

VIABILITY OF FROZEN-THAWED BOVINE IVM/IVF EMBRYOS IN  
RELATION TO AGING USING VARIOUS CRYOPROTECTANTS

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ABSTRACT

Bovine IVF embryos developed on Days 7, 8 and 9 were equilibrated with 1.6 M propylene glycol (PG), 1.8 M ethylene glycol (EG), 1.1 M diethylene glycol (DEG) or 1.3 M ethylene glycol monomethyl ether (EME) for 10 to 20 min in modified phosphate buffered saline (mPBS) supplemented with 10% superovulated cow serum. The embryos were loaded into 0.25-ml plastic straws and were placed directly into a 0°C alcohol bath chamber and held for 2 min. They were cooled from 0°C to -5.5°C at 1°C/min and then seeded, followed by a 10-min holding period at -5.5°C. The straws were then cooled to -30°C at 0.3°C/min before plunging into liquid nitrogen. Embryos were thawed and placed directly into the culture medium and washed 3 times. The survival rates of the Day-9 embryos based on reappearance of blastocoele, expansion, and hatching after 48 h of post-thaw culture were significantly lower ( $P < 0.01$ ) than those of the Day-7 and 8 embryos, in all of the cryoprotectants tested. On the other hand, while the reappearance of blastocoele and expansion of blastocysts after 48 h of post-thaw culture were not significantly different among each cryoprotectant, the percentage of hatching blastocysts were significantly different between DEG and EME ( $P < 0.05$ ), between DEG and EG ( $P < 0.01$ ) and between PG and EG ( $P < 0.05$ ). These findings demonstrate that the age of the embryo (Day 7 and 8) is very important for the successful freezing of IVF bovine embryos. Also, as to the hatching rates, EME and EG are superior as cryoprotectants than the other 2 cryoprotectants tested.

Key words: bovine, IVF embryo, freezing, cryoprotectants

INTRODUCTION

Recently, advances in embryo transfer technology have enabled us to produce bovine embryos through the fertilization and development of in vitro matured oocytes derived from slaughtered cattle. Blastocysts thus produced have the ability to develop into live calves after transfer into recipient cows (5,6). Therefore,

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the cryopreservation of these embryos will have an important role in the practical application of this technique. However, there have been only a few preliminary reports on the cryopreservation of bovine embryos produced in vitro (4,7,16). The survival rates of frozen-thawed IVF bovine embryos are affected by the quality or age of the embryos, cryoprotectant used, the cooling rates during freezing, and the method of equilibration or removal of the cryoprotectant (4,7,16-18). Mukojima et al. (11) reported that the pregnancy rate of the recipient receiving fresh IVF bovine embryos was not affected by embryo age. However, Tachikawa et al (16) reported that the in vitro developmental rate of fresh IVF blastocysts obtained on Day 9 after insemination was lower than that obtained on Day 7 or Day 8.

In frozen-thawed bovine embryos, the cryoprotectant must be diluted or removed before culture or transfer into the uterine horn. Until now, a stepwise method has been used to remove the cryoprotectant (1,19,20). Direct transfer methods (one-step or two-step method) have also been described for in vivo bovine embryos (8,12-14).

Recently, some investigators have reported direct transfer methods without dilution or removal of the cryoprotectant, with or without sucrose, using glycerol (9), propylene glycol (15) and ethylene glycol (17,18).

In this study, we examined whether embryo age after insemination affected the survival rate of the post-thaw IVF embryos frozen in various cryoprotectants (ethylene glycol, diethylene glycol, propylene glycol and ethylene glycol monomethyl ether) and thawed by direct transfer methods.

#### MATERIALS AND METHODS

##### In Vitro Fertilization of Ovarian Oocytes

Cumulus-oocyte complexes (COCs) were aspirated from small antral follicles (1 to 5 mm in diameter) in bovine ovaries obtained from a local slaughterhouse. After collection, only oocytes with an intact, unexpanded cumulus oophorus were washed twice with the maturation medium (25 mM-Hepes TCM-199 with Earle's salts; Gibco, NY, USA) supplemented with 5% serum collected from superovulated cows on Day 7 of their cycle (SCS; 10), 0.01 AU/ml FSH (Denka, Kawasaki, Japan) and 50 µg/ml of Gentamicin sulfate (Sigma, St.Louis, USA). Thereafter the COCs were placed into the maturation medium and cultured for 21 to 22 h at 38.5°C with 5% CO<sub>2</sub> in air.

Frozen semen from a single bull was used. The semen was washed twice with BO medium (2), without bovine serum albumin, and supplemented with 5mM-caffeine, by centrifugation, at 500 x g for 5 min. The sperm pellet was resuspended in BO medium containing bovine serum albumin (5 mg/ml, Sigma, St.Louis, USA) and 2.5 mM caffeine for a sperm concentration of  $5 \times 10^6$  /ml. A 100-µl aliquot of the sperm suspension was incubated for 1 h at 38.5°C with 5% CO<sub>2</sub> in air prior to insemination.

Insemination and subsequent culture were performed according

to the method reported by Kajihara et al (6). The COCs cultured for 21 to 22 h were transferred to each sperm microdroplet for insemination. After the incubation of spermatozoa and oocytes for 5 h, each oocyte was transferred to culture medium (25 mM Hepes TCM-199 supplemented with 5% of SCS, 5µg/ml insulin (Sigma, St.Louis, USA), and 50 µl/ml gentamicin sulfate;18) and cultured for up to 9 d after insemination at 38.5°C in 5% CO<sub>2</sub> in air.

Morphologically normal blastocysts and expanded blastocysts which had developed to that stage on Days 7, 8, 9 following in vitro fertilization were used in this experiment.

#### Freezing-Thawing and Culture

Embryos were suspended in Dulbecco's phosphate buffered saline (D-PBS; Gibco, NY, USA) supplemented with 10% SCS and 1.6 M propylene glycol (PG; Wako Pure Chemical, Osaka, Japan), 1.8 M ethylene glycol (EG; Wako Pure Chemical, Osaka, Japan), 1.1 M diethylene glycol (DEG; Boron Laboratory, Saitama, Japan) or 1.3 M ethylene glycol monomethyl ether (EME; Boron Laboratory, Saitama, Japan). The cryoprotectants were added directly at room temperature (20 to 25°C).

After 10 to 20 min equilibration, the embryos were loaded into 0.25-ml plastic straws, placed directly into a 0°C alcohol bath chamber, and held for 2 min. Then the embryos were cooled from 0°C to -5.5°C at 1°C/min and seeded at -5.5°C. After seeding, the straws were held for 10 min at -5.5°C, and cooled at a rate of 0.3°C/min to -30°C. Finally, the straws were plunged and stored in LN<sub>2</sub>.

Embryos were thawed by placing the straws in a 30°C water bath, and the contents drained into a sterile petri dish. The embryos were then placed into the culture medium for rehydration and washed 3 times. Embryos were cultured on feeder layers of bovine cumulus cells in TCM-199 supplemented with 5% SCS and 5µg/ml insulin under 38.5°C in 5% CO<sub>2</sub> in air.

Embryos were evaluated microscopically at 24 h intervals for 48 h. Survival rates were assessed at 48 h culture, by 3 parameters: 1) re-appearance of the blastocoele 2) attainment of a fully expanded stage (including the hatching and hatched blastocyst) and 3) ability to hatch in vitro.

The effect of the cryoprotectant on subsequent development into above criterions for each age group was tested by an analysis of variance (ANOVA). A probability of 0.05 was considered significant.

#### RESULTS

Table 1 shows the survival rates of in vitro produced bovine embryos frozen in various cryoprotectants, assessed by re-appearance of the blastocoele after 48 h of culture. The survival rates in the Day-9 embryos were significantly lower than those in the Day-7 and 8 embryos among 4 cryoprotectants (P<0.01).

Table 1. Survival rates (%) of in vitro produced bovine blastocysts frozen with various cryoprotectants. Survival rates were assessed by re-appearance of the blastocoele after 48 hours of culture.

| Cryoprotectant | Age of embryo |           |           | ANOVA |
|----------------|---------------|-----------|-----------|-------|
|                | Day 7         | Day 8     | Day 9     |       |
| EME            | 94.4 (71)     | 76.3 (38) | 65.2 (23) | a     |
| E G            | 85.3 (68)     | 86.1 (36) | 60.5 (43) | a     |
| DEG            | 81.7 (60)     | 78.1 (32) | 29.2 (24) | a     |
| P G            | 81.4 (43)     | 82.5 (40) | 55.0 (20) | a     |
| ANOVA          | a             | a         | b         |       |

Values in rows and columns with no letters in common are significantly different ( $P < 0.01$ ). EME=Ethylene glycol monomethyl ether; EG=Ethylene glycol; DEG=Diethylene glycol; PG=Propylene glycol. Figures in parenthesis indicate number of embryos.

Table 2 shows the survival rates based on attainment of fully expanded stage after 48 h culture. The rate of expansion of blastocysts in the Day-9 embryos was significantly lower than those in the Day-7 and 8 embryos among 4 cryoprotectants ( $P < 0.01$ ).

Table 2. Survival rates (%) of in vitro produced bovine blastocysts frozen with various cryoprotectants. Survival rates were assessed by attainment of fully expanded stage after 48 hours culture.

| Cryoprotectant | Age of embryo |           |           | ANOVA |
|----------------|---------------|-----------|-----------|-------|
|                | Day 7         | Day 8     | Day 9     |       |
| EME            | 87.3 (71)     | 55.3 (38) | 43.5 (23) | a     |
| E G            | 76.5 (68)     | 80.6 (36) | 39.5 (43) | a     |
| DEG            | 68.3 (60)     | 57.1 (21) | 20.8 (24) | a     |
| P G            | 65.1 (43)     | 70.0 (40) | 35.0 (20) | a     |
| ANOVA          | a             | a         | b         |       |

Values in rows and columns with no letters in common are significantly different ( $P < 0.01$ ). EME=Ethylene glycol monomethyl ether; EG=Ethylene glycol; DEG=Diethylene glycol; PG=Propylene glycol. Figures in parenthesis indicate number of embryos.

Table 3 shows the survival rates based on the ability to hatch from the zona pellucida after 48 h of culture. As to the age of embryo, the hatching blastocysts rates in the Day-9 embryos were significantly lower than those in the Day-7 and 8 embryos among 4 cryoprotectants ( $P < 0.05$ ). As to the cryoprotectants, there were significant differences between DEG and EME, between DEG and EG and between PG and EG ( $P < 0.05$ ).

Table 3. Survival rates (%) of in vitro produced bovine blastocysts frozen with various cryoprotectants. Survival rates based on the ability to hatch from the zona after 48 hours of culture.

| Cryoprotectant | Age of embryo |           |           | ANOVA |
|----------------|---------------|-----------|-----------|-------|
|                | Day 7         | Day 8     | Day 9     |       |
| EME            | 66.2 (71)     | 44.7 (38) | 34.8 (23) | a     |
| EG             | 60.3 (68)     | 66.7 (36) | 34.9 (43) | a, c  |
| DEG            | 51.7 (60)     | 33.3 (21) | 16.7 (24) | b     |
| PG             | 51.2 (43)     | 40.0 (40) | 20.0 (20) | d     |
| ANOVA          | a             | a         | b         |       |

Values in rows with no letters in common are significantly different ( $P < 0.05$ ). a-b, c-d Values in columns are significantly different ( $P < 0.05$ ). EME=Ethylene glycol monomethyl ether; EG=Ethylene glycol; DEG=Diethylene glycol; PG=Propylene glycol. Figure in parenthesis indicate number of embryos.

#### DISCUSSION

Massip et al. (9) and Suzuki et al. (15) have already succeeded in freezing in vivo bovine embryos by a direct transfer method. Since then, many researchers have applied these methods for in vivo or in vitro fertilized embryos. Recently, Voelkel et al. (17,18) used ethylene glycol to freeze in vivo embryos and obtained high survival and pregnancy rates by direct transfer.

In the present experiment, bovine IVF embryos on Days 7, 8 and 9 were frozen in PG, EG, DEG and EME. The methods of direct rehydration into holding medium without step-wise or sucrose-mediated dilution of each cryoprotectant were examined to determine the effects of different embryo ages. Tachikawa et al. (16) reported that the average percentage of hatching embryos was decreased with the delay of development to the blastocyst stage. Fukushima et al. (4) reported that the pregnancy rate of IVF bovine embryos on Day 8 was lower than on Day 7. Similarly, in our study the average survival rate and the percentages of expanding and hatching blastocysts were decreased with the progression of days in 3 of 4 cryoprotectants (excluding EG). The present findings suggest that the survival rate of the frozen-thawed bovine embryos is affected by the embryo quality and not the type of the cryoprotectant used.

Voelkel et al. (17,18) reported that poor in vivo (16%) and in vitro (30.2%) embryo viability was observed when PG was used as a cryoprotectant. However, in our experiment, the average rate of reappearance of blastocoele (81.4%), and the percentages of fully expanded (65.1%) and hatching (51.2%) blastocysts in the frozen-thawed IVF bovine embryos on Day 7, using PG as a cryoprotectant, were not significantly different from those obtained using EG (85.3%, 76.5% and 60.3%, respectively) as a cryoprotectant. The difference observed in the viability of frozen-thawed bovine embryos may be due to the different freezing methods used (with different seeding point, cooling rate etc.). The IVF embryos used in this experiment were co-cultured with cumulus cells according

to the methods of Goto et al. (5). Fukui et al. (3) reported that the cell number of the blastocysts would be effected depending on the in vitro culture condition. This suggests that the differences in survival rates between our experiment and that of Voelkel et al. (17,18) may be dependent on the differences in culture conditions, embryo quality, embryo age and whether they are fertilized in vivo or in vitro.

When IVF embryos on Days 7 and 9 were frozen using EME as the cryoprotectant, the average rate of reappearance of blastocoele and the percentages of expanding and hatching blastocysts were higher than those obtained using the other cryoprotectants. These findings indicate that EME may be a useful cryoprotectant for bovine embryos, with direct rehydration performed by placing thawed embryos into a holding medium. However, more research is needed to determine possible negative effects of EME.

In conclusion, the present findings suggested that embryo age (Day 7 and 8) is a very important factor for successful freezing of IVF bovine embryos. As regards hatching rate of frozen thawed bovine IVF embryos, after in vitro culture, EME and EG may be superior as cryoprotectants than PG and DEG.

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