Successful cryopreservation of mouse blastocysts using a new vitrification solution

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Summary. Mouse blastocysts were exposed to solutions containing four concentrations (10, 20, 30 and 40% v/v) of six permeating cryoprotectants (glycerol, ethylene glycol, propylene glycol, dimethyl sulfoxide, 1,3-butanediol and 2,3-butanediol) in phosphate-buffered saline (PBS) with calf serum (CS) at room temperature (20–22°C). Blastocysts were exposed to these solutions for various periods, diluted into PBS plus CS with or without 1 mol trehalose l⁻¹ solution and their subsequent survival *in vitro* was examined. Two-way ANOVA showed a significant interaction ($P < 0.01$) between cryoprotectant type, concentration of cryoprotectant and method of dilution. However, no significant interaction was observed between cryoprotectant type and duration of exposure. Results suggest that cryoprotectant-induced injury to nonfrozen blastocysts is variable and depends on the cryoprotectant used. On the basis of toxicity assays, ethylene glycol was the least harmful and was combined with dimethyl sulfoxide and 1,3-butanediol to produce a new vitrification solution. Mouse blastocysts were successfully cryopreserved using a vitrification solution (designated as VSv) consisting of 20% ethylene glycol, 20% dimethyl sulfoxide and 10% 1,3-butanediol (v/v). Embryos were equilibrated in two steps, first in an equilibration solution (designated as ESv: 10% ethylene glycol, 10% dimethyl sulfoxide and 5% 1,3-butanediol; v/v) and then to VSv or one-step in VSv at different exposure times at room temperature, and then vitrified by direct plunging into liquid nitrogen. High developmental rates were obtained *in vitro* when the embryos were exposed to ESv and VSv for 3 and 0.5 min, respectively (96.2%) or exposed to VSv for 0.5 min (95.4%). Prolonged exposure time proved detrimental to subsequent embryo development *in vitro*. When vitrified warmed embryos were transferred immediately to pseudopregnant recipients, the rate of development to normal fetuses did not significantly differ from that of the nonvitrified control (two-step, 54.2 and one-step, 45.0 versus 60.0%, $P > 0.05$).

These results suggest that the simple vitrification solution described in this study is effective for the cryopreservation of mouse blastocysts.

Keywords: mouse; blastocyst; vitrification; cryoprotectant; equilibration

Introduction

The development of vitrification solutions represents a new concept in embryo cryopreservation. Vitrification solutions are aqueous cryoprotectant solutions that do not form ice crystals when cooled at moderate rates to very low temperatures (Fahy *et al.*, 1987), and thus avoid the potentially deleterious effects of intra- and extracellular freezing (Mazur, 1984). However, to achieve good survival after vitrification, embryos must be able to tolerate exposure and dehydration to these highly concentrated solutions (Rall, 1987).

Preimplantation mouse embryos have been successfully cryopreserved using vitrification solutions with different compositions. The first vitrification solution (VS1; Rall & Fahy, 1985) was composed of permeating (dimethyl sulfoxide, acetamide and propylene glycol) and nonpermeating (polyethylene glycol) cryoprotectants. This was followed by the development of a simpler solution composed of two permeating (glycerol and propylene glycol) cryoprotectants (Scheffen *et al.*, 1986). Rall (1987) also reported the use of either glycerol or propylene glycol in combination with polyethylene glycol. Kasai *et al.* (1990) developed a solution composed of a single permeating (ethylene glycol) and two nonpermeating (ficoll and sucrose) cryoprotectants.

However, previous studies (Scheffen *et al.*, 1986; Bielanski, 1987; Valdez *et al.*, 1990b) showed that mouse embryos at different stages differ in their sensitivity when exposed to vitrification solutions. These investigators reported that vitrified warmed mouse blastocysts had very low viability compared with other embryo developmental stages. This paper describes a simple vitrification solution composed of three permeating cryoprotectants that is effective for the cryopreservation of mouse blastocysts.

Materials and Methods

Source and recovery of embryos

Female ICR mice, four to seven weeks old and weighing at least 20 g, were used as embryo donors, and eight to twelve week old female mice of the same strain weighing at least 30 g were used as recipients for embryo transfer.

The mice were superovulated by intraperitoneal injection of 5 iu of pregnant mares' serum gonadotrophin (PMSG) (Teikoku Zoki, Tokyo, Japan), followed by 5 iu of human chorionic gonadotrophin (hCG; Teikoku Zoki) 48 h later. The females were mated singly with adult males from the same strain and were inspected the following morning for the presence of vaginal plugs (day 1).

Blastocyst-stage embryos were flushed from the excised uteri at 92 to 94 h after hCG injection using Dulbecco's phosphate-buffered saline (PBS[−]; Nissui Pharmaceutical Co., Tokyo, Japan) in which $0.9 \text{ mmol CaCl}_2 \cdot 2\text{H}_2\text{O l}^{-1}$ and $0.49 \text{ mmol MgCl}_2 \cdot 6\text{H}_2\text{O l}^{-1}$ were added (PBS) and supplemented with 10% heat-inactivated calf serum (CS; Gibco Lab., Life Technologies Inc., NY). After recovery, morphologically normal embryos were washed three times in fresh PBS plus CS, then pooled and held at room temperature (20–22°C) until used.

Cryoprotectant toxicity assays on nonfrozen blastocysts

Embryos were pipetted into a plastic culture dish containing 3 ml cryoprotectant, 2,3-butanediol (>98% mixture of meso-, D- and L-form; 2,3-butanediol, Merck-Schuchardt, Germany), 1,3-butanediol, dimethyl sulfoxide, propylene glycol, ethylene glycol or glycerol (Kanto Chemicals, Tokyo, Japan) in PBS plus CS at room temperature. After exposure, the embryos were pipetted into 3 ml 1 mol trehalose l^{-1} (Sigma, St Louis, MO, USA) in PBS plus CS for 5 min to dilute the cryoprotectant (Valdez *et al.*, 1991). After dilution, embryos were washed three times in PBS plus CS, placed in microdroplets of Whitten's medium (Biggers *et al.*, 1971) and then cultured *in vitro* at 37°C in 5% CO_2 in air for 24 h. Control embryos were not exposed to cryoprotectant, but were directly cultured *in vitro*. Survival of the embryos was assessed by their ability to subsequently develop into expanded or hatching blastocysts.

In Expt 1, embryos were exposed to increasing concentrations of cryoprotectants, 0, 10, 20, 30 or 40% (v/v) in PBS plus CS for 10 min at room temperature. In Expt 2, embryos were exposed for 20 or 40 min to 20% (v/v) concentration of cryoprotectant in PBS plus CS at room temperature. In Expt 3, embryos were either directly diluted in PBS plus CS or 1 mol trehalose l^{-1} solution for 5 min after exposure to (20% v/v) concentration of cryoprotectant for 5 min at room temperature.

Vitrification solutions

On the basis of toxicity assays, three permeable cryoprotectants, ethylene glycol, dimethyl sulfoxide, and 1,3-butanediol were selected as the components of a new vitrification solution. In this study, the equilibration solution (designated as ESv) consisted of 10% ethylene glycol, 10% dimethyl sulfoxide and 5% 1,3-butanediol (v/v), and the vitrification solution (designated as VSv) consisted of 20% ethylene glycol, 20% dimethyl sulfoxide and 10% 1,3-butanediol (v/v) in PBS plus CS.

Confirmation of vitrification

Vitrification of rapidly cooled VSv was confirmed by visual inspection of straws after cooling and during warming (Scheffen *et al.*, 1986) and by freeze-fracture electron microscopy (Valdez *et al.*, 1990a). In the first method, straws

were filled with either ESv or VSv, heat sealed and then plunged directly into liquid nitrogen ($2500^{\circ}\text{C min}^{-1}$). The straws were then warmed by transfer into water at 20°C . Straws were inspected for visible crystallization (whitening) after cooling and during warming.

Examination of vitrification solutions by freeze-fracture electron microscopy was as described by Valdez *et al.* (1990a). Twenty blastocysts were first equilibrated in ESv for 3 min, and then exposed to VSv for 40 s at room temperature. A small drop ($1.5\text{ }\mu\text{l}$) of VSv containing the embryos is pipetted on top of a small sample holder. It was then directly plunged into liquid nitrogen ($3000^{\circ}\text{C min}^{-1}$). Embryos that were cryo-fixed in isotonic PBS plus CS served as control. Each frozen specimen was transferred into a freeze-etching apparatus kept at a temperature of -110°C in a vacuum of 1×10^{-6} torr or lower. The specimen was fractured with a cold knife, etched for 1 min and replicated with platinum-carbon. After dissolving the specimen with a commercial bleach, the freeze-fracture replica was examined with a JEM 1200 EX electron microscope at an accelerating voltage of 100 kV.

Vitrification procedure

The vitrification procedure was similar to that described by Scheffen *et al.* (1986) and Valdez *et al.* (1990b). Embryos were equilibrated by the two-step (ESv-VSv) or one-step (VSv) procedure at room temperature. In the two-step procedure, embryos were first equilibrated in ESv and then transferred to $20\text{ }\mu\text{l}$ of VSv in a 0.25 ml French straw (IMV, L'Aigle, France). The column of VSv in the straw was separated by two air bubbles from the 1 mol trehalose l^{-1} solution placed on each side. In the one-step procedure, embryos were directly transferred to $20\text{ }\mu\text{l}$ of VSv in the manner described above. The end of the straw was heat sealed, and the straw was then held vertically and plunged directly into liquid nitrogen. Vitrified embryos were stored for 1 h to 21 days in liquid nitrogen.

Warming and cryoprotectant dilution

The straws were warmed by gently shaking in a 20°C water bath until ice had disappeared from the trehalose solution (about 15 s). The contents of each straw were then expelled into a sterilized plastic culture dish and held for 5 min at room temperature to dilute the cryoprotectant (Valdez *et al.*, 1991). The embryos were then collected and washed three times in PBS plus CS. Embryo survival was assessed by *in vitro* culture as described above.

In vitro experiments with or without vitrification

In Expt 4, embryos were pipetted into plastic culture dishes containing 3 ml of ESv or VSv and then exposed in the former for 3, 20 or 60 min or in the latter for 0.5, 1 or 3 min at room temperature. After exposure, embryos were immediately diluted in 1 mol trehalose l^{-1} , washed in PBS plus CS and then cultured *in vitro*. Nontreated control embryos were directly cultured *in vitro*.

In Expts 5 and 6, embryos were first equilibrated in ESv for 3 min before exposure to VSv for 0.5, 1, 3 or 5 min (Expt 5), or embryos were first equilibrated in ESv for 5, 10 or 20 min before exposure to VSv for 0.5 min (Expt 6) at room temperature. In Expt 7, embryos were exposed directly to VSv for 0.5, 1, 3, 5 or 10 min at room temperature. Embryos were then vitrified by direct immersion into liquid nitrogen.

In vivo development experiment

In Expt 8, ten to fifteen embryos per straw were vitrified after two-step or one-step exposure. After warming and cryoprotectant dilution, the embryos were washed in PBS plus CS and maintained in this medium on a warm plate at 37°C until transfer.

All vitrified warmed embryos from the same straw, irrespective of their morphological appearance were transferred within 20 min after warming into the uterine horn of a day 4 pseudopregnant recipient that had been mated to a vasectomized male (day of vaginal plug = day 1). The day 4 recipients were in synchrony on the same day as the donors. The uterine transfer procedure was as described by Hogan *et al.* (1986) with modifications by Abas Mazni *et al.* (1990). Five to eight embryos were transferred into each uterine horn of recipients under anaesthesia. In the case of nonvitrified embryos (control), five to seven fresh embryos that were flushed on the same day as the vitrified embryos were transferred into each uterine horn.

All recipients were necropsied on day 17 of pregnancy, and the number of implantation sites, normal or abnormal fetuses were recorded.

Statistical analysis

Cryoprotectant toxicity data were analysed by two-way ANOVA to determine interactions among cryoprotectant type, concentration, duration of exposure and dilution. Duncan's multiple range test was used to determine differences between mean ($\pm$ SEM) viability rates. Survival rates of nonvitrified and vitrified warmed embryos *in vitro* and *in vivo* were analysed using the χ^2 test.

Results

Effects of cryoprotectants on nonfrozen blastocysts

Expt 1: Effect of cryoprotectant concentration. There was a significant interaction between concentration and cryoprotectant type as shown by two-way ANOVA ($P < 0.01$) (Table 1). At 10% (v/v) concentration, high survival rates were obtained in all embryos exposed to cryoprotectants. At 20% (v/v) concentration, survival rates significantly decreased ($P < 0.05$) for embryos exposed to 2,3-butanediol, propylene glycol and glycerol. At 30% concentration, embryos treated with ethylene glycol showed significantly higher survival rates ($P < 0.05$) than those exposed to the other cryoprotectants. At 40% concentration, very low or no survival was observed in all embryos exposed to the various cryoprotectants.

Table 1. Effect of cryoprotectant concentration on the development ($\pm$ SEM)* of nonfrozen mouse blastocysts *in vitro* after exposure for 10 min (Expt 1)

Cryoprotectant	Cryoprotectant concentration (% v/v)			
	10	20	30	40
2,3-butanediol	100.0 $\pm$ 0 ^a	66.0 $\pm$ 13.6 ^{bd}	8.7 $\pm$ 5.3 ^{cde}	0 ^{cd}
1,3-butanediol	100.0 $\pm$ 0 ^a	96.7 $\pm$ 3.3 ^{ac}	1.7 $\pm$ 1.7 ^{bd}	0 ^{bd}
Dimethyl sulfoxide	100.0 $\pm$ 0 ^a	98.3 $\pm$ 1.7 ^{ac}	25.0 $\pm$ 9.6 ^{bc}	0 ^{cd}
Propylene glycol	100.0 $\pm$ 0 ^a	95.0 $\pm$ 1.7 ^{bc}	2.1 $\pm$ 2.1 ^{cd}	0 ^{cd}
Ethylene glycol	100.0 $\pm$ 0 ^a	95.9 $\pm$ 2.4 ^{ac}	74.6 $\pm$ 2.8 ^{bf}	2.1 $\pm$ 2.1 ^{cd}
Glycerol	100.0 $\pm$ 0 ^a	80.9 $\pm$ 8.5 ^{bde}	21.9 $\pm$ 6.2 ^{ce}	4.2 $\pm$ 5.2 ^{ce}
Control†				

*Values were computed as number of embryos viable: number of embryos recovered (total of 44–60 embryos per treatment group; averaged over four replicates).

†Embryos not exposed to cryoprotectant had 100.0 $\pm$ 0 survival rate after 24 h culture.

Values within rows with different superscripts (a, b, c) are significantly different ($P < 0.05$).

Values within columns with different superscripts (d, e, f) are significantly different ($P < 0.05$).

Expt 2: Effect of duration of exposure. There was no significant interaction between duration of exposure and cryoprotectant type as shown by two-way ANOVA (Table 2). At 20 min exposure, 2,3-butanediol showed significantly lower survival rate ($P < 0.05$) than those exposed to the other cryoprotectants. At 40 min exposure, both 2,3-butanediol and 1,3-butanediol showed significantly lower survival rates than those exposed to the other cryoprotectants ($P < 0.05$).

Table 2. Effect of duration of exposure on the development ($\pm$ SEM)* of nonfrozen mouse blastocysts *in vitro* exposed to 20% (v/v) concentration of cryoprotectant (Expt 2)

Cryoprotectant (20% v/v)	Duration of exposure (min)	
	20	40
2,3-butanediol	35.9 $\pm$ 21.8 ^a	8.3 $\pm$ 5.0 ^a
1,3-butanediol	81.2 $\pm$ 9.3 ^b	64.2 $\pm$ 12.1 ^b
Dimethyl sulfoxide	93.8 $\pm$ 2.2 ^b	96.7 $\pm$ 1.9 ^c
Propylene glycol	91.3 $\pm$ 6.3 ^b	87.5 $\pm$ 4.4 ^{bc}
Ethylene glycol	96.7 $\pm$ 3.3 ^b	95.8 $\pm$ 2.5 ^{bc}
Glycerol	79.2 $\pm$ 8.9 ^{bc}	81.7 $\pm$ 11.4 ^{bc}

*Values were computed as number of embryos viable: number of embryos recovered (total of 50–55 embryos per treatment group; averaged over four replicates).

Values within columns with different superscripts (a, b, c) are significantly different ($P < 0.05$).

Expt 3: Effect of cryoprotectant dilution. There was a significant interaction between dilution method and cryoprotectant type as shown by two-way ANOVA ($P < 0.01$) (Table 3). It was observed that the survival of embryos exposed to glycerol and then directly diluted in PBS plus CS or 1 mol trehalose l^{-1} was significantly different ($P < 0.05$). However, the survival of embryos exposed to the other cryoprotectants and then directly diluted in PBS plus CS or 1 mol trehalose l^{-1} were not significantly different.

Table 3. Effect of method of dilution on the development ($\pm$ SEM)* of nonfrozen mouse blastocysts *in vitro* exposed to 20% (v/v) concentration of cryoprotectant for 5 min (Expt 3)

Cryoprotectant (20% v/v)	Dilution method	
	PBS + CS	Trehalose
2,3-Butanediol	92.5 $\pm$ 4.8 ^c	95.0 $\pm$ 2.9
1,3-Butanediol	98.8 $\pm$ 1.2 ^c	100.0 $\pm$ 0.0
Dimethyl sulfoxide	98.8 $\pm$ 1.2 ^c	100.0 $\pm$ 0.0
Propylene glycol	98.8 $\pm$ 1.2 ^c	100.0 $\pm$ 0.0
Ethylene glycol	100.0 $\pm$ 0.0 ^c	100.0 $\pm$ 0.0
Glycerol	28.8 $\pm$ 11.3 ^{ad}	96.3 $\pm$ 2.4 ^b

*Values were computed as number of embryos viable: number of embryos recovered (total of 59–60 embryos per treatment group; averaged over four replicates).

Values within rows with different superscripts (a, b) are significantly different ($P < 0.05$).

Values within columns with different superscripts (c, d) are significantly different ($P < 0.05$).

PBS: phosphate-buffered saline; CS: calf-serum.

Confirmation of vitrification. Upon visual inspection of the straws after cooling and warming, ESv appeared milky and therefore, did not vitrify, whereas VSv appeared transparent, which indicated vitrification. A freeze replica image of a vitrified mouse blastocyst after exposure to ESv and VSv showed that there were no intracellular ice crystals in the cytoplasm. No extracellular ice crystals were observed (Fig. 1a). However, a freeze replica image of a mouse blastocyst cryofixed in PBS plus CS showed intracellular ice crystals in the cytoplasm (Fig. 1b).

Development *in vitro*

Expt 4: Effect of duration of exposure to ESv or VSv without vitrification. The *in vitro* survival rates of embryos exposed to ESv for 3 and 20 min at room temperature without vitrification were significantly higher than those exposed for 60 min (100.0 and 97.7% versus 53.3%, $P < 0.001$) (Table 4). However, the survival rates of embryos exposed to VSv for 0.5 and 1 min *in vitro* were significantly higher than those exposed for 3 min (100.0 and 100.0% versus 0%, $P < 0.001$). Nontreated control embryos showed a 100.0% survival rate *in vitro*.

Expts 5 and 6: Effect of duration of equilibration in ESv and VSv (two-step) followed by vitrification. In Expt 5, the survival rates of vitrified warmed embryos that were exposed to VSv *in vitro* for 0.5 and 1 min after 3 min equilibration time in ESv at room temperature were significantly higher than those exposed to VSv for 3, 5 and 10 min (96.2 and 89.3% versus 25.9, 9.5 and 0% ($P < 0.001$) (Table 5).

In Expt 6, the *in vitro* survival rates of vitrified warmed embryos that were equilibrated in ESv for 5 min at room temperature before exposure to VSv for 0.5 min were significantly higher than those equilibrated in ESv for 10 and 20 min (91.3% versus 55.6 and 43.6% ($P < 0.001$) (Table 5).

Fig. 1. (a) A freeze replica photograph of a vitrified mouse blastocyst. Note the absence of ice crystals in the cytoplasm (C) and extracellular solution (ES). Scale bar = 0.2 μ m. (b) A freeze replica photograph of a mouse blastocyst cryofixed in isotonic phosphate-buffered saline. Note the presence of ice crystals in the cytoplasm (arrows). Scale bar = 0.5 μ m.

Expt 7: Effect of duration of exposure in VSv (one-step) followed by vitrification. The survival rates of vitrified warmed embryos exposed to VSv only for 0.5 and 1 min *in vitro* were significantly higher than those exposed to VSv for 3, 5, and 10 min (95.4 and 94.1% versus 29.0, 6.7 and 0%, respectively, $P < 0.001$) (Table 5).

Development in vivo. In Expt 8, a total of 16 out of 22 recipients became pregnant after transfer of vitrified and nonvitrified embryos. Pregnancy rates of 66.7 (6/9), 80.0 (4/5), and 75.0% (6/8) were obtained for two-step, one-step, and fresh (control) recipients, respectively (Table 6). The rates of implantation and development to normal fetuses of vitrified warmed embryos after two-step

Table 4. Effect of duration of exposure to ESv or VSv on the development of mouse blastocysts *in vitro* without vitrification (Expt 4)

Exposure time (min)	Number of embryos*	Development after 24 h <i>in vitro</i> (%)
ESv		
3	45	45 (100.0) ^a
20	43	42 (97.7) ^a
60	45	24 (53.3) ^b
VSv		
0.5	38	38 (100.0) ^a
1	40	40 (100.0) ^a
3	45	0 (0) ^b
Nontreated Control	45	45 (100.0)

*Total of three to four replicates per treatment group. Values with different superscripts are significantly different ($P < 0.001$).

ESv: equilibration solution; VSv: vitrification solution.

Table 5. Effect of duration of equilibration in ESv and VSv (two-step) or VSv (one-step) on the development of vitrified warmed mouse blastocysts *in vitro* (Expts 5, 6 and 7)

Expt	Equilibration time (min)		Number of embryos*	Development after 24 h <i>in vitro</i> (%)
	ESv	VSv		
Expt 5	3	0.5	104	100 (96.2) ^a
	3	1	56	50 (89.3) ^a
	3	3	54	14 (25.9) ^b
	3	5	42	4 (9.5) ^c
	3	10	30	0 (0.0) ^c
Expt 6	5	0.5	92	84 (91.3) ^a
	10	0.5	90	50 (55.6) ^b
	20	0.5	39	17 (43.6) ^b
Expt 7	0	0.5	65	62 (95.4) ^a
	0	1	51	48 (94.1) ^a
	0	3	69	20 (29.0) ^b
	0	5	30	2 (6.7) ^c
	0	10	32	0 (0.0) ^{cd}

*Total of three to eight replicates per treatment group. Values with different superscripts are significantly different (a, b; a, c; b, d: $P < 0.001$) (b, c: $P < 0.05$).

ESv: equilibration solution; VSv: vitrification solution.

exposure (65.3 and 54.2%, respectively) did not significantly differ from that of the nonvitrified control group (78.6 and 60.0%, respectively). Although a significant difference ($P < 0.05$) was observed between the rate of implantation of vitrified warmed embryos after one-step exposure (60.0%) and that of the control (78.6%), no significant difference ($P > 0.05$) was observed in the rate of development to normal fetuses (60.0 versus 45.0%).

Table 6. *In vivo* development of vitrified warmed mouse blastocysts transferred to pseudopregnant recipients* (Expt 8)

Treatment group	Pregnant recipients	Number of embryos transferred	Number of implantations (%)†	Number of normal fetuses (%)‡
Vitrified				
two-step	6	72	47 (65.3) ^{ab}	39 (54.2)
one-step	4	40	24 (60.0) ^a	18 (45.0)
Nonvitrified fresh (control)	6	70	55 (78.6) ^b	42 (60.0)

*Necropsied at day 17 of pregnancy.

†Represents normal, premature or resorbing fetuses and implantation sites.

‡Number of normal fetuses: number of embryos transferred.

Values with different superscripts in each column are significantly different (a, b; $P < 0.05$).

Discussion

This study shows that a combination of three permeating cryoprotectants, ethylene glycol, dimethyl sulfoxide and 1,3-butanediol is effective for the cryopreservation of mouse blastocysts by vitrification.

In the process of composing a new vitrification solution, toxicity assays were first conducted to determine the least harmful cryoprotectants that could be used. Results showed that cryoprotectants can induce injury to nonfrozen mouse blastocysts to varying degrees at room temperature depending on the type of cryoprotectant used. A two-way interaction between cryoprotectant type, concentration and method of dilution had a significant effect on subsequent embryo survival *in vitro* ($P < 0.01$). Ethylene glycol seemed the least harmful of the six cryoprotectants examined for mouse blastocysts. This result is consistent with previous reports (Kasai *et al.*, 1981; Kasai *et al.*, 1990) that ethylene glycol is less toxic to nonfrozen mouse morulae at room temperature than other cryoprotectants. Hence, ethylene glycol was selected as the first component of the solution under study. In addition, ethylene glycol was reported to provide considerable protection against freezing damage to mouse and rat embryos (Miyamoto & Ishibashi, 1978) and has been successfully used for the quick freezing (Abas Mazni *et al.*, 1990) and vitrification (Kasai *et al.*, 1990) of mouse morulae and *Drosophila* embryos (Steponkus *et al.*, 1990). Ethylene glycol has also been shown to protect rye protoplasts from dehydration injury during vitrification (Langis & Steponkus, 1991) and may have a similar effect on mouse embryos. However, ethylene glycol has been reported to be a weak glass former compared with dimethyl sulfoxide and 1,3-butanediol (Fahy *et al.*, 1987). Since vitrification relies on a sufficiently concentrated solution that solidifies as glass during cooling without appreciable ice formation, dimethyl sulfoxide and 1,3-butanediol were included as additional components of the new vitrification solution to take advantage of their strong glass forming abilities to achieve vitrification at a low concentration of cryoprotectants thus minimizing chemical toxicity (MacFarlane & Forsyth, 1990). Moreover, dimethyl sulfoxide has been reported to be efficient for ultrarapid freezing of mouse embryos (Trownson *et al.*, 1987; Shaw & Trownson, 1989), and is one of the main components of the first vitrification solution for mouse embryos (VS1; Rall & Fahy, 1985). On the other hand, 1,3-butanediol has been used successfully for the cryopreservation of human red blood cells (Mehl & Boutron, 1988), but to our knowledge this is the first time it was used for the cryopreservation of mouse embryos.

Our results confirm earlier reports (Rall & Fahy, 1985; Scheffen *et al.*, 1986; Valdez *et al.*, 1990b) that exposure to vitrification solutions produces time-dependent injury, i.e. prolonged exposure was detrimental to subsequent embryo survival.

Prolonged exposure of embryos to ESv or VSv at room temperature without vitrification results in reduced survival rates *in vitro*. However, VSv is more detrimental to embryo survival than ESv.

On the basis of these results, we adopted a two-step exposure period before vitrification by rapid cooling to -196°C . In addition, it has been suggested that the use of a stepwise equilibration procedure controls the extent of cryoprotectant permeation, and thus prevents injury due to chemical toxicity or excessive osmotic stresses during equilibration or subsequent dilution (Rall, 1987).

Our results show high survival rates of vitrified warmed embryos *in vitro* after two-step exposure when the embryos are first equilibrated in ESv for 3 to 5 min before exposure to VSv for 0.5 min. When the embryos are equilibrated in ESv for 3 min and exposed to VSv for increasing periods, a high survival rate is observed *in vitro* only up to 1 min exposure in VSv, but sharply decreased after 3 min. These results suggest that prolonged exposure in both ESv and VSv is detrimental to subsequent embryo survival of vitrified warmed embryos. However, the exposure time in the latter is more critical than in the former.

Our results further show that it is possible to achieve high survival rates *in vitro* after one-step exposure to VSv even without full equilibration in ESv provided the embryos are exposed for only a short period, i.e. 0.5 or 1 min. Prolonged exposure to VSv for 3, 5 or 10 min appeared very damaging to the embryos as shown by the very low survival rates. However, it is difficult to explain why some vitrified warmed embryos exposed to VSv for 3 min survived, whereas none of the embryos exposed under similar condition without vitrification survived (Expt 4). This is probably related to differences in the method of dilution or volume of diluent during dilution between vitrified and nonvitrified exposed embryos.

Results of the exposure experiments agree with the previous conclusions that some permeation is necessary for successful cryopreservation by vitrification, but complete permeation is not desirable because it increases the likelihood of injury due to chemical toxicity or excessive osmotic swelling during dilution (Rall, 1987).

The *in vitro* results obtained in this study either by two-step (96.2%) or one-step (95.4%) exposure to the vitrification solution at room temperature were higher than those reported for mouse blastocysts in previous studies (Scheffen *et al.*, 1986; Bielanski, 1987; Valdez *et al.*, 1990b) using the vitrification solution developed by Scheffen *et al.* (1986). Our results were also higher than those reported for rat blastocysts by Kono *et al.* (1988) using the solution (VS1) developed by Rall & Fahy (1985).

Our results also demonstrate that normal fetuses could be obtained from vitrified warmed blastocysts that are transferred immediately after warming to pseudopregnant recipients. This observation indicates that it is not necessary to culture the embryos *in vitro* to obtain high *in vivo* development. Moreover, our results represent a conservative estimate of survival *in vivo* since all vitrified embryos recovered after warming, regardless of their morphology, were transferred to recipients. Although a higher rate of development to normal fetuses was obtained for embryos vitrified after two-step compared with one-step exposure (54.2 versus 45.0%), no significant difference was observed. Our development results from experiments *in vivo* are higher than those of Kono *et al.* (1988) for vitrified warmed rat blastocysts (41%), and those of Scheffen *et al.* (1986) and Rall *et al.* (1987) for vitrified warmed day 4 (30%) and day 3 (17%) mouse embryos, respectively. Kasai *et al.* (1990) and Valdez *et al.* (1990c) reported similar results after transfer of vitrified warmed mouse morulae (51 and 53.6%, respectively). Our results, however, are lower than those obtained after transfer of frozen-thawed mouse blastocysts cryopreserved by a simplified freezing method (60.3%; Landa, 1982) or by conventional freezing (63.2%; Whittingham *et al.*, 1979).

The vitrification solution developed in this study is simple and does not require precooling to achieve high survival rate. It can be used effectively for the cryopreservation of mouse blastocysts by vitrification. However, the ultrastructural changes that occur when embryos are exposed to vitrification solutions need to be investigated so that more effective and reliable vitrification solutions that are suitable for each embryo developmental stage or species can be developed.

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References

- Abas Mazni, O., Valdez, C.A., Takahashi, Y., Hishinuma, M. & Kanagawa, H. (1990) Quick freezing of mouse embryos using ethylene glycol with lactose or sucrose. *Animal Reproduction Science* **22**, 161–169.
- Bielanski, A. (1987) Survival *in vitro* of zona pellucida-free mouse embryos after cooling by conventional two-step or vitrification methods. *Cryo-Letters* **8**, 294–301.
- Biggers, J.D., Whitten, W.K. & Whittingham, D.G. (1971) The culture of mouse embryos *in vitro*. In *Methods in Mammalian Embryology*, pp. 110–112. Ed. J. C. Daniels Jr. W. H. Freeman and Co., San Francisco.
- Fahy, G.M., Levy, D.I. & Ali, S.E. (1987) Some emerging principles underlying the physical properties, biological actions, and utility of vitrification solutions. *Cryobiology* **24**, 196–231.
- Hogan, B., Constantini, F. & Lacy, E. (1986) Recovery, culture and transfer of embryos. In *Manipulating the Mouse Embryo*, pp. 142–145. Cold Spring Harbor, NY.
- Kasai, M., Niwa, K. & Iritani, A. (1981) Effects of various cryoprotective agents on the survival of unfrozen and frozen mouse embryos. *Journal of Reproduction and Fertility* **63**, 175–180.
- Kasai, M., Komi, J.H., Takakamo, A., Tsudera, H., Sakurai, T. & Machida, T. (1990) A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *Journal of Reproduction and Fertility* **89**, 91–97.
- Kono, T., Suzuki, O. & Tsunoda, Y. (1988) Cryopreservation of rat blastocysts by vitrification. *Cryobiology* **25**, 170–173.
- Landa, V. (1982) A simplified method for freezing mouse blastocysts. *Folia Biologica (Praha)* **28**, 266–273.
- Langis, R. & Steponkus, P.L. (1991) Vitrification of isolated rye protoplasts: protection against dehydration injury by ethylene glycol. *Cryo-Letters* **12**, 107–112.
- MacFarlane, D.R. & Forsyth, M. (1990) Recent insights on the role of cryoprotective agents in vitrification. *Cryobiology* **27**, 345–358.
- Mazur, P. (1984) Freezing of living cells: mechanism and implications. *American Journal of Physiology* **247** (Cell Physiology 16:C125–C142).
- Mehl, P. & Boutron, P. (1988) Cryoprotection of red blood cells by 1,3 butanediol and 2,3 butanediol. *Cryobiology* **25**, 44–54.
- Miyamoto, H. & Ishibashi, T. (1978) The protective action of glycols against freezing damage of mouse and rat embryos. *Journal of Reproduction and Fertility* **54**, 427–432.
- Rall, W.F. (1987) Factors affecting the survival of mouse embryos cryopreserved by vitrification. *Cryobiology* **24**, 387–402.
- Rall, W.F. & Fahy, G.M. (1985) Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature* **313**, 573–575.
- Rall, W.F., Wood, M.J., Kirby, C. & Whittingham, D.G. (1987) Development of mouse embryos cryopreserved by vitrification. *Journal of Reproduction and Fertility* **80**, 499–504.
- Scheffen, B., Van Der Zwalmen, P. & Massip, A. (1986) A simple and efficient procedure for preservation of mouse embryos by vitrification. *Cryo-Letters* **7**, 260–269.
- Shaw, J.M. & Trounson, A.O. (1989) Effect of dimethyl sulfoxide and protein concentration on the viability of two-cell mouse embryos frozen with a rapid freezing technique. *Cryobiology* **26**, 413–421.
- Steponkus, P.L., Myers, S.P., Lynch, D.V., Gardner, V., Bronhteyn, V., Leibo, S.P., Rall, W.F., Pitt, R.E., Lin, T.T. & MacIntyre, R.J. (1990) Cryopreservation of *Drosophila melanogaster* embryos. *Nature* **345**, 170–172.
- Trounson, A., Peura, A. & Kirby, C. (1987) Ultrarapid freezing: a new low-cost and effective method of embryo cryopreservation. *Fertility and Sterility* **49**, 843–850.
- Valdez, C.A., Abas Mazni, O., Kanagawa, H. & Fujikawa, S. (1990a) Confirmation by cryo-electron microscopy of the absence of crystallization using a vitrification solution. *Cryo-Letters* **11**, 351–358.
- Valdez, C.A., Abas Mazni, O., Takahashi, Y., Hishinuma, M. & Kanagawa, H. (1990b) Effects of equilibration time, precooling and developmental stage on the survival of mouse embryos cryopreserved by vitrification. *Theriogenology* **33**, 627–636.
- Valdez, C.A., Abas Mazni, O., Taniguchi, T., Hishinuma, M. & Kanagawa, H. (1990c) *In vitro* and *in vivo* development of vitrified-thawed mouse morulae. *Proceedings 7th Congress of the Federation of Asian Veterinary Association*, pp. 844–850. Eds P. Poomvises & P. Ingkaninum. Chulalongkorn University Press, Bangkok, Thailand.
- Valdez, C.A., Hishinuma, M., Takahashi, Y. & Kanagawa, H. (1991) Effect of trehalose dilution on the survival of vitrified-thawed mouse morulae. *Japanese Journal of Veterinary Research* **39**, 23–26.
- Whittingham, D.G., Woods, M.J., Farrant, J., Lee, H. & Halsey, J.A. (1979) Survival of frozen mouse embryos after rapid thawing from -196°C . *Journal of Reproduction and Fertility* **56**, 11–21.

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