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Survival of Mouse Embryos Frozen to −196° and −269°C

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"Frozen-thawed" mouse pups and the albino foster mother who gave birth to them. The pups developed from eight-cell embryos that had been surgically removed from brown genetic mothers, frozen to −196°C for 21 hours, thawed, and then transferred to the oviduct of the foster mother. See page 411. [P. Whittingham, S. Leibo, and P. Mazur, Oak Ridge National Laboratory]

Survival of Mouse Embryos Frozen to −196° and −269°C

Abstract. *Mouse embryos survived freezing to −196°C. Survival required slow cooling (0.3° to 2°C per minute) and slow warming (4° to 25°C per minute). Depending on the specific rates used, 50 to 70 percent of more than 2500 frozen and thawed early embryos developed into blastocysts in culture after storage at −196°C for up to 8 days. When approximately 1000 of the survivors, including some frozen to −269°C (4°K), were transferred into foster mothers, 65 percent of the recipients became pregnant. More than 40 percent of the embryos in these pregnant mice gave rise to normal, living full-term fetuses or newborn mice.*

Most attempts to preserve multicellular mammalian systems such as tissues and organs by freezing have failed, partly because the approaches have been empirical. However, an understanding of the mechanisms of freezing injury in single cells is emerging, and we applied that understanding to the freezing of one such multicellular system—early mouse embryos.

The ability to preserve viable embryos by freezing them to low temperatures would have applications in genetics and developmental biology and could facilitate the upgrading of domestic animals. Before 1971, attempts to freeze mammalian embryos below −20°C had, with rare exceptions, failed (*1*). Then it was reported that 55 to 65 percent of mouse eight-cell embryos and early blastocysts survived freezing to −79°C for 30 minutes if they were suspended in a 7.5 percent solution of polyvinylpyrrolidone in a modification of Dulbecco's phosphate-buffered salt solution (PBS medium) and cooled at 60°C min^{-1} (*2*). However, the embryos would not survive longer than 30 minutes at that temperature, and two-cell embryos did not survive at all.

Because of these limitations, we explored the cryobiological factors—chiefly suspending medium, cooling rate, final temperature, and warming rate—that might influence survival of preimplantation stages of mouse embryos (one-, two-, and eight-cell embryo, and blastocyst). The criteria of survival (*2*) were development to expanded blastocysts in culture and development to living mice in the uterus of a foster mother.

Embryos were obtained from randombred Swiss-Webster albino (specific pathogen-free, National Laboratories) or F_1 hybrid [(C57BL ♀) × (C3H/AN ♂)F_1 Cum] virgin female mice (6 to 12 weeks old) mated with F_1 hybrid males. (Parental source did not appear to affect survival after freezing and thawing.) The females were induced to superovulate by the intraperitoneal injection of 5 international units (I.U.) of serum gonadotropin from pregnant mares and 5 I.U. of human chorionic gonadotropin given approximately 48 hours apart, and were then mated individually. One-, two-, and eight-cell embryos and early blastocysts were recovered from the reproductive tracts at 22, 44 to 46, 66 to 70, and 90 to 96 hours, respectively, after the injection of human chorionic gonadotropin (*3*). Embryos of a given age were pooled and washed twice in 2 ml of PBS medium at room temperature (*2, 4*). Cumulus cells were removed from one-cell embryos by exposure to hyaluronidase (150 U.S. Pharmacopeia units per milliliter) in PBS medium for 3 to 5 minutes before washing.

Ten to 40 embryos in ~0.001 ml of medium were pipetted into tubes (10 by 100 mm) containing 0.1 ml of PBS medium. Except where noted, the tubes were cooled to 0°C, and 0.1 ml of 2*M* dimethyl sulfoxide (DMSO) or glycerol at 0°C was added. After 15 minutes the samples were transferred to a bath at −3.5° to −4.5°C, and seeded 2 minutes later with a minute ice crystal. After another 5 minutes, they were transferred to baths cooling at 0.3° to 40°C min^{-1} (*5*). Samples were cooled to −78°, −196°, or −269°C, kept at these temperatures for 1 minute to 192 hours, and thawed in four ways: (i) a 35°C water bath for 35 seconds (rate 450°C min^{-1}); (ii) an ice bath (rate 215°C min^{-1}); (iii) air at room temperature (rate 25°C min^{-1}); or (iv) 20 ml of ethanol in tubes (38 by 200 mm), the ethanol, initially at −110°C, warming by contact with room temperature air (rate 4°C min^{-1}). (Cooling and warming rates were measured between −65° and −10°C.)

Thawed samples were immediately diluted at 0°C by the addition of 0.2, 0.2, and 0.4 ml of medium at 45-second intervals. Each sample tube was emptied into an embryological watch glass containing 1 ml of medium and rinsed with 1 ml of medium. The embryos were washed by transfer through two changes of fresh medium at room temperature. They were then examined at × 50 magnification to determine the number recovered, the number exhibiting normal or abnormal morphology, and the number that had degenerated. All embryos except those that had totally degenerated were transferred to a modified Krebs-Ringer bicarbonate medium (*6*) and cultured by Brinster's method (*7*) in small droplets of culture medium under mineral oil at 37°C in a mixture of 5 percent CO_2 in air. Except for one-cell embryos (*8*), they were cultured until they developed into blastocysts or expanded blastocysts (24 to 90 hours, depending on the developmental stage at the time of freezing). The number of embryos recovered after thawing was 90.3 percent of the total frozen. Survival is defined as the percentage of recovered embryos that developed into blastocysts. Of the surviving embryos, 9.7 percent exhibited one or more damaged blastomeres after thawing, but were also capable of developing into fetuses (first footnote, Table 1) and into viable young (*9*).

Dimethyl sulfoxide, one of the more effective protective agents, received the chief emphasis (*9a*). The reported toxicity for embryos (*2, 4*) was nearly

eliminated by the stepwise dilution method, as shown by the survival of 84 ± 2 percent of unfrozen control embryos suspended in 1*M* DMSO and 0°C for up to 90 minutes and at 20°C for at least 18 minutes.

A major cause of freezing injury is intracellular ice formation, and to prevent it, the cooling must be slow enough to allow all freezable water to flow out of the cell during cooling (*10*). Thermodynamic and kinetic calculations (*11*) indicate that "slow enough" for cells the size of these embryos (70 μm diameter) is about 1°C min^{-1} or less. Hence, survival of eight-cell embryos suspended in 1*M* DMSO and frozen to −78°C was tested at cooling rates from 0.3° to 40°C min^{-1} and at four warming rates (Fig. 1). Although rapid thawing of animal cells is generally equal or superior to slow thawing (*12*), we found that slow thawing (25° or 4°C min^{-1}) which may prevent injurious osmotic events in these large multicellular systems, yielded far more survivors, provided that cooling was between 0.3° to 1.9°C min^{-1}. Cooling at 7° or 16°C min^{-1} was completely lethal even with lower thawing rates.

Storage of the eight-cell embryos in 1*M* DMSO at −78°C for 3 days did not decrease viability significantly, but to reduce uncertainty about long-term stability we examined the effects of cooling embryos to −196°C (Fig. 1). The dependence of survival on cooling rate was similar, but survivals were slightly lower than in samples cooled to −78°C. Cooling to −110°C before transfer to liquid nitrogen appeared somewhat better than direct transfer from −80° to −196°C (59 ± 3 percent compared to 51 ± 6 percent) (*13*). And warming at 4°C min^{-1} appeared slightly better than warming at 25°C min^{-1} (58 ± 4 percent compared to 54 ± 6 percent) (*13*). The maximum survivals of 70 to 80 percent are absolute values; relative to unfrozen DMSO controls the survivals are 83 to 95 percent.

Eight-cell embryos were also exposed to 1*M* DMSO at 0°C for various periods before freezing (100 embryos, single samples). Survivals after exposure for 2.4, 6.5, 16.7, 31.5, and 61.6 minutes and subsequent freezing at 1.8°C min^{-1} were 33, 65, 44, 62, and 75 percent, respectively. Survival of embryos cooled at 0.8°C min^{-1} after a 17-minute exposure depended somewhat on the concentration of DMSO (34 ± 10, 50 ± 4, 49 ± 7, and 36 ± 5 percent in 0.75, 1.0, 1.25, and 1.5*M* DMSO, respectively). A concentration of 1 to 1.25*M* appears best.

Glycerol (1*M*) was also able to protect eight-cell embryos from freezing damage. Survivals were about half of those obtained with 1*M* DMSO, but the dependence on cooling rate was similar. Viability after cooling at 1.2, 1.4, 1.9, and 5.5°C min^{-1} was 34 ± 9, 30 ± 1, 2 ± 2, and 0 percent, respectively. Embryos equilibrated with glycerol at 0°C survived freezing at 1.2° and 1.4°C min^{-1} as well as or better than embryos equilibrated at 20°C (38 ± 6 percent compared to 27 ± 2 percent, $P < .3$, 80 embryos frozen).

These latter results and possibly those for time in DMSO suggest that permeation of additive may not be required for protection. The amount of additive in cells at the time of freezing was not determined, but was probably small, especially for cells in glycerol at 0°C. Sherman (*14*) showed that unfertilized mouse eggs in hyperosmotic solutions of glycerol (5 to 35 percent) at 5°C remain shrunken indefinitely, and even at 37°C they require 8 to 10 minutes to regain their normal volume. Whittingham and Wales (*4*) observed that two-cell embryos at room temperature require 4 to 5 minutes and 1 to 2 hours to return to normal volume in hyperosmotic DMSO and glycerol, respectively. Protection without permeation would be consistent with results on mouse marrow stem cells (*5*), hamster tissue culture cells (*15*), and bovine red cells (*16*).

The results for one- and two-cell embryos frozen to −196°C at various rates and warmed at 5°C min^{-1} are summarized in Fig. 2. Again, survival (*8*) was highest (49 and 68 percent,

Table 1. In vivo development of frozen-thawed mouse embryos to full-term fetuses and live young. Embryos in 1*M* DMSO were frozen at 0.3° to 2°C min^{-1} to minimum temperatures (MT), held for various times, and thawed at 4° or 25°C min^{-1}. They were then transferred to pseudopregnant females, either immediately or after developing to blastocysts in culture. The number of embryos in pregnant recipients was used to calculate percentages of fetuses, resorptions, and live-born.

MT (°C)	Time at MT (hours)	Embryo stage: When frozen	Embryo stage: When transferred	Transferred (No.)	Recipients (No.)	Pregnancies (No.)	Embryos in pregnant recipients (No.)	Fetuses (No.)	Resorptions (No.)	Live-born (No.)	Live-born + fetuses (%)
− 78	0.2–2	2-cell	Blast	48	8	4	25	5	15		20
− 196	0.2–4	1-cell	1-cell	60	5	3	36	8	2		22
− 196	72	2-cell	2-cell	136	11	7	92	29	8		33
				26	2	2	26			10	
− 196	0.5–72	2-cell	Blast	122	20	12	73	26	29		38
				7	1	1	7			4	
− 196	21–78	8-cell	8-cell	62	5	2	24	9	0		38
				52	5	5	52			20	
− 196	0.5–95	8-cell	Blast*	347	52	35	245	131	61		54
				36	4	2	16			11	
− 269	0.2†	2-cell	2-cell	12	2	1	6	2	0		61
				12	2	2	12			9	
− 269	0.2‡	8-cell	Blast	7	1	1	7			3	43
Total§				927	118	77 (65%)	621 (67%)				43
Total, nonlittered recipients						64	501	210 (42%)	115 (23%)		
Total, littered recipients						13	120			57 (48%)	

* In addition, ten eight-cell embryos that had had one or more blastomeres damaged after freezing and had developed into blastocysts were transferred into one recipient. All ten implanted, and one yielded an 18-day living fetus. Also, 21 eight-cell embryos frozen in 1*M* glycerol developed into blastocysts and were transferred to four recipients, three of which became pregnant. Of the 15 embryos in these animals, 5 developed into fetuses and 9 implanted but were resorbed. † Plus 141 hours at −196°C. ‡ Plus 192 hours at −196°C. § If damaged embryos plus embryos frozen in glycerol are included, the total number of embryos transferred is 958, and the total number of fetuses plus live-born is 273.

respectively) at a cooling rate of 0.3°C min^{-1}, lower between 0.6 and 1.9°C min^{-1}, and close to zero at 6°C min^{-1}. When 190 early blastocysts were frozen at various rates after contact with 1*M* DMSO at 0°C for 15 minutes, survivals were low, but maximal (18 percent) at cooling rates of 0.6° and 1.3°C min^{-1} (Fig. 2). The blastocysts did not survive freezing after 15 minutes in 1*M* glycerol at 0°C, a fact which suggests that additive may have to pass through the cells and into the blastocoel to prevent injury of the cell surfaces that line the blastocoelic cavity.

The in vitro survival data (Figs. 1 and 2) support the view that for maximal survival embryos must be cooled slowly enough (< 2°C min^{-1}) to avoid extensive intracellular ice. This value is consistent with theoretical calculations (*10, 11*), and with the observation that cooling rates of this order produce internal ice in unfertilized mouse eggs (*1*).

The optimum cooling rate (0.3 to 0.4°C min^{-1}) is with one exception (*17*) the lowest reported for any animal cell. It demonstrates that these sensitive multicellular systems can withstand more than 3 hours of dehydration and contact with concentrated solutions of electrolytes as they cool to −70°C [DMSO solutions remain partly liquid down to this temperature (*18*)]. The high sensitivity of the embryos to rapid thawing has not been reported for animal cells; its cause is unknown. These results in a "simple" multicellular system may be significant in the freezing of more complex mammalian tissues and organs.

Survivals for one-, two-, and eight-cell embryos cooled at 0.3° to 1.9°C min^{-1} in DMSO did not appear to decline with time at −196°C. Survivals were 53 ± 4, 55 ± 6, 44 ± 6, and 55 ± 3 percent after 0.1 to 5, 20 to 28, 38 to 50, and 71 to 96 hours, respectively. Eight of 19 eight-cell embryos also survived after being cooled at 1.3°C min^{-1} to −196°C, held for 188 hours, cooled to −269°C in liquid helium and held for 15 minutes, returned to −196°C for 4 hours, and then thawed. Thus, there is no detectable decay after 8 days at −196°C.

Of more than 2800 frozen-thawed embryos, approximately 600 that had developed to blastocyst in culture were transferred into pseudopregnant females, and an additional 360 were transferred into animals immediately after thawing. The recipients were albino Swiss-Webster females that had been rendered pseudopregnant by natural mating with vasectomized albino Swiss-Webster males (proved sterile). Frozen-thawed embryos that had developed from the two- or eight-cell stage to blastocyst were pooled and transferred to the uterine horns on day 3 of pseudopregnancy (*19*). (Day 1 is the day on which the vaginal plug is found.) For immediate transfer, frozen-thawed embryos that appeared morphologically normal were pooled and introduced into the ampullar region of the oviduct on day 1 of pseudopregnancy (*9*). The in vitro data had shown that about 97 percent of morphological "normals" subsequently developed into blastocysts.

Most pregnant mice were examined on day 18 of pregnancy (occasionally days 14 to 17) to determine the number of embryos that had developed into nearly full-term live fetuses and the number that had implanted but had been subsequently resorbed; the remainder of the pregnant mice were allowed to deliver at term (Table 1). All fetuses and newborn mice had dark eyes, a genetic marker indicating that all had been derived from frozen-thawed embryos (*20*) and not from the mating with the vasectomized albino males. Furthermore, all the young mice developed agouti or black coats.

Sixty-five percent of the recipients became pregnant, a value in the normal range for virgin females. In these pregnant animals 43 percent of the frozen-

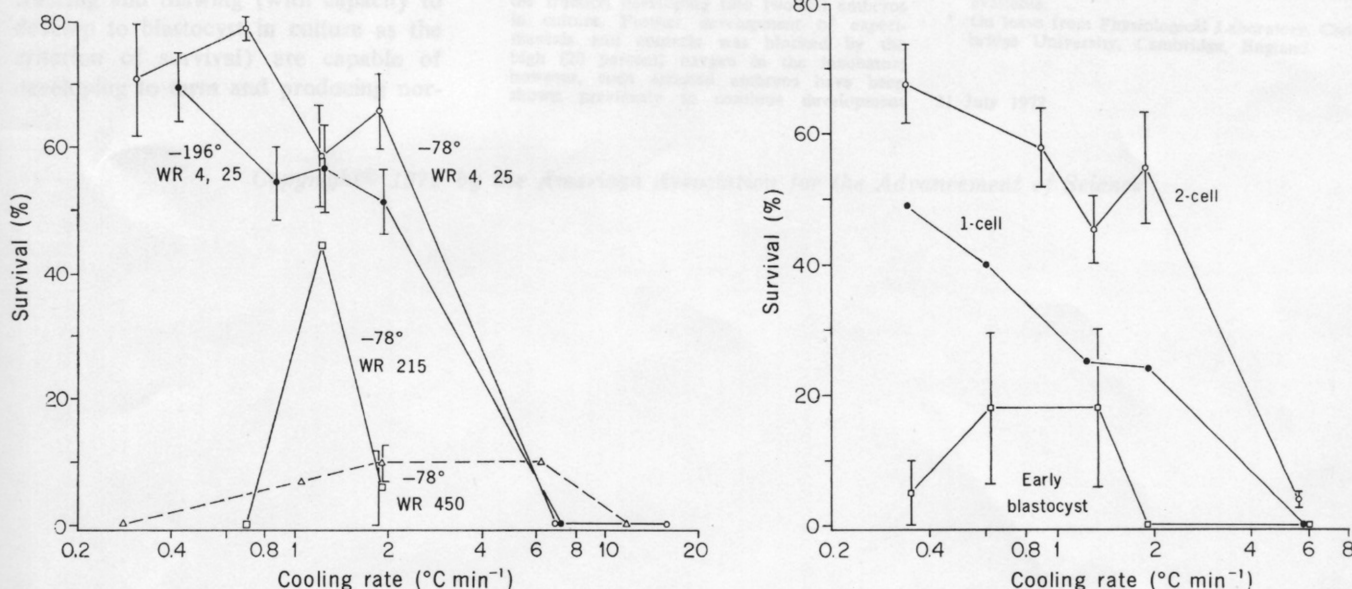

Fig. 1 (left). In vitro survival of eight-cell mouse embryos in 1*M* DMSO as a function of cooling rate, final temperature, and warming rate. The numbers on the curves refer to final temperatures and warming rates (*WR*) in degrees Celsius per minute. Embryos cooled to −196°C were cooled at the indicated rates to −80° or −110°C before transfer to liquid nitrogen. The numbers of replicate samples and total embryos frozen (given in parentheses) for each curve, reading from the lowest cooling rate, were 2 (30), 4 (50), 2 (20), 5 (70), 1 (30), and 2 (30) (○); 7 (108), 7 (118), 9 (128), 12 (188), and 3 (60) (●); 1 (10), 1 (10), and 2 (30) (□); and 2 (30), 1 (30), 3 (60), 1 (30), and 2 (30) (△). Fig. 2 (right). In vitro survival of various developmental stages of mouse embryos as a function of cooling rate. Embryos were suspended in 1*M* DMSO, cooled to −110°C at the indicated rates, transferred to liquid nitrogen, and warmed at 4°C min^{-1}. The numbers of replicate samples and total embryos frozen (in parentheses) for each curve, reading from the lowest cooling rate, were 4 (70), 4 (70), 3 (50), 4 (70), and 6 (104) (○); 1 (40) for each rate (●); and 3 (40) for each rate (□).

thawed embryos transferred developed into either dark-eyed living fetuses or newborn mice that appeared entirely normal. None appeared abnormal. In the 13 animals allowed to litter, 48 percent of 120 transferred embryos developed into black or agouti pups. (These mice have yielded normal litters, both when mated with each other and with a normal control mouse). In the 64 females examined before term, 42 percent of the transferred embryos had developed into fetuses and 23 percent more had implanted but had been resorbed. Thus 65 percent of the 501 embryos in pregnant females underwent implantation. Fetuses and live-born mice developed normally from embryos cooled to −78°, −196°, or −269°C, regardless of whether transfer was immediately after thawing or after development to blastocysts. Since 43 percent of the embryos in pregnant females underwent complete development and since the frozen thawed embryos had been pooled before transfer, it is statistically unlikely that any animal failed to become pregnant because the six to ten embryos it received were all nonviable. Rather, the nonpregnancy was probably a physiological failure of the foster mother.

The results in Table 1 compare favorably with those obtained for the transfer of normal, unfrozen embryos into mice (*21*). Thus, embryos that survive freezing and thawing (with capacity to develop to blastocyst in culture as the criterion of survival) are capable of developing to term and producing normal, living young. Our results imply (*2*) that storage of mutant strains of mice not in current use but of potential interest is now possible. As needed, the stored embryos could be thawed and transferred to foster mothers, and the resulting offspring used to reestablish the mutant strain. If these procedures prove applicable to embryos of large domestic animals, they would also facilitate worldwide dissemination of stock with an optimal genetic background for a particular use or geography. Finally, the success of cryobiological theory in suggesting the proper approach to the freezing of these sensitive embryos increases the likelihood that ways can be found to freeze complex mammalian systems for medical use.

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