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

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Abstract. Mouse embryos have for the first time survived freezing to -196°C. Survival required slow cooling (0.3 to 2°C/min) and slow warming (4 to 25°C/min). Depending on the specific rates used, 50 to 70 percent of over 2500 frozen-thawed early embryos developed into blastocysts in culture after storage at -196°C for up to 8 days. When ~ 1000 of the survivors, including some frozen to -269°C (4°K), were transferred into foster mothers, 65 percent of the recipients became pregnant. Over 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 should provide a rational basis for renewed efforts. The present report deals with the application of cryobiological theory 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. Prior to 1971, attempts to freeze mammalian embryos below -20°C had, with rare exceptions, failed (1). Then in 1971 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 (PVP) in a modified Dulbecco's phosphate-buffered salt solution (PBS medium) and cooled at 60°C/min (2). The embryos were apparently in an unstable state, however, for they would not survive longer than 30 minutes at that temperature. Two-cell embryos did not survive this procedure.

Because of the temporal sensitivity at -79°C of eight-cell embryos in PVP and because of the sensitivity of two-cell embryos to freezing in general, we decided to explore the cryobiological factors that might influence survival (chiefly suspending medium, cooling rate, final temperature, and warming rate) of the various preimplantation stages of mouse embryos (one-, two-, and eight-cell, and blastocyst). The criteria of survival were those used previously (2) — ability to develop to expanded blastocysts in culture and ability to develop into living mice in the uterus of a foster mother.

Embryos were obtained from random-bred Swiss-Webster albino (S.P.F., National Labs.) or F_1 hybrid [(C57BL ♀) X (C3H/AN ♂)F_1 Cum] virgin female mice (6 to 12 weeks old) mated with F_1 hybrid males. (A few direct comparisons did not suggest any effect of parental source

on the percentage of embryos surviving freezing and thawing.) The females were induced to superovulate by the intraperitoneal injection of 5 I.U. of pregnant mare's serum gonadotropin and 5 I.U. of human chorionic gonadotropin (HCG) 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 HCG by procedures described elsewhere (3). Embryos of a given stage 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.P. units/ml) in PBS medium for 3 to 5 minutes prior to washing.

A known number of embryos (10 to 40, depending on the total number available) in ~0.001 ml of medium were then pipetted into tubes (10 by 100 mm) containing 0.1 ml of PBS medium. The usual procedure was to cool the tubes to 0°C and add 0.1 ml of 2M dimethyl sulfoxide (DMSO) or glycerol at 0°C. Fifteen minutes later the samples were transferred to a bath at -3.5 to -4.5°C, held 2 minutes, and then seeded with a minute ice crystal. They were held an additional 5 minutes before being transferred to baths cooling at rates of 0.3 to 40°C/min. The baths and cooling procedures were based on those described elsewhere (5). Samples were cooled to -78, -196, or -269°C for 1 minute to 192 hours and thawed in one of four ways: (i) immersed in a water bath at 35°C for 35 seconds (rate 450°C/min); (ii) immersed in an ice bath (rate 215°C/min); (iii) held in air at room temperature until thawed (rate 25°C/min); or (iv) immersed in 20 ml of ethanol at -110°C in tubes (38 by 200 mm), the ethanol then being allowed to warm by contact with room temperature air (rate 4°C/min). (The cooling and warming rates were measured between -65 and -10°C.)

Immediately upon completion of thawing, the samples were diluted at 0°C by the addition of 0.2, 0.2, and 0.4 ml of PBS at 45-second intervals. The contents of each sample tube were

emptied into an embryological watchglass containing 1 ml of PBS medium, and the tubes were rinsed with an additional 1 ml of medium to ensure maximum recovery of embryos. After collection, the embryos were washed by transfer through two changes of fresh PBS medium at room temperature. They were then examined at 50 X 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 then 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). Percentage survival was defined as 100 X (the number developing to blastocyst/the total number recovered after thawing). The total number recovered was 90.3 percent of the total frozen; the remainder presumably were lost by adhesion to glass walls, etc. Of the embryos that developed to blastocyst, 9.7 percent exhibited one or more damaged blastomeres after thawing. These also were scored as survivors, for they are capable of developing into fetuses (see footnote to Table 1) and into viable young (9).

DMSO is one of the more effective protective agents known, and so it received the chief emphasis. It was previously found to be toxic to embryos above 0°C, especially when removed rapidly (2, 4). We found, however, that when dilution was carried out stepwise in the manner described, 84 ± 2 percent of unfrozen control embryos survived contact with 1 to 1.5 M DMSO at 0°C for up to 90 minutes and contact at 20°C for at least 18 minutes.

Previous experimental and theoretical work has shown that a major cause of freezing injury is intracellular ice formation, and that to prevent internal ice formation cells must be cooled slowly enough to allow all freezable water to flow out of the cell during cooling (10).

Thermodynamic and kinetic calculations (11) indicate that to avoid intracellular ice cells similar in size to these embryos (70 μm diameter) must be cooled at about 1°C/min or less. Hence, in the initial experiments eight-cell embryos were suspended in 1M DMSO and frozen to -78°C at rates ranging from 0.3 to 40°C/min. Rapid warming (450°C/min) was used because we and others have nearly always found rapid thawing of animal cells to be equal or superior to slow thawing (12). The results (bottom curve of Fig. 1) were low survivals (7, 10, and 10 percent, respectively) at cooling rates of 1.0, 1,9, and 6.2°C/min, and no survival at lower or higher rates. Suspicious that injurious osmotic events might be occurring during the rapid thawing of these large multicellular systems, we decreased the warming rate to 215°C/min. The result (Fig. 1) was 44 percent survival in one sample cooled at 1.2°C/min but only 0 and 6 percent at cooling rates of 0.7 and 1.9°C/min. When the warming velocity was further lowered to 25 or 4°C/min, however, freezing injury was greatly reduced, provided that cooling was between 0.3 to 1.9°C/min (top curve of Fig. 1). Cooling at 7 or 16°C/min continued to be completely lethal.

Storage of the eight-cell embryos in 1M DMSO at -78°C for up to 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). Viability was maximal at a cooling rate of 0.4°C/min, dropped slightly at cooling rates up to 1.9°C/min, and was abolished at a rate of 7°C/min. Survivals appear to be somewhat lower than in samples cooled only to 78°C. Samples cooled to -110°C before transfer to liquid nitrogen showed slightly higher mean survivals than did samples transferred directly from -80 to -196°C (59 ± 3 vs. 51 ± 6 percent) (13). Samples warmed at 4°C/min from -196°C showed slightly higher mean survivals than those warmed at 25°C/min (58 ± 4 vs. 54 ± 6 percent) (13). But neither difference is significant at the 95 percent confidence level ($P > 0.2$ and 0.6,

respectively). Note that the maximum survivals of 70 to 80 percent are absolute values; relative to unfrozen DMSO controls the survivals are 83 to 95 percent.

In one experiment involving 100 embryos and single samples, eight-cell embryos were exposed to 1M DMSO at 0°C for various lengths of time before freezing. Survivals after exposure for 2.4, 6.5, 16.7, 31.5, and 61.6 minutes and subsequent freezing at 1.8°C/min were 33, 65, 44, 62, and 75 percent, respectively. Survival of embryos cooled at 0.8°C/min 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 1.5M DMSO, respectively). A concentration of 1 to 1.25M appears best.

Glycerol (1M) was also able to protect eight-cell embryos from freezing damage. Although survivals were about half of those obtained with 1M DMSO, the dependence on cooling rate was similar. Viability after cooling at 1.2, 1.4, 1.9, and 5.5°C/min 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 as well as or better than embryos equilibrated at 20°C (38 ± 6 vs. 27 ± 2 percent, $P < 0.3$, 80 embryos frozen).

The results of this experiment and possibly those of the experiment on time in DMSO suggest that permeation of additive may not be required for protection. We have no direct estimates of the amount of additive in the cells at the time of freezing; however, it was probably not much, especially in the case of cells in glycerol at 0°C. Sherman (14) has shown 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) have observed that two-cell embryos require 4 to 5 minutes and 1 to 2 hours to return to normal volume in hyperosmotic DMSO and glycerol,

respectively, at room temperature. Protection without permeation would be consistent with recent findings on hamster tissue culture cells (15), mouse marrow stem cells (5), 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 are summarized in Fig. 2. Again, survival (8) is maximum at a cooling rate of 0.3°C/min, somewhat lower at rates between 0.6 and 1.9°C/min, and 0 or close to 0 percent, at 6°C/min. Figure 2 also gives results for the survival of 190 early blastocysts frozen at various rates after contact with 1M DMSO at 0°C for 15 minutes. Survivals are low, but they are maximal (18 percent) at cooling rates of 0.6 and 1.3°C/min. The blastocysts did not survive freezing after 15 minutes in 1M 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 in Figs. 1 and 2 support the view that for maximal survival embryos must be cooled slowly enough to avoid extensive intracellular ice. "Slowly enough" appears to be < 2°C/min, a value that is consistent with theoretical calculations (10, 11). Although we do not have direct evidence that embryos cooled at 6°C/min or more freeze internally, Sherman (1) has evidence that cooling rates of this order produce internal ice in unfertilized mouse eggs.

The optimum cooling rate (0.3 to 0.4°C/min) is with one exception (17) the lowest reported for any animal cell. It demonstrates that these sensitive multicellular systems can withstand over 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)]. Especially interesting is the high sensitivity of the embryos to rapid thawing. This has heretofore not been reported for animal cells. Its cause is unknown. These findings of

insensitivity to very slow cooling and high sensitivity to rapid thawing in a "simple" multicellular system may be significant in the freezing of more complex mammalian tissues and organs.

Although we made no specific study of stability as a function of time in the frozen state, embryos in various experiments were held at -196°C for as long as 96 hours. Pooled mean survivals for one-, two-, and eight-cell embryos cooled at 0.3 to 1.9°C/min in DMSO and held at -196°C for 0.1 to 5, 20 to 28, 38 to 50, and 71 to 96 hours were 53 ± 4, 55 ± 6, 44 ± 6, and 55 ± 3 percent, respectively. In addition, two groups of eight-cell embryos were cooled at 1.3°C/min to -196°C, held for 188 hours, and then cooled to -269°C in liquid helium and held for 15 minutes. After they had been returned to liquid nitrogen for 4 hours and then thawed, eight of 19 survived. Thus, there is no detectable decay after 8 days at -196°C.

The preceding data were based on the in vitro culture of over 2500 frozen-thawed embryos. Approximately 600 of the embryos that had developed to blastocyst in culture were also transferred into pseudopregnant females to determine their ability to develop in vivo. As a further test of viability after freezing and thawing, an additional 360 embryos were transferred into animals immediately after thawing. Thus a total of over 950 frozen-thawed embryos were transferred to recipients.

The recipients were albino Swiss-Webster females that had been rendered pseudopregnant by natural mating with vasectomized albino Swiss-Webster males proven 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.) In the case of frozen-thawed embryos to be transferred immediately after thawing, those appearing morphologically normal in a given experiment

were pooled and introduced into the ampullar region of the oviduct on day 1 of pseudo-pregnancy (9). The in vitro data had shown that, on the average, 97 percent of morphological "normals" subsequently developed into blastocysts.

The majority of pregnant mice were examined on day 18 of pregnancy (occasionally days 14 to 17), in order 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 shows the numbers of embryos transferred and the numbers of pregnant females, implantation sites, fetuses, and live young born. 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 albino recipients became pregnant, a value that is in the normal range, allowing for possible loss of embryos during transfer and for the fact that the females were virgins. In these pregnant animals 43 percent of the frozen-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, which will subsequently be weaned and tested for fertility. In the case of the 64 females examined on day 18 or earlier, 42 percent of the transferred embryos had developed into fetuses and 23 percent more had implanted but had been subsequently resorbed. Thus 65 percent of the 501 embryos transferred to females that became pregnant underwent implantation. Fetuses and live-born mice developed normally from embryos cooled to all three minimum temperatures of exposure (-78, -196, and -269°C), regardless of whether the embryos had been transferred immediately

Table 1. In vivo development of frozen-thawed mouse embryos to full-term fetuses and live young. Embryos in 1M DMSO were frozen at 0.3 to 2°C/min, held for various times, and thawed at 4 or 25°C/min. They were then transferred to pseudopregnant females, either immediately or after developing to blastocysts in culture.

Min temp (°C)	Time at min temp (hr)	Embryo stage: When frozen	Embryo stage: When transferred	No. transferred	No. recipients	No. pregnancies	No. embryos in pregnant recipients	Fetuses No.	Fetuses (%)*	Resorbtions No.	Resorbtions (%)*	Liveborn No.	Liveborn (%)*	Liveborn + fetuses (%)*
-78	0.2-2	2-cell	blast	48	8	4	25	5	(20)	15	(60)	-	-	20
-196	0.2-4	1-cell	1-cell	60	5	3	36	8	(22)	2	(6)	-	-	22
-196	72	2-cell	2-cell	136	11	7	92	29	(32)	8	(9)	-	-	33
				26	2	2	26	-	-	-	-	10	(38)	
-196	0.5-72	2-cell	blast	122	20	12	73	26	(36)	29	(40)	-	-	38
				7	1	1	7	-	-	-	-	4	(57)	
-196	21-78	8-cell	8-cell	62	5	2	24	9	(38)	0	0	-	-	38
				52	5	5	52	-	-	-	-	20	(38)	
-196	0.5-95	8-cell	blast†	347	52	35	245	131	(53)	61	(25)	-	-	54
				36	4	2	16	-	-	-	-	11	(69)	
-269	0.2‡	2-cell	2-cell	12	2	1	6	2	(33)	0	0	-	-	61
				12	2	2	12	-	-	-	-	9	(75)	
-269	0.2§	8-cell	blast	7	1	1	7	-	-	-	-	3	(43)	43
	Total‖			927	118	77 (65%)	621 (67%)	-	-	-	-	-	-	-
	Total for nonlittered recipients			-	-	64	501	210	(42)	115	(23)	-	-	43.0
	Total for littered recipients			-	-	13	120	-	-	-	-	57	(48)	

Footnotes to Table 1

*100 X (number of fetuses, resorptions, or liveborn/number of embryos transferred into females that subsequently became pregnant). †In addition, 10 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 1M glycerol developed into blastocysts and were transferred to four recipients, three of which became pregnant. Of the 15 embryos in these animals, five developed into fetuses and nine implanted but were resorbed. ‡Plus 141 hr at -196°C. §Plus 192 hr at -196°C. ‖The total number of embryos transferred was 958, and the total number of fetuses plus liveborn was 273.

after thawing or after having developed into blastocysts. Since 43 percent of the embryos transferred into females that subsequently became pregnant 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 it can be concluded that embryos that survive freezing and thawing (with ability to develop to blastocyst in culture as the criterion of survival) are capable of developing to term and producing normal, living young. As mentioned elsewhere (2), one implication of our findings is that the storage of mutant strains of mice not in current use but of potential interest is now possible. When desirable, the stored embryos could be transferred to foster mothers, and the resulting offspring could be used to reestablish the mutant strain. A second implication is that if these procedures prove applicable to embryos of large domestic animals, they would facilitate world-wide dissemination of stock with an optimal genetic background for a given use or a given geography. Finally, and more generally, 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 that are of direct medical interest.

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22. We thank Dr. Hugh Long, Thermonuclear Division, ORNL, for making helium facilities available.

FIGURE LEGENDS

Fig. 1. In vitro survival of eight-cell mouse embryos in 1M 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). 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 numbers of embryos frozen at each rate, respectively, reading from the lowest to the highest rate, were 2,30; 4,50; 2,20; 5,70; 1,30; 2,30 (○); 7,108; 7,118; 9,128; 12,188; 3,60 (●); 1,10; 1,10; 2,30 (□); and 2,30; 1,30; 3,60; 1,30; 2,30 (△- - - △).

Fig. 2. In vitro survival of various developmental stages of mouse embryos as a function of cooling rate. Embryos were suspended in 1M DMSO, cooled to -110°C at the indicated rates, transferred to liquid nitrogen, and warmed at 4°C/min. The numbers of replicate samples and the numbers of embryos frozen at each cooling rate, respectively, reading from the lowest to the highest rate, were 4,70; 4,70; 3,50; 4,70; 6,104 (○); 1,40 for each rate (●); and 3,40 for each rate (□).

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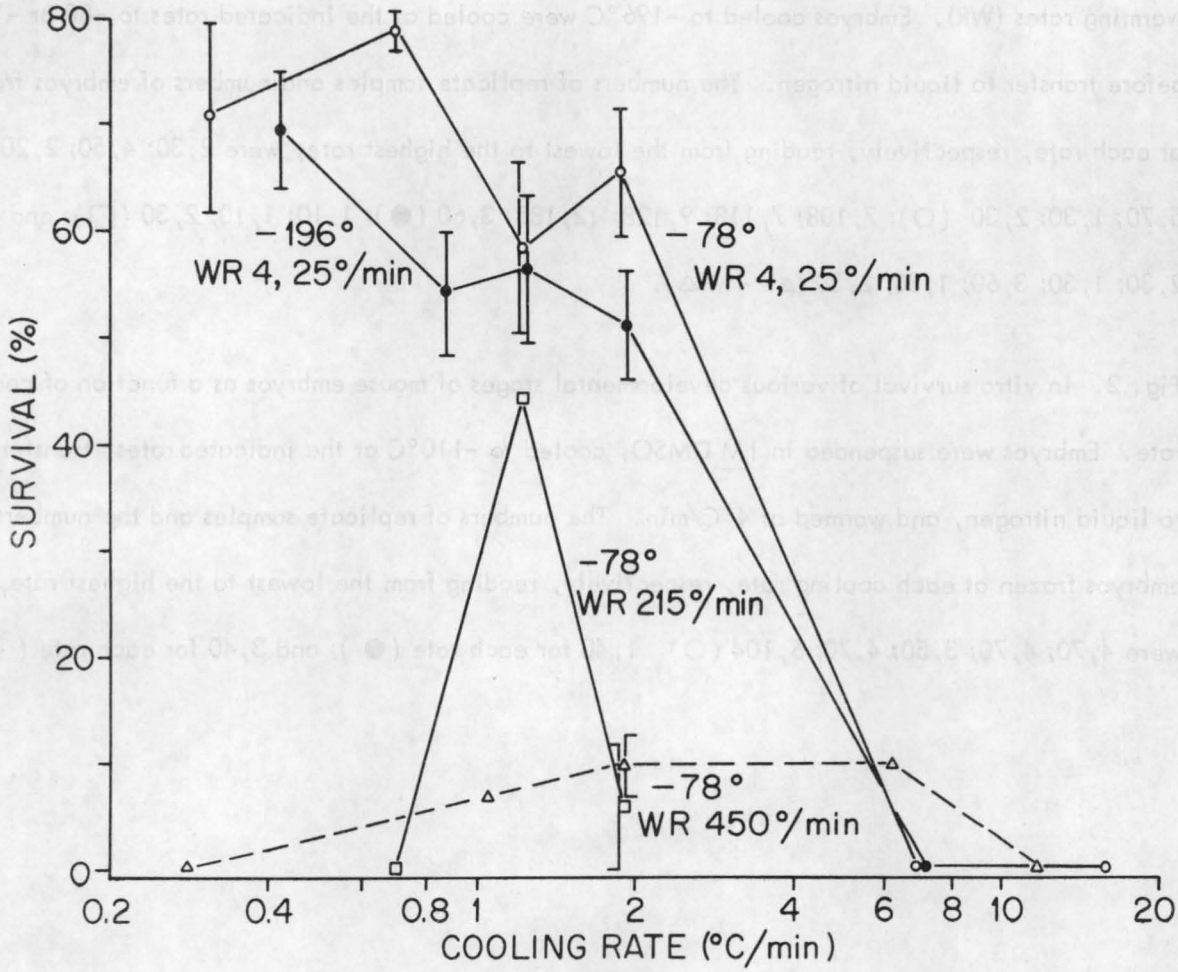

Figure 1

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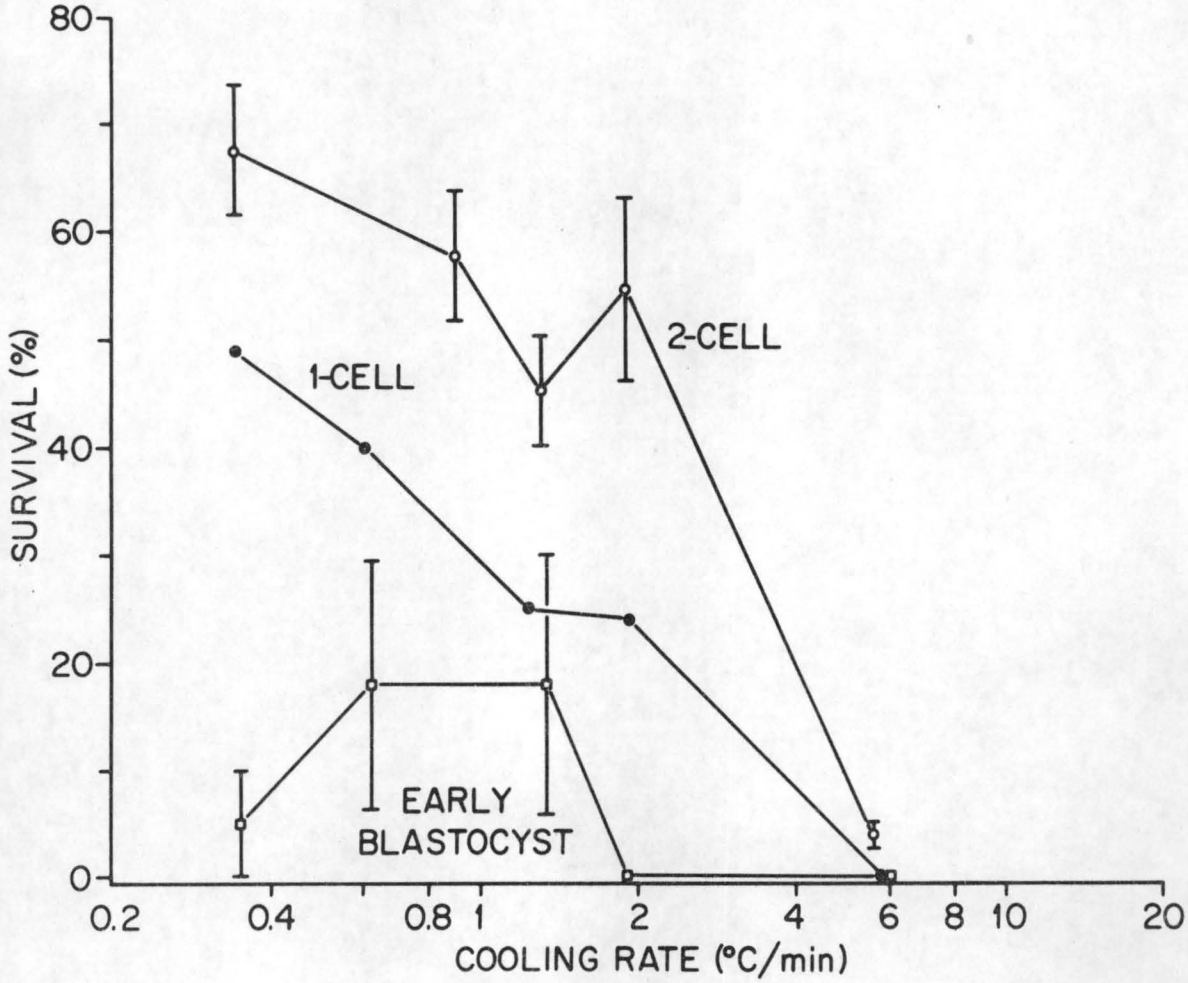

Figure 2