Stability of mouse oocytes at -80°C : the role of the recrystallization of intracellular ice

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Abstract

The germplasm of mutant mice is stored as frozen oocytes/embryos in many facilities worldwide. Their transport to and from such facilities should be easy and inexpensive with dry ice at -79°C . The purpose of our study was to determine the stability of mouse oocytes with time at that temperature. The metaphase II oocytes were cryopreserved with a vitrification solution (EAFS10/10) developed by M Kasai and colleagues. Two procedures were followed. In one, the samples were cooled at $187^{\circ}\text{C}/\text{min}$ to -196°C , warmed to -80°C , held at -80°C for 1 h to 3 months, and warmed to 25°C at one of three rates. With the highest warming rate ($2950^{\circ}\text{C}/\text{min}$), survival remained at 75% for the first month, but then slowly declined to 40% over the next 2 months. With the slowest warming ($139^{\circ}\text{C}/\text{min}$), survival was only $\sim 5\%$ even at 0 time at -80°C . In the second procedure, the samples were cooled at $294^{\circ}\text{C}/\text{min}$ to -80°C (without cooling to -196°C) and held for up to 3 months before warming at $2950^{\circ}\text{C}/\text{min}$. Survival was $\sim 90\%$ after 7 days and dropped slowly to 35% after 3 months. We believe that small non-lethal quantities of intracellular ice formed during the cooling and that the intracellular crystals increased to a damaging size by recrystallization during the 3 month's storage at -80°C . From the practical point of view, this protocol yields sufficient stability to make it feasible to ship oocytes worldwide in dry ice.

Reproduction (2011) **141** 407–415

Introduction

A major cause of death in cells subjected to freezing and warming is the formation of intracellular ice (IIF). Or put differently, to survive freezing, a cell must be cryopreserved in ways that avoid or minimize it. If during freezing the cells contain freezable water after being cooled below their nucleation temperature, they will undergo IIF either by homogeneous nucleation or by heterogeneous nucleation. Homogeneous nucleation occurs when small numbers of liquid water molecules randomly arrange themselves in an ice-like structure of sufficient size to be stable. In objects the size of mouse oocytes it occurs at about -40°C . In some cases, however, the cells undergo IIF at much higher temperatures. In such cases, the ice nucleation is catalyzed by specific substances and is referred to as heterogeneous nucleation. An excellent heterogeneous nucleator of supercooled intracellular water is extracellular ice. We have argued that, in some cases, supercooled intracellular water may be nucleated by external ice passing through aquaporin channels in the plasma membrane (Seki & Mazur 2010). We and others have given further evidence that ice can pass from one cell to the supercooled contents of an adjoining cell through the pores in the gap junctions between the two cells (Acker *et al.* 2001, Irimia & Karlsson 2002, Seki & Mazur 2010).

One way to avoid lethal IIF is to cool cells slowly enough to eliminate any supercooling below the nucleation temperature. An alternative way is to load the cells with high concentrations of so-called glass-inducing solutes and cool them rapidly enough so that their interior cannot freeze even though the cells become supercooled. The supercooled water in such cells becomes extremely viscous, and below the glass transformation temperature (T_g), it converts or vitrifies into a non-crystalline solid. While the vitreous state can exist for centuries or millennia at sufficiently low temperatures, it is nevertheless thermodynamically unstable and, when warmed, it can convert to crystalline ice, the thermodynamically stable form. The conversion can take two forms referred to as devitrification and recrystallization. Devitrification is the conversion above T_g from the vitreous state to the crystalline state. Recrystallization is the conversion of small ice crystals into larger ones. It occurs because this process is thermodynamically favorable. Devitrification occurs a few degrees above T_g . Recrystallization is a kinetic process that occurs with increasing rapidity as the temperature rises from ~ -80 to $\sim -40^{\circ}\text{C}$. We have determined that the recrystallization rate in mouse oocytes in 1.5–2 M ethylene glycol (EG) increases four- to fivefold for each five-degree rise in temperature

(Seki & Mazur 2008a). This corresponds to an activation energy of 26 kcal/mol.

The transition from a supercooled viscous liquid to a glass at T_g is accompanied by sizeable and abrupt changes in the heat capacity of the sample (Wowk 2010), changes that can be readily detected by differential scanning calorimetry. In EG–water solutions, T_g varies from -134 to -131 °C as the EG concentration varies from 35 to 60 wt. % (Hayes & Pegg 1996). Generally, cryopreserved oocytes and embryos are frozen to and maintained at -196 °C (liquid nitrogen) or ~ -140 °C (liquid nitrogen vapor). These temperatures are well below the T_g of the solutions in which they are frozen and in all probability below the T_g of the cytoplasm. Consequently, cellular and external unfrozen water will vitrify during cooling and remain vitrified during storage; and it will devitrify and probably recrystallize during warming.

In some cases, however, users may freeze embryos and oocytes to and maintain them at -80 °C, the temperature of dry ice and the temperature of most ultra-low mechanical freezers. Even more common may be the situation where for ease and cost reasons, one desires to ship embryos/oocytes at -80 °C in dry ice regardless of whether they have been initially processed and stored at ≤ -140 °C or at -80 °C. Oocytes/embryos frozen to and maintained at -80 °C and no lower almost certainly have not vitrified. Oocytes/embryos frozen to ≤ -140 °C probably have vitrified and probably devitrify during warming to and storage at -80 °C.

The studies we are reporting consisted of two groups. In one group, MII oocytes were suspended in a vitrification solution (EAFS10/10), cooled rather rapidly to -196 °C, and then warmed to and held at -80 °C for 0–3 months. The oocytes in the other group were cooled only to -80 °C and then held at -80 °C for 0–3 months. The purpose of our study was twofold. The first purpose was the mechanistic one of determining whether survival at low temperatures was affected by whether or not the cells and medium had vitrified, and, if death occurred during low-temperature storage, what was its cause? The second purpose was the practical one of whether the oocytes are stable enough at -80 °C to permit their being shipped worldwide at that temperature.

Two programs, the North American Conditional Mouse Mutagenesis project (NorCOMM) and the European Conditional Mouse Mutagenesis Program (EUComm), have been established recently to coordinate the generation of tens of thousands of mouse mutations, the storage of such mutants as cryopreserved embryos/oocytes/sperm, and the distribution of them to interested parties. The Federation of international mouse resources (Davisson 2006) is the operating arm of these programs. It is an affiliation of 17 major resource centers from four continents (Asia, Australia, Europe, and North America). With this increased emphasis on dissemination, the transportation of frozen oocytes/embryos in dry

nitrogen shippers will be expensive and cumbersome. Transportation in dry ice would be much more economical even with the added 'hazardous substance' charge, if the cells survive the shipping times.

Results

Relation between cooling rate and the vitrification or freezing of EAFS10/10 cooled to -196 °C

Dr Kasai, Dr Edashige, and colleagues have developed an effective procedure for cryopreserving mouse oocytes in their EAFS10/10 vitrification solution. As described in Materials and Methods, the procedure yields a cooling rate of about 187 °C/min to -196 °C.

Figure 1A shows that, with that cooling rate, a change in slope of the temperature–time curve occurs at -67 °C (Table 1). A marked change in slope also occurs in samples cooled at 37 °C/min. These are a consequence of ice formation in the EAFS10/10 solution. In contrast, no change in slope is observed in EAFS10/10 samples cooled at 522 °C/min, or 1827 °C/min to -196 °C (Seki & Mazur 2009) indicating that the EAFS10/10 vitrifies at these higher cooling rates. These conclusions are also confirmed by the differential scanning calorimeter (DSC) thermograms in Fig. 2A and B, and the data in Table 1. With cooling rates of 37 and 187 °C/min, exotherms began to be generated at -45 and -75 °C. At a cooling rate of 500 °C/min, no DSC exotherms were visible indicating that the medium vitrified (data not shown).

Figure 1 Cooling kinetics of EAFS10/10 vitrification solutions in 0.25 ml straws with five procedures. Each cooling procedure was repeated five times. (A) Samples cooled with LN_2 ; (B) samples cooled in a deep freezer at -80 °C. Details of the procedures are given in Table 4. The arrows point to the regions where a change in slope indicates ice formation. The precise values are given in Table 1. A is modified, with permission from Elsevier, from Fig. 1 of Seki S & Mazur P 2009 The dominance of warming rate over cooling rate in the survival of mouse oocytes subjected to a vitrification procedure. *Cryobiology* 59 75–82. © 2009 Elsevier Inc.

Table 1 Temperature of ice formation in EAFS as a function of cooling rate to -196°C .

Method	Cooled to -196°C at			Cooled to -80°C or $-150^{\circ}\text{C}^{\text{a}}$ at	
	37 $^{\circ}\text{C}/\text{min}$	187 $^{\circ}\text{C}/\text{min}$	500 $^{\circ}\text{C}/\text{min}$	29 $^{\circ}\text{C}/\text{min}$	294 $^{\circ}\text{C}/\text{min}$
Kinetic	-40°C	-67°C	Not detected	-43°C	-68°C
DSC	-45°C	-75°C	Not detected	-39°C	-97°C

^aIn the kinetic time/temperature measurements (Fig. 1), the samples were cooled to -80°C . With DSC measurements, the samples were cooled to -150°C , because there is a sizeable lag between the real temperature of samples and the temperature read on the DSC monitor.

Extracellular ice formation in EAFS10/10 as a function of the cooling rate to -80°C

Figures 1B and 2C and D show comparable results for EAFS10/10 samples cooled to a final temperature of -80°C at a rate of 29 $^{\circ}\text{C}/\text{min}$ (placement of straws into the chilled air of the deep freezer) and 294 $^{\circ}\text{C}/\text{min}$ (direct immersion of straws into an ethanol bath pre-chilled to -80°C in the deep freezer). Pronounced changes in the time–temperature slopes occurred at -43 and -68°C respectively (Table 1). And in DSC (Fig. 2C and D), exotherms due to freezing of the medium began to be generated at -39 and -97°C . For a cooling rate of 29 $^{\circ}\text{C}/\text{min}$, the onset of freezing occurred at -43 and -39°C with the thermocouple and DSC approaches respectively. With a cooling rate of 294 $^{\circ}\text{C}/\text{min}$, it occurred at -68 and -97°C respectively. These time–temperature curves and DSC exotherms were carried out with EAFS10/10 alone, and therefore give no information on whether the oocytes themselves vitrified or underwent IIF. Even if oocytes had been presented in the medium, their response would have yielded no information on this point. Their contribution to the heat of fusion of freezing would be far too small to be detectable. There are reasons to believe, however, that oocytes and other cells can vitrify under conditions where bulk samples of the medium freeze.

Survival of oocytes after cooling in EAFS10/10 at 187 $^{\circ}\text{C}/\text{min}$ to -196°C , holding for up to 3 months at -80°C followed by warming to 25°C at various rates

We decided to restrict our survival measurements to oocytes that had been cooled at 187 $^{\circ}\text{C}/\text{min}$ to -196°C , followed by warming to and holding at -80°C for various times before warming at each of three rates to 25°C . There were three reasons for the restriction. One was that much of the prior work in Drs Kasai and Edashige's laboratories and in our laboratory had used that cooling rate. A second reason was that the highest rate attainable with straws cooled to -80°C (294 $^{\circ}\text{C}/\text{min}$) was rather close to the 187 $^{\circ}\text{C}/\text{min}$ to -196°C and, like that rate, it also resulted in freezing of the medium. The third reason is that the use of a rather

high cooling rate may minimize injury from chilling. Metaphase II oocytes possess a meiotic spindle composed of the protein tubulin. Tubulin undergoes depolymerization in 2–4 min at around 0°C (Magistrini & Szöllösi 1980, Zenzes *et al.* 2001), and the spindle becomes disorganized and can disappear. Rapid cooling may prevent that process from occurring. Figure 3 and Table 2 show the survival of oocytes frozen to -196°C with liquid nitrogen (LN_2) at a cooling rate of 187 $^{\circ}\text{C}/\text{min}$, held at -80°C for 0–3 months (0–93 days), and warmed and thawed at rates ranging from 139 to 2950 $^{\circ}\text{C}/\text{min}$. The figure addresses three questions. First, does survival decline with time at -80°C ? Secondly, does the rate of warming from -80°C affect survival or the rate of decline in survival with time? And thirdly, is survival after direct warming from -196°C any different from two-step warming involving warming from -196 to -80°C followed immediately by rapid warming from -80°C ?

With respect to the first point, survival decreased with time at -80°C for all three warming rates, but the negative slope was significantly different from zero only in the case of samples warmed at 2950 $^{\circ}\text{C}/\text{min}$. With that warming rate, survival dropped from 85% immediately after exposure to -80°C to 42% after 3 month's storage. With respect to the second point, the rate of warming had a major effect on survival at all storage times. The average survival over the 3 month's storage was $\sim 60\%$ for those warmed at 2950 $^{\circ}\text{C}/\text{min}$ and $\sim 10\%$ for those warmed at 139 $^{\circ}\text{C}/\text{min}$. The survivals after warming at the intermediate rate of 476 $^{\circ}\text{C}/\text{min}$ were intermediate.

Figure 2 DSC thermograms of a drop of EAFS10/10 cooled at 37 (A), 187 (B), 29 (C), and 294 (D) $^{\circ}\text{C}/\text{min}$. The term 'corrected temperature' on the abscissa refers to the fact that to obtain the true temperature of the sample, the indicated temperature had to be corrected for thermal lags in the instrument. The procedures for doing so are described in Kleinhans *et al.* (2006). A and B are modified, with permission from Elsevier, from Fig. 2A of Seki S & Mazur P 2009 The dominance of warming rate over cooling rate in the survival of mouse oocytes subjected to a vitrification procedure. *Cryobiology* 59 75–82. © 2009 Elsevier Inc.

Figure 3 The survival of oocytes initially cooled in EAFS10/10 to -196°C at $187^{\circ}\text{C}/\text{min}$, warmed to -80°C , and held at -80°C for 1 h, 7, 14, 21, 31 (1 month), 62 (2 months), and 93 (3 months) days, at which points the oocytes were warmed and thawed at $139^{\circ}\text{C}/\text{min}$ (closed triangles), $476^{\circ}\text{C}/\text{min}$ (closed squares), and $2950^{\circ}\text{C}/\text{min}$ (closed circles). As a control, samples were also warmed directly from -196 to 25°C (without holding at -80°C), at $139^{\circ}\text{C}/\text{min}$ (open triangle), $476^{\circ}\text{C}/\text{min}$ (open square), and $2950^{\circ}\text{C}/\text{min}$ (open circle). The survival curve is based on 18–54 oocytes for each time point (Table 2). The mean slopes of the survival versus time curves for 139, 476, and $2950^{\circ}\text{C}/\text{min}$ were -0.2143 ± 0.1310 , -0.0675 ± 0.0638 , and -0.3884 ± 0.0915 respectively. Only the last was significantly different from zero.

With respect to the third point, there was little or no difference in the survival of oocytes warmed and thawed immediately after freezing to -196°C (open symbols) and the survivals of those warmed in two quick steps from -196 to -80°C and from -80 to $+25^{\circ}\text{C}$ (closed symbols at zero time).

Survival of oocytes after cooling in EAFS10/10 at $294^{\circ}\text{C}/\text{min}$ to -80°C , holding time for up to 3 months at -80°C followed by warming to 25°C at $2950^{\circ}\text{C}/\text{min}$

The data just presented in Fig. 3 and Table 2 were the survivals of oocytes frozen to -196°C and then warmed to and held at -80°C for up to 3 months. Figure 4 and Table 3 show similar data except the initial freezing to -196°C was omitted; i.e. the oocytes were frozen to -80°C at $294^{\circ}\text{C}/\text{min}$ by immersing the straws in ethanol pre-chilled to -80°C in a deep freezer, held for 0–3 months (0–93 days), and thawed at $2950^{\circ}\text{C}/\text{min}$. The effect of storage time on survival was very similar to that observed in Fig. 3 for samples warmed at that rate. Survival dropped from 90% for embryos held 0–7 days at -80°C to 33% after 3 months.

As indicated in Materials and Methods, a viable oocyte was considered to be one that shrank in hypertonic sucrose/isotonic PB1, reexpanded in PB1-lacking sucrose, and maintained normal morphology after 2 h incubation in M16 medium. Of the non-survivors stored 1–3 months at -80°C , 11–19% shrank

in the PB1 that contained hypertonic sucrose but did not regain normal volume when transferred to PB1-lacking sucrose.

Discussion

The experimental results in this paper can be summarized succinctly. When ICR mouse oocytes are suspended in the vitrification solution EAFS10/10, cooled at $187^{\circ}\text{C}/\text{min}$ to -196°C , warmed to -80°C , stored at -80°C for 0–3 months, and rapidly warmed and thawed at $2950^{\circ}\text{C}/\text{min}$, survivals are 85% with zero storage and drop progressively to 40% after 3 month's storage (Fig. 3). If the warming rate is decreased, survivals are much lower at all storage times. With a warming rate of $139^{\circ}\text{C}/\text{min}$, they lie between 0 and 20%. Time-temperature curves and DSC show that with this cooling rate of $187^{\circ}\text{C}/\text{min}$, ice forms in the EAFS10/10/medium itself.

In experiments that were parallel except that the initial cooling to -196°C was omitted, the results were very similar (Fig. 4). When the oocytes were warmed rapidly, survival was about 90% over the first 7 days of storage at -80°C and dropped smoothly to 33% over the ensuing 86 days. Here too, the external EAFS10/10 froze during the initial cooling.

We would like to divide this discussion into two parts: the mechanistic and the applied.

Table 2 Survival of mouse oocytes frozen to -196°C in EAFS10/10 solution, warmed to -80°C , held at -80°C for various times, and then warmed to 25°C at the indicated rates.

Time kept at -80°C	Warming rate ($^{\circ}\text{C}/\text{min}$)	No. of oocytes used	No. of oocytes recovered (%)	No. of oocytes survived (% $\pm$ S.E.M.)
0 min	2950	42	40 (95.2)	33 (79.1 $\pm$ 9.7)
	476	18	16 (88.9)	8 (44.4 $\pm$ 11.1)
	139	18	17 (94.4)	2 (11.1 $\pm$ 11.1)
60 min	2950	30	28 (93.3)	25 (83.3 $\pm$ 6.1)
	476	30	29 (96.7)	7 (33.3 $\pm$ 10.0)
	139	30	30 (100.0)	2 (6.7 $\pm$ 6.7)
7 days	2950	36	35 (97.2)	25 (71.4 $\pm$ 8.7)
	476	30	30 (100.0)	11 (36.7 $\pm$ 16.2)
	139	24	21 (87.5)	1 (4.2 $\pm$ 4.2)
14 days	2950	30	28 (93.3)	20 (66.7 $\pm$ 13.9)
	476	30	30 (100.0)	17 (56.7 $\pm$ 8.5)
	139	24	24 (100.0)	2 (8.3 $\pm$ 4.8)
21 days	2950	30	29 (96.7)	16 (53.3 $\pm$ 19.3)*
	476	30	27 (90.0)	12 (40.0 $\pm$ 10.0)
	139	24	22 (91.7)	0 (0.0 $\pm$ 0.0)
1 month (31 days)	2950	30	28 (93.3)	22 (73.3 $\pm$ 11.3)
	476	30	28 (93.3)	14 (46.7 $\pm$ 13.3)
	139	30	28 (93.3)	5 (16.7 $\pm$ 7.5)
2 months (62 days)	2950	54	43 (79.6)	29 (53.7 $\pm$ 12.4)*
	476	42	41 (97.6)	19 (45.2 $\pm$ 13.0)
	139	42	40 (95.2)	2 (4.8 $\pm$ 3.1)
3 months (93 days)	2950	30	25 (83.3)	12 (40.0 $\pm$ 17.2)*
	476	30	30 (100.0)	4 (13.3 $\pm$ 6.2) *
	139	30	27 (90.0)	0 (0.0 $\pm$ 0.0)

Survival was assessed by morphological/osmotic observations after thawing. Values with an asterisk are significantly different from those for oocytes not held at -80°C ($P < 0.05$).

Figure 4 The survival of oocytes in EAFS10/10 after cooling to -80°C at $294^{\circ}\text{C}/\text{min}$ and holding at that temperatures for 1 h to 93 days before warming and thawing them at $2950^{\circ}\text{C}/\text{min}$. The survival curve is based on 23–48 oocytes for each time point (Table 3). The mean slope of the survival curve is -0.5620 ± 0.1157 . This is significantly different from 0 ($P=0.02$).

Mechanistic aspects

When the oocytes are equilibrated in EAFS10/10, their water content drops to 28% of isotonic because of the osmotic effects of the hypertonic non-permeating sucrose (Seki & Mazur 2008a). The subsequent cooling rates of $294^{\circ}\text{C}/\text{min}$ or $187^{\circ}\text{C}/\text{min}$ to -80°C or -196°C are far too high to allow the loss of further cell water. Consequently, the oocytes arrive at -80°C with a total intracellular concentration of the permeating solutes EG and acetamide of 6.5 molal (Seki & Mazur 2008a, Table 4). If all this were EG, it would translate to ~ 30 wt. %. According to Hayes & Pegg (1996), the T_g for 35 wt.% EG in water is -134°C . This would mean that supercooled water in oocytes cooled in EAFS10/10 to -196°C would vitrify at about -134°C , and supercooled water in oocytes cooled no lower than -80°C would not vitrify but would remain in a viscous supercooled state. We have no direct information on whether these oocytes are supercooled or frozen upon initially cooling to -80°C , but indirect information indicates that they are not frozen, at least initially. First, if they had undergone extensive intracellular freezing, they would have almost certainly been dead upon thawing. Secondly, after exposure to EAFS10/10 for 2 min, the water content of the oocytes will be reduced below the equilibrium value of 28% and will likely be close to the 20% water content that prevents IIF in oocytes cooled in 1 or 1.5 M EG (Mazur *et al.* 2007). Thirdly, the cooling rate used here is about ten times higher than that used in Mazur *et al.* (2007), and this rapid cooling and the high viscosity of the cell solutes would further suppress the likelihood of IIF.

We know from our thermal measurements that the EAFS10/10 medium does freeze when cooled at 200 – $300^{\circ}\text{C}/\text{min}$. But that fact is not inconsistent with

lack of intracellular freezing. Cells contain high concentrations of macromolecules that may well enhance supercooling and vitrification.

The next question is what is responsible for the slow loss in survival with time at -80°C ? It would not appear to be devitrification. Devitrification is usually (and we think erroneously) defined as the conversion of vitreous water or solution to crystalline ice that occurs when the temperature rises above T_g . Vitrification cannot have occurred in oocytes cooled no lower than -80°C since T_g lies some 54°C lower. If they did not vitrify, they could not have devitrified. Yet the kinetics of death at -80°C was almost identical to that of oocytes that had been cooled initially to -196°C and therefore must have vitrified during that cooling and devitrified during the subsequent warming to -80°C .

We believe that the source of the damage during storage is the slow crystallization or recrystallization of small intracellular ice crystals formed during the initial cooling. Ice nuclei form in the supercooled cell water during cooling. But because the cooling rate and intracellular solute concentrations are high, the intracellular viscosity rises to very high values with the rapid drop in temperature, and the growth of these ice nuclei is greatly impeded. If the cells are cooled below T_g , that growth is completely stopped. If the cells are then warmed to and held at -80°C , or if the cells are cooled no lower than -80°C in the first place, the growth of the intracellular ice nuclei slowly resumes or continues.

There are two types of growth. One is the growth of the ice nuclei into the viscous supercooled matrix causing it to crystallize. The other is the growth of larger ice crystals at the expense of smaller ones because the former are thermodynamically favored. This latter form of growth is referred to as recrystallization. We have observed recrystallization and its kinetics in mouse oocytes frozen in 1.5 and 2.0 M EG/PBS (Seki & Mazur 2008a, 2008b). It is manifested as a blackening of the oocytes during warming or during holding at intermediate temperatures during the warm. It is initially observable at -80°C , and its rate increases about fivefold for each five-degree rise in temperature. This corresponds to an activation energy of 26 kcal/mol. The

Table 3 Survival of mouse oocytes frozen and kept at -80°C in EAFS10/10 solution for various times followed by immersion in water at 25°C .

Time kept at -80°C	No. of oocytes used	No. of oocytes recovered	No. of oocytes survived (% $\pm$ S.E.M.)
60 min	48	48 (100.0)	36 (75.0 $\pm$ 8.9)
7 days	24	23 (95.3)	22 (91.7 $\pm$ 4.8)
1 month (31 days)	48	45 (93.8)	30 (62.5 $\pm$ 10.8)
2 months (62 days)	48	45 (93.8)	22 (45.8 $\pm$ 12.5)*
3 months (93 days)	66	48 (72.7)*	22 (33.3 $\pm$ 12.1)*

Survival was assessed by the morphological/osmotic observation after thawing. *Values with an asterisk are significantly different from those for oocytes kept at -80°C for 60 min ($P<0.05$).

Table 4 Protocol of each cooling procedure.

Protocol	1/4 ml straw was covered with	Cooled by	Cooling rate $\pm$ S.E.M. ($^{\circ}$ C/min)
1	1/2 ml straw + double glass ^a	LN ₂ vapor ^b	37 $\pm$ 0 ^c
2 ^d	None	LN ₂ vapor ^b	187 $\pm$ 6 ^c
3	1/2 ml straw ^e	LN ₂	522 $\pm$ 54 ^c
4	None	Air at -80° C	29 $\pm$ 1 ^f
5	None	Ethanol at -80° C	294 $\pm$ 10 ^f

^a1/4 ml sample straw was covered with a 1/2 ml straw, a 7 mm OD $\times$ 90 mm glass tube, and a 10 mm OD $\times$ 90 mm glass tube. ^bThe straw was placed horizontally on a Styrofoam disk (14 cm diameter and 1.5 cm thick) floated on the surface of LN₂ in Dewar flask for > 5 min before being immersed in LN₂. ^cThe cooling rates were determined from 20 to -120° C. *N* = 5. ^dThis procedure is the standard procedure used in our laboratory, and Kasai's and Edashige's laboratory. ^e1/4 ml sample straw was covered with a 1/2 ml straw. ^fThe cooling rates were determined from 20 to -70° C. *N* = 5. The procedures in protocols 1, 2, and 3 were developed by Seki & Mazur (2009).

blackening becomes evident at a lower temperature when warming is slower simply because slower warming translates to more time. In Fig. 3, we see that slow warming is much more damaging than rapid warming even in the absence of storage at -80° C. This we find consistent with ascribing the storage damage to the recrystallization of intracellular ice.

One matter of divergence is that here (Figs 3 and 4), some 50% of the oocytes are viable after 2 months at -80° C, whereas we reported previously (Fig. 6 in Seki & Mazur 2008a) that oocytes frozen in 2.0 M EG/PBS blacken almost completely after 36 h at -80° C. Complete blackening is lethal. A problem in resolving the discrepancy is that we cannot visually observe the oocytes frozen in EAFS10/10 (the medium is too opaque) and we cannot determine what degree of blackening corresponds to what degree of survival, although we believe that even small amounts are lethal. It could be, however, that intracellular recrystallization proceeds more slowly for cells in EAFS than for cells in EG alone.

Applied aspects

Our data indicate clearly that long-term storage at -80° C of oocytes vitrified in EAFS10/10 is not feasible. Although survival drops by 60% in 3 months, we pointed out in the introduction that practical advantages might accrue to the ability to ship-frozen oocytes packed in dry ice at -79° C. Based on our data, that would be an acceptable procedure since survivals remain high with storage at that temperature for far longer than a week. There are several caveats, however. First, we have based survivals on morphological/osmotic criteria – not on functional criteria – such as the ability to be fertilized and develop *in vitro*. Secondly, we have demonstrated that the warming rate is critical. The oocytes must be warmed rapidly to achieve high survivals. Thirdly, the storage or shipping temperature could be critical,

although we have no direct information on this point. We do know that the rate of recrystallization can rise fivefold with a five-degree rise in temperature. That means that if the temperature of the samples inadvertently rose to -70° C, the killing rate might increase 25-fold. Although warming rate is clearly very important, the cooling rate is much less so (Seki & Mazur 2009). One effect of cooling rate, however, is that it influences whether or not the suspending medium freezes. The EAFS10/10 solution used here did freeze with the cooling rates used, but we have definitive evidence in a slightly different context that oocyte survival in EAFS10/10 is not affected by whether or not the medium freezes (Seki & Mazur 2009). We have no information on the criticality of the exact composition of the vitrification solution, but we doubt that small differences are important.

We have not determined the developmental ability of oocytes after cooling to -196° C and holding at -80° C. Dr M Kasai, however, has preliminary unpublished developmental data on oocytes in EAFS10/10 cooled to and warmed directly from -196° C at a cooling rate and warming rate similar to the 187 $^{\circ}$ C/min and 2950 $^{\circ}$ C/min used here. He found that the percentages of oocytes that cleaved to the two-cell stage after fertilization was as high as in unfrozen controls provided that the zonae were removed (M Kasai, personal communication). Presumably, this will also be true of oocytes held at -80° C for a number of days. However, we cannot ensure whether these oocytes can be fertilized and develop to term. Therefore, the developmental ability needs to be confirmed before -80° C storage is used in practice.

Finally, there is the question of whether stability at -80° C is affected by the developmental stage of the oocytes/embryo. The work reported here was all with oocytes. Jin *et al.* (2008) have carried out a somewhat similar study with mouse morulae vitrified in media containing three or four times the concentration of EG but no acetamide. Based on both an osmotic assay and a functional assay, survival of the morulae drops appreciably in <24 h of holding at -80° C. But if the concentration of sucrose in the vitrification solution was increased threefold, a high percentage survived being held at -80° C for 4–10 days after initial cooling to -196° C (Jin *et al.* 2010).

Materials and Methods

Animals

ICR mice were purchased from Harlan Sprague–Dawley, Inc., Indianapolis, IN, USA. The procedures for obtaining and manipulating the mouse oocytes were carried out under the University of Tennessee Institutional Animal Care and Use Committee protocol 911-0607, approved 28 June 2007.

Collection of oocytes

Mature female ICR mice (8–12 weeks of age) were induced to superovulate with i.p. injections of 5 IU of equine chorionic gonadotropin (Sigma) and 5 IU of human chorionic gonadotropin (hCG) (Sigma) given 48 h apart. Ovulated unfertilized oocytes were collected from the ampullar portion of the oviducts at 13 h after hCG injection and were freed from cumulus cells by suspending them in modified PBS (PB1) containing 0.5 mg/ml hyaluronidase followed by washing with fresh PB1 medium.

Composition of freezing solutions

The freezing solution (EAFS10/10) was developed by [Pedro et al. \(1997\)](#) for cryopreservation of mouse oocytes at metaphase II stage. It consists of 10% (v/v) EG and 10.7% (w/v) acetamide dissolved in a stock consisting of 30% (w/v) Ficoll 70 and 0.5 M sucrose in PB1 medium. The final concentrations of sucrose and Ficoll are 0.4 M and 24% (w/v) respectively. The letters E, A, F, and S stand for EG, acetamide, Ficoll, and sucrose. The 10/10 refers to the weight % of EG and acetamide. Its mass composition was given in a previous report ([Seki & Mazur 2008a](#)); namely, 3.23 molal EG and 3.27 molal acetamide as the highly permeating cryoprotectants ([Pedro et al. 2005](#)), and 0.150 molal salts (as NaCl) and 0.720 molal sucrose as impermeable solutes, plus 20.68 wt.% of Ficoll and 0.166 wt.% of BSA.

Preparing oocytes for experiments

With the oocytes and solutions maintained at 25°C , the following solutions were successively aspirated into 0.25 ml straws (IMV Technologies, L'Aigle, France): a 60 mm column of PB1 medium containing 0.5 M sucrose, a 20 mm column of air, a 5 mm column of EAFS10/10, a 5 mm column of air, and a 12 mm column of EAFS10/10 to contain the oocytes. The oocytes were transferred to successive drops of EAFS10/10 at 25°C , and three to six oocytes were transferred by micropipette into the 12 mm column of EAFS10/10 in each straw. The end of the straw opposite the polyvinyl alcohol plug was then heat sealed. Two minutes after first introducing the oocytes into EAFS10/10, freezing was initiated using the following procedures.

Achieving various cooling rates to -196°C

The procedures for achieving the various cooling and warming rates used in this study and the measured rates are summarized in [Tables 4 and 5](#).

Cooling procedures 1–3 involved cooling in LN_2 vapor or in LN_2 . In procedures 1 and 2, naked 0.25 ml straws or straws, surrounded by 0.5 ml straws and two concentric glass tubes (7 mm OD $\times$ 90 mm and 10 mm OD $\times$ 90 mm), were placed horizontally on a 1.5 cm thick Styrofoam disk floating on the surface of LN_2 in a Dewar flask. Six or more minutes later, the straws and tube assembly were immersed in LN_2 . In procedure 3, 0.25 ml straws covered by 0.5 ml straws were immersed directly into LN_2 . After more than 24 h at -140 or -196°C ,

the straws were removed from the covering straw and glass tubes and quickly transferred to an empty Dewar that had been pre-chilled to -80°C in the mechanical freezer (American Scientific -80°C Cryo-fridge). We confirmed using a calibrated thermocouple that the deep freezer maintained -80°C over the storage period. They were pulled out of the Dewar periodically over the ensuing 3 months, and warmed as indicated in the second section below.

Achieving various cooling rates to -80°C

Cooling procedures 4 and 5 involved directly cooling the sample straws in the deep freezer at -80°C . The 0.25 ml straws were inserted into an empty Dewar pre-chilled to -80°C (procedure 4), or into a Dewar containing ethanol pre-cooled to -80°C (procedure 5).

Achieving various warming rates from -80°C

The experimental plan was to store the samples at -80°C for various periods over the ensuing 3 months, and then warm the straws and oocytes to room temperature using the three rates shown in [Table 5](#): namely, 139, 476, and $2950^{\circ}\text{C}/\text{min}$. The procedure to achieve the rate of $2950^{\circ}\text{C}/\text{min}$ is the standard method developed and used by Dr Kasai and Dr Edashige's laboratories, as well as by us. In warming procedure 1, the 0.25 ml straws were removed from a Dewar flask at -80°C and steadily fanned in 23°C air until the contents warmed above 0°C . In procedure 2, the 0.25 ml straw was inserted into an IMV 0.5 ml straw that had been pre-cooled to -80°C in the deep freezer, and the assembly was then immersed in 25°C water with stirring. In procedure 3, the 0.25 ml straw removed from a Dewar flask at -80°C and immersed in a 25°C water bath. The warming rates in [Table 5](#) differ somewhat from those previously reported because the starting temperature here was -80°C rather than the -196°C used previously ([Seki & Mazur 2008a, 2008b](#)).

Measurement of the cooling rates and warming rates, and thermal events during cooling

To determine the cooling and warming rates with each procedure, a 36-gauge copper–constantan thermocouple was inserted into a standard IMV 0.25 ml straw containing a 50 mm column of EAFS10/10 solution. Concentric outer tubes were applied as required for the needed insulation. The thermocouple was attached to a Fluke (2190A) Digital

Table 5 Protocol of each warming procedure after holding at -80°C .

Protocol	1/4 ml straw was covered with	Warmed with	With stirring ^b	Warming rate ^a $\pm$ S.E.M. ($^{\circ}\text{C}/\text{min}$)
1	None	Air at 25°C	—	139 ± 7
2	Straw ^c	Water at 25°C	+	476 ± 18
3	None	Water at 25°C	+	2950 ± 119

^aThe warming rates were calculated between -70 and -35°C . $N=4-5$.

^b+, yes; —, no. ^cThe 1/4 ml sample straws were inserted into pre-cooled (-80°C) 1/2 ml straws before being plunged into water at 25°C .

Thermometer (Seattle, WA, USA). It outputs about three temperature readings per second (the exact output rate was determined by stopwatch for each run). The outputs were fed into a PC computer with Windows XP using the Hyperterminal program provided, and transferred to the computer hard drive. Changes in temperature versus time were recorded from +25 to about −180 °C, but the cooling rates listed in the right-hand column of Table 4 were based on the time taken to traverse the range of +20 to −120 °C. Plots of the resulting temperatures and times permitted calculations of the cooling rates. At many of the cooling rates, there were rather abrupt changes in slope that were evidence of freezing of the EAFS10/10 solution.

To confirm that these changes in slope were in fact due to ice formation, cooling protocols 1, 2, 4, and 5 were replicated in our DSC. The set-up and procedures for DSC have been described previously (Kleinhans *et al.* 2006, Seki & Mazur 2009, Seki *et al.* 2009). The thermal curves in Fig. 1 were generated by measuring and plotting the temperatures during cooling as a function of time. Ice formation releases heat and produces a change in slope. The thermal curves in Fig. 2 were generated by the DSC. Basically, they show the amount of heat that has to be added to the reference sample to keep its temperature the same as that of the experimental sample. This generates an exotherm when ice forms in the latter. There is no correspondence in the sizes of the exotherms in the two figures.

Post-thaw manipulations and determination of post-thaw viability

After being frozen, held at −80 °C, and thawed at each of the three warming rates, the contents of each straw were expelled into a watch glass by flushing the straw with 0.8 ml of a solution of 0.5 M sucrose in PB1. The oocytes were then pipetted into fresh 0.5 M sucrose/PB1 solution. Approximately, 10 min later, they were transferred to fresh PB1 medium lacking sucrose, and then transferred to and cultured in modified M16 medium (Pedro *et al.* 1997) for 2 h (for simplicity, this will henceforth be referred to as M16).

Viability was assessed at three time points based on osmotic responsiveness and morphological normality. First, the oocytes were examined during the 10 min in 0.5 M sucrose/PB1. Membrane-intact oocytes were expected to shrink with time because the sucrose is hypertonic. Secondly and thirdly, they were examined immediately after being placed in M16 medium and after 2 h incubation. They fall into two binary groups after the incubation. One group consists of degenerate oocytes that are clearly non-viable. The others are indistinguishable from fresh oocytes, and we know from past experiments that the plasma membranes in this latter group are intact and function normally with respect to their osmotic response to hypertonic and hypotonic media, and with respect to their ability to remain supercooled in the presence of external ice. The ability to manifest these characteristics after 2 h in M16 is considered a rather stringent test of viability. Still other criteria of normality are given in Mazur *et al.* (2005, pp. 48–49). They mostly deal with measures of the integrity of the plasma membrane.

Statistical analysis

Error figures in tables and error bars in graphs are S.E.M. (S.D. of the mean). Tests of significance were carried out using χ^2 tests. Statistics for the mean slopes were calculated by Instat v. 3. 1 (Graph Pad Software, San Diego, CA, USA).

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

This research was supported by National Institutes of Health grant R01-RR 18470 (PI is P Mazur).

Acknowledgements

We appreciate that Dr M Kasai provided important data on the developmental ability of cryopreserved mouse oocytes by personal communication.

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Received 22 October 2010

First decision 14 December 2010

Revised manuscript received 4 January 2011

Accepted 14 January 2011