Journal of Reproduction and Development, Vol. 53, No. 4, 2007

—Technical Note—

Effect of Cryoprotectant Composition on *In Vitro* Viability of *In Vitro* Fertilized and Cloned Bovine Embryos Following Vitrification and In-Straw Dilution

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Abstract. In this study the efficacy of the combination of glycerol (GLY) and ethylene glycol (EG) as cryoprotectants in a vitrification method developed for direct embryo transfer was evaluated by *in vitro* development of *in vitro* fertilized (IVF) and somatic cell nuclear transfer (SCNT) embryos after vitrification. The IVF and SCNT blastocysts were vitrified in either 40% GLY, 30% GLY + 10% EG, or 20% GLY + 20% EG using French straws. After warming, the straws were held vertically for 1 min without shaking and were then placed horizontally for 5 min to dilute the cryoprotectants. After washing, the vitrified-warmed embryos were cultured *in vitro* for 72 h. There were no differences among the vitrification solutions with respect to the rates of vitrified-warmed IVF and SCNT embryos surviving and developing to the hatched blastocyst stage. However, the rates of development to the hatched blastocyst stage of the SCNT embryos vitrified with 40% GLY tended to be higher than those vitrified with 30% GLY + 10% EG or 20% GLY + 20% EG (26% *vs.* 7–8%, respectively). The development rates to the hatched blastocyst stage of the IVF and SCNT embryos vitrified with solution containing EG were significantly lower ($P<0.05$) than those of non-vitrified embryos. These results suggest that use of the combination of GLY and EG as cryoprotectants had no beneficial effect on the viability of embryos after in-straw dilution. However, this method is so simple that it can be used for practical direct transfer of vitrified embryos in the field.

Key words: Bovine, Cryoprotectant, Direct transfer, *In vitro* fertilization (IVF), Somatic cell nuclear transfer (SCNT), Vitrification

(**J. Reprod. Dev. 53:** 963–969, 2007)

Recent reports have confirmed that vitrification of oocytes and embryos is at least as efficient as conventional freezing [1–5]. Vitrification reduces the time commitment and equipment expense associated with cryopreservation when compared with conventional freezing. Direct transfer of vitrified embryos may be more practical in the field because manipulation of embryos through dilution of the cryoprotectant at the time of transfer is not necessary. Direct transfer following in-straw dilution of cryoprotectants has become a widely adopted practice for transfer of bovine embryos frozen by the conventional slow rate procedure [6]. In development of direct transfer procedures, switching from glycerol [7] to ethylene glycol [8, 9] resulted in simplification of the process after thawing. Although many vitrification methods

Accepted for publication: March 6, 2007
Published online: April 10, 2007
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have been developed for bovine embryos [10], most of these protocols require the step-wise removal of the cryoprotectant before embryo transfer. Recently, the open-pulled straw (OPS) technology was proven successful for in-straw cryoprotectant dilution following vitrification of bovine embryos [11, 12]. However, this technique for direct transfer of vitrified embryos requires skillful operators to handle the embryo container properly during in-straw warming. Therefore, an alternative method has been required to facilitate application of direct transfer of bovine vitrified embryos to field conditions.

As production of a large number of identical cloned animals from somatic cells is becoming more feasible, it is necessary to develop a simple and efficient freezing method that allows direct transfer of embryos on a large industrial scale. *In vitro* and *in vivo* development of bovine somatic cell nuclear transfer (SCNT) embryos after vitrification and warming have been demonstrated [12, 13], but little information is available concerning direct transfer of vitrified SCNT embryos.

The objective of this study was to develop an in-straw dilution method suitable for direct transfer of vitrified bovine embryos. In addition, we examined the effect of use of combinations of ethylene glycol (EG) and glycerol (GLY) as a cryoprotectant on the viability of bovine *in vitro* fertilized (IVF) and SCNT embryos after vitrification and warming.

Materials and Methods

Preparation of IVF embryos

Preparation of bovine IVF embryos was conducted according to the methods described by Mori *et al*. [14]. Briefly, cumulus-oocyte complexes (COCs) were obtained from ovaries collected from a local abattoir. The COCs were cultured in a maturation medium containing TCM-199 medium (Earle's salts) buffered with 25 mM HEPES buffer (TCM-199; Invitrogen, Carlsbad, CA, USA) and supplemented with 5% (v/v) fetal bovine serum (FBS; Invitrogen) and antibiotics (50 μg/ml gentamicin; Sigma-Aldrich, St. Louis, MO, USA) for 21 h at 38.5 C in an atmosphere of 5% CO_2 and 95% air with saturated humidity. After maturation, the COCs were fertilized *in vitro* with frozen-thawed spermatozoa (5×10^6 cells/ml) in a Brackett-Oliphant medium [15] that contained bovine serum albumin (BSA; 3 mg/ml, Sigma), heparin (10 μg/ml; Novo Industry A/S, Osaka, Japan), and 2.5 mM caffeine (Sigma). After incubation with spermatozoa for 5 h, the oocytes were transferred to culture medium (TCM-199 supplemented with 5% FBS, antibiotics, and 5 μg/ml insulin; Sigma) in a 4-well culture dish (Nunc, Roskilde, Denmark) and incubated at 38.5 C in 5% CO_2 in air. Forty-eight hours after insemination (day 2; the day of insemination was defined as day 0), the culture medium was replaced with fresh medium, and the cumulus cells surrounding the embryos were removed by pipetting. Morphologically normal blastocysts were harvested on day 7 or 8. To eliminate sampling biases, embryos that were morphologically similar in size were allocated to each experimental group.

Preparation of SCNT embryos

A fibroblast-like cell line was established from a bovine fetus collected at a slaughterhouse. The fetal lung cells were cultured in α-MEM (Invitrogen) supplemented with 10% FBS, 1% (v/v) MEM Non-Essential Amino Acid solution (Invitrogen), and antibiotics. Once the fetal cells reached complete confluence, the cells were trypsinized and either frozen for storage (in liquid nitrogen) or used as donors for nuclear transfer (NT). The methods used for preparation of recipient oocytes and NT were modified from the procedure used by Fahrudin *et al*. [16]. Briefly, the COCs collected from the bovine ovaries were matured for 19 h and denuded mechanically in 0.1% (w/v) hyaluronidase (Sigma). The first polar body and adjacent nuclear material were visualized with Hoechst 33342 (5 μg/ml; Sigma) and removed from the oocytes by aspirating a minimal amount of the surrounding cytoplasm into a bevelled pipette. A single donor cell was placed into the perivitelline space of the enucleated oocyte. Couplets were transferred into a drop of fusion buffer [0.3 M mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.5 mM Hepes, and 0.4% (w/v) BSA] and then manually aligned between the two electrode needles connected to the micromanipulator (MO-202D; Narishige, Tokyo, Japan). A single simultaneous fusion and activation electrical pulse of 2.3 kV/cm was applied for 30 μs to the couplets using an electro cell fusion generator (LF101; Nepa Gene, Chiba, Japan). The fused couplets were activated in

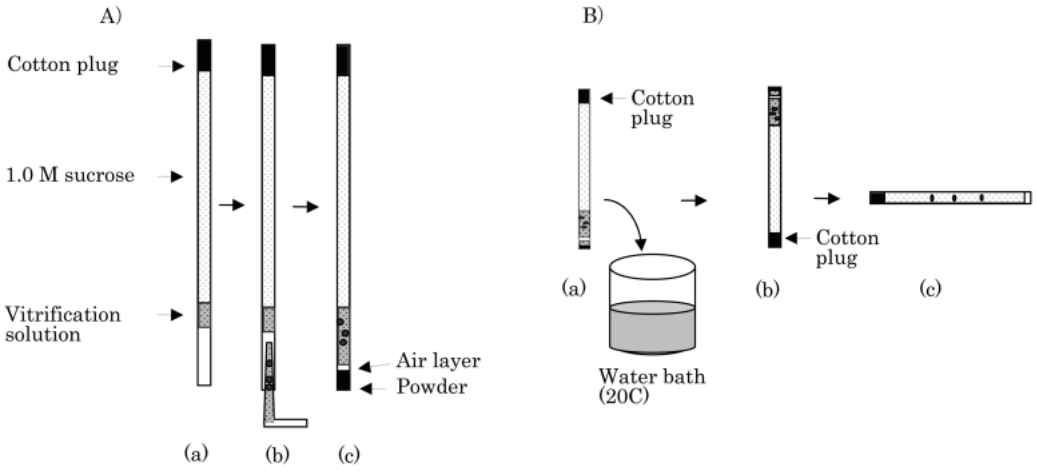

Fig. 1. Steps involved in the procedures of embryo loading and in-straw dilution. A) The straw was loaded in the following manner. (a) It was preloaded with the TCM-199 solution containing 1 M sucrose (8.0 cm) followed by the vitrification solution (approximately 1.0 cm). (b) The vitrification solution (30 μl) containing embryos was loaded using a pipette. (c) The rest of the straw was filled with an air bubble (approximately 0.1 cm) and vitrification solution (0.2 cm), and then it was sealed with powder. B) The cryoprotectants were diluted in the following manner. (a) The straw was plunged vertically into a 20 C water bath for warming until the TCM-199 solution melted (approximately for 10 sec). (b) It was then inverted and maintained in a vertical position for 1 min to allow mixture of the cryoprotectant and sucrose solutions. (c) After cutting off the layer of powder, the straw was placed in a horizontal position for 5 min to dilute the cryoprotectants.

10 μg/ml cycloheximide (Sigma) for 5 h and then cultured in modified synthetic oviduct fluid (mSOF) medium [17] supplemented with 0.4% BSA in a portable incubator. On day 3 after fusion, embryos that had cleaved were co-cultured with bovine cumulus cells in mSOF supplemented with 5% FBS at 38.5 C under 5% CO_2 in air. Morphologically normal blastocysts were harvested on day 7 or 8 after fusion. The bovine cumulus cells were derived from the recipient COCs and prepared at the time of removal of the cumulus cells. To eliminate sampling biases, embryos that were morphologically similar in size were allocated to each experimental group.

Vitrification of IVF and SCNT embryos

The IVF and SCNT embryos were washed in TCM-199 supplemented with 0.3% (w/v) BSA and antibiotics (TCM-199 solution). It has been shown that bovine blastocysts are less permeable to GLY than to EG and that the permeating speed of the former is slower than that of the latter [18]. Permeation of a certain amount of cryoprotectant into the embryo, which increases as the duration of exposure increases, has been suggested to be an essential factor in cryosurvival [3]. Therefore, the embryos were first equilibrated at room temperature (23–25 C) for 2 min in TCM-199 solution supplemented with 10% (v/v) GLY (Wako Pure Chemical, Osaka, Japan) and then placed into TCM-199 solution supplemented with 20% GLY for 2 min before exposure to a GYL-containing vitrification medium. After equilibration at each step (10% and then 20% GLY), the embryos were exposed to three different vitrification solutions to examine the effect of combinations of EG (Wako) and GLY on the viability of vitrified-warmed IVF and SCNT embryos. The vitrification solutions consisted of a TCM-199 solution supplemented with 0.6 M sucrose and either 40% GLY, 30% GLY + 10% (v/v) EG, or 20% GLY + 20% EG. In each vitrification solution group, the embryos were first suspended in a microdroplet (30 μl) of ice-cold vitrification solution and then consecutively transferred into different fresh microdroplets twice for equilibration. The vitrification solution (about 30 μl) containing the embryos (approximately 2–5 embryos per straw) was loaded into a 0.25-ml French plastic straw (I.M.V., L'Aigle, France) using a pipette (Fig. 1A). The end of the straw was sealed

Table 1. Effect of vitrification solution on the survival and development of IVF embryos after vitrification and warming[†]

Vitrification solution	No. of vitrified embryos	Mean ± SEM (No.) of survived embryos[††]	Mean ± SEM (No.) of embryos developed to the hatched blastocyst stage[†††]
40% GLY	55	74.2 ± 6.6 (40)	33.1 ± 3.2 (18)[a,b]
30% GLY + 10% EG	55	63.7 ± 9.5 (36)	29.5 ± 8.6 (18)[a]
20% GLY + 20% EG	55	59.0 ± 8.1 (31)	24.2 ± 13.1 (11)[a]
Non-vitrified control	65	–	57.0 ± 6.7 (40)[b]

[†]Five replicates.

[††]After warming, the embryos were cultured *in vitro* for 48 h, and the survival rate of the embryos was assessed based on the re-expansion of the blastocoele.

[†††]After warming, the embryos were cultured *in vitro* for 72 h, and the rate of hatched blastocysts was assessed.

[a,b]Values with different superscripts within the same column are significantly different ($P < 0.05$).

with powder. After the vitrification solutions and embryos were loaded into the straw, the straw was placed vertically to prevent mixing of the vitrification and sucrose solutions. Approximately half of the straw, including the vitrification solution containing the embryos, was rapidly immersed in liquid nitrogen, and the rest of the straw was then immersed slowly to prevent bursting. The embryos were vitrified in liquid nitrogen within 2 min after exposure to the vitrification solution.

In-straw cryoprotectant dilution and in vitro assessment

After storage for more than 5 days in liquid nitrogen, the straws were warmed in a 20 C water bath. They were then inverted and maintained in a vertical position for 1 min to allow mixture of the cryoprotectant and sucrose solutions without shaking. Subsequently, the layer of powder was cut off and the straw was placed in a horizontal position for 5 min to dilute the cryoprotectants (Fig. 1B). After dilution, the contents of the straws were expelled into a culture dish. The embryos were immediately transferred into fresh culture medium and washed twice. After washing, the embryos were cultured for 72 h on a feeder layer of cumulus cells in the culture medium in which the IVF embryos were cultured for 9 days.

The embryos were evaluated microscopically at 24-h intervals for 72 h. The survival rate of the embryos was assessed based on re-expansion of the blastocoele after 48 h of culture. The rate of blastocysts hatched after 72 h of culture was also determined. For controls, IVF and SCNT embryos were cultured in a similar manner without vitrification.

Statistical analysis

Data are expressed as means ± SEM. Among the cultured embryos, the proportions that survived and developed to the hatched blastocyst stage were subjected to arcsin transformation and analyzed by analysis of variance. The significance of differences between means was compared with the post-hoc Fisher's protected least significant difference test (PLSD test) using the Statview program (Abacus Concepts, Berkeley, CA, USA). Differences with a probability value (P) of 0.05 or less were considered significant.

Results

As shown in Table 1, there were no differences among the vitrification solutions with respect to the rates of survival and development to the hatched blastocyst stage of vitrified-warmed IVF embryos after *in vitro* culture. Similarly, there were no differences in the rates of survival and development of vitrified-warmed SCNT embryos among the vitrification solutions (Table 2). However, the rate of development of the SCNT embryos tended to be higher in the 40% GLY group than in the 30% GLY + 10% EG and 20% GLY + 20% EG groups. The rates of development of the IVF embryos vitrified with 30% GLY + 10% EG or 20% GLY + 20% EG were significantly lower ($P<0.05$) than those of non-vitrified embryos. Moreover, the

Table 2. Effect of vitrification solution on the survival and development of SCNT embryos after vitrification and warming[†]

Vitrification solution	No. of vitrified embryos	Mean ± SEM (No.) of survived embryos[††]	Mean ± SEM (No.) of embryos developed to the hatched blastocyst stage[†††]
40% GLY	23	76.4 ± 9.9 (18)	26.1 ± 11.5 (6)[a]
30% GLY + 10% EG	22	71.8 ± 11.4 (16)	7.1 ± 7.1 (2)[a]
20% GLY + 20% EG	22	50.3 ± 17.0 (11)	8.6 ± 5.1 (2)[a]
Non-vitrified control	22	–	68.9 ± 17.4 (12)[b]

[†]Four replicates.

[††]After warming, the embryos were cultured *in vitro* for 48 h, and the survival rate of the embryos was assessed based on the re-expansion of the blastocoele.

[†††]After warming, the embryos were cultured *in vitro* for 72 h, and the rate of hatched blastocysts was assessed.

[a,b]Values with different superscripts within the same column are significantly different ($P < 0.05$).

rates of development of the SCNT embryos after vitrification and warming significantly decreased ($P<0.05$) compared with those of non-vitrified embryos.

Discussion

Although use of the combination of GLY and EG as cryoprotectants had no beneficial effect on viability of the vitrified embryos after warming and direct dilution in straws. Dilution of cryoprotectants can be performed in this unique method by holding the warmed straw vertically without shaking. When the straw was maintained in a vertical position for 1 min after warming, the cryoprotectant layers within the straw gradually settled at the bottom as a result of the higher specific gravities of the cryoprotectants and then mixed with the sucrose layer. The cryoprotectants were observed to be completely mixed with the sucrose solution within 1 min, irrespective of the cryoprotectants used. However, this method requires a sticky cryoprotectant such as glycerol to prevent mixing of the vitrification and sucrose solutions before plunging into liquid nitrogen because the vitrification solutions within the straws were not separated by air bubbles. In preliminary experiment, use of vitrification solution containing more than 30% EG resulted in the solutions tending to mix with the sucrose solution before vitrification. Moreover, the rates of survival of the IVF embryos vitrified with 10% GLY + 30% EG and 40% EG significantly ($P<0.05$) decreased (16/54, 30.3 ± 4.2%, and 10/50, 19.3 ± 4.7%, respectively) compared with those of vitrification solution containing less than 20% EG (data not shown). These findings indicate that the in-straw dilution method without shaking the straw after warming requires a sticky cryoprotectant to prevent mixing of the vitrification and sucrose solutions before the straws plunged into liquid nitrogen. Therefore, in the present study, less than 20% EG was used in combination with GLY, and the combined effect of the cryoprotectants on the viability of IVF and SCNT embryos after vitrification and warming was evaluated.

Use of two permeable cryoprotectants rather than only one enables use of a lower concentration of each cryoprotectant in the vitrification solution. It has been shown that bovine embryos are more permeable to EG than to GLY, but the former is more toxic [3, 18]. Therefore, the combination of EG and GLY is assumed to reduce not only the toxicity of each cryoprotectant but also the osmotic damage at warming because EG is more likely to diffuse out of the cell rapidly. However, our results showed that there were no differences among the vitrification solutions with respect to the rates of survival and development of the vitrified-warmed IVF embryos. These results indicate that use of the combination of EG and GLY as cryoprotectants in the vitrification method developed for direct embryo transfer had no beneficial effect on the viability of vitrified-warmed embryos. On the other hand, the SCNT embryos are considerable to be more sensitive to freeze-thaw damage than IVF embryos [13, 19, 20] because the integrity of the zona pellucida in SCNT embryos is lost due to micromanipulation during enucleation and cell

injection. Therefore, the zona pellucida integrity of the embryos may be related to development of the vitrified-warmed embryos after *in vitro* culture. In the present study, the rate of development of the SCNT embryos vitrified with 30% GLY + 10% EG or 20% GLY + 20% EG tended to be lower compared with those vitrified with 40% GLY, whereas no detrimental effects were observed for the combination of EG and GLY in the IVF embryos. These observations indicate that the high sensitivity of SCNT embryos to freeze-thaw damage might clarify the detrimental effects of the combination of EG and GLY on the viability of vitrified embryos after warming. However, our results showed that when the SCNT embryos were vitrified in a solution of 40% GLY, the viability of the embryos after warming was similar to that of IVF embryos vitrified in the same solution. This indicates that the SCNT embryos could survive cryopreservation even when the cryoprotectants were diluted within the straw for direct transfer.

In conclusion, addition of EG to a GLY-containing vitrification medium had no beneficial effect on the survival and development of vitrified IVF and SCNT embryos after in-straw dilution. The viability of vitrified IVF and SCNT embryos decreased as compared with that of fresh embryos. However, this method (involving no shaking of the straw after warming) is so simple that it can be used for direct transfer of vitrified embryos in the field.

Acknowledgements

The authors thank the staff of the Meat Inspection Office of Kitakyushu City (Kitakyushu, Japan) for supplying the bovine fetus and ovaries. This work was supported by a Grant-in-Aid for Scientific Research (No. 16310140) from Japan Society for the Promotion of Science.

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