Different Concentrations of Trehalose

Concentration of trehalose (*M*)	No. of embryos treated	No. viable at 12 h (%)	No. expanding <250 μm (%)
0	26	15 (57.7)[a]	5 (19.2)[a]
0.05	39	29 (74.4)[a]	15 (38.5)[b]
0.1	36	25 (69.4)[a]	13 (36.1)[b]
0.2	36	15 (41.7)[b]	5 (13.9)[a]

[a,b] Values within columns with different superscripts differ significantly ($P < 0.05$).

nificantly higher than those cryopreserved without PVP (55.2 and 20.7%). The viability and the percentage of embryos that formed blastocysts >250 μm following cryopreservation in 5, 10, and 20% PVP with 0.05 *M* T and 1.8 *M* EG were 85.2 and 37.0%, 91.7 and 41.7%, 93.9 and 69.7%. This was significantly higher than those cryopreserved without PVP (64.3 and 35.7%) (Table 4). Significantly ($P < 0.05$) more nonbiopsied blastocysts cryopreserved in 1.8 *M* EG + 0.05 *M* T and 20% PVP formed blastocysts >250 μm than the biopsied blastocysts (Table 5). The mean numbers of viable ICM cells of control (unfrozen) biopsied blastocysts and those cryopreserved in 1.8 *M* EG + 0.05 *M* T with 0, 5, 10, or 20% PVP (53.6 ± 16.6, 35.8 ± 24.0, 37.8 ± 11.5, 44.5 ± 19.1, 45.3 ± 20.6, respectively) were not different. No significant differences were observed in the mean number of trophectoderm cells among treatment groups (Table 6) ($P > 0.05$).

TABLE 3

Development of Biopsied Blastocysts Following Cryopreservation in 1.8 *M* Ethylene Glycol Supplemented with Different Concentrations of Polyvinylpyrrolidone (PVP)

Concentration of PVP (%)	No. of embryos treated	No. viable at 12 h (%)	No. expanding >250 μm (%)
0	29	16 (55.2)[a]	6 (20.7)[a]
5	36	28 (77.8)[b]	18 (50.0)[b]
10	32	25 (78.1)[b]	14 (43.8)[b]
20	26	20 (76.9)[b]	17 (65.4)[b]

[a,b] Values within columns with different superscripts differ significantly ($P < 0.05$).

TABLE 4

Development of Biopsied Blastocysts Following Cryopreservation in 1.8 *M* Ethylene Glycol Supplemented with 0.05 *M* Trehalose and Different Concentrations of Polyvinylpyrrolidone (PVP)

Concentration of PVP (%)	No. of embryos treated	No. viable at 12 h (%)	No. expanding >250 μm (%)
0	28	18 (64.3)[a]	10 (35.7)[a]
5	27	23 (85.2)[b]	10 (37.0)[a]
10	36	33 (91.7)[b]	15 (41.7)[a]
20	33	31 (93.9)[b]	23 (69.7)[b]

[a,b] Values within columns with different superscripts differ significantly ($P < 0.05$).

DISCUSSION

At 48 h culture *in vitro,* most normal or cryopreserved hatched embryos had developed to more than 300 μm in diameter. These embryos continued to expand, and the number of cells increased (unpublished data). In this study we assessed as viable those biopsied embryos that were larger than 250 μm in diameter, because the number of cells is decreased by biopsy.

Experiment 1 showed that embryos were not harmed by exposure to EG + T with different concentrations of PVP and subsequent dilution of the cryoprotectants. It is well known that sugars can be used as osmotic buffers to maintain osmotic equilibrium between embryonic cells and the external concentration of the cryoprotectant.

When the biopsied blastocysts were cryopreserved in 1.8 *M* EG and various concentrations of trehalose, the postthaw survival rate of biopsied blastocysts frozen in 0.05 or 0.1 *M* T was significantly higher than control (EG only) or 0.3 *M* T. This indicates that a low concentration

TABLE 5

Development of Biopsied or Nonbiopsied Blastocysts Cryopreserved in 1.8 *M* EG Supplemented with 0.05 T and 20% PVP

Treatment	No. of embryos treated	No. viable at 12 h (%)	No. expanding >250 μm (%)
Biopsied	20	20 (100.0)	18 (90.0)[a]
Nonbiopsied	20	16 (80.0)	12 (60.0)[b]

[a,b] Values within columns with different superscripts differ significantly ($P < 0.05$).

TABLE 6

The Mean (±SD) Inner Cell Mass (ICM) and Trophectoderm (TE) Cell Number of Biopsied Blastocyst (Developed >250 μm in Diameter) Cryopreserved in 1.8 *M* Ethylene Glycol (EG) Supplemented with 0.05 *M* Trehalose (T) and Different Concentrations of Polyvinylpyrrolidone (PVP)

Treatment	No. of embryos assessed	ICM No.	TE No.	Total
Unfrozen	18	53.6 ± 16.6	116.2 ± 27.0	169.8 ± 31.1
A	20	35.8 ± 17.8	113.4 ± 24.5	149.2 ± 28.6
B	22	37.8 ± 11.5	94.5 ± 35.5	132.3 ± 39.2
C	18	44.5 ± 19.1	100.3 ± 20.6	144.8 ± 24.7
D	19	45.3 ± 20.6	93.3 ± 28.7	138.6 ± 32.8

[a] Treatment A, 1.8 *M* EG + 0.05 *M* T; B, 1.8 *M* EG + 0.05 *M* T + 5 % PVP, C 1.8 *M* EG + 0.05 *M* T + 10% PVP; D, 1.8 *M* EG + 0.05 *M* T + 20% PVP (EG, ethylene glycol; PVP, polyvinylpyrrolidone; T, trehalose).

of trehalose is most beneficial for the cryopreservation of biopsied blastocysts. Trehalose probably reduces or prevents osmotic shock while the biopsied blastocysts are suspended in the holding medium for direct rehydration. In previous report (21), we suggested that 1.8 *M* EG is ideal for the cryopreservation of bovine embryos and for direct transfer into the female reproductive tract. However, the present findings indicate that inclusion of a low concentration of trehalose in the EG-based cryoprotectant may improve the success rate.

Experiment 3 showed that PVP has a beneficial effect on the cryopreservation of biopsied blastocysts. Viability of postthaw biopsied blastocysts frozen with PVP in EG (1.8 *M*) was significantly higher than that of blastocysts frozen with PVP. Optimum viability and cell numbers in biopsied blastocysts were obtained when the embryos were cryopreserved in 1.8 *M* EG + 0.05 *M* T and 20% PVP. Ren *et al.* (16) reported that PVP, polyvinylalcohol (PVA), etc., promoted vitrification. Kasai *et al.* (9) used Ficoll for vitrification of mouse embryos. Basically, the explanation of high survival with vitrification is that no ice forms either inside or outside the cells, which results in high embryo survival. In the present study, embryos were slowly cooled in a low concentration of EG with PVP. Leibo and Oda (10) reported that when PVP was combined with a relatively low concentration of EG, it enhanced the cryoprotective properties of EG solutions, yielding high survival of zygotes and embryos frozen either slowly or rapidly. Carroll *et al.* (5) reported that fertilization of mouse oocytes frozen in the presence of PVA was significantly increased.

We also determined the viability of ICM and trophectoderm cells using immunosurgery and the differential staining technique described earlier (8, 23). Takagi *et al.* (23) reported that the viability of ICM cells of frozen–thawed bovine embryos tends to be lower than that of cells of unfrozen IVF embryos, irrespective of the cryoprotectant used. In the present study, the total numbers of viable ICM and TE cells of biopsied blastocysts frozen in 1.8 *M* EG + 0.05 *M* T with 0, 5, 10, and 20% PVP were significantly lower than control. However, the numbers of biopsied blastocysts that expanded up to 250 μm in diameter following exposure to 20% PVP were significantly higher than those for other concentrations of PVP. These results suggest that 20% PVP may play a beneficial role, probably by forming a protective coating around biopsied blastocysts during freezing and thawing.

In conclusion, we have shown that (a) exposure of biopsied blastocysts to 1.8 *M* EG + 0.05 *M* T and different concentrations of PVP did not affect their development in culture and (b) the cryoprotectant mixture containing 1.8 *M* EG + 0.05 *M* T and 20% PVP is better for the cryopreservation of biopsied blastocysts in the bovine.

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