A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability

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Summary. Mouse morulae were exposed to solutions containing 30–50% of permeable agents (ethylene glycol, glycerol, propylene glycol) in modified phosphate-buffered saline (PB1 medium) at 20°C for 20 min. A high percentage of them developed to expanded blastocysts in culture, after exposure to 30% and 40% ethylene glycol (98 and 84%, respectively), or 30% glycerol (88%). Ethylene glycol and glycerol were diluted to 30 and 40% with PB1 medium or with PB1 containing 30% Ficoll or 30% Ficoll + 0.5 M-sucrose, immersed in liquid nitrogen in straws and warmed in 20°C water. Solutions containing 40% of a permeable agent with Ficoll did not crystallize during cooling or warming. Mouse morulae were exposed to 40% ethylene glycol in PB1 medium containing 30% Ficoll (EF) or PB1 medium + 30% Ficoll + 0.5 M-sucrose (EFS) for 5–20 min at 20°C. EFS solution was non-toxic to the embryos during 5 min of exposure. When embryos, equilibrated in EFS solution for 2 or 5 min at 20°C, were vitrified at –196°C and were warmed rapidly, nearly all embryos developed in culture (97–98%), and 51% developed to live young at term after transfer. This method, which results in virtually no decrease in embryonic viability, may be of practical use for embryo preservation.

Keywords: mouse; embryo; vitrification; ethylene glycol; sucrose; Ficoll

Introduction

The embryo freezing technique first reported by Whittingham *et al.* (1972) has been one of the most reliable methods for embryo preservation, and is widely used (Wood *et al.*, 1987). Most attempts to improve the technique, therefore, have been directed to simplification of procedures (Wood & Farrant, 1980; Kasai *et al.*, 1980; Miyamoto & Ishibashi, 1986; Széll & Shelton, 1986a; Trounson *et al.*, 1987). Among such attempts, vitrification (Rall & Fahy, 1985) is a significant advance, in which embryos are suspended in a highly concentrated solution which can be cooled to liquid nitrogen temperatures without crystallizing. However, the initial vitrification solution (VS1) was highly toxic and embryos must be exposed to this solution at ~4°C, which reduces the practicality of the technique. In addition, embryos must be equilibrated in VS1 in a stepwise manner for 20–35 min at 2 different temperatures before being plunged into liquid nitrogen.

Vitrification, however, has potential advantages over conventional methods in that it takes only a few seconds for cooling embryos, and there is no extracellular crystallization, which is one of the major causes of cell injury. Besides simplification of the procedures, the most important requirement of an embryo preservation technique must be to maintain high survival. In this paper, we describe a simple method for embryo storage by vitrification, which results in extremely high survival rates.

Materials and Methods

Embryos. Female ICR mice (6–12 weeks old; CLEA Japan, Inc., Tokyo, Japan) were induced to superovulate by i.p. injections of 5 i.u. PMSG (Serotropin; Teikokuzoki, Tokyo, Japan) and 5 i.u. hCG (Puberogen; Sankyozoki, Tokyo, Japan) given 48 h apart. Females were mated with ICR male mice, except for the embryo transfer experiment, for which C3H males were used. Embryos were flushed from the upper half of the excised uteri and a region of the oviducts with modified phosphate-buffered saline (PB1; Whittingham, 1971) 77–79 h after the injection of hCG. The embryos were washed in fresh PB1 medium and only morphologically normal, compacted morulae were used.

Experiments involving suspension of embryos in cryoprotectant solutions were conducted in a room at $20 \pm 0.5^\circ\text{C}$.

Vitrification solutions. As candidates for a basic agent to compose a new vitrification solution, 3 permeable cryoprotectants, ethylene glycol, glycerol and propylene glycol, were examined. Non-permeable agents were Ficoll 70 (average molecular weight 70 000; Pharmacia, Uppsala, Sweden) as a macromolecule to assist vitrification, and sucrose as a low molecular weight compound which causes embryo shrinkage by osmosis.

Toxicity test of cryoprotectants. In the first series of experiments, the toxicity of individual permeable cryoprotectants was tested. Embryos (9–10) were suspended in 100 μl PB1 medium containing a permeable cryoprotectant at a concentration of 30, 40 or 50% (v/v) under paraffin oil (Nacalai Tesque, Inc., Kyoto, Japan) in a culture dish (35×10 mm, Nunc Inc., Naperville, IL, USA) at 20°C . After 20 min, embryos were transferred to PB1 medium containing the same agent (the same concentration) + 0.5 M-sucrose, then to PB1 medium containing 0.5 M-sucrose (S-PB1), and then to fresh PB1 medium at 5-min intervals at 20°C .

In the second series of experiments, the toxicity of 2 vitrification solutions was tested. Embryos (9–10) were suspended in 40% ethylene glycol diluted in PB1 medium containing either 30% Ficoll (EF solution) or 30% Ficoll + 0.5 M-sucrose (EFS solution) under paraffin oil in a culture dish at 20°C . Final concentrations of Ficoll and sucrose were, therefore, 18% and 0.3 M, respectively. After holding for 5, 10, 15 or 20 min at 20°C , embryos in EF solution were transferred successively to EFS solution, S-PB1 medium and fresh PB1 medium at 5-min intervals. Those in EFS solution were transferred directly to S-PB1 medium and then to fresh PB1 medium 5 min later.

Recovered embryos were washed in a modified Krebs–Ringer–bicarbonate (KRB) medium (Toyoda & Chang, 1974) and were cultured in ~ 0.3 ml of the medium under paraffin oil in 5% CO_2 in air at 37°C . The survival of the embryos was assessed by their ability to develop into expanded blastocysts during 48 h of culture.

Experiments were replicated 4–5 times, with 39–50 embryos being tested for each treatment.

Vitrification test of media. Ethylene glycol and glycerol were diluted to a concentration of 30 or 40% with PB1 medium or PB1 medium containing 30% Ficoll or 30% Ficoll + 0.5 M-sucrose. The solutions were introduced into 0.25-ml plastic straws, and were immersed directly into liquid nitrogen. After being held for about 10 sec, they were quickly transferred into 20°C water and agitated gently. According to the appearance of the straws in liquid nitrogen and in 20°C water, samples which remained transparent were considered uncrystallized, and those which turned opaque were considered crystallized.

Experiments were replicated 6 times using two types of plastic straws (Fujihirakogyo, Tokyo, Japan; I.M.V., L'Aigle, France). A solution was scored as crystallizing if the solution turned opaque one or more times.

Vitrification of embryos. EFS solution was prepared in a 0.25-ml plastic straw by successive aspiration of S-PB1 medium (~ 100 μl), air (~ 20 μl), EFS solution (~ 6 μl), air (~ 6 μl) and EFS solution (~ 40 μl), with a 1-ml syringe connected by a small silicone tube, and the straw was held horizontally at 20°C (Fig. 1a). Embryos (8–11) were suspended in EFS solution at 20°C in a watch glass, and were washed in the solution once. They were transferred to the larger column of EFS solution (~ 40 μl), and then air (~ 6 μl), EFS solution (~ 6 μl), air (~ 15 μl) and S-PB1 medium (~ 20 μl) were aspirated successively (Fig. 1b) before being sealed with the powder. At 2, 5 or 10 min exposure of embryos to the solution, straws were immersed in liquid nitrogen vertically in two steps, in order to prevent bursting of straws: first, about a half of the straw, including the EFS solution, was immersed rapidly, and then the rest of the straw, including the larger column of S-PB1 (~ 100 μl), was immersed slowly. After storage for 2–125 days, the straws were warmed rapidly in 20°C water. As soon as the crystallized S-PB1 medium in the straw began to melt (about 5 sec), embryos in the vitrification solution were expelled into a watch glass by flushing the straw with ~ 0.8 ml S-PB1 medium, agitated gently, and then transferred into fresh S-PB1 medium at 20°C . At about 5 min after flushing out, embryos were transferred to fresh PB1 medium.

In-vitro survival of the embryos was assessed as described above. To examine the developmental potential *in vivo*, F1 embryos (C3H $\times$ ICR) were vitrified after exposure to EFS solution for 2 or 5 min. They were warmed approximately 12 h in advance of the time when synchrony between embryos and recipient uteri was expected, so that embryos were slightly older than uteri. They were cultured for 1–6 h before being transferred into one or both uterine horns (4–8 per horn (average of 6) and 6–15 per recipient (average of 10.5)) of recipient mice of the ICR strain on Day 3 of pseudopregnancy, induced by mating with a vasectomized ICR male. The recipients were allowed to litter.

Statistics. Data were analysed by χ^2 tests.

Fig. 1. Diagram of a 0.25-ml straw just before loading embryos (a), and just before cooling in liquid nitrogen (b), showing the configuration of EFS solution, S-PB1 medium, air and plug.

Results

Toxicity test of cryoprotectants

As shown in Table 1, when mouse morulae were exposed to a 30% solution of permeable cryoprotectants for 20 min at 20°C, the percentage of embryos developing to expanded blastocysts in culture was high in ethylene glycol (98%) and glycerol (88%), but was low ($P < 0.001$) in propylene glycol (16%). After exposure to 40% solution of permeable agents, only embryos exposed to ethylene glycol showed a high percentage development (84%). However, embryos did not survive exposure to 50% ethylene glycol.

Table 1. Survival of mouse morulae exposed to permeable cryoprotectants for 20 min at 20°C

	No. of embryos developed/no. of embryos treated (%)		
	Concentration of cryoprotectants		
Cryoprotectants	30%	40%	50%
Ethylene glycol	48/49 (98) ^a	42/50 (84) ^a	0/40 (0) ^b
Glycerol	44/50 (88) ^a	1/40 (3) ^b	N.t.
Propylene glycol	8/49 (16) ^b	0/40 (0) ^b	N.t.

Values with different superscripts are significantly different ($P < 0.001$).

N.t. = not tested.

Vitrification test of media

As shown in Table 2, when ethylene glycol and glycerol were diluted to 30% with PB1 or PB1 medium containing Ficoll or Ficoll + sucrose, all the media remained transparent after cooling in liquid nitrogen. However, within 1 sec of immersion in 20°C water, straws turned opaque, which is a sign of crystallization. Solutions containing 40% ethylene glycol or glycerol also remained transparent when plunged into liquid nitrogen. At warming, solutions diluted in PB1 medium without non-permeable agents again turned opaque, but media containing Ficoll remained transparent. There was no difference between the effects of ethylene glycol and glycerol as permeable agents on the occurrence of crystallization.

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Table 2. Occurrence of crystallization in various media during rapid cooling in liquid nitrogen and warming in 20°C water*

Media		Concentration of permeable cryoprotectants			
		30%		40%	
Permeable cryoprotectants	Diluents	Cooling	Warming	Cooling	Warming
Ethylene glycol	PB1	—	+	—	+
	30% Ficoll in PB1	—	+	—	—
	30% Ficoll + 0.5 M-sucrose in PB1	—	+	—	—
Glycerol	PB1	—	+	—	+
	30% Ficoll in PB1	—	+	—	—
	30% Ficoll + 0.5 M-sucrose in PB1	—	+	—	—

*Transparent samples were considered uncrystallized and were scored (—), and opaque samples were considered crystallized and were scored (+).

Toxicity test of vitrification solutions

The toxicity of vitrification solutions containing ethylene glycol and the impermeable solutes Ficoll and sucrose are shown in Table 3. Survival rates of mouse embryos decreased with increasing durations of exposure in either solution. However, the toxicity of EFS solution was significantly lower than that of EF solution, and virtually no decrease in in-vitro survival of the embryos was observed after exposure to EFS solution for 5 min at 20°C (98% survival).

Table 3. Survival of mouse morulae exposed to vitrification solutions at 20°C

Solution	No. of embryos developed/no. of embryos treated (%)			
	Duration of exposure (min)			
	5	10	15	20
EF	26/50 (52) ^{ab}	12/50 (24) ^{cd}	0/48 (0) ^{ef}	0/39 (0) ^g
EFS	49/50 (98) ^a	40/50 (80) ^{bc}	39/50 (78) ^{de}	10/50 (20) ^{fg}

Values with the same superscripts are significantly different (a–a, c–c, d–d, e–e, f–f, $P < 0.001$; b–b, g–g, $P < 0.01$).

Vitrification of embryos

In total, 274 ICR mouse morulae were vitrified in EFS solution, and 261 (95%) were recovered after warming. When embryos had been equilibrated for 10 min, the proportion of embryos which developed in culture was 77% (Table 4), but when they were vitrified after 2 or 5 min of equilibration, nearly all the recovered embryos were morphologically intact and a high percentage of them developed to expanded blastocysts in culture (97–98%, Table 4). Of 185 vitrified F1 embryos, 178 (96%) were recovered, 175 (98%) of which were morphologically normal and were considered transferable: 167 of them were successfully transferred to 16 recipient mice, 11 of which delivered young (5 embryos were lost during transfer, and 3 were not transferred). Of 112 embryos which had been transferred to recipients that established pregnancy, 57 (51%) developed to live young and 6 (5%) were stillbirths. There was no difference among groups treated for 2 min and 5 min before cooling. All of them had pigmented eyes, confirming that they were derived from vitrified embryos.

Table 4. Survival of mouse morulae stored at -196°C by vitrification in EFS solution

Equilibration period (min)	No. of embryos stored	No. of embryos recovered	No. of embryos developed to expanded blastocysts in culture (%) [*]
2	104	99	97 (98) ^a
5	111	106	103 (97) ^a
10	59	56	43 (77) ^b

^{*}Percentage of recovered embryos.

Values with different superscripts are significantly different (a–b, $P < 0.001$).

Discussion

Vitrification is defined as “the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling” (Fahy *et al.*, 1984). Because the vitrified solution is normally supercooled at a low temperature, it can be crystallized (devitrified) during warming, which leads to the death of the cells suspended in it (Rall, 1987). In order to make a solution vitrify in liquid nitrogen and prevent crystallization during warming, the presence of high concentrations of permeable cryoprotective agents is essential. The first successful solution for vitrifying mouse embryos (VS1; Rall & Fahy, 1985) contained dimethyl sulphoxide, acetamide and propylene glycol as permeable agents. Thereafter, glycerol and propylene glycol were reported effective for vitrifying mouse embryos both in combination (Scheffen *et al.*, 1986) or as single permeating cryoprotectants (Rall, 1987).

For the present experiment on the toxicity of individual cryoprotectants, a selection was made of glycerol and propylene glycol, along with ethylene glycol, which was proved effective for freezing mouse embryos (Miyamoto & Ishibashi, 1977; Kasai *et al.*, 1981). As the toxicity of cryoprotectants is related to temperature, the ambient temperature for embryo manipulation was strictly controlled at $20 \pm 0.5^{\circ}\text{C}$. The results show that ethylene glycol is the least toxic of the three (Table 1) in spite of the higher molecular concentration of the solution (40% of ethylene glycol, glycerol and propylene glycol are approximately 7.2, 5.5 and 5.5 M, respectively).

Definitive evidence for vitrification requires the use of physical procedures such as X-ray diffraction, calorimetry, or freeze-fracture ultramicroscopy (Rall, 1987), because ice crystals smaller than the wavelength of light will not be detected by visual inspection (Meryman, 1958). However, it has been reported that when several vitrification solutions are cooled and warmed rapidly, there is no evidence of crystallization according to appearance or calorimetry (Rall, 1987). In the present study, therefore, apparent transparency was the criterion for non-crystallization.

It has been shown that the inclusion of a macromolecule in a solution facilitates vitrification (Fahy *et al.*, 1984), and polyethylene glycol is widely used for this purpose (Rall & Fahy, 1985; Rall, 1987). Here, Ficoll 70 was used as a macromolecule, because it has high solubility with low viscosity. The present experiment on vitrification of various media indicates that Ficoll facilitates non-crystallization by preventing devitrification during warming (Table 2). Preliminary data showed that 30% Ficoll in PBI medium is non-toxic to mouse embryos during 20 min of exposure at 20°C (in-vitro survival was 100%, data not shown).

However, when Ficoll is added to 40% ethylene glycol (EF solution), it increased the toxicity of the ethylene glycol solution: 30% Ficoll (w/v) seems non-toxic but Ficoll occupies about 11% (v/v) of the EF and EFS solutions. If Ficoll were excluded from the EF and EFS solutions, the concentration of ethylene glycol would, therefore, be about 45% in both solutions, which would have increased toxicity. Fortunately, the further addition of 0.3 M-sucrose (EFS solution) decreased this

toxic injury (Table 3). In our toxicity test of the EF and EFS solutions (Table 3), embryos treated in EF solution were suspended in EFS solution for 5 min before removing cryoprotectants in S-PB1, whereas embryos in EFS solution were directly transferred to S-PB1 after treatment. Embryos in the EF solution were therefore exposed to ethylene glycol + Ficoll 5 min longer than those in EFS solution at the same equilibration period. However, survival rates of embryos exposed to EF solution for 5, 10 or 15 min were significantly lower ($P < 0.01$, 0.001 and 0.001, respectively) than for those exposed to EFS solution for 10, 15 or 20 min, respectively. Sucrose may reduce toxicity associated with Ficoll by causing the embryos to shrink rapidly and reducing the amount of ethylene glycol in the cells. It has been reported that the addition of sucrose reduces glycerol permeation (Széll & Shelton, 1986b).

After vitrification and warming, in-vitro survival rates of the embryos which had been equilibrated for 5 and 10 min in EFS solution (97 and 77%, respectively, Table 4) were as high as those of embryos exposed to EFS solution for the same periods without cooling (98 and 80%, respectively, Table 3). Therefore, there seems to be no decrease in in-vitro viability by vitrifying embryos. Although the proportion of live young was not very high (51%), it may be possible to elevate it by refinement of the transfer technique, considering the high in-vitro viability.

The new vitrification solution described here (EFS solution) is based on ethylene glycol as the permeating cryoprotectant and two non-permeating agents, Ficoll and sucrose. Although ethylene glycol is less commonly used for embryo freezing than dimethyl sulphoxide and glycerol, the present study indicates that it has an advantage of lower toxicity. Ficoll was found effective for vitrification of embryos for the first time. Sucrose, however, has been successfully used for freezing eggs and embryos in combination with dimethyl sulphoxide (Kasai *et al.*, 1979; Trounson *et al.*, 1987), propylene glycol (Renard *et al.*, 1984), and glycerol (Széll & Shelton, 1986a). Sucrose also facilitates removal of intracellular cryoprotectants during dilution (Leibo & Mazur, 1978; Kasai *et al.*, 1980).

For successful embryo preservation by vitrification, permeation of a cryoprotectant is considered essential, although full permeation is not necessary but rather harmful (Rall, 1987). In the original method of vitrification (Rall & Fahy, 1985; Rall, 1987), embryos are first equilibrated in 25% of a vitrification solution at $\sim 20^\circ\text{C}$ for permeation of cryoprotectants before dehydration at 4°C . In a supplementary experiment, we have observed that mouse morulae suspended in 40% ethylene glycol in PB1 medium at 20°C shrink rapidly and regain most of their volume within 5 min (data not shown), indicating rapid permeation of ethylene glycol into the embryos. In EFS solution, on the other hand, embryos remain shrunken because of the presence of sucrose. As discussed above, sucrose may reduce the amount of intracellular cryoprotectant (Széll & Shelton, 1986b), but in the present conditions, the amount of ethylene glycol which penetrates into the embryos during 2–5 min of equilibration in EFS solution at 20°C seems enough for survival.

Besides giving high survival, this method for embryo preservation, using EFS solution, would be very simple. A time of 2–5 min would be enough to pretreat embryos for equilibration and loading into a straw before plunging into liquid nitrogen. Furthermore, embryos can be manipulated at room temperature (20°C). This new method of vitrification may offer a simple and reliable means for embryo preservation.

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