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Effect of one-step dilution procedures on the viability of bovine embryos frozen-thawed in ethylene glycol

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INTRODUCTION

Embryos frozen in glycerol showed a low viability after being directly placed in an isotonic solution or transferred to recipient animals without removing the cryoprotectant[12]. Frozen-thawed embryos will be vulnerable to osmotic injuries owing to excessive expansion, if they are frozen in a cryoprotectant which has a low permeability to embryo cells such as glycerol and are directly placed in an isotonic condition. However, osmotic injuries could be reduced when embryos were frozen in high permeable cryoprotectants even if embryos were diluted directly in an isotonic solution without dilution. The direct dilution of cryoprotectants from frozen-thawed embryos have been reported for dimethyl sulfoxide (DMSO)[6,20], methanol[1,10], propylene glycol (PG)[12] and ethylene glycol (EG)[8,9,11,17].

Highly permeable cryoprotectants can be used alone for direct transfer of bovine embryos without the addition of nonpermeable agents. Suzuki et al.[12] reported a high pregnancy rate when embryos frozen in PG were transferred directly to the recipient animals. Voelkel and Hu[17,18] reported that EG could be used effectively for direct transfer of bovine embryos. We also confirmed that EG was an effective cryoprotectant for direct transfer of bovine embryos[2]. Direct transfer is of much more practical use under on-farm conditions because no laboratory equipment is needed for dilution of cryoprotectants. Also direct transfer can eliminate technical errors made during the dilution of cryoprotectants. In addition, embryos frozen-thawed in such cryoprotectant show high survival rates when the embryos are transferred directly into an isotonic solution after thawing. It is possible that cryopotectants can be diluted directly by placing embryos in an isotonic solution. The direct dilution method is commonly used for in vitro produced bovine embryos frozen-thawed in EG[3-5,14,15,22]. From a practical standpoint, the freezing method using high permeable cryoprotectants can be effectively used when confirming the embryos viability before transferring them to recipient animals, or when multiple frozen-thawed embryos are used in various experiments. The possibility exists that the viability of frozen-thawed embryos will vary among individuals if frozen embryos are diluted directly in an isotonic solution under varying field conditions. As far as we know, no reports have been examined the effect of direct dilution conditions on the viability of bovine in vivo embryos frozen-thawed in ethylene glycol. The present study was conducted to examine the effect of the direct dilution conditions on the viability of bovine embryos frozen-thawed in 1.8M EG.

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MATERIALS AND METHODS

Embryos

Embryos were collected nonsurgically from superovulated Japanese Black and cross-breed cows on day 7 or 8 after estrus. Follicle stimulating hormone (FSH; Antrin Pharmaceutical Ind., Denka, Kawasaki, Japan) was injected intramuscularly (i.m.) at decreasing doses twice daily over 4 days (total 28 AU; 5/5, 4/4, 3/3, 2 and 2 AU). Luteolysis was induced by injecting 750 μg cloprostenol (Estrumate, Sumitomo Pharmaceutical Ind., Osaka, Japan) i.m. 48 h after the initial injection of FSH. Artificial insemination was performed on the day of estrus and again the following morning with frozen semen from a Japanese Black bull. The embryos were examined microscopically to determine their morphological quality and developmental stage as previously described[7]. Two hundred twenty-four embryos of excellent to fair quality ranging from the compacted morula to blastocysts stage were used in the present study. The embryos were washed with several changes of modified Dulbecco's phosphate-buffered saline containing 1g/l of glucose, 0.036 g/l Na pyruvate (Gibco, Laboratories, Grand Island, NY, U.S.A), and supplemented with 20% heat-inactivated calf serum (Gibco, Laboratories, Grand Island, NY, U.S.A) (PBS-CS). The embryos were then maintained in PBS-CS at room temperature (25℃ to 30℃) or on a hot plate at 36℃ until used.

Freezing and thawing of the embryos

The cryoprotectant used in the present study was 1.8M EG (Wako pure chemical Ind., Osaka, Japan) dissolved in PBS-CS, and the embryos were frozen as previously described[2]. The embryos were transferred into the freezing medium first, then each embryo was loaded into a 0.25 ml French plastic straw (I.M.V., L'Aigle, France) filled only with cryoprotectant solution. The embryos were equilibrated for 10 to 33 min (total time in the dish plus the straw) at room temperature. The straws were then placed directly into the precooled chamber of a programmable freezer (ET-1, Fujihira, Tokyo, Japan, and ET-UM, Fujiya Yano Science, Hokkaido, Japan) at −7℃. After 2 min, the straws were seeded, maintained at −7℃ for another 8 min, and cooled to −30℃ at 0.3℃/min before being plunged into liquid nitrogen. The straws were thawed by immersion in a 30℃ water bath until all the ice had disappeared.

Experiment 1

The effect of dilution temperature on the viability of embryos frozen-thawed in 1.8M EG was examined. The frozen-thawed embryos along with 0.1 ml of the freezing medium, were each transferred directly into 2 to 3 ml of PBS-CS, then held for 10 to 30 min at 22℃ or 38.5℃. PBS-CS was kept at 22℃ or 38.5℃ until use, respectively. The embryos were maintained in an incubator at 38.5℃ or in room at 22℃ during dilution. The embryos were washed with several changes of fresh PBS- CS, then placed in a 100 μl drop of TCM-199 (Gibco, Laboratories, Grand Island, NY, U.S.A) medium supplemented with 10% CS on a petri dish under paraffin oil. They were incubated at 38.5℃ in an atmosphere of 5% CO_2 in humidified air. The viability of the embryos was assessed by their ability to develop from compacted morula to blastocysts and from blastocysts to expanding blastocysts after a 48 hr culture.

Experiment 2

This experiment was conducted to examine the effect of PBS-CS volume for direct dilution on viability when the frozen-thawed embryos in 1.8M EG were diluted directly in PBS-CS. Dilution of EG was performed using the following three methods. The frozen-thawed embryos along with 0.1 ml of the freezing medium, were transferred directly into 0.5 ml (6-fold) of PBS-CS or 5 ml (51-fold) PBS-CS, then held for 10 min, and diluted step-wise (6% EG, 3% EG, 0% EG). PBS-CS was warmed to 38.5℃ until use in this experiment, and embryos were maintained in an incubator at 38.5℃ during dilution. The culture and assessment of embryonic development were performed in the same manner as Experiment 1.

Statistical analysis

The statistical significance of the results was evaluated by χ^2 test.

RESULTS

The viability of frozen-thawed embryos after culture when the EG was diluted directly in PBS-CS at 22℃ or 38.5℃ are shown in Table 1. The viability after culture was significantly lower for embryos diluted at 22℃ (26%) than for those at 38.5℃ (50%) or stepwise (50%)($P<0.05$). The viability of frozen-thawed embryos were affected by dilution temperature, and significantly improved at 38.5℃ as compared with 22℃ (Experiment 1). The viability frozen-thawed embryos after culture when the EG was diluted by the three methods are shown in Table 2. There were no significant differences in viability after culture between the dilution methods. The PBS-CS volume for direct dilution did not influenced viability of frozen-thawed embryos (Experiment 2).

Table 1 In vitro development of embryos frozen-thawed in 1.8 M ethylene glycol after one-step dilution: Effect of one-step dilution temperatures of ethylene glycol

Experimental groups[a]	No. of embryos	Culture period (hours)		
		0h	24h	48h
22℃	46	30(65.2)*	17(36.9)	12(26.0)[b]
38.5℃	44	35(79.5)	22(50.0)	22(50.0)[c]
Stepwise	44	32(72.7)	27(61.3)	22(50.0)[c]

* No. of viable embryos (%)

[a] Embryos frozen in 1.8M EG were diluted in PBS-CS at 22℃, 38.5℃, or by stepwise.

[b,c] Different superscripts denote significant difference ($P<0.05$).

Table 2 In vitro development of embryos frozen-thawed in 1.8 M ethylene glycol after one-step dilution: Effect of PBS-CS volume as diluent for one-step dilution of ethylene glycol

Experimental groups[a]	No. of embryos	Culture period (hours)		
		0h	24h	48h
Method A	30	24(80.0)*	21(70.0)	17(56.7)[b]
Method B	30	25(83.3)	19(63.3)	18(60.0)[b]
Stepwise	30	25(83.3)	19(63.3)	17(56.7)[b]

* No. of viable embryos (%)

[a] Embryos frozen in 1.8M EG were diluted 6-fold (Method A) and 51-fold (Method B) in PBS-CS at 38.5℃ or by stepwise.

[b] Values are not different.

DISCUSSION

Miyamoto and Ishibashi[8,9] reported that EG can be effectively used for freezing and one-step dilution in an isotonic solution out of cryoprotectant in mouse and rat embryos. Songsasen et al.[11] reported that EG was superior to propylene glycol or DMSO when sheep embryos were frozen and then directly diluted in PBS. Kojima et al.[6] reported that the survival rate of rabbit embryos frozen-thawed in 1.5M DMSO after one-step dilution in PBS and direct transfer to recipient animals was equivalent to that of embryos diluted by the stepwise method. However, low pregnancy rates were obtained after direct transfer to recipient animals of bovine embryos frozen-thawed with 1.5M DMSO[19]. Suzuki et al.[12] demonstrated that 1.6M PG can be effectively used for freezing, one-step dilution in PBS, and direct transfer to recipient animals. Voelkel and Hu[18] reported that post-thaw viability was higher for embryos frozen in 1.5M EG and diluted directly in PBS than for embryos frozen with 1.4M glycerol, 1.5M DMSO or 1.5M PG. They also demonstrated that the pregnancy rate after direct transfer of embryos frozen-thawed in 1.5M EG was equivalent to that of embryos frozen-thawed in 1.4 M glycerol followed by stepwise dilution. According to these reports, it appears that there is variation among species or cryoprotectant types in the viability of embryos subjected to one-step dilution and direct transfer after thawing. However, it is probable that EG is more effective than other cryoprotectants for direct transfer and one-step dilution in an isotonic solutions for mouse, rat[8,9], sheep[11] and bovine embryos[18]. The high permeability to embryo cells is an advantage of EG over other cryoprotectants[11,13]. Recently, the one-step dilution method in which embryos are directly diluted out of a cryoprotectant is commonly used for bovine IVF (in vitro fertilization) embryos frozen-thawed in EG[3-5,14,15,22].

The present study was conducted to examine the effects of conditions for one-step dilution in PBS-CS on the viability of bovine in vivo embryos frozen-thawed in 1.8M EG. In Experiment 1, the

viability of frozen-thawed embryos after one-step dilution in PBS-CS at 38.5℃ was significantly improved compared with embryos diluted at 22℃. This result clearly demonstrates that the dilution temperature had a considerable effect on the viability of embryos frozen-thawed in 1.8M EG when dilution was performed by one-step dilution in PBS-CS. The embryos frozen-thawed in 1.8M EG could be diluted directly in PBS-CS safely if dilution was performed at 38.5℃. The excessive expansion of embryonic cells during dilution of cryoprotectants is a serious problem for embryo viability. In particular, the viability of embryos may be adversely affected by osmotic injury when frozen-thawed embryos are placed directly into an isotonic solution. The effectiveness of the elevation of temperature during dilution has been reported in mouse[20] and rabbit embryos[16,21], when DMSO was diluted from frozen-thawed embryos by addition of PBS in a stepwise manner. In these reports, higher viability was obtained when DMSO was diluted from frozen-thawed embryos at 37℃ rather than 0℃ or 20℃ in mouse embryos. The results of Experiment 1 were supported by these reports.

Voelkel and Hu[18] reported that high post-thaw viability was obtained after one-step dilution of embryos frozen in 1.25 to 1.75 M EG at room temperature. Our results were consistent with those of Voelke and Hu[18], who held the embryos in PBS for 5 min at 20℃ to 24℃ after thawing, then transferred the embryos into the culture medium. In the present study, we held the embryos in PBS-CS for 10 to 30 min at 22℃. These differences in dilution procedure and freezing conditions may have contributed to the variation in results. Further experiments are needed to determine the differences.

Although the dilution mechanism of embryos transferred into the uterine horn of the recipient animal is still unknown, EG is probable diluted by very a small quantity of uterine fluid. Therefore, the effect of PBS-CS volume on embryo viability should be considered in addition to the dilution temperature when frozen-thawed embryos are diluted directly in PBS-CS. For this reason, the effect of PBS-CS volume for direct dilution on the viability of embryos frozen-thawed in 1.8M EG was examined in Experiment 1. The frozen-thawed embryos along with 0.1 ml of the freezing medium, were added to 0.5 ml (6-fold) or 5 ml (51-fold) of PBS-CS at 38.5℃. There were no significant differences between the 6-fold and 51-fold. The viability rates of both one-step dilution methods were equivalent to that of embryos diluted by the stepwise method. The results of Experiment 2 demonstrate that the viability of frozen-thawed bovine embryos in 1.8 M EG after one-step dilution in PBS-CS was not affected by the solution volume.

In conclusion, the present study demonstrates that dilution temperature is an important factor when frozen-thawed bovine in vivo embryos in 1. 8 M EG are diluted directly in an isotonic solution. Furthermore, the present study shows that EG can be effectively used in the dilution of frozen-thawed embryos by directly transferring embryos into the isotonic solutions and that dilution procedures can be simplified if EG is used as a cryoprotectant for bovine in vivo embryos.

Summary

The present study was conducted to examine the effect of the one-step dilution procedures on the viability of bovine embryos frozen-thawed in 1.8 M ethylene glycol (EG). Embryos were collected from superovulated cows on Day 7 or 8 after estrus. The embryos were transferred into the freezing medium first, then each embryo was loaded into a 0.25 ml straw filled only with freezing medium. The embryos were equilibrated for 10 to 33 min at room temperature. The straws were then placed directly into the precooled chamber of a programmable freezer at −7℃. After 2 min, the straws were seeded, maintained at −7℃ for another 8 min, and cooled to −30℃ at 0.3℃/min before being plunged into liquid nitrogen. The straws were thawed by immersion in a 30℃ water bath until all the ice had disappeared. In Experiment 1, embryos frozen in 1.8M EG were diluted in PBS-CS at 22℃, 38.5℃, or by stepwise. The dilution temperature significantly influenced the viability of embryos (26%, 50% and 50%, respectively) ($P<0.05$). In Experiment 2, the

frozen-thawed embryos along with 0.1 ml of the freezing medium, were diluted 6-fold and 51-fold in PBS-CS at 38.5℃ or by stepwise. There were no differences in the viability of embryos between the dilution procedurees (57%, 60% and 57%, respectively). These results demonstrate that the dilution temperature is an important factor when bovine embryos frozen-thawed in 1.8M EG are directly diluted in isotonic solution.

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要　　約

等張液を用いた凍害防止剤の一段階希釈が，エチレングリコール（EG）を用いて凍結したウシ胚の生存性に及ぼす影響を調べた。供試胚は，過剰排卵処置牛から回収した後期桑実胚および胚盤胞を用いた。胚は，子牛血清を20%加えた修正ダルベッコリン酸緩衝液（PBS－CS）で洗浄したのち，1.8 M EG添加PBS－CS中で凍結した。1.8 M　EGで凍結した胚を一段階でPBS－CSに移して希釈した場合の温度（22および38.5℃）が胚の生存率に及ぼす影響を調べた結果，38.5℃（50%）の生存率は22℃（26%）に比べて有意に高く（$P<0.05$），段階的希釈法（50%）と同等であった。また，1.8 M　EGで凍結した胚を38.5℃下で一段階希釈した場合の希釈倍率（6倍，51倍）を検討した結果，両区（57%，60%）に生存率の差はなく段階的希釈法（57%）と同等であった。以上のことより，1.8 M　EGで凍結した胚を等張液を用いて一段階希釈する場合，希釈時の温度が胚の生存率に影響する重要な要因であり，38.5℃に加温することによって高い生存率が得られることが明らかになった。