High *in vitro* and *in vivo* survival of day 3 mouse embryos vitrified or frozen in a non-toxic solution of glycerol and albumin

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A vitrification solution consisting of 6.5 mol glycerol l⁻¹ and 6% (w/v) BSA in a modified Dulbecco's PBS (designated solution VS3a) was examined for the cryopreservation of 8–12-cell mouse embryos. Solution VS3a vitrified when cooled to -196°C at rates of 10–2500°C min⁻¹ and vitrified suspensions did not crystallize when warmed at 200 or 2000°C min⁻¹. However, slow cooling at 5°C min⁻¹ or slow warming at 20°C min⁻¹ resulted in visible crystallization of solution VS3a. Embryos were equilibrated in solution VS3a in three steps at room temperature and placed into a 0.25 ml plastic straw in a way that permitted in-straw dilution with 1 mol sucrose l⁻¹. Embryos equilibrated in solution VS3a and diluted immediately exhibited high rates of development *in vitro* to blastocysts (> 90%) if the total time of exposure to 100% solution VS3a did not exceed 5 min. Embryos exhibited high rates of development *in vitro* (75–97%) when equilibrated in 100% solution VS3a for 1 min and then cryopreserved using all combinations of three rates of cooling (5200 or 2500°C min⁻¹) and three rates of warming (20 200 or 2000°C min⁻¹). Although embryo suspensions visibly crystallized during slow cooling at 5°C min⁻¹, the rate of cooling was not a significant source of variance ($P > 0.26$). However, the rate of warming was found to have a small but significant effect on embryo survival ($P < 0.05$). Vitrified embryos exhibited a high rate of development *in vivo* after transfer to foster mothers (63%). A paired embryo transfer study comparing vitrification in VS3a with conventional slow freezing in 1.5 mol glycerol l⁻¹ showed no difference in the rate of development *in vivo* after either cryopreservation method ($P > 0.12$). These results demonstrate that embryos can be vitrified in solution VS3a by a simple procedure that includes the widest range of cooling and warming conditions reported to date.

Introduction

In the two decades since the first successful cryopreservation of mouse embryos (Whittingham *et al.*, 1972; Wilmut, 1972), basic and applied research has resulted in the cryopreservation of embryos of at least 13 other mammalian species (Rall, 1992a). Improvements have been made in nearly all steps of the cryopreservation process, resulting in considerable simplification of the original slow freezing and thawing procedure. These advances include the use of plastic insemination straws as the sample container (Tsunoda and Sugie, 1977), a reduction of the time needed for controlled freezing to the storage temperature (Willadsen, 1977; Whittingham *et al.*, 1979; Wood and Farrant, 1980), cryoprotectant solutions containing sucrose that osmotically dehydrate embryos before cooling (Massip *et al.*, 1984; Takeda *et al.*, 1984; Szll and Shelton, 1986), transfer of thawed embryos into recipient females without a period of culture *in vitro* (Renard and Babinet, 1984), the use of

sucrose to reduce osmotic swelling during dilution of the cryoprotectant (Leibo and Mazur, 1978), and vitrification procedures that prevent the formation of ice in the embryo suspension during cooling and warming (Rall and Fahy, 1985; Scheffen *et al.*, 1986). These and other studies using mammalian embryos have increased our understanding of the basic mechanisms of cryoprotection and cryoinjury (Leibo, 1986; Mazur, 1990; Rall, 1992a).

Vitrification procedures offer two important advantages over conventional slow freezing approaches. First, potential injury associated with the formation of ice is eliminated because no ice forms in vitrified suspensions during the cooling, storage or warming steps. Second, vitrification permits a substantial reduction in the time and equipment required for cryopreservation. However, rapid cooling is not essential for successful vitrification, although in practice most suspensions are vitrified using ultrarapid cooling procedures. Recent studies indicate that when embryos are dehydrated and vitrified in a mixture of 6.5 mol glycerol l⁻¹ and 6% (w/v) polyethylene glycol in a Hepes-buffered saline (VS3), high

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survival is obtained using a wide range of cooling rates ($15\text{--}2500^{\circ}\text{C min}^{-1}$) and warming rates ($10\text{--}2000^{\circ}\text{C min}^{-1}$) (Rall, 1987).

The greatest challenge for successful vitrification is the formulation of a vitrification solution and an effective equilibration procedure to produce the necessary degree of cellular dehydration and avoid significant toxic or osmotic injury (Rall, 1987). Generally, the choice of vitrification solution (composition, concentration) and equilibration conditions (time, temperature) depend on the permeability characteristics of the cells in question. Considerable progress has been made in reducing toxicity since the first vitrification solution (VS1; Rall and Fahy, 1985) was developed, which required low temperatures (about 4°C) during the final dehydration steps of equilibration. Two vitrification solutions permit all equilibration steps to be performed at room temperature. (i) A mixture of 25% (v/v) glycerol and 25% (v/v) propylene glycol in PBS yields high survival of mouse (Scheffen *et al.*, 1986) and cattle (Massip *et al.*, 1986) embryos after vitrification. (ii) Similarly, a mixture of 40% (v/v) ethylene glycol, 18% (w/v) Ficoll and $0.3\text{ mol sucrose l}^{-1}$ in PBS yields high survival of mouse morulae (Kasai *et al.*, 1990) and bovine blastocysts (Tachikawa *et al.*, 1993) after vitrification. In all four of these studies, embryos were recovered from the straw container before the vitrification solution was diluted. Vitrification solutions containing other mixtures of cryoprotectants have been reported (e.g. Shaw *et al.*, 1991; Ishimori *et al.*, 1992; Tada *et al.*, 1993).

The present study examined the survival of 8–12-cell mouse embryos after: (i) equilibration at room temperature in a vitrification solution (VS3a) consisting of $6.5\text{ mol glycerol l}^{-1}$ and 6% (w/v) BSA in Dulbecco's PBS; (ii) dilution using the one-step procedure (Leibo, 1984) within the straw container in which the embryos were cryopreserved; and (iii) various combinations of a wide range of cooling and warming rates after equilibration in solution VS3a. Finally, we used a paired experimental design to compare the rate of development of embryos *in vivo* after cryopreservation by vitrification in VS3a with that after conventional slow freezing in $1.5\text{ mol glycerol l}^{-1}$. Preliminary reports of these data appeared elsewhere (Rall, 1992b; Wood and Rall, 1992).

Materials and Methods

Source and collection of embryos

Outbred, 3–9-week-old ICR mice (Harlan–Sprague Dawley, Indianapolis, IN) were induced to superovulate by an injection i.p. of 5 iu pregnant mares' serum gonadotrophin G4877; Sigma Chemical Co., St Louis, MO) and 5 iu hCG (CG2; Sigma Chemical Co.) given 44–46 h apart. Females were paired with C57BL/6J $\times$ DBA/2J F_1 hybrid males immediately after injecting hCG and examined the next morning for the presence of a copulation plug (day 1 of pregnancy). On the morning of day 3 of pregnancy (66 h after hCG injection), embryos were flushed from the excised uteri and oviducts using either a modified Dulbecco's phosphate-buffered medium (PB1; Whittingham, 1974) supplemented with 3 mg BSA (Miles Pentex, Kankakee, IL) ml^{-1}

or a modified Krebs–Ringer medium with Hepes buffer (M2; Fulton and Whittingham, 1978) supplemented with 4 mg BSA ml^{-1} . After collection, 8–12-cell embryos were washed in fresh collection medium, pooled and kept at about 4°C until used.

Vitrification solutions

The glycerol-based vitrification solution (VS3a) was similar to that described previously (VS3; Rall, 1987) with three exceptions: (i) BSA replaced polyethylene glycol; (ii) solution PB1 was used as the solution base; and (iii) the solution was adjusted to pH 7.2. Solution VS3a, therefore, consisted of a mixture of glycerol (6.5 mol l^{-1}) and BSA (6% w/v) in PBS (final concentrations: $136.9\text{ mmol NaCl l}^{-1}$, $2.68\text{ mmol KCl l}^{-1}$, $0.49\text{ mmol MgCl}_2\text{ l}^{-1}$, $1.47\text{ mmol KH}_2\text{PO}_4\text{ l}^{-1}$, $8.09\text{ mmol Na}_2\text{HPO}_4\text{ l}^{-1}$, $0.9\text{ mmol CaCl}_2\text{ l}^{-1}$, $5.56\text{ mmol glucose l}^{-1}$, $0.33\text{ mmol sodium pyruvate l}^{-1}$, 100 iu penicillin G ml^{-1} and 0.01% phenol red). Dilutions of solution VS3a were prepared using a solution of PB1 that contained 6% (w/v) BSA to yield solutions with 25% and 65% of the glycerol concentration in solution VS3a (1.6 and $4.2\text{ mol glycerol l}^{-1}$, respectively).

Preparation of one-step vitrification straws

Columns of a diluent solution ($1.0\text{ mol sucrose l}^{-1}$ in PB1) and solution VS3a were placed into a plastic insemination straw (0.25 ml, No. A201; IMV, L'Aigle), as shown in Fig. 1. (i) The cotton–polyvinyl alcohol plug was pushed a total of 9 mm into the straw to facilitate heat sealing. (ii) A column of the diluent solution, 7.5 cm long, was placed in the straw using a 1 ml syringe fitted with a 27 gauge, 3.8 cm hypodermic needle. Care was taken to avoid wetting the wall in other sections of the straw when the needle was inserted and withdrawn. (iii) A 1 cm column of solution VS3a was placed into the straw using a second needle and syringe. The diluent and VS3a solutions were separated by an air bubble gap of 0.5 cm. (iv) The end of the straw closest to the cotton plug was heat-sealed, and a handle (0.5 ml plastic insemination straw) was slip-fitted over the heat seal.

Exposure of embryos to the vitrification solution

Groups of between six and 12 embryos at the 8–12-cell stage were washed in PB1 containing 6% (w/v) BSA and then transferred into a solution of $1.6\text{ mol glycerol l}^{-1}$ and 6% BSA in PB1 (25% VS3a) at room temperature ($20\text{--}26^{\circ}\text{C}$) for 20 min. Embryos were then rinsed in a solution of $4.2\text{ mol glycerol l}^{-1}$ and 6% BSA in PB1 (65% VS3a) and pipetted into the column of solution VS3a in a straw prepared as described above. The total time between placing embryos into 65% VS3a and pipetting into the straw did not exceed 1.25 min. The straw was then heat-sealed and held horizontally at room temperature before either cooling to -196°C or immediately diluting and recovering the embryos (see below). Care was taken during loading and sealing to prevent warming of the embryo suspension.

Fig. 1. Diagram of the final configuration of solutions and mouse embryos in 0.25 ml plastic insemination straws for (a) conventional slow freezing in 1.5 mol glycerol l^{-1} and (b) vitrification in solution VS3a.

Cooling and warming procedures for vitrified embryos

After exposure of embryos to solution VS3a for a total of 1 min, straws were cooled using one of three methods. (i) The straws were cooled slowly ($5^{\circ}\text{C min}^{-1}$). Straws were placed in the chamber of a controlled-rate biological freezer (No. R202; Planer, Sunbury-on-Thames) at -11°C . The diluent column was seeded 3–10 min later by touching the wall of the straw with a spatula precooled in liquid nitrogen (-196°C). Five minutes later, the chamber was cooled at a rate of $5^{\circ}\text{C min}^{-1}$, and when the temperature reached -130°C the straws were immersed in liquid nitrogen. (ii) The straws were cooled rapidly (at approximately $200^{\circ}\text{C min}^{-1}$). Straws were placed in cold nitrogen vapour ($< -170^{\circ}\text{C}$) for at least 3 min and then plunged into liquid nitrogen. (iii) The straws were cooled very rapidly (at approximately $2500^{\circ}\text{C min}^{-1}$). The column of solution VS3a and about 1 cm of the column of diluent were immersed in liquid nitrogen for about 20 s. The straw was then placed into cold nitrogen vapour (as described above) for 3 min to allow the diluent column to crystallize slowly. Straws were stored submerged in liquid nitrogen for periods of between 1 day and 13 months.

Vitrified embryos were warmed using one of three methods. (i) They were warmed very rapidly (at approximately $2000^{\circ}\text{C min}^{-1}$, over the range -100 to -30°C). Straws were removed from liquid nitrogen, held horizontally in the air at 20°C for 10 s and then immersed in water at 20°C for 10 s. The short period in air reduces the likelihood of fracture of the embryo suspension (Rall and Meyer, 1989). (ii) The embryos were warmed rapidly (at approximately $200^{\circ}\text{C min}^{-1}$). Straws were held horizontally in the air for 1 min (to allow the embryo suspension to warm to about -20°C), and then immersed in water at 20°C for 6 s to melt the diluent. (iii) The embryos were warmed slowly (at approximately $20^{\circ}\text{C min}^{-1}$). Straws were transferred into a 17 mm $\times$ 180 mm Pyrex tube previously

cooled to approximately -170°C in liquid nitrogen and allowed to warm in air. When the measured temperature of replicate straws containing a 40 gauge thermocouple warmed to about -20°C , straws were immersed in water at 20°C for 6 s to melt the diluent.

Preparation of one-step straws for controlled slow freezing

Columns of a diluent (1.0 mol sucrose l^{-1} in PB1) and cryoprotectant solution (1.5 mol glycerol l^{-1} in PB1) were placed into a 0.25 ml plastic insemination straw using a modification of the procedure described by Rall (1992a). (i) The cotton-polyvinyl alcohol plug was pushed a total of 9 mm into the straw to facilitate heat-sealing. (ii) A 6.8 cm column of the diluent solution was aspirated into a straw, followed by an air bubble 0.8 cm long, a 0.8 cm column of cryoprotectant solution, a 0.8 cm air bubble, a 1 cm column of the cryoprotectant solution, and finally air until the first column contacted and wetted the cotton plug. (iii) The end of the straw closest to the cotton plug was heat-sealed and a handle (0.5 ml plastic insemination straw) was slip-fitted over the heat seal (Fig. 1).

Controlled slow freezing and thawing of embryos

Embryos were placed in a solution of 1.5 mol glycerol l^{-1} in PB1 for 10 min at room temperature. Groups of embryos (10–20 per group) were then pipetted into the final column of 1.5 mol glycerol l^{-1} in straws prepared as described above, and the straws were heat-sealed. Twenty minutes after placing the embryos in the glycerol (1.5 mol l^{-1}) solution, straws were placed in an ethanol bath (Biocool; FTS Systems, Stone Ridge, NY) precooled to -7°C . After 0.5 min, straws were partially withdrawn from the ethanol and the sucrose column was seeded by touching the wall of the straw with a spatula

precooled in liquid nitrogen. Straws were held at the seeding temperature for 10 min and then the bath was cooled at a rate of $0.5^{\circ}\text{C min}^{-1}$ to -40°C . After 10 min at -40°C , straws were transferred into liquid nitrogen and stored submerged in liquid nitrogen for periods between 1 day and 13 months.

Slowly frozen embryos were thawed rapidly (at approximately $2000^{\circ}\text{C min}^{-1}$) by removing the straw from liquid nitrogen, holding it horizontally in air at room temperature for 10 s and then immersing it in water at 20°C for 10 s.

One-step dilution of embryos

Vitrified and frozen embryos were diluted from the cryoprotectant solution using a modification of the one-step method described by Leibo (1984). (i) After warming and removing the handle, the straw was held firmly at the plugged end and shaken vigorously to dislodge the air bubbles and mix the contents. (ii) The straw was incubated plug end up in water at 36°C for 3 min and then placed plug end down in water at 20°C for 1 min. (iii) The straw was held at the end opposite to the plug and shaken until the air bubbles migrated to the opposite end. (iv) The straw was placed in water at 20°C for 2–5 min and the contents were then expelled into an empty Petri dish. (v) The shrunken embryos were collected and placed in Medium M2 for 10 min to allow the cytoplasm to rehydrate. Embryo survival was assayed *in vitro* or *in vivo*.

Survival assays

In vitro. Embryos recovered from a single straw were cultured together in a $10\text{ }\mu\text{l}$ drop (< 20 embryos per drop) of a bicarbonate-buffered Krebs-Ringer medium (M16; Whittingham, 1971) supplemented with 4 mg BSA ml^{-1} (M16 plus BSA), in the well of a microwell culture plate (No. 452256; Nunc, Roskilde) under silicone oil at 38°C in an atmosphere of 7% CO_2 , 5% O_2 and 88% N_2 . Embryonic development was determined by examination using a microscope ($\times 100$) at intervals of 24 h for 72 h. Survival was defined as the percentage of recovered embryos developing to expanded blastocysts after 48 h.

In vivo. Embryos were cultured overnight (18–24 h) in drops of M16 plus BSA under light liquid paraffin oil (BDH; Poole) in a plastic Petri dish (Falcon, Becton Dickinson; Lincoln Park, NJ) at 37°C in a humidified atmosphere of 5% CO_2 in air. Embryos that developed to the morula or early blastocyst stage were transferred surgically to the uterine horns (4–7 embryos per horn) of recipient females on day 3 of pseudopregnancy. Some recipients received frozen embryos in one horn and vitrified embryos in the contralateral horn (Expt 1). Vitrified embryos were assigned alternately to the right and left horns of consecutive recipients as a precaution against bias due to technical differences in the ease of transfer into uterine horns. Other recipients received vitrified or frozen embryos in both uterine horns (Expt 2).

Recipient females were adult C57BL/6Lac $\times$ CBA/CaLac F_1 hybrid mice mated with sterile males heterozygous for the translocation T145H (Lyon and Meredith, 1966) to induce pseudopregnancy. The day on which a copulation plug was

found was designated day 1 of pseudopregnancy. Embryo recipients were allowed to produce litters or were autopsied on day 15 or 16 of gestation. When recipients produced litters, the number and sex of normal pups were recorded; when autopsy was performed on day 15 or 16, the number of normal fetuses and resorbing implantations were recorded.

Statistical analyses

The statistical significance of differences in the survival of embryos following cryopreservation treatments was determined using two-way analysis of variance (ANOVA) by procedure GLM (SAS Institute, 1990) or chi-squared analysis with Yates' correction for continuity. Data from a paired-comparison of cryopreservation treatments were analysed either by two-way ANOVA for paired comparisons or by *t* test for paired comparisons (Sokal and Rohlf, 1981). In all cases, data were transformed using the angular method for proportions, $\theta = \arcsin \sqrt{p}$, where *p* is the proportion of embryos surviving a treatment.

Results

Vitrification and crystallization of solution VS3a during cooling and warming

Straws prepared for vitrification but without embryos were cooled at rates of 5, 10, 200 or $2500^{\circ}\text{C min}^{-1}$ to -196°C . Vitrification was assessed by visually inspecting the contents of the straws at -196°C . Solutions cooled at rates between 10 and $2500^{\circ}\text{C min}^{-1}$ were transparent in appearance and were assumed to be vitrified. However, slow cooling at $5^{\circ}\text{C min}^{-1}$ yielded a mottled, milky white suspension, which is indicative of substantial crystallization. Straws containing vitrified solution VS3a were warmed at approximately 20, 200 or $2000^{\circ}\text{C min}^{-1}$, and the occurrence of crystallization was identified by the transient whitening of the solution. Solutions warmed at 200 or $2000^{\circ}\text{C min}^{-1}$ remained transparent; that is, they exhibited no evidence of devitrification (crystallization). However, vitrified solution VS3a whitened visibly (crystallized) when warmed slowly at $20^{\circ}\text{C min}^{-1}$. Visual estimates of the extent of whitening suggested that less ice formed during slow warming than during slow cooling.

Injury associated with prolonged equilibration in solution VS3a

Embryos exposed to solution VS3a for 1 or 5 min before dilution from the cryoprotectant without cooling exhibited high rates of development *in vitro* to expanded blastocysts (94%, $n = 146$ and 90%, $n = 69$, respectively). Prolonged exposure of embryos to solution VS3a for 10, 20 or 30 min before dilution resulted in progressively lower rates of development (69%, $n = 61$, 52%, $n = 59$ and 31%, $n = 68$, respectively).

Development of vitrified embryos in vitro

The effect of the rate of cooling to -196°C and the rate of warming on the survival of embryos in solution VS3a is shown

Table 1. Survival of 8–12-cell mouse embryos cooled and warmed in solution VS3a to -196°C at various rates

Cooling conditions	Warming conditions		
	Slow ($20^{\circ}\text{C min}^{-1}$)	Rapid ($200^{\circ}\text{C min}^{-1}$)	Very rapid ($2000^{\circ}\text{C min}^{-1}$)
Slow ($5^{\circ}\text{C min}^{-1}$)	75.2 ± 4.4 (11)	88.8 ± 4.3 (9)	92.9 ± 2.1 (13)
Rapid ($200^{\circ}\text{C min}^{-1}$)	80.9 ± 4.7 (12)	86.1 ± 3.7 (14)	90.9 ± 2.5 (24)
Very rapid ($2500^{\circ}\text{C min}^{-1}$)	81.5 ± 4.2 (13)	97.1 ± 1.6 (15)	91.1 ± 2.2 (15)

Values are means $\pm$ SEM survival of embryos (%) developing to expanded blastocysts in culture. Number of replicate samples is given in parentheses.

Table 2. Estimate of overall survival *in vivo* following cryopreservation by vitrification in VS3a or controlled slow freezing of 8–12-cell mouse embryos

Treatment	Number of embryos thawed	Number of morulae and blastocysts after culture (%)	Number of embryos transferred (number of recipients)	Number of recipients establishing pregnancy	Number of implantations on day 15 of pregnancy	Number of normal fetuses on day 15 of pregnancy (%)	Number of normal pups (%)	Estimated overall % survival ^d
Vitrification	206	199 (97%)	127 (22) ^a	22	97 (76%)	93 (73%)	—	63%
			26 (2) ^b	2	20 (77%)	17 (65%)	—	
			46 (4) ^b	3	—	—	19 (41%)	
			Total:	199 (28)	27	110	19	
Slow freezing	157	155 (99%)	129 (22) ^a	22	110 (85%)	105 (81%)	—	76%
			26 (3) ^c	2	—	—	14 (54%)	
			Total:	155 (25)	24	110	14	

^aVitrified embryos were transferred into one uterine horn and slowly frozen embryos into the contralateral horn (Expt 1).

^bVitrified embryos were transferred into both uterine horns (Expt 2).

^cSlowly frozen embryos were transferred into both uterine horns (Expt 2).

^dOverall estimated survival based on the number of embryos thawed.

in Table 1. A high rate of development *in vitro* (86–97%) was obtained when the embryo suspensions were cooled and warmed at rates of $200^{\circ}\text{C min}^{-1}$ or higher; that is, in conditions that ensure vitrification and prevent devitrification. Similarly, high rates of survival (75–93%) were obtained after slow cooling at $5^{\circ}\text{C min}^{-1}$ or slow warming at $20^{\circ}\text{C min}^{-1}$ or both treatments, even though the formation of ice was observed in those suspensions. Statistical analysis of these data indicated that there was no significant effect of the cooling rate or the interaction of cooling and warming rates on the survival of embryos (two-way ANOVA, $F_s = 1.34$, $P > 0.26$). However, the warming rate had a significant effect on survival ($F_s = 11.02$; $P < 0.005$). Multiple pairwise comparisons of all combinations of warming rates indicated that only slow warming at $20^{\circ}\text{C min}^{-1}$ resulted in a small but statistically significant reduction in survival (Tukey's studentized range test, $P < 0.05$).

Implantation and development of vitrified and slowly frozen embryos

Embryos were either vitrified in solution VS3a using a cooling rate of $200^{\circ}\text{C min}^{-1}$ or frozen slowly in 1.5 mol

glycerol l^{-1} (Table 2). A total of 206 vitrified and 157 slowly frozen embryos were warmed rapidly ($2000^{\circ}\text{C min}^{-1}$), diluted from the cryoprotectant and cultured *in vitro* for 24 h. Virtually all (97%) of 363 cryopreserved embryos developed to morulae or blastocysts. A total of 199 vitrified and 155 frozen embryos were transferred to 31 pseudopregnant recipients, of which 29 females (94%) established pregnancy. Overall, 63% of vitrified embryos and 76% of frozen embryos developed to form normal late-stage fetuses or liveborn offspring (Table 2).

Embryos were transferred into recipient females using one of two experimental designs. Expt 1 yielded a direct comparison of the relative abilities of vitrified and slowly frozen embryos to develop *in vivo*. A total of 22 recipient females received vitrified embryos in one uterine horn and slowly frozen embryos in the contralateral horn. A total of 127 vitrified and 129 slowly frozen embryos were transferred and all 22 recipients established pregnancy (Table 2). Analysis using the two-way ANOVA for paired comparisons (Table 3) indicated that the rates of development to normal fetuses of vitrified and slowly frozen embryos did not differ (73 versus 81%, respectively; $F_s = 2.51$, $P > 0.12$). Furthermore, differences between embryo transfer recipients were not a significant source of variation in this experiment ($F_s = 0.85$, $P > 0.5$). Similarly, no

Table 3. Two-way ANOVA table of paired comparison of the development of mouse embryos to normal fetuses *in vivo* following cryopreservation by vitrification or slow freezing

Source of variation	Degrees of freedom (d.f.)	Sum of squares (SS)	Mean square (MS)	F_s
Cryopreservation method	1	1481.2	1481.2	2.51 ^a
Recipient females	21	10 533.0	501.6	0.85 ^a
Remainder (error)	21	12 404.8	590.7	
Total	43	24 419.0		

^aNot significant.

Statistical table F values: $F_{0.1[1,21]} = 2.96$; $F_{0.05[1,21]} = 4.32$; $F_{0.01[1,21]} = 8.02$.

statistically significant differences were detected in the rates of implantation ($F_s = 1.76$, $P > 0.19$) and post-implantation mortality ($F_s = 0.59$, $P > 0.45$).

In Expt 2, a total of 46 vitrified embryos were transferred to both uterine horns of four recipients, of which three established pregnancy and delivered 19 normal pups (13 females, 6 males) (Table 2). A total of 26 slowly frozen embryos were transferred into both uterine horns of three recipients, of which two established pregnancy and delivered 14 normal pups (6 females, 8 males) (Table 2). The proportion of embryos developing to liveborn offspring following either cryopreservation method was not different (Chi-squared analysis, $P > 0.3$).

Finally, the remaining 26 vitrified embryos were transferred into two recipients. On day 15 of gestation, 17 normal fetuses and three resorbing implantations were observed (Table 2).

Discussion

High rates of development *in vitro* and *in vivo* were obtained when 8–12-cell mouse embryos were cryopreserved by a simple vitrification procedure that used a solution containing glycerol and BSA, step-wise equilibration at room temperature and dilution with sucrose within the straw. Cooling and warming conditions did not markedly affect the survival of embryos, even when ice formed in the suspension during slow cooling (5°C min^{-1}) or slow warming ($20^\circ\text{C min}^{-1}$). However, the combination of slow cooling and slow warming resulted in a small (10–15%) but statistically significant decrease in survival. These results confirm and expand previous reports that embryos frozen or vitrified in glycerol solutions tolerate crystallization of the suspension during warming (Rall and Polge, 1984; Rall, 1987). By contrast, none of the embryos vitrified in solutions based on dimethylsulfoxide or propylene glycol survive when ice forms in the suspension during slow warming (Rall and Fahy, 1985; Rall, 1987).

Solution VS3a is similar to that reported previously (VS3, Rall, 1987), with three exceptions. First, the pH of the solution was adjusted to 7.2. Second, BSA was substituted for the synthetic polymer polyethylene glycol. This eliminates the possibility of cell fusion associated with exposure to high concentrations of polyethylene glycol (Pontecorvo, 1975). BSA was selected because it is a common component of embryo handling and culture media, and the concentration of albumin added to solution VS3a, 6% (w/v), is similar to the concentration of protein normally present in serum (Kaneko, 1989).

Third, solution VS3a was prepared using a modified PBS solution (PB1; Whittingham, 1974) without reducing the concentrations of calcium, magnesium or phosphate ions. Unlike vitrification solutions containing polyethylene glycol and dimethylsulfoxide or propylene glycol, solution VS3a does not reduce the solubility of divalent cations. This property may be related, in part, to the mechanism proposed by Timasheff and colleagues (Gekko and Timasheff, 1981; Timasheff, 1993) to explain the ability of glycerol and chemically similar non-electrolytes to protect proteins from denaturation.

Solution VS3a was prepared in a manner that maintained the concentration of saline components of PB1 on a molar basis (moles salt litre⁻¹ solution). This contrasts with the methods reportedly used to prepare other vitrification solutions, which maintain a constant concentration of salts on a molal basis (moles salt kg⁻¹ H₂O) but which reduce the concentration of saline components by about 50% on a molar basis (Scheffen *et al.*, 1986; Kasai *et al.*, 1990). Such a reduction may result in osmotic imbalances when cells are equilibrated in solutions of permeating cryoprotectants prepared by the molal method. On the basis of studies with human erythrocytes, Meryman and Douglas (1982) suggest that the molar definition is preferable when the cryoprotectant concentration is greater than 4 mol l⁻¹. Recently, Papis *et al.* (1993) compared both methods for maintaining the concentration of saline components in a rabbit embryo vitrification solution. They report that the molar approach yields higher embryo viability after vitrification than does the molal approach.

The total concentration of solutes in solution VS3a was adjusted to yield a stable vitreous state when cooled and warmed at moderate rates. Our observations indicate that this goal was achieved. No visible ice crystallization occurred when the solution was cooled at rates of $100^\circ\text{C min}^{-1}$ or more, or when vitrified samples were warmed at rates of $200^\circ\text{C min}^{-1}$ or more. However, either slow cooling at 5°C min^{-1} or slow warming at $20^\circ\text{C min}^{-1}$ resulted in visible crystallization. The fact that more than 75% of embryos equilibrated in solution VS3a developed normally *in vitro* after cooling and warming at these low rates indicated that, under these conditions, crystallization of the embryo suspension was not inconsistent with high survival after thawing. The mechanism of survival is presumably similar to that proposed to explain the similarly high survival of embryos cryopreserved by the so-called ultrarapid freezing procedure (Széll and Shelton, 1986; Trounson *et al.*, 1987; Leibo, 1989; Mazur, 1990). According to this view, osmotic dehydration before cooling and warming

increases the concentration of cytoplasmic solutes, which prevents the formation of lethal quantities of intracellular ice.

The formation of ice in a straw containing embryos suspended in solution VS3a does not necessarily indicate that ice has formed in the cytoplasm of embryos. Indeed, knowing the glass-forming tendency of a bulk vitrification solution provides little or no information regarding the likelihood of vitrification within a cell or embryo. Experimental evidence suggests that rapidly cooled embryos vitrify as defined by the lack of intracellular ice when examined using electron microscopy (Valdez *et al.*, 1990). Rall (1987) proposed that cellular dehydration during the step-wise equilibration procedure described here may yield a concentrated cytoplasm that has greater glass-forming abilities than that of the suspending solution. According to this view, osmotic dehydration during exposure to 65% VS3a and solution VS3a of the equilibration procedure results in the exosmosis of cytoplasmic water and a concomitant increase in the concentration of endogenous macromolecules and glycerol that permeate into the cytoplasm during equilibration in 25% VS3a. Since the cytoplasm of preimplantation embryos is reported to contain approximately 15% (w/v) protein in normal isotonic conditions (see Rall, 1987), osmotic dehydration produces approximately a doubling of the initial concentration of endogenous macromolecules. Such an increase ought to result in a more stable cytoplasmic glass than the extracellular glass (solution VS3a) that contains 6% (w/v) BSA. The high rates of survival of vitrified embryos in this study are consistent with this suggestion.

The paired-comparison experimental design provided a powerful method for comparing the efficacy of vitrification and conventional slow freezing procedures. We analysed our results using a two-way ANOVA (Sokal and Rohlf, 1981) because this approach allows two components of variance (cryopreservation method and recipient females) to be measured. The more traditional method for analysing such data, the *t* test for paired comparisons, yields the same conclusion with respect to the cryopreservation method but does not allow the variance component among recipient females to be measured. Our analysis indicated that the developmental rates of vitrified and slowly frozen embryos to normal fetuses were statistically similar. Furthermore, there was no significant added variance among recipient females. This indicated that there were no significant differences (such as genetic, environmental or technical) in the ability of recipient females used in our experiment to establish pregnancies and gestate cryopreserved embryos.

Estimates of the overall efficiency of any embryo cryopreservation procedure is best expressed in terms of overall survival (i.e. the ratio of the number of normal pups or fetuses to the number of embryos thawed). Expt 1 provided a direct estimate of overall efficiency of vitrification in VS3a and slow freezing (Table 2). The overall estimates of the development of vitrified and slowly frozen embryos reported here compare favourably to other reports. For example, Wood *et al.* (1987) and Glenister *et al.* (1990) provided estimates of 16–66% survival for slowly frozen mouse embryos from various inbred, hybrid and outbred genotypes. Overall survival rates following the vitrification of mouse embryos range from 18 to 62% (Scheffen *et al.*, 1986; Rall *et al.*, 1987; Kasai *et al.*, 1990, 1992).

This study has defined a simple cryopreservation procedure for 8–12-cell mouse embryos that yields high overall rates of survival *in vitro* and *in vivo*. Other important features of our vitrification procedure include: (i) a nontoxic vitrification solution containing glycerol and BSA; (ii) step-wise equilibration at room temperature; (iii) the widest range of cooling and warming rates of any embryo cryopreservation procedure reported to date; and (iv) sucrose dilution of cryoprotectant within the straw container in which the embryos were cryopreserved.

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