Solid CO₂ freezing of mouse embryos

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Summary. Eight-cell mouse embryos were frozen rapidly to -196°C in the presence of glycerol by a two-step procedure; the lower two-thirds of each tube containing embryos was placed directly from 0°C into crushed solid CO₂ for some time before transfer to liquid nitrogen. The cooling rate between -10 and -60°C was $\sim 30^{\circ}\text{C}/\text{min}$. Suitable conditions for the survival of embryos in this fashion were found to be: stepwise addition of glycerol and no seeding; 1.8 M-glycerol; 5–30 min equilibration time; 10–30 min holding time in crushed solid CO₂; $\sim 500^{\circ}\text{C}/\text{min}$ thawing rate. A relatively high proportion (62%) of frozen embryos survived after rapid thawing, but none survived slow thawing. Eight-cell embryos frozen-thawed in this fashion and transferred to recipients developed into normal young.

Introduction

The successful preservation of mammalian embryos at low temperatures has been accomplished with suitably slow rates of cooling ($0.2\text{--}0.8^{\circ}\text{C}/\text{min}$) and thawing ($4\text{--}25^{\circ}\text{C}/\text{min}$) in the presence of dimethylsulphoxide (DMSO) as the cryoprotectant (Whittingham, Leibo & Mazur, 1972; Wilmut, 1972). These rates of cooling and thawing have to be maintained over temperatures from -4 to -60°C and from -70 to -20°C respectively for optimal survival of mouse embryos (Leibo, Mazur & Jackowski, 1974). Rapid thawing has generally been found to be much more damaging for the survival of mouse embryos than slow thawing (Whittingham *et al.*, 1972; Wilmut, 1972). By contrast, sheep and cow embryos can survive rapid thawing when they are slowly cooled at $0.3^{\circ}\text{C}/\text{min}$ to temperatures between -30 and -45°C before transfer to liquid nitrogen (Willadsen, 1977; Willadsen, Polge & Rowson, 1978; Polge & Willadsen, 1978).

Eight-cell mouse embryos can survive rapid thawing if slow cooling has been stopped at -35 and -40°C by transfer to liquid nitrogen (Whittingham, Wood, Farrant, Lee & Halsey, 1979). Mouse embryos can also be preserved in DMSO by using a simple technique based upon the two-step freezing procedure (Wood & Farrant, 1980; Kasai, Niwa & Iritani, 1980, 1981). The present paper describes a solid CO₂ method of freezing mouse embryos.

Materials and Methods

Female ICR mice, aged 5–10 weeks, were induced to superovulate by intraperitoneal injections of 5 i.u. PMSG and 5 i.u. hCG, given 44–48 h apart, and were mated. The 8-cell embryos were flushed from the oviducts with a modified Dulbecco's phosphate-buffered salt solution (PBS: Whittingham, 1971a) at 67–70 h after the injection of hCG.

The embryos were washed in several changes of PBS and 11–25 embryos were transferred to each freezing tube (10×100 mm glass test-tube) containing 0.1 ml PBS. Except where noted, the tubes were cooled to 0°C at $2^{\circ}\text{C}/\text{min}$ in an ethanol bath, and glycerol in PBS was added to samples

at 0°C in two increments of 0.075 ml at 10-min intervals. The final concentration of glycerol was usually 1.8 M. After 10 min, the lower two-thirds of each tube was placed directly from 0°C into crushed solid CO₂ in a Dewar flask without seeding. To obtain close contact between the crushed solid CO₂ and the tube wall, the former was gently pressed by fingers towards the tube wall and then the Dewar flask was sealed. After samples were held for 10–30 min in crushed solid CO₂, they were transferred to liquid nitrogen and stored for 1 h to 3 days before thawing. The average cooling rate was ~30°C/min between –10 and –60°C. In some experiments, samples were seeded at –7°C with an ice crystal to induce ice formation. After 5 min, samples were placed into crushed solid CO₂ or cooled to –70°C at 0.5°C/min before transfer to liquid nitrogen. Samples were usually thawed rapidly but some were thawed slowly. Rapidly thawed samples were agitated in a 40°C water bath (~500°C/min). Slowly thawed samples were placed in 600 ml of –70°C ethanol in 1000 ml beaker (~0.7°C/min) or suspended in air at room temperature (~20°C/min). The rates of thawing were calculated between –70 and –10°C.

After thawing, the embryos were washed at 25–30°C in three changes of PBS (2 ml/wash) at 3-min intervals and cultured by the microdrop method in a modified Krebs–Ringer bicarbonate medium at 37°C for 48 h in 5% CO₂ in air (Whittingham, 1971b). Survival of the frozen–thawed 8-cell embryos was assessed by their ability to develop to blastocysts during culture. Experiments were replicated 3–6 times and data were analysed for statistical significance using χ^2 tests after confirmation that the results for each treatment were homogeneous. To examine the further development of 8-cell embryos frozen by the solid CO₂ procedure, embryos which had developed normally to blastocysts after being cultured for 40–48 h were transferred to the uterine horns (5–8 embryos/horn) of females on Day 4 of pseudopregnancy, induced by mating with a vasectomized male. The recipients were allowed to litter.

Results

Experiment 1. The effects of the number of times glycerol was added and ice-seeding on the survival of frozen–thawed mouse embryos were examined (Table 1). In this study, we did not examine at what temperature nucleation occurred when seeding was not induced. A higher survival rate was obtained when glycerol was added to embryos in two increments at 10-min intervals and seeding was omitted, and a significant difference ($P < 0.01$) was found only between single (not seeded) and stepwise addition (not seeded). This schedule was used for subsequent experiments.

Experiment 2. Glycerol concentration affected survival of embryos after solid CO₂ freezing before transfer to liquid nitrogen (Table 2). The survival of embryos after freezing and thawing was highest with 1.8 M-glycerol ($P < 0.05$); higher or lower concentrations of glycerol afforded less protection or were themselves deleterious. We used 1.8 M-glycerol for subsequent experiments.

Experiment 3. Embryos were kept in 1.8 M-glycerol at 0°C for various periods after the final addition of glycerol and then frozen to –196°C before rapid thawing (Table 2). Embryos exposed to glycerol at 0°C for 0.1 min appeared more sensitive to freezing than those exposed for longer times (5–30 min) ($P < 0.02$). The higher survival rates were found after exposure to glycerol for 5–30 min and prolonged exposure (60 min) appeared to be detrimental. The equilibration time to 1.8 M-glycerol at 0°C before freezing was 10 min for subsequent studies.

Experiment 4. Embryos were held in crushed solid CO₂ for various periods before transfer to liquid nitrogen and thawed rapidly (Table 2). The highest survival rates were obtained with a holding time of 10–30 min.

Experiment 5. The survival of mouse embryos after being frozen and thawed at several rates in the presence of glycerol is summarized in Table 3. When embryos were frozen by the solid CO₂ procedure before rapid cooling to liquid nitrogen, a relatively high survival (62%) was obtained

Table 1. Effects of the number of times glycerol is added and ice-seeding on the survival of 8-cell mouse embryos after solid CO₂ freezing before transfer to liquid nitrogen and rapid thawing

No. of additions of glycerol	Seeding	No. of trials	No. of embryos recovered	No. of embryos developing to blastocysts after 48 h in culture	% Survival
1*	Not seeded	4	63	18	29
	Seeded	4	68	30	44
2†	Not seeded	4	75	46	61
	Seeded	4	80	32	40

* Equilibration time of embryos to 1.8 M-glycerol at 0°C was 15 min.

† Glycerol was added to embryos at 10-min intervals and equilibration time was 10 min after the final addition of glycerol.

Table 2. Factors affecting the survival of 8-cell mouse embryos after solid CO₂ freezing before transfer to liquid nitrogen and rapid thawing

	No. of trials	No. of embryos recovered	No. of embryos developing to blastocysts after 48 h in culture	% Survival
Glycerol conc. (M)				
0.5	3	52	0	0
1.0	5	57	9	16
1.5	5	68	28	41
1.8	5	85	55	65
2.0	5	77	37	48
2.5	5	81	34	42
3.0	5	62	22	35
Equilibration time (min) at 0°C*				
0.1	6	97	33	34
5	6	79	45	57
10	6	85	53	62
30	6	83	44	53
60	6	82	18	22
Holding time (min) in CO ₂ *				
3	5	71	24	34
5	5	82	37	45
10	5	93	53	57
30	5	97	52	54
60	5	79	25	32
120	5	73	17	23

* Glycerol was added to embryos at 0°C in two increments of 0.075 ml at 10-min intervals to make a final concentration of 1.8 M.

with rapid thawing whereas no embryos survived slow thawing. By contrast, a 77% survival rate was obtained when embryos were frozen slowly and thawed rapidly. With slow cooling higher survival rates were obtained after rapid (77%) than slow (55–61%) thawing ($P < 0.02$).

Experiment 6. The survival rates for embryos stored for 1–3 h, 30–35 or 115–123 days at –196°C after freezing by the solid CO₂ procedure were 56 (35/63), 51 (36/70), and 50% (29/58), respectively.

Experiment 7. Of the 35 blastocysts transferred to 5 pseudopregnant recipients, 2 females littered 6 normal young at term from 15 blastocysts transferred.

Table 3. Effects of freezing and thawing rates on the survival of 8-cell mouse embryos frozen in the presence of glycerol

Freezing rates	Thawing rates (°C/min)	No. of trials	No. of embryos recovered	No. of embryos developing to blastocysts after 48 h in culture	% Survival
Rapid*	~0.7	3	63	0	0
	~20	4	68	0	0
	~500	5	95	59	62
Slow†	~0.7	5	86	47	55
	~20	5	97	59	61
	~500	5	105	81	77

* 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.

Discussion

Two-step freezing was first used for the low-temperature preservation of bull spermatozoa by Luyet & Keane (1955) and has since been applied to a number of types of cells, including embryos (Asahina, 1959; Rapatz & Luyet, 1973; Farrant, Knight, McGann & O'Brien, 1974; Farrant, Walter, Lee & McGann, 1977; Wood & Farrant, 1980; Kasai *et al.*, 1980, 1981). Such a method has been exploited successfully to elucidate interactions between freezing and thawing rates (Farrant *et al.*, 1977). Our two-step freezing procedure (rapid cooling to solid CO_2 followed by rapid cooling to liquid nitrogen) for preserving mouse embryos is simpler and quicker and eliminates the necessity of having elaborate and expensive freezing equipment.

The present results demonstrated that 8-cell mouse embryos frozen in glycerol by solid CO_2 procedure before transfer to liquid nitrogen can survive rapid but not slow thawing. These findings essentially agree with previous reports on the survival of mouse embryos frozen by a two-step method (Wood & Farrant, 1980; Kasai *et al.*, 1980, 1981) and are also consistent with those on the survival of rapidly thawed mouse (Whittingham *et al.*, 1979), sheep (Willadsen, 1977) and cattle (Willadsen *et al.*, 1978) embryos when slowly cooled in DMSO to relatively high subzero temperatures before rapid cooling to liquid nitrogen.

The major factor for cell damage at subzero temperature is generally the formation of intracellular ice during freezing and its growth and recrystallization during thawing (Mazur, 1966). If mouse embryos are cooled at $\sim 2^{\circ}\text{C}/\text{min}$ no intracellular ice forms, but at cooling rates exceeding $2^{\circ}\text{C}/\text{min}$ intracellular ice forms between -40 and -50°C (Leibo, 1977). The present results with solid CO_2 freezing gave good survival after rapid but not slow thawing and suggest that some intracellular ice is formed (Whittingham *et al.*, 1979; Wood & Farrant, 1980; Kasai *et al.*, 1980).

The survival rate of embryos frozen in glycerol by the two-step procedure described here was similar to that of embryos frozen and thawed slowly but lower than that of embryos frozen slowly and thawed rapidly (Table 3). The cooling and thawing rates required for optimal survival of cell types change with the concentration of the cryoprotectant, and also differ for different cryoprotectants. Greater survival of mouse embryos with solid CO_2 freezing might, therefore, be obtained by changes of the freezing and thawing procedures and cryoprotectants.

It was originally reported that the survival of mouse embryos frozen slowly with DMSO required slow rather than rapid thawing, although small percentages of embryos frozen slowly could survive rapid thawing (Whittingham *et al.*, 1972; Wilmut, 1972). In contrast to previous findings, it has now been shown that 8-cell mouse embryos frozen slowly with glycerol can survive both slow and rapid thawing and that a higher survival rate is obtained after rapid than slow thawing. Our results are consistent with the general belief that rapid thawing of frozen mammalian

cells is equal or superior to slow thawing (Meryman, 1966; Leibo, 1976). When mouse morulae were frozen slowly with glycerol and thawed in PBS containing glycerol + 0.5 M-sucrose, survival for slowly and rapidly thawed embryos was similar (Kasai *et al.*, 1981). The previous (Whittingham *et al.*, 1972; Wilmut, 1972; Kasai *et al.*, 1981) and present results suggest that there may be some differences in the protective effects of DMSO and glycerol against damage of mouse embryos during freezing and thawing.

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