Cryopreservation of expanded mouse blastocysts by vitrification in ethylene glycol-based solutions

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Experiments were conducted to find optimal conditions for obtaining high survival of expanded mouse blastocysts after vitrification. The vitrification solutions used were designated EFS20, EFS30 and EFS40, and contained 20%, 30% and 40% ethylene glycol, respectively, diluted in PB1 medium containing 30% Ficoll plus $0.5 \text{ mol sucrose l}^{-1}$. In the toxicity test of the solutions and each cryoprotectant, ethylene glycol was found to be toxic to embryos. For vitrification, expanded blastocysts were exposed to the vitrification solutions at 10, 20 or 25°C for various periods; they were then cooled rapidly in liquid nitrogen, after which they were warmed rapidly. When the embryos were directly exposed to EFS40 at 20°C for 2 min before vitrification, 66% of them re-expanded during 48 h of post-warming culture. The re-expansion rates decreased when exposure time was shortened (0.5 min), when exposure temperature was lowered (10°C), or when embryos were vitrified in EFS20 and EFS30, although these conditions should be less toxic. When embryos had been pretreated in a dilute (10–20%) ethylene glycol solution for 5 min, followed by short exposure (0.5 min) to EFS40 at 20°C, post-vitrification survival rate increased to 83–84%; furthermore, the rate reached 94% when the temperature was increased to 25°C. Expanded blastocysts cryopreserved by this two-step method developed into live young as well as control embryos after transfer. The results suggest that permeation of ethylene glycol, possibly into the blastocoel, during pretreatment in a dilute ethylene glycol solution prevented lethal ice formation, while reduced exposure of the embryos to EFS solution decreased toxic injury. It is expected that this simple and efficient method of cryopreservation will be applicable to expanded blastocysts of various species.

Introduction

In recent years, attention has focused on vitrification as a rapid and efficient method for cryopreservation of biological systems (Fahy *et al.*, 1984). The first successful vitrification of mammalian embryos was achieved by Rall and Fahy (1985) with eight-cell mouse embryos. The vitrification solution they used (VS1) was composed of dimethyl sulfoxide (DMSO), acetamide, propylene glycol and polyethylene glycol as cryoprotectants, and embryos were exposed to the solution at a low temperature (4°C) after stepwise equilibration, to avoid its toxicity. This method was applied to the cryopreservation of mouse embryos at various developmental stages, but the survival of blastocysts was lower than that of eight-cell embryos (Hsu *et al.*, 1986; Kono and Tsunoda, 1987; Matsumoto *et al.*, 1987). In addition, manipulating embryos under a microscope at a low temperature was not practical, and the whole procedure before rapid cooling required 25–40 min. Several attempts have therefore been made to improve the vitrification solution and the procedure for embryo treatment (Scheffen *et al.*, 1986; Rall, 1987; Nakagata, 1989; Schiewe *et al.*, 1991).

Mouse morulae have been vitrified by a simple method, without appreciable loss of viability (Kasai *et al.*, 1990). This method using a new vitrification solution based on ethylene glycol, which permeates the cell rapidly and has low toxicity requires embryo treatment for only 2 min at 20°C before the sample is plunged into liquid nitrogen. This simple method developed for morulae was examined to determine whether it was effective for mouse embryos at other developmental stages; it was found that blastocysts with a small blastocoel could also be vitrified without significant decrease in survival (91%). As the blastocoel began to enlarge, however, survival decreased, the rate being 57% in expanded blastocysts (Miyake *et al.*, in press).

In mice, few attempts have been made to cryopreserve expanded blastocysts, even by conventional freezing. However, in livestock embryos, the expanded blastocyst is a stage of great importance. The first successful embryo freezing in cows (Wilmut and Rowson, 1973) and goats (Bilton and Moore, 1976) was accomplished at this stage, and it has been found that embryo tolerance to freezing increases after expansion of the blastocoel in pigs (Nagashima *et al.*, 1989). Furthermore, recent techniques have made it possible to produce expanded cattle blastocysts through an *in vitro* process of maturation, fertilization and development (Goto *et al.*, 1988; Leibfried-Rutledge *et al.*,

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Fig. 1. When fresh expanded blastocysts (a) were suspended in PB1 medium containing 20% (v/v) ethylene glycol at 20°C, the embryos shrank within the zona pellucida, and the zona also slightly decreased in diameter. After 2 min of suspension, the embryos were still shrinking (b), but after 5 min (c) and 10 min (d), they had generally re-expanded within the zona. Bar: 100 μ m.

1989; Gordon and Lu, 1990). However, attempts to vitrify bovine blastocysts have been disappointing (Massip *et al.*, 1986; Douchi *et al.*, 1990). Survival of mouse blastocysts after cryopreservation has also been lower than that of eight-cell embryos to morulae, not only with various methods of vitrification (Scheffen *et al.*, 1986; Bielanski, 1987; Kono and Tsunoda, 1987; Matsumoto *et al.*, 1987; Nakagata, 1989; Valdez *et al.*, 1990), but also with conventional freezing (Whittingham *et al.*, 1972; Massip *et al.*, 1984). The present study was conducted to find optimal conditions for obtaining high survival of expanded mouse blastocysts, as a model for embryos with a large blastocoel, after vitrification in solutions based on ethylene glycol.

Materials and Methods

Collection of expanded blastocysts

Female ICR mice (6–12 weeks old, CLEA Japan, Inc., Tokyo) kept under a 14 h light:10 h dark cycle were mated with ICR or BDF1 (C57BL/6N $\times$ DBA/2N) male mice. Some of the females were mated naturally, but others were induced to superovulate with i.p. injections of 5 iu PMSG (Serotropin: Teikokuzoki, Tokyo) and 5 iu hCG (Puberogen: Sankyozoki, Tokyo) given 48 h apart. Morulae and expanded blastocysts were collected in modified phosphate-buffered saline (PB1) (Whittingham, 1971) from the uteri of mated animals at 78–80 and 95–100 h, respectively, after the presumed endogenous LH surge (in naturally mated animals) or hCG injection (in superovulated animals). Morulae and unexpanded blastocysts were cultured in a modified Krebs–Ringer–bicarbonate (KRB) medium (Toyoda and Chang, 1974) for 3–40 h under paraffin oil in a culture dish in a CO₂ incubator (5% CO₂ in air at 37°C). Embryos were used when they had developed into expanded blastocysts, which had an apparently expanded blastocoel and zona pellucida, but were not hatching or had not hatched from the zona (Fig. 1a). Thus, the effects of embryonic age, which ranged from 95 to 120 h after LH/hCG, were disregarded.

Vitrification solutions

Ethylene glycol was diluted to 20, 30 and 40% (v/v) in PB1 medium containing 30% (w/v) Ficoll 70 (average molecular weight 70 000; Pharmacia, Uppsala) plus 0.5 mol sucrose l⁻¹. The respective solutions were designated EFS20, EFS30 and EFS40, after the initials of the three cryoprotectants together with the concentration of ethylene glycol. Although the final molar concentrations of Ficoll and sucrose in the three solutions were different, their molal concentrations remained constant. When these solutions were aspirated in a 0.25 ml plastic straw (I.M.V., L'Aigle) and transferred into liquid nitrogen, they all remained transparent; on being transferred to 20°C water, EFS40 remained transparent, whereas EFS30 and EFS20 became opaque, which was a sign of devitrification.

Toxic effects of cryoprotectants on expanded blastocysts

Cryoprotectant-induced injury during exposure was examined by suspending ten expanded blastocysts in 100 μ l of an EFS solution (EFS20, EFS30 or EFS40), or in PB1 medium containing a constituent cryoprotectant, under paraffin oil in a culture dish at 20 $\pm$ 0.5°C; they were kept there for 2, 5, 10 or 20 min. Cryoprotectants tested were 20%, 30% and 40% (v/v) ethylene glycol, 50% (w/v) Ficoll, 1.0 mol sucrose l⁻¹, and 30% Ficoll plus 0.5 mol sucrose l⁻¹. Without being cooled, embryos in EFS solutions were transferred to PB1 medium containing 0.5 mol sucrose l⁻¹ (S-PB1) and then to fresh PB1 medium, at 5 min intervals, at 20°C. Embryos in ethylene glycol were transferred to PB1 medium containing the same concentration of ethylene glycol plus 0.5 mol sucrose l⁻¹, then to S-PB1, and then to fresh PB1, at 5 min intervals. Those in Ficoll or sucrose were transferred directly to fresh PB1 medium. Experiments were replicated 3–4 times for each treatment.

Vitrification of expanded blastocysts

Embryos were vitrified by the procedure described by Kasai *et al.* (1990), with some modifications. A large column (about

55 mm) of S-PB1 medium, and two columns (about 3 and about 13 mm) of EFS solution (EFS20, EFS30 or EFS40) were aspirated in a 0.25 ml plastic straw and separated by air; the straw was then kept horizontal at 10 ± 1 , 20 ± 0.5 or $25 \pm 0.5^\circ\text{C}$.

In the first series of vitrification experiments, expanded blastocysts were exposed to EFS solutions in one step from PB1 medium. In the second series of experiments, however, embryos were vitrified by a two-step method; embryos were first pre-treated by suspension in PB1 medium containing 10%, 20% or 30% (v/v) ethylene glycol at 20 ± 0.5 or $25 \pm 0.5^\circ\text{C}$ for 5 or 10 min before being exposed to EFS solutions.

When the embryos were vitrified after 0.5 or 1 min of exposure to an EFS solution, they were directly transferred to the larger column of the EFS solution (about 13 mm) via a pipette, with a small volume (about 0.2 μl) of PB1 medium (one-step method) or PB1 medium containing 10–30% ethylene glycol (two-step method). When the exposure time to EFS solution before vitrification was 2 or 5 min, the embryos were first suspended in the same EFS solution in a watch glass, washed in the solution twice, and then transferred to the EFS column in the straw.

The EFS solution (about 3 mm) and S-PB1 medium (about 12 mm) were then aspirated and separated by air, before being sealed with straw powder. About ten embryos were loaded in each straw. The configuration of the straw was described by Kasai *et al.* (1990). At 0.5, 1, 2 or 5 min after exposure of the embryos to EFS solution, the straws were plunged into a Dewar vessel (inner diameter 100 mm) containing 5–7 cm depth of liquid nitrogen, so as to immerse about half of the straw, which included the EFS solution (powder side), in liquid nitrogen; the rest of the straw, including the larger column of S-PB1 (cotton plug side) was in the gas. This method of cooling prevented the bursting of straws caused by rapid freezing of the large column of S-PB1 medium. After being kept in the Dewar vessel for at least 1 min, straws were stored in a liquid nitrogen tank.

For embryo recovery, straws treated at 10 and 20°C were warmed rapidly in water at 20°C , and those treated at 25°C were warmed in water at 25°C . As soon as the crystallized S-PB1 medium in the straw began to melt (about 5 s), the contents of the straw were expelled into a watch glass by flushing the straw with about 0.8 ml S-PB1 medium at the same temperature as the water. The embryos were then pipetted into fresh S-PB1 medium. At about 5 min after being flushed out, the embryos were transferred to fresh PB1 medium. Experiments were replicated at least four times for each treatment.

Assessment of survival

Treated embryos were washed in KRB medium and cultured in about 0.2 ml of the medium under paraffin oil in a culture dish in a CO_2 incubator at 37°C . They were examined at 10- to 16-h intervals and at 48 h; the criteria for *in vitro* survival was re-expansion of the blastocoel (A) within 16 h and (B) within 48 h of culture. As a control, fresh expanded blastocysts were cultured after being kept in PB1 medium for 30 min at 10, 20 and 25°C .

Embryos from pigmented mice (ICR $\times$ BDF1) were used to examine their developmental potential *in vivo*. After recovery from vitrification, embryos were cultured in KRB medium for 3–9 h, and five or six re-expanded blastocysts were transferred to each uterine horn of recipient mice (10–12 embryos per

animal). As a control, fresh expanded blastocysts were transferred likewise. Recipients were female ICR mice on day 3 of pseudopregnancy, which had been induced by mating them with vasectomized ICR males (day 1 was the day the copulation plug was found in the morning). The recipients were allowed to produce litters.

Statistical analysis

Survival rate after each treatment was compared with that in the control group, with χ^2 tests; when expected frequency was less than 5, Fisher's exact probability test was used. In the vitrification of embryos, the data were further analysed by analysis of variance to determine the significance of treatments and the interaction between them; for the analysis, the percentage of embryos that survived was transformed to radians to normalize the results.

Results

Toxicity test

When embryos were exposed to vitrification solutions containing various concentrations of ethylene glycol at 20°C , 93–100% of the embryos could re-expand their blastocoel during culture (for 16 h and 48 h) after 5–10 min exposure to EFS20 and EFS30 (Table 1). With EFS40, however, the survival assessed during 16 h of culture began to decrease after only 2 min of exposure (80%). Quite similar events were observed when embryos were treated in PB1 medium containing the same concentration of ethylene glycol alone; the survival decreased with increasing concentrations of ethylene glycol (Table 2). On the other hand, when embryos were suspended in PB1 medium containing 50% Ficoll, 1.0 mol sucrose l^{-1} , or 30% Ficoll plus 0.5 mol sucrose l^{-1} for 20 min, 100% (50 of 50), 94% (47 of 50) and 96% (48 of 50), respectively, of the embryos re-expanded within 16 h of the following culture, and all the embryos regained their volume within 48 h.

Vitrification of embryos

The survival of embryos after vitrification in EFS solutions in one step is summarized in Table 3. For analysis of variance, data for EFS20, which resulted in very low levels of survival, were excluded, because the solution was not evaluated at 10°C . In the survival rate, assessed by the re-expansion of the blastocoel during 48 h of culture, there was an interaction among exposure temperature, vitrification solution and exposure time ($P < 0.05$). The highest survival rate (66%) was obtained when embryos were vitrified after 2 min of exposure to EFS40 at 20°C . Further exposure (5 min) to the solution decreased survival, as expected from the results of the toxicity test (Table 1). The survival rates of vitrified embryos were also reduced, when they were exposed for a shorter period (0.5 min) and when they were vitrified in EFS20 and EFS30. Embryos were exposed to another low toxic condition, EFS solutions at 10°C . Post-warming survival rate, assessed by 48-h culture, increased up to 50–51% as the duration of the exposure increased. The proportions of embryos that had re-expanded during 16 h of culture were low in any treatment (0–31%), and by this criterion,

Table 1. Percentage survival of expanded mouse blastocysts exposed to various vitrification solutions at 20°C*

Vitrification solution	Exposure time					
	2 min		5 min		10 min	
	A	B	A	B	A	B
EFS20	100	100 (30)	100	100 (30)	100	100 (40)
EFS30	100	100 (30)	100	100 (40)	93	95 (40)
EFS40	80 ^a	90 (40)	13 ^a	18 ^a (40)	0 ^a	30 ^a (30)

*Survival was assessed by determining the percentage of blastocysts re-expanded (A) within 16 h and (B) within 48 h of culture. Figures in parentheses are the numbers of blastocysts treated.

^a $P < 0.01$; significantly different from control, in which 100% (40 of 40) of embryos survived after being kept at 20°C for 30 min.

Table 2. Percentage survival of expanded mouse blastocysts exposed to PB1 medium containing various concentrations of ethylene glycol at 20°C*

Concentration of ethylene glycol (%)	Exposure time					
	2 min		5 min		10 min	
	A	B	A	B	A	B
20	100	100 (30)	100	100 (40)	100	100 (40)
30	100	100 (30)	100	100 (40)	83 ^a	90 (40)
40	100	100 (40)	25 ^a	25 ^a (40)	0 ^a	0 ^a (40)

*Survival was assessed by determining the percentage of blastocysts re-expanded (A) within 16 h and (B) within 48 h of culture. Figures in parentheses are the numbers of blastocysts treated.

^a $P < 0.01$; significantly different from control, in which 100% (40 of 40) of embryos survived after being kept at 20°C for 30 min.

Table 3. Percentage survival of expanded mouse blastocysts vitrified in various solutions after one-step exposure to various vitrification solutions at 10 and 20°C*

Exposure temperature	Vitrification solution	Exposure time					
		0.5 min		2 min		5 min	
		A	B	A	B	A	B
20°C	EFS20	0	0 (41)	7	12 (41)	0	6 (35)
	EFS30	4	4 (46)	26	48 (42)	10	33 (40)
	EFS40	31	47 (45)	24	66 (50)	6	13 (48)
10°C	EFS30	8	16 (38)	13	18 (39)	20	51 (41)
	EFS40	10	10 (40)	7	44 (54)	16	50 (38)

*Survival was assessed by determining the percentage of blastocysts re-expanded (A) within 16 h and (B) within 48 h of culture. Seven to ten embryos were placed in each straw, and the number of replicates was 4–6. Figures in parentheses are the total numbers of blastocysts treated.

All the rates were significantly ($P < 0.01$) different from control, in which 100% (40 of 40) of embryos survived after being kept at each temperature for 30 min.

Table 4. Percentage survival of expanded mouse blastocysts vitrified in EFS40 by a two-step method after pretreatment at 20°C*

Pretreatment		Exposure time in EFS40					
Concentration of ethylene glycol (%)	Time (min)	0.5 min		1 min		2 min	
		A	B	A	B	A	B
10	5	69	83 (58)	56	82 (55)	32	49 (41)
	10	61	69 (54)	48	74 (46)	40	56 (50)
20	5	49	84 (57)	30	59 (44)	22	29 (45)
	10	36	80 (66)	28	47 (43)	25	35 (40)
30	5	24	71 (41)	40	63 (40)	9	16 (45)
	10	19	33 (42)	12	21 (43)	3	3 (40)

*Survival was assessed by determining the percentage of blastocysts re-expanded (A) within 16 h and (B) within 48 h of culture. Seven to ten embryos were placed in each straw and the number of replicates were 4–7. Figures in parentheses are the total numbers of embryos treated. All the rates were significantly ($P < 0.01$) different from controls, in which 100% (40 of 40) of embryos survived after being kept at 20°C for 30 min.

Table 5. Percentage survival of expanded mouse blastocysts vitrified in EFS40 by a two-step method after pretreatment at 25°C*

Pretreatment		Exposure time in EFS40					
Concentration of ethylene glycol (%)	Time (min)	0.5 min		1 min		2 min	
		A	B	A	B	A	B
10	5	92	94 (68)	80 ^a	84 ^a (51)	28 ^a	30 ^a (43)
	10	86 ^b	88 ^b (49)	80 ^a	88 ^b (49)	22 ^a	27 ^a (41)
20	5	72 ^a	87 ^b (67)	51 ^a	64 ^a (47)	7 ^a	14 ^a (42)
	10	45 ^a	50 ^a (40)	7 ^a	12 ^a (43)	7 ^a	11 ^a (45)

*Survival was assessed by determining the percentage of blastocysts re-expanded (A) within 16 h and (B) within 48 h of culture. Nine to 12 embryos were placed in each straw and the number of replicates was 4–7. Figures in parentheses are the numbers of embryos treated.

^a $P < 0.01$, ^b $P < 0.05$: significantly different from controls, in which 100% (40 of 40) of embryos survived after being kept at 25°C for 30 min.

there was no significance in each variable and in the interaction between the variables.

The survival of embryos after two-step vitrification in EFS40 is summarized in Tables 4 and 5. Embryos suspended in dilute ethylene glycol solution shrank within the zona (Fig. 1a, b) and then regained their volume within 5–10 min (Fig. 1c, d). When embryos were treated at 20°C, post-warming survival was significantly affected by the three variables, namely, concentration of ethylene glycol ($P < 0.01$), pretreatment time ($P < 0.05$, when assessed by 48 h of culture) and exposure time in EFS40 ($P < 0.01$), although there was no interaction between these variables. Compared with the survival after one-step vitrification (Table 3), higher survival rates were obtained, especially when embryos had been pretreated in a dilute ethylene glycol solution (10 or 20%) for 5 min, followed by brief exposure (0.5 min) to EFS40 (49–69% when assessed by 16 h of culture, and 83–84% when assessed by 48 h of culture) (Table 4). When the temperature was increased to 25°C, post-warming survival

was again affected by concentration of ethylene glycol ($P < 0.01$), pretreatment time ($P < 0.05$) and exposure time in EFS40 ($P < 0.01$), with an interaction between ethylene glycol concentration and pretreatment time ($P < 0.05$). Compared with the data at 20°C (Table 4), higher proportions (80–92%) of the embryos that had been pretreated in 10% ethylene glycol followed by short (0.5 or 1 min) exposure to EFS40 re-expanded quickly (within 16 h of culture). The highest value (92%) was obtained when the embryos had been pretreated in 10% ethylene glycol for 5 min followed by 0.5 min of exposure to EFS40, and the rate reached 94% when assessed after 48 h of culture (Table 5); these rates were not significantly different from those of control embryos.

To examine developmental potential *in vivo*, embryos were pretreated with 10% ethylene glycol for 5 min at 25°C and vitrified in EFS40 after 0.5 min of exposure. In total, 188 cryopreserved embryos were transferred to 16 recipients, of which 13 (81%) became pregnant; 12 of these animals delivered 84 (36

female and 48 male) live young and 1 stillborn fetus on days 19–21. One pregnant recipient was killed on day 22, and two well-developed live fetuses and one dead fetus were found in the uterus. In controls, 132 fresh embryos were transferred to 11 recipients, in total; all of them became pregnant, and they delivered 78 (33 female and 45 male) live young and two stillborn fetuses. The proportion of full-term young/embryos in the cryopreserved group was 58% (88 of 152), when expressed on the basis of the number of embryos transferred to recipients that became pregnant. This proportion was not significantly different from that for the control (61%; 80 of 132).

Discussion

Expanded blastocysts were first collected from superovulated animals. However, the number of normal embryos was small and the developmental stage was variable. Expanded blastocysts were then collected from naturally mated females; the developmental stage was rather uniform, but the number of collected embryos was restricted. Morulae were therefore collected from superovulated animals, and cultured until they expanded. Data were presented disregarding the source of embryos, as no effects of superovulation and embryo culture on survival were found.

Compared with the results of a similar toxicity test on mouse morulae, which were able to survive up to 5 min of exposure to EFS solution (Kasai *et al.*, 1990), expanded blastocysts must be more sensitive to this solution, namely a high concentration of ethylene glycol, than are morulae. This injury must have been caused either by the chemical toxicity of ethylene glycol or by the osmotic stress that occurred during removal of the permeated ethylene glycol from the embryos. However, as the permeability of a cryoprotectant increases as the developmental stage proceeds (Mazur *et al.*, 1976), and as the volume of each cell constituting the blastocyst is smaller than that in the morula, osmotic stress is a less probable explanation for the injury. Rather, the intracellular concentration of ethylene glycol in the blastocyst would be expected to increase more rapidly than in the morula, and this might promote the toxic effect.

The highest survival rate obtained in the one step vitrification method was 66% with 2 min of exposure to EFS40 at 20°C (Table 3), but this rate is lower than that obtained with morulae vitrified in the same conditions (98%; Kasai *et al.*, 1990). One factor restricting the survival would be the toxic effects of EFS40, which began to appear during 2 min of exposure at 20°C. The other factor would be insufficient permeation of ethylene glycol, since less permeable conditions, such as shorter exposure, lower temperature and lower concentrations of ethylene glycol, resulted in poorer survival after vitrification. The blastocyst differs structurally from the morula in that it has a fluid-filled blastocoel. When mouse blastocysts with various blastocoel volumes were vitrified or underwent very rapid freezing, survival rates decreased as the blastocoel enlarged (Shaw *et al.*, 1991; Miyake *et al.*, in press). It has been shown that the inclusion of a macromolecule in a solution facilitates vitrification (Fahy *et al.*, 1984). There are various macromolecules in the cytoplasm, whereas Ficoll is present outside the embryo. In the blastocoel, however, there must be few macromolecules, and, possibly, sufficient permeation of ethylene glycol is required for the vitrification of the cavity; insufficient permeation is likely to

lead to the formation of ice crystals in the cavity, which must reduce the post-warming survival. For the permeation, however, ethylene glycol must first penetrate into the cells and then into the cavity, which may require a certain time. Yet, the toxic effects of the cryoprotectant to the embryos must be avoided during this exposure time.

In the next series of vitrification tests, we therefore first suspended the embryos in a dilute, and thus less toxic, ethylene glycol solution for permeation of ethylene glycol into the blastocoel. Embryos suspended in 10% or 20% ethylene glycol generally regained their isotonic volume within 5–10 min, both at 20 and 25°C, which indicates that ethylene glycol permeated fully into the blastocoel during this pretreatment period. As expected, higher levels of survival were obtained after vitrification if the following exposure time to EFS40 was limited. These results strongly support the interpretation outlined above, i.e. that expanded blastocysts can survive vitrification if (1) the toxic action of the cryoprotectant is avoided, and (2) sufficient ethylene glycol permeates into the embryo, possibly into the blastocoel.

The ambient temperature of 25°C was more suitable than 20°C for embryo treatment by the two-step method, if the periods of pretreatment and exposure to EFS40 were short (5 min and 0.5–1 min, respectively). Because the permeability of a cryoprotectant increases as the temperature rises, further permeation of ethylene glycol after exposure of the embryos to EFS40 would appear to be favourable for their survival. However, the reduced survival noted after longer (2 min) exposure to EFS40 at 25°C (19–30%) suggests that the toxic effect of the cryoprotectant is also greater at this temperature than at 20°C.

Using a vitrification solution containing 25% glycerol and 25% propylene glycol, various investigators have attempted to vitrify different stages of mouse embryos by a similar two-step method; however, the survival of blastocysts was found to be lower than that of morulae (Scheffen *et al.*, 1986; Bielanski, 1987; Valdez *et al.*, 1990). Although these authors did not examine precise conditions for the pretreatment and exposure to the vitrification solution, it would appear that the ethylene glycol-based vitrification solution used in the study reported here, which yielded high survival, has the advantages of lower toxicity and higher permeability than the solution used by other workers.

Landa (1982) reported that the optimal day for pseudo-pregnant animals to receive transfers of day 4 mouse blastocysts was day 2 for frozen–thawed embryos and day 3 for fresh embryos, since frozen–thawed embryos required about 48 h for the resumption of normal developmental potential. In the study reported here, although all the embryos were transferred to day 3 recipients, the proportion of embryos that developed into full-term young was not different from that of controls, showing that embryos were less stressed by our two-step method of vitrification. The fact that these embryos had re-expanded quickly (within 16 h) also indicates that they were less stressed.

The present results show that it is possible to vitrify expanded mouse blastocysts without significant loss of viability, both *in vitro* and *in vivo*. For high survival, embryos have to be exposed to cryoprotectants in two steps; however the 5 min pretreatment followed by 0.5 min exposure to the vitrification solution at room temperature is a simple procedure. It is expected that the present vitrification method using EFS40 will be suitable for embryos of various species which have large blastocoels.

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