Liquid nitrogen vapour freezing of mouse embryos

H. Miyamoto and T. Ishibashi

Department of Animal Science, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

Summary. Mouse morulae were frozen rapidly to -196°C in the presence of glycerol by a two-step procedure; the embryos were transferred directly from -7°C after seeding into liquid nitrogen vapour at -170 to -180°C and then into liquid nitrogen 10–15 min later. Suitable conditions for the survival of embryos frozen with liquid nitrogen vapour were found to be: 2 M-glycerol, 2 M-propylene glycol, 2 M-ethylene glycol; 5–30 min equilibration time at 0°C ; 3–60 min holding time in liquid nitrogen vapour; dilution of glycerol with sucrose out of the frozen-thawed embryos; morula and early blastocyst stage embryos. Relatively high survival rates (69–74%) were obtained after rapid freezing by liquid nitrogen vapour. Morulae frozen in this fashion, cultured and transferred to recipients developed into normal young.

Introduction

Slow cooling (0.2 – $0.8^{\circ}\text{C}/\text{min}$) and slow thawing (4 – $25^{\circ}\text{C}/\text{min}$) have been reported as necessary to obtain optimal survival of mouse embryos (Whittingham *et al.*, 1972; Wilmut, 1972). However, a high proportion of cattle and sheep embryos survived if slow cooling at $0.3^{\circ}\text{C}/\text{min}$ was terminated between -30 and -36°C by direct transfer to liquid nitrogen and embryos were thawed rapidly at $360^{\circ}\text{C}/\text{min}$ (Willadsen, 1977; Polge & Willadsen, 1978). Mouse embryos responded in a similar fashion (Whittingham *et al.*, 1979).

Moreover, several workers have recently reported the preservation of mammalian embryos using the simple technique based upon the two-step freezing procedure (Wood & Farrant, 1980; Kasai *et al.*, 1980; Bouyssou & Chupin, 1982; Miyamoto & Ishibashi, 1983a; Renard *et al.*, 1984; Széll & Shelton, 1986). Kasai *et al.* (1980) reported that 8% of mouse embryos in dimethyl sulphoxide (DMSO) survived direct transfer from -7°C after seeding into liquid nitrogen vapour at -100°C and then into liquid nitrogen. More recently a relatively high survival of mouse embryos was obtained when the freezing medium in the straw contained glycerol + sucrose and embryos were frozen in liquid nitrogen vapour (Széll & Shelton, 1986). Rall & Fahy (1985) reported that a high proportion of mouse embryos pre-equilibrated in vitrification solution survived after rapid cooling to liquid nitrogen. Renard *et al.* (1984) showed that high survival of rabbit embryos was obtained in the presence of propanediol (propylene glycol) when embryos pretreated with sucrose were transferred directly into liquid nitrogen vapour at -30°C and held for 30–240 min at this temperature before rapid cooling in liquid nitrogen.

In a previous paper reviewing embryo preservation, Miyamoto (1986) has shown that a relatively high survival was obtained when mouse morulae in glycerol were frozen with liquid nitrogen vapour at -170 to -180°C before transfer into liquid nitrogen. The present study reports in detail all the experiments performed using a liquid nitrogen vapour method.

Materials and Methods

Female ICR mice (4–5 weeks old) were induced to superovulate by intraperitoneal injection of 5 i.u. PMSG and 5 i.u. hCG, given 48 h apart, and were then mated. The morulae were flushed from the

reproductive tracts at 75–77 h after the hCG injection with a modified Dulbecco's phosphate-buffered salt solution (PB1; Whittingham, 1971a). Embryos at other stages were recovered at various times after the hCG injection.

The embryos were washed once with fresh Medium PB1, and 10–25 embryos were pipetted into each freezing tube (10 × 100 mm glass test-tube) containing 0.1 ml Medium PB1 and cooled to 0°C at 2°C/min in an ethanol bath. Except as noted, glycerol was added to the samples at 0°C in two increments of 0.075 ml at 10-min intervals. The final concentration of glycerol was usually 2 M. In one experiment the embryos were placed into test-tubes containing 0.1 ml 0.5 M-sucrose in Medium PB1 at room temperature, and held at this temperature for 5 min. They were cooled to 0°C and then glycerol + 0.5 M-sucrose in Medium PB1 was added in two steps. Therefore the embryos were frozen in Medium PB1 containing 2 M-glycerol + 0.5 M-sucrose. Except for this experiment in which the embryos were pretreated with sucrose before freezing, the sucrose was absent in the freezing medium both before and during freezing.

The samples were held at 0°C for 10 min after the final addition of glycerol, cooled to –7°C at 1°C/min, and seeded with an ice crystal to induce ice formation. After 5 min they were transferred directly into liquid nitrogen vapour at –170 to –180°C in a liquid nitrogen refrigeration tank and held at that temperature in vapour for 10–15 min before being immersed in the liquid nitrogen. The average cooling rate was ~32°C/min between –10 and –70°C. The samples for slow freezing were cooled to –70°C at 0.5°C/min by adding solid CO₂ to the ethanol, and then they were immersed in the liquid nitrogen.

After storage at –196°C for 1–4 days, the samples were usually thawed rapidly but some were thawed slowly. Rapidly thawed samples were agitated in a 40°C water bath (~500°C/min) and slowly thawed samples were suspended in air at room temperature (18–24°C) (~20°C/min). After thawing, the embryos were usually placed into Medium PB1 containing 2 M-glycerol + 0.5 M-sucrose and then placed into Medium PB1 containing 0.5 M-sucrose at 3-min intervals at room temperature, followed by two washes in fresh Medium PB1 (Kasai *et al.*, 1980). In some experiments, the frozen-thawed embryos were diluted rapidly by direct transfer of embryos into 2 ml Medium PB1 at room temperature or slowly by stepwise addition of 0.2, 0.2 and 0.4 ml Medium PB1 at 2-min intervals.

The frozen-thawed morulae were cultured by the microdrop method in a modified Krebs–Ringer–bicarbonate medium at 37°C for 40–48 h in 5% CO₂ in air (Whittingham, 1971b). In one experiment, embryos at stages other than the morulae were cultured for 24–68 h, depending on the developmental stage at the time of freezing. Survival of the embryos was assessed by their ability to develop to expanded blastocysts after culture. Experiments were replicated 4–6 times and data were analysed for statistical significance by χ^2 test after confirmation that the results for each treatment were homogeneous.

To examine the further development of the frozen embryos, embryos were cultured for 20–24 h and 5–8 embryos that had developed normally to blastocysts were transferred to each uterine horn of 9 recipients on Day 4 of pseudopregnancy, induced by mating with a vasectomized male. The recipients were allowed to litter.

Results

The effect of various factors on the survival of mouse embryos frozen in liquid nitrogen vapour before transfer into liquid nitrogen is summarized in Tables 1 and 2.

Glycerol concentration

Glycerol concentration was found to be the most important factor affecting survival of morulae frozen in liquid nitrogen vapour. The optimum glycerol concentration was 2 M ($P < 0.05$); higher

Table 1. Factors affecting the survival of mouse morulae frozen in liquid nitrogen vapour at -170 to -180°C before transfer into liquid nitrogen

	No. of embryos recovered	No. of embryos developing to expanded blastocysts after culture	% Survival
Glycerol conc. (M)			
0.5	65	5	8
1.0	75	25	33
1.5	69	40	58
2.0	73	62	85
3.0	78	52	67
4.0	71	51	72
Holding time (min) in vapour			
3	68	51	75
5	66	48	73
10	75	54	72
15	72	54	75
30	68	49	72
60	74	48	65
Glycerol dilution out of frozen-thawed embryos			
Direct dilution	66	7	11
Stepwise dilution	74	45	61
Dilution with sucrose	71	53	75
Pretreatment with sucrose before freezing			
Not pretreated with sucrose	83	62	75
Pretreated with sucrose	97	76	78
Cryoprotectant (conc.)			
Glycerol (2 M)	75	60	80
DMSO (2 M)	83	15	18
Ethylene glycol (2 M)	70	48	69
Propylene glycol (2 M)	77	54	70
Erythritol (2 M)	77	44	57
Methanol (3 M)	76	0	0
Sucrose (0.5 M)	53	0	0
Embryonic stage			
2-cell	51	0	0
4-cell	62	0	0
8-cell	73	36	49
Morula	77	54	70
Early blastocyst	84	61	73
Expanding blastocyst	71	29	41

Usually the embryos were exposed to 2 M-glycerol at 0°C for 10 min after the two-step addition of glycerol, seeded and then frozen in liquid nitrogen vapour before being immersed in liquid nitrogen.

or lower concentrations of glycerol afforded less protection or were themselves deleterious. We used 2 M-glycerol for subsequent studies.

Equilibration time of embryos in glycerol

To determine whether the time of exposure to glycerol at 0°C before freezing had any effect upon survival of frozen-thawed embryos, the embryos were kept in 2 M- or 4 M-glycerol at 0°C for various periods after the single step addition of glycerol (Table 2). In one group, the embryos were

Table 2. The effect of equilibration time in glycerol at 0°C before freezing on the survival of mouse morulae frozen with liquid nitrogen vapour at -170 to -180°C before transfer into liquid nitrogen

Glycerol conc. (M)	Seeding	Equilibration time (min)	No. of embryos recovered	No. of embryos developing to expanded blastocysts after culture	% Survival
2	No	0.1	66	0	0
		1	82	26	32
		3	78	36	46
		5	83	64	77
		10	75	63	84
		20	74	59	80
		30	81	68	84
		60	75	36	48
2	Yes	0.1	63	49	78
		5	66	51	77
		10	61	52	85
		20	58	42	72
		30	65	52	80
		60	60	30	50
4	No	0.1	63	0	0
		1	76	15	20
		3	83	46	55
		5	66	46	70
		10	74	54	73
		30	69	53	77
		60	75	41	55

The embryos were exposed to glycerol at 0°C for various periods after the single-step addition of glycerol and transferred into liquid nitrogen vapour after or without seeding.

then transferred directly from 0°C without seeding into liquid nitrogen vapour at -170 to -180°C and held for 10–15 min at that temperature before being immersed in liquid nitrogen. In the other group the embryos were cooled to -7°C at 1°C/min, seeded at this temperature and 5 min later they were transferred directly from -7°C into liquid nitrogen vapour before being immersed in liquid nitrogen. When the embryos were frozen in 2 M- or 4 M-glycerol by transferring them from 0°C without seeding into liquid nitrogen vapour, no embryos survived freezing after exposure to glycerol at 0°C for 0.1 min and a low proportion of the embryos (32–46% and 20–55%) was protected following exposure for 1–3 min. Embryos exposed to glycerol at 0°C for 0.1–3 min appeared more sensitive to freezing than those exposed for longer times (5–30 min). The higher survival rates were found after exposure to glycerol for 5–30 min ($P < 0.01$) and prolonged exposure (60 min) appeared to be detrimental. When the embryos were frozen in 2 M-glycerol by transferring them from -7°C after seeding into liquid nitrogen vapour, a high proportion (78%) of the embryos was protected after exposure to glycerol at 0°C for only 0.1 min and exposure time (0.1–30 min) had little effect on survival.

Holding time in liquid nitrogen vapour before transfer into liquid nitrogen

The embryos were equilibrated in 2 M-glycerol at 0°C for 10 min and after seeding they were frozen in liquid nitrogen vapour. Embryos were held in liquid nitrogen vapour at -170 to -180°C for various periods before transfer into liquid nitrogen. Holding time (3–60 min) in vapour appeared to have little effect on survival and for the subsequent experiments a 10–15 min holding period was used.

Dilution of glycerol from frozen–thawed embryos

The survival of embryos frozen in glycerol was compared after diluting glycerol from the frozen–thawed samples by rapid dilution, stepwise dilution or dilution with Medium PB1 containing sucrose and glycerol. The survival (11%) of embryos after rapid dilution was lower ($P < 0.001$) than that of embryos after stepwise dilution (61%) or dilution with sucrose (75%). Although the survival (75%) of embryos after dilution with sucrose appeared higher than that after stepwise dilution (61%) the difference was not significant. A sucrose-dilution procedure was used for the present study.

Pretreatment with sucrose before freezing

We examined the effect of exposure to 0.5 M-sucrose at room temperature for 5 min before freezing on the survival of frozen embryos. The embryos were frozen in Medium PB1 containing 2 M-glycerol plus 0.5 M-sucrose. The pretreatment with 0.5 M-sucrose before freezing did not improve the embryo survival under the present experimental conditions.

Cryoprotectant

The effect of various cryoprotectants on the survival of embryos frozen in liquid nitrogen vapour was examined. Glycerol (2 M), propylene glycol (2 M) and ethylene glycol (2 M) were effective cryoprotectants. Although the survival rate of embryos frozen in glycerol (80%) appeared higher than that of embryos frozen in propylene glycol (70%) or ethylene glycol (69%), the difference was not significant. Considerable protection against freezing damage was also afforded by erythritol (2 M). However the degree of protection given by DMSO (2 M) was low and methanol (3 M) and sucrose (0.5 M) did not afford cryoprotection.

Embryonic stage of development at the time of freezing

The effect of embryonic stage of development on survival of frozen–thawed embryos was examined. The embryos at various stages were equilibrated in 2 M-glycerol at 0°C for 10 min and after seeding they were frozen in liquid nitrogen vapour. Survivals for 2-cell, 4-cell, 8-cell, morula, early blastocyst and expanding blastocyst stage embryos were 0, 0, 49, 70, 73 and 41%, respectively. The survival of morula and early blastocyst stage embryos was higher ($P < 0.01$) than survival of other stages.

Freezing and thawing rates

The survival of embryos after being frozen and thawed at several rates in the presence of glycerol or propylene glycol is summarized in Table 3. When embryos in glycerol were frozen rapidly by liquid nitrogen vapour before rapid cooling in liquid nitrogen, a relatively high survival (74%) was obtained with rapid thawing whereas a low proportion (38%) of frozen embryos survived slow thawing ($P < 0.01$). By contrast, high survival rates (86–88%) were obtained when embryos were frozen in glycerol slowly and thawed slowly or rapidly. The survival of embryos frozen–thawed rapidly in glycerol (74%) was lower ($P < 0.05$) than that of embryos frozen slowly (86–88%). When embryos were frozen in propylene glycol by vapour, a 69% survival rate was obtained with rapid thawing but no embryos survived slow thawing. With slow cooling in the presence of propylene glycol, 80 and 76% survival rates were obtained after slow and rapid thawing, respectively.

Table 3. Effects of freezing and thawing rates on the survival of mouse morulae frozen in the presence of glycerol or propylene glycol

Cryoprotectants (conc.)	Freezing rates	Thawing rates (°C/min)	No. of embryos recovered	No. of embryos developing to expanded blastocysts after culture	% Survival
Glycerol (2 M)	Rapid*	~ 20	112	43	38
		~ 500	105	78	74
	Slow†	~ 20	91	78	86
		~ 500	83	73	88
Propylene glycol (2 M)	Rapid	~ 20	72	0	0
		~ 500	77	53	69
	Slow	~ 20	82	66	80
		~ 500	85	65	76

* For details of procedures, see text.

† Samples were cooled to -70°C at $0.5^{\circ}\text{C}/\text{min}$ before transfer to liquid nitrogen.

In-vivo development of frozen-thawed embryos

The morulae equilibrated in 2 M-glycerol for 10 min were frozen in liquid nitrogen vapour before transfer into liquid nitrogen. They were thawed rapidly and sucrose was used for the glycerol dilution. As a further test of viability, the embryos which developed to blastocysts after culture were transferred to pseudopregnant recipients. Of the 118 blastocysts transferred to 9 recipients, 4 females littered 36 (31%) normal young at term from 50 blastocysts transferred.

Discussion

Our findings support a previous anecdotal report that mouse morulae in DMSO survived direct transfer from -7°C after seeding into liquid nitrogen vapour at -100°C before rapid cooling to liquid nitrogen (Kasai *et al.*, 1980). Furthermore, the present results demonstrate that mouse embryos frozen in glycerol by our rapid freezing technique based upon the two-step cooling procedure can survive quite well. This finding is in agreement with the results of Széll & Shelton (1986) that a relatively high proportion of mouse embryos survived rapid freezing in liquid nitrogen vapour. The present freezing procedure provides a quick and easily performed technique for preserving mouse embryos with a minimum of specialized equipment and makes it possible to freeze a large number of embryo samples in a short time.

It was reported that mouse embryos fully equilibrated in glycerol or DMSO were killed when the embryos were cooled at $\sim 20^{\circ}\text{C}/\text{min}$ to temperatures below -40°C (Rall *et al.*, 1983). The difference in survival between embryos frozen rapidly in the present study and those in the previous study (Rall *et al.*, 1983) may be due to the differences in the embryonic stage, thawing rate and method for removal of cryoprotectant from frozen-thawed embryos.

The exposure time of embryos to glycerol was found to be the important factor if the embryos were frozen in glycerol by transferring them from 0°C without seeding into liquid nitrogen vapour. No embryos survived freezing after exposure to glycerol for 0.1 min and higher survivals were found after exposure to glycerol at 0°C for 5–30 min. It has been shown that relatively little DMSO (Whittingham *et al.*, 1972; Leibo *et al.*, 1974; Schneider & Maurer, 1983), glycerol (Whittingham *et al.*, 1972; Mazur *et al.*, 1976; Jackowski *et al.*, 1980) or ethylene glycol (Miyamoto & Ishibashi, 1977) permeates mouse ova and embryos even after extended periods of equilibration at 0 – 4°C and permeation of cryoprotectant may not be required for protection. Therefore, in the present study embryos exposed to glycerol would not have been fully equilibrated even after 60 min at 0°C and

would contain a small amount of intracellular cryoprotectant when freezing was started. In previous (Whittingham *et al.*, 1972; Leibo *et al.*, 1974; Miyamoto & Ishibashi, 1977; Schneider & Maurer, 1983) and present studies, after equilibration in the cryoprotectant at 0°C, the samples were usually transferred to a -3.5/-6°C bath held for 2 min, seeded and 5 min later they were cooled slowly (0.3-1.8°C/min): it took about 7 min from the end of equilibration to the start of continuous slow cooling. It seems reasonable, therefore, to assume that the cryoprotectant entered the blastomeres to some extent during these 7 min and also during the interval of continuous slow cooling even at high subzero temperatures. Further experiments are needed to determine whether a cryoprotectant that protects embryos against freezing damage permeates them to exert its beneficial effect.

Although DMSO has been the cryoprotectant of choice for deep-freezing of mammalian embryos (Whittingham, 1980), the degree of protection by DMSO was very low under the present experimental conditions. Ethylene glycol and propylene glycol are also effective cryoprotectants for mouse embryos cooled slowly to very low temperatures or high subzero temperatures before rapid cooling to liquid nitrogen (Miyamoto & Ishibashi, 1977, 1978, 1983b; Kasai *et al.*, 1981; Rall & Polge, 1984; Renard & Babinet, 1984). In contrast, only very low survival was obtained when mouse embryos were frozen in ethylene glycol by the two-step method (Kasai *et al.*, 1981) whereas high protection was afforded by ethylene glycol under the present conditions. Although methanol has been shown to protect mouse embryos from freezing damage if slow cooling is terminated between -30 and -40°C and followed by rapid cooling to liquid nitrogen (Rall *et al.*, 1984), embryos in methanol did not survive liquid nitrogen vapour freezing in the present study. It is concluded that the ability of the cryoprotectant to protect mouse embryos from the deleterious effects of freezing and thawing depends on the cooling and thawing conditions.

The sensitivity of embryos to freezing was greatly affected by the embryonic stage of development. The survival of morulae and early blastocysts was high compared to that of 2-cell, 4-cell, 8-cell stage embryos and expanding blastocysts, suggesting that morulae and early blastocysts are the least susceptible to damage by the present freezing procedures. These findings agree with the report of Massip *et al.* (1984) on the survival of mouse embryos at various stages frozen slowly to -25°C before rapid cooling to -196°C. Although mouse embryos at all the preimplantation stages have been successfully frozen by slow cooling (Whittingham *et al.*, 1972), the present results indicate that a differential sensitivity of embryos at various stages to freezing is observed when rapid freezing procedures are used. Since the optimum conditions required to freeze mammalian embryos at various stages are different (Bank & Maurer, 1974; Schneider & Maurer, 1983; Massip *et al.*, 1984; present study), cooling and thawing conditions should be adapted to the embryonic stage to obtain high survival.

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