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Cryobiology 55 (2007) 158–166

CRYOBIOLOGY

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Intracellular ice formation in mouse oocytes subjected to interrupted rapid cooling ☆

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Received 8 May 2007; accepted 23 June 2007
Available online 4 July 2007

Abstract

The formation of ice crystals within cells (IIF) is lethal. The classical approach to avoiding it is to cool cells slowly enough so that nearly all their supercooled freezable water leaves the cell osmotically before they have cooled to a temperature that permits IIF. An alternative approach is to cool the cell rapidly to just above its ice nucleation temperature, and hold it there long enough to permit dehydration. Then, the cell is cooled rapidly to −70 °C or below. This approach, often called interrupted rapid cooling, is the subject of this paper. Mouse oocytes were suspended in 1.5 M ethylene glycol (EG)/PBS, rapidly cooled (50 °C/min) to −25 °C and held for 5, 10, 20, 30, or 40 min before being rapidly cooled (50 °C/min) to −70 °C. In cells held for 5 min, IIF (flashing) occurred abruptly during the second rapid cool. As the holding period was increased to 10 and 20 min, fewer cells flashed during the cooling and more turned black during warming. Finally, when the oocytes were held 30 or 40 min, relatively few flashed during either cooling or warming. Immediately upon thawing, these oocytes were highly shrunken and crenated. However, upon warming to 20 °C, they regained most of their normal volume, shape, and appearance. These oocytes have intact cell membranes, and we refer to them as survivors. We conclude that 30 min at −25 °C removes nearly all intracellular freezable water, the consequence of which is that IIF occurs neither during the subsequent rapid cooling to −70 °C nor during warming.
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Keywords: Intracellular ice; Recrystallization; Oocytes; Mouse; Freezing

The success of the classical slow cooling approach to cryopreservation relies on the fact that lethal intracellular ice formation (IIF) can be avoided if cells are cooled slowly enough so that osmotic dehydration can reduce the amount of unfrozen intracellular water to near the equilibrium value, and to do so before the cells reach the temperature where intracellular nucleation becomes probable. Many cell types can be preserved by this method once "slow enough" has been defined by experiment or modeling [8]. But some can not. Included among the latter are cells or cell systems that exhibit a high sensitivity to chilling injury; i.e., sensitivity to the lowering of temperature in the absence of ice formation. Examples are *Drosophila* embryos [7] porcine embryos [12] and oocytes of rhesus monkeys and humans [16,20].

But there is an alternative approach often referred to as interrupted rapid cooling. In this procedure, a cell is cooled rapidly to a temperature slightly above the IIF nucleation zone, and is then held at that temperature long enough for it to dehydrate isothermally to near its equilibrium water content before it is cooled rapidly to −70 °C or below. The method has been successfully applied to a variety of cells including Chinese hamster tissue culture cells [1] and mouse embryos [2,17,18]. It has been analyzed in some detail by Mazur [6] and Toner et al. [17]. Papers 2, 17, and 18 reported that when the sub-zero holding temperature and time at that temperature were appropriate, a high percentage of the embryos survived the subsequent step of

☆ This research was supported by NIH Grant R01-RR-18470.
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0011-2240/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cryobiol.2007.06.007

rapid cooling in liquid nitrogen (LN_2). Mazur [6] and Toner et al. [17] on the basis of modeling, inferred that an inappropriate holding temperature or too short a holding time caused lethality because of IIF. Farrant et al. [1] published micrographs of freeze-substituted hamster tissue-culture cells supporting that view, but neither he or the other authors have tested this proposition directly in mouse oocytes or embryos. A major purpose of our study was to address this omission.

There are two significant aspects to this two-step rapid freezing approach. The first is that cell dehydration occurs isothermally at a sizably reduced subzero temperature as opposed to the classical method in which dehydration occurs progressively as cooling progresses slowly (commonly 1 °C/min) from the freezing point to −30 °C. As a consequence, in interrupted rapid cooling, the cells spend considerably less time at temperatures near zero. This could be important if a cell is especially sensitive to chilling injury near 0 °C, but exhibits reduced sensitivity at, say, −25 °C.

The second point is that since the dehydration is occurring isothermally, it allows one to calculate the water permeability, L_p, at the sub-zero holding temperature (e.g., −25 °C) without having to know the change in cell volume over that time. Comparison of that L_p with an L_p derived at 0 °C by other methods allows one to calculate the activation energy of water permeability (E_a) over the range of 0 to −25 °C and compare that E_a with that obtained by classical volume measurements at temperatures between 0 and +30 °C.

Knowledge of L_p and its E_a at subzero temperatures is essential in defining and avoiding cooling rates that are too high to preclude lethal IIF, and in predicting the optimum cooling rate. But, heretofore, modeling this process has usually assumed that L_p and their E_a's obtained from above-zero measurements can be extrapolated to sub-freezing temperatures. This assumption may not be correct. The work reported here permits an estimate of the subzero L_p. The basis of that estimate will be reported elsewhere [4].

The present paper describes the response of the oocytes as a function of the isothermal holding period and the isothermal temperature in terms of whether or not IIF occurs and when it occurs.

Methods

Many of the methods used were described in detail in [9]; consequently, here we give details only for those aspects that differed.

Source of oocytes

MII oocytes from ICR mice were harvested in Japan in Dr. Keisuke Edashige's laboratory in Kochi University, Japan, loaded into straws, vitrified in an ethylene glycol–acetamide–Ficoll–sucrose mixture, and express shipped to Tennessee. For an experiment, the oocytes in two to four straws were thawed rapidly, and mixed with 0.5 M sucrose. Some 10 min later, the oocytes were transferred to PB1 lacking sucrose, and then to previously prepared droplets of M16 medium for some 2 h. On pp. 48–49 of [9], we give eight points of evidence that the vitrified-thawed—M16 incubated oocytes are normal with respect to plasma membrane integrity and osmotic response. One indication of normality is their morphological appearance. A second is that they shrink or swell in anisosmotic solutions of glycerol/PBS or EG/PBS in quantitative accord with that calculated from fundamental osmotic equations (For example, see Table 7 of Mazur et al.[11].

Experimental media and sample preparation

For an experiment, two to three oocytes are transferred from an M16 droplet to 1 ml of Dulbecco's phosphate buffered saline (PBS) containing the desired concentration of cryoprotective agent (1.5 M ethylene glycol [EG]) and Snomax (a commercial preparation of freeze-dried *Pseudomanas syringii*, the ice nucleating bacterium). Snowmax is introduced to minimize the supercooling of the suspending medium. Then, 15 min later, a 1.5 μl droplet of this medium is placed in the center of a 75 μm thick spacer in a Linkam quartz sample cuvette, the oocytes pipetted in a minimum volume to that droplet, and a coverglass applied. The sample cuvette is then inserted in a Linkam BCS 196 cryostage and the freezing–thawing run initiated. The stage was attached to a Zeiss microscope, and the sample observed with a 20 × objective for a displayed magnification of 500×. The images are displayed at 40 frames/s on a monitor and captured on a computer hard drive at desired intervals as short as 1 image/10 s.

The initial freezing medium has a total weight percent of EG and salts (W_T^o) of 9.94% and a weight ratio of the EG to salt (R) of 11.51. The molality of the salt and the EG are 0.151 m and 1.636 m, respectively (From [11] for solution R12-1×-EG9 in Table 1).

The Linkam cryostage, freezing protocols, and ramps

The protocol was designed to rapidly cool oocytes to holding temperatures that were slightly above those known in our prior studies [9,10] to cause IIF in the great preponderance of oocytes. The primary hold temperature was −25 °C. Using liquid nitrogen vapor for cooling and electrical resistors for heating, the Linkam cryostage with its associated Pax-it software allows samples to be subjected to sequential ramps in which cooling rate, limiting temperature, holding time, and warming rate can be specified. The ramps used here were as follows:

Ramp 1: Rapid cooling (50 °C/min) to −8.0 °C.
Ramp 2: Slow cooling (5 °C/min) to −10 °C. EIF occurs at −9.09 ± 0.06 °C, a supercooling of ∼5 degrees in the external medium. Hold 2 min at −10 °C (This is an important step in maximizing the subse-

Table 1
Ice formation in mouse oocytes suspended in 1.5 M EG, rapid cool to EIF, 2-min hold after EIF, rapid cool to −20, −25, or −30 °C, variable hold, and rapid cool to −70 °C

Hold temperature (°C)	Hold time (min)	# Oocytes	% Flash during Ramp 3 or hold **Class 1** (%)	% Flash during Ramp 4 **Class 2 & 3** (%)	% Blacken during warm **Class 4** (%)	% Normal after thaw **Class 5** (%)
−25	5	10	10	80	10	0
−25	10	8	0	12	88	0
−25	20	8	25	0	38	38
−25	30	25	8	0	12	80
−25	40	9	22	0	0	78
−20	30	9	0	0	89	11
−30	30	11	45	0	36	18

Ramp 3 is a 50 °C/min cool to hold temperature; Ramp 4 is a 50 °C/min cool from the hold temperature to −70 °C.

quent supercooling of the cellular water [10], probably because it allows time for the previously supercooled extracellular medium to equilibrate).

Ramp 3: Rapid cool (50 °C/min) to −25 °C (or, in some cases, to −20 °C or −30 °C). Hold 5, 10, 20, 30, or 40 min.

Ramp 4: Rapid cool (50 °C/min) to −70 °C.

Ramp 5: Moderately rapid warming (10 °C/min) to +20 °C.

This sequence of ramps and the values assigned to them differ considerably from that used previously [9] in that the cooling rates in Ramps 1, 3, and 4 of 50 °C/min are considerably higher than those we used previously. As reported in [10], the 2-min hold at −10 °C in Ramp 2 just after EIF is important in maximizing the extent to which the subsequently rapidly cooled oocytes supercool before undergoing IIF. If the 2 min post-EIF hold time is omitted, the mean IIF (flash) temperature of oocytes in 1.5 M EG rises from −41 to −29 °C.

Results

Consequences of holding at −25 °C

The observations fall into five classes:

Class 1. The oocytes abruptly flash black during the rapid cooling in Ramp 3 or during the ensuing hold period at times ranging from 1 to 14 min. Since they undergo IIF before the rapid cooling in Ramp 4, they are not especially relevant to the present study.

Class 2. The oocytes abruptly flash black during the rapid cooling in Ramp 4 after the completion of the hold. The mean flash temperature was -47.1 ± 1.6 °C ($n = 17$).

Class 3. The oocytes turn brown somewhat more slowly during the rapid cool in Ramp 4 and complete blackening during warming in Ramp 5.

Class 4. There is no observable flashing during the rapid cool in Ramp 4, but the oocytes blacken during the warming at 10 °C/min. Unequivocal blackening commences at -53.2 ± 1.0 °C ($n = 21$), and progresses during several degrees of subsequent warming (Fig. 1).

Class 5. There is no blackening or browning during either cooling or warming.

Classes 1–4 result in severely and lethally damaged oocytes after thawing (Fig. 1). However, **Class 5** oocytes, which are extremely shrunken and crenated immediately after thawing (Fig. 2, middle), presumably are viable, for by the time they have warmed to +20 °C, the crenation disappears and in 2–3 min, the oocytes approach normal appearance and volume (Fig. 2, bottom) [also see Second Freezing, below].

The actual numerical observations are given in Table 1; namely,

Hold 5 min @ −25 °C: 80% undergo IIF during the rapid cooling in Ramp 4 (**Classes 2& 3**), whereas 10% show no flashing during cooling but undergo blackening during warming (**Class 4**). None are **Class 5**; i.e., none survive.

Hold 10 min: The percent flashing during the rapid cool in Ramp 4 drops to 12% (**Classes 2& 3**), but the percent undergoing blackening during warming rises to 88% (**Class 4**). Again, none are **Class 5**; i.e., none survive.

Hold 20 min: The percent flashing during the rapid cool in Ramp 4 drops to 0%, the percent that turn black during warming is 38% (**Class 4**), and, now, 38% survive (**Class 5**).

Hold 30 min: Again, none undergo IIF during the rapid cool in Ramp 4, and only 12% blacken during warming. Some 80% show no browning or blackening during either cooling or warming; i.e., they are **Class 5** survivors.

Hold 40 min: The results are quite similar to the 30 min hold; namely, 78% survived (i.e., are **Class 5**).

In summary, as the holding time increases from 5 to 30 min at −25 °C, the response of the population shifts from most undergoing abrupt visible flashing during cooling to an increasing fraction that darken only during warming, to a population where some 80% show no flashing or darkening either during cooling or warming, and survive. Fig. 3 shows the survival pattern.

2nd Freeze: As a measure of the membrane integrity of the "survivors", eleven of them (six after a 30 min hold; five

after a 40 min hold) were subjected to a repeat rapid freeze at 50 °C/min to −70 °C, but with no hold at −25 °C: They flashed at the expected low temperature for an oocyte with an intact plasma membrane. (−43.6 ± 1.9 °C) [compare the −40.6 °C that Mazur et al. [9] reported for oocytes cooled at 50 °C/min.]

Consequences of carrying out a 30 min hold at −20 °C

The percent surviving drops to 11% (vs. 80% for a 30-min hold at −25 °C), and the remaining 89% blacken during warming.

Consequences of carrying out a 30-min hold at −30 °C

Again, the survival (18%) is much lower than that after a 30-min hold at −25 °C. But, now nearly half undergo IIF during the hold [**Class 1**]; and 36% blacken during warm [**Class 4**].

Morphological appearance of Class 5 oocytes immediately after thawing

The middle photograph in Fig. 2 shows the appearance immediately after thawing of three oocytes subjected to sufficient dehydration during freezing to have avoided IIF. The appearance is dramatic, with the shrunken cells exhibiting sharp spines and points. This appearance is in striking contrast to that of an oocyte osmotically dehydrated at −25 °C in a completely ice-free EG/PBS solution with solute concentrations nearly equal to that in the liquid portion of solutions frozen to those temperatures. In this unfrozen case, the oocytes have extensively shrunken but have done so isotropically (Fig. 4).

The suspending medium for these ice-free experiments was prepared to have the same composition as that of a frozen solution at −23 °C (half way between −20° and −25 °C). The total solute weight percent concentration (W_T) at −23 °C is obtained from the ternary phase diagram in Fig. 5. It is 38.42%. From that value and the value of R (11.51), one can compute the weight percents of EG and PBS (33.35% and 3.073%) and their molalities (9.25 and 0.854 molal, respectively). Snomax was omitted.

Discussion

In 1977, Mazur [5] conjectured that cells that lose 90% of their water prior to being cooled to their ice nucleation temperature, will not undergo IIF when cooled to still lower temperatures. This conjecture was loosely based on the fact that about 10% of the water in cells is bound in the sense of being non-freezable.

Here, we see that 80% of oocytes held for 30 min at −25 °C show no evidence of IIF either during the subsequent rapid cool to −70 °C, or during warming, and they appear normal after thawing and warming to 20 °C, with intact cell membranes. The clear interpretation is that during that 30-min period, the oocytes lost all their freezable water osmotically; i.e., during the hold, all the supercooled water flowed out of the cell and froze externally.

Phase diagram data on EG/NaCl/water allow one to calculate the equilibrium fraction of unfrozen water in a 1.5 M EG/0.15 M PBS solution at specified subzero temperatures. One experimental set of such ternary data has been published by Woods et al. [19]; however, Kleinhans and Mazur's comparison of these data with that of ternary phase diagrams synthesized by adding the freezing points of binary solutions of EG/water and NaCl/water [3] shows substantial discrepancies. These discrepancies between experimental and synthesized ternary phase diagrams are not present in glycerol/NaCl/water, sucrose/NaCl/water, or DMSO/NaCl/water. For that reason, the estimates of equilibrium unfrozen fractions of water given below are based on the synthesized ternary plot in [3].

The ternary phase diagram for the EG/PBS solution used here ($R = 11.51$) is shown in Fig. 5. It depicts the freezing point of a solution as a function of the total weight percent of solute (W_T) in that solution, or conversely it shows the equilibrium weight percent concentration of solutes (W_T) in the unfrozen portion of a solution frozen to a given sub-zero temperature. The vertical dotted tie line on the left depicts the initial wt% concentration of solute (W_T^o), here 9.94%. The vertical tie-line on the right depicts the total solute (EG + salt) concentration (W_T) in the unfrozen portion at −25 °C; namely, 40.3%. The fraction of the original water in the solution that remains unfrozen (U) is given by [13, equations 4 and 7])

$$U = [(100 - W_T)(W_T^o/W_T)]/(100 - W_T^o)$$

The curve labeled Eq in Fig. 6 shows that at −25 °C, the equilibrium unfrozen fraction of water is 16.4%. That value should apply within the cell as well as outside since the oocytes are highly permeable to EG and had equilibrated with EG during the 15 min room-temperature exposure prior to initiating the experiments.

The upper curve in Fig. 6 depicts the changes in the relative volume of water in oocytes subjected to our interrupted rapid cooling procedure and compares them with those occurring during slow equilibrium cooling. With interrupted rapid cooling, the length of the hold at −25 °C, and therefore, the water content of the oocytes at the termination of the isothermal hold period has a major effect on the type of IIF manifested and whether it is manifested. If the hold period is only 5 min, at which time the calculated cell water content is 56% of that in the isotonic cell, the 90% of the oocytes that remained unfrozen at the end of the hold all underwent flashing during the subsequent rapid cooling. Of these, 56% turn black and 44% turn chocolate brown and complete blackening during the warm. If the hold time is doubled to 10 min, at which time the calculated water content is 40%, only 12% flash during cooling, the remaining 88% blacken during warming. Doubling the hold time to 20 min further reduces the water content to 23% and it reduces the percent that flash during Ramp 4

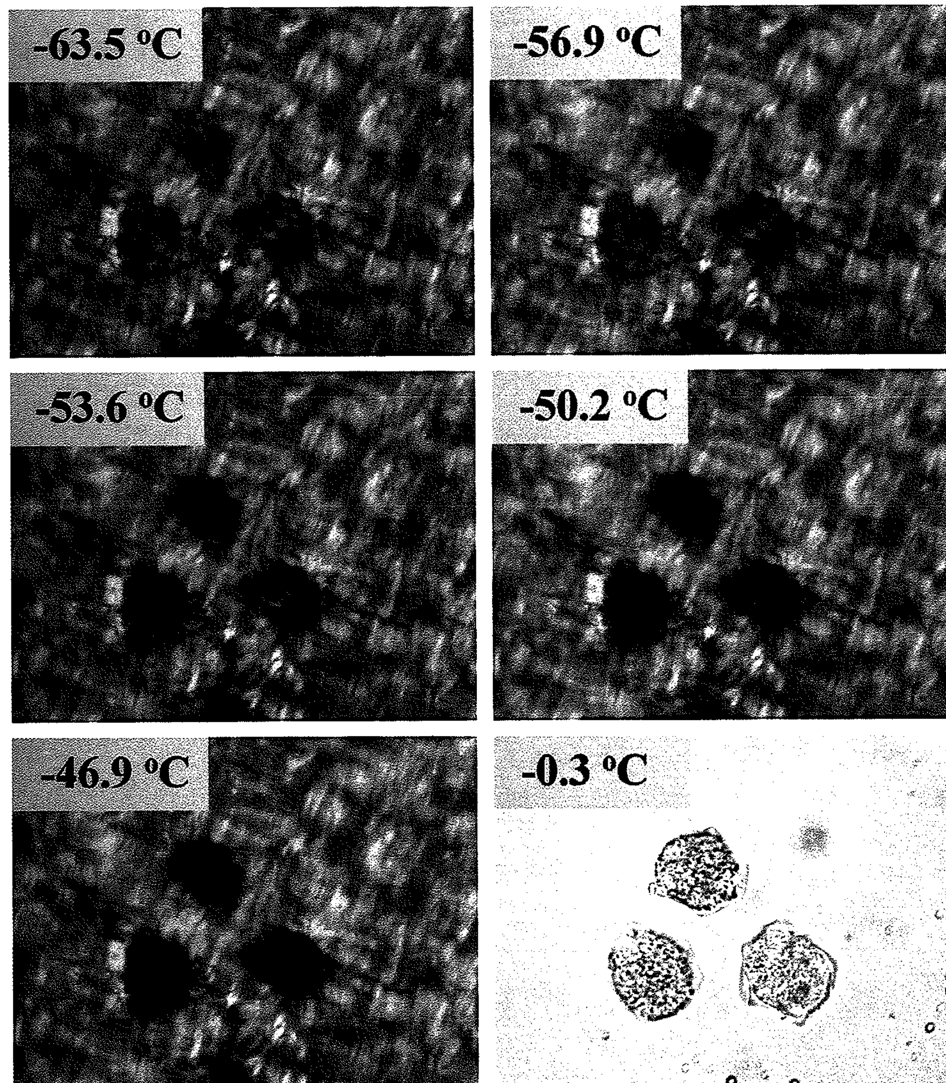

Fig. 1. Gradual darkening of three **Class 4** oocytes as the temperature warmed from −56.9 to −46.9 °C at 10 °C/min. These oocytes had been rapidly cooled to and held at −25 °C for 10 min before further rapid cooling to −70 °C. **Class 4** oocytes are those that show no darkening or flashing during the prior cooling, but only during warming. The bottom photo at −0.3 °C depicts the extensive damage immediately after thawing. That appearance remains unchanged after warming to +20 °C. These and the photographs in Figs. 2 and 4 are at the same magnification. The normal oocyte is 75 μm in diameter (see **Fig. 2**, top photo).

rapid cooling to zero, and reduces the percent that blacken during warming to 38%. Now, we see appreciable survival (38%). If the hold time is further increased to 30 min, the cell water content is reduced to 19.7% and 80% do not flash or blacken during either Ramp 4 rapid cooling or during subsequent warming; i.e., survival has risen to 80%. Increasing the hold time to 40 min only drops the water content by 0.3%; i.e., to 19.4%, and the survival remains the same as with the 30 min hold. Note that even after 40 min, the cell water volume has not quite dropped to the equilibrium value of 16.4%.

Presumably, when the water content of the oocyte has been reduced to intermediate levels by holding at −25 °C for 5–10 min, either the size of any intracellular ice crystals formed during the subsequent rapid cooling to −70 °C or their growth rate is diminished in some oocytes so that they reflect light less and appear undarkened or slightly brown. However, during warming, the ice crystal growth resumes at −53 °C, and cell blackening is completed.

If the water content is still further reduced, no blackening or browning occurs during cooling, but it does occur during warming as evidenced by slow blackening, beginning again at −55 to −53 °C (**Class 4** oocytes). This blackening could either be a consequence of the devitrification of a glass that had formed during the cooling or it could be the recrystallization of very small crystals that formed during cooling—crystals so small that they do not reflect or scatter light [Recrystallization is the growth of larger ice crystals at the expense of smaller ones as a consequence of differences in their surface energies]. In theory, it might

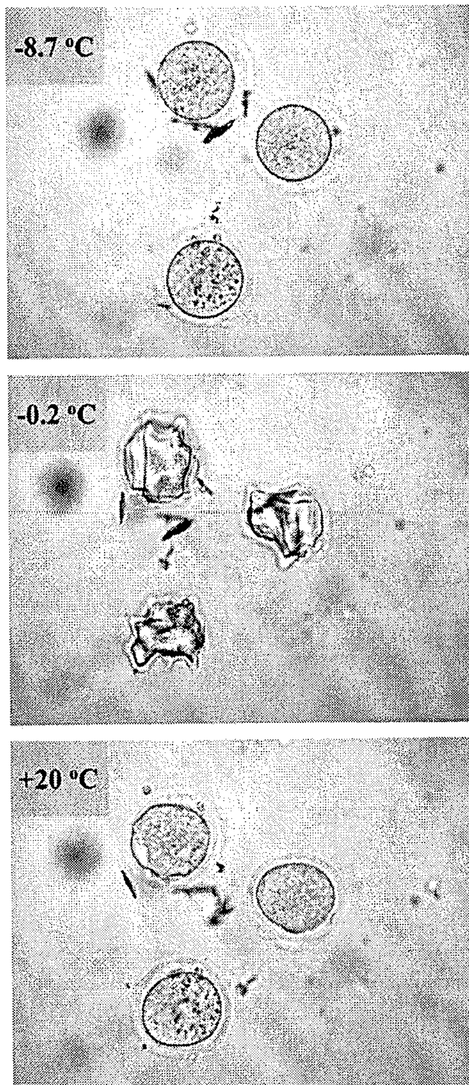

Fig. 2. Appearances of three **Class 5** oocytes immediately before EIF at −9.9 °C, immediately after being warmed from −70 °C and thawed, and after being warmed to +20 °C for 3 min. **Class 5** oocytes are those that exhibit no darkening or flashing during either cooling or warming. These particular oocytes had been rapidly cooled to and held at −25 °C for 30 min before further rapid cooling to −70 °C. The oocyte diameter in the top photograph is 75 μm.

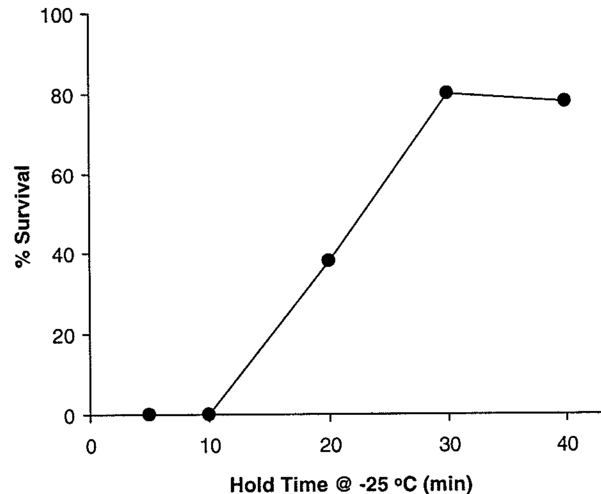

Fig. 3. Survival of mouse oocytes subjected to interrupted rapid cooling as a function of the holding time at −25 °C. "Survival" is synonymous with **Class 5** oocytes; i.e., those that do not manifest IIF during either cooling or warming, are highly crenated immediately after thawing, and nearly return to normal volume and appearance 2–5 min later at 20 °C.

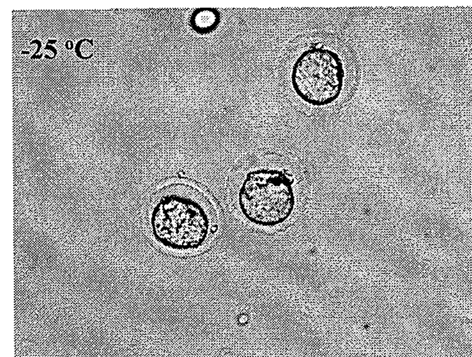

Fig. 4. The isotropically shrunken appearance of oocytes at −25 °C after suspension for 15 min in a solution of 9.3 molal EG and 0.85 molal PBS followed by rapid cooling to −25 °C. The shrinkage is manifested by the large interzonal space. The isotropic nature of the shrinkage is emphasized by a comparison with the middle photograph in Fig. 2. No external ice is present. This solution has the same composition as exists in the unfrozen portions of a 1.5 M EG/0.15 M PBS solution frozen to −23 °C.

be possible to distinguish between the two possibilities using differential scanning calorimetry (DSC). If the blackening during warming is a consequence of devitrification of a glass, the full latent heat of ice formation should be released. If the blackening is a consequence of recrystallization of existing small ice crystals, very little heat should be evolved. It is possible that oocytes in this category (**Class 4**) would survive if warming could be carried out at a high enough rate to prevent devitrification or recrystallization. [A paper by Rall and Polge supports this view. They reported [14] that mouse 8-cell embryos frozen in 1.5 M glycerol underwent blackening when warmed at 2 °C/min but not when warmed at 250 °C/min. (See more below) Furthermore, most of the latter were viable after thawing on the basis of invitro development.] Finally, we find that if the water content is still further reduced to an estimated 20% of the isotonic value, no visible intracellular ice forms during either cooling or warming (**Class 5**). Thus, the cell water content is critical with respect to IIF. Lowering it by 3% from 23% to 20% decreases the percentage undergoing IIF from 62% to 20%. This five-fold reduction from the water content of normal isotonic oocytes results in a five-fold increase in the molality of intracellular EG to 8.18 molal. That appears sufficient to induce vitrification during further rapid cooling to −70 °C and sufficient to prevent devitrification and recrystallization during warming.

Rall et al. [15] and Rall and Polge [14] reported that 8-cell mouse embryos cooled at 0.5 °C/min to −42 °C and then cooled rapidly to −150 °C underwent an initial flash-

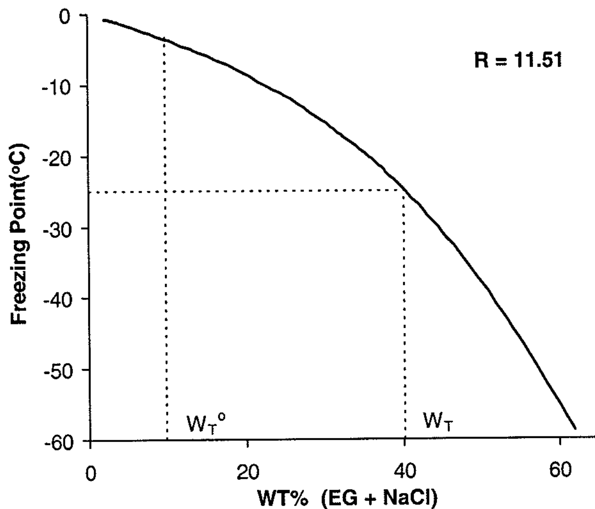

Fig. 5. Ternary phase diagram of EG/NaCl/ water (from [3], Fig. 6C, upper curve).

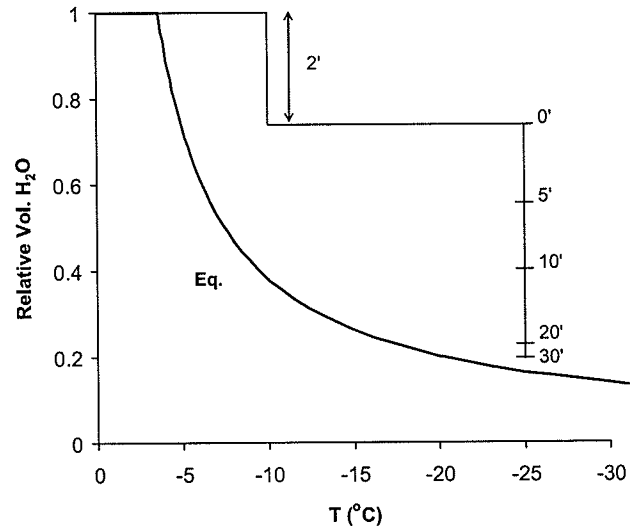

Fig. 6. The fraction of water in the EG/PBS medium and in the oocytes that remains unfrozen as a function of sub-zero temperature in our interrupted rapid cooling procedure. The lower curve labeled Eq. is the equilibrium curve derived from Fig. 5 using the equation for U in the text. The upper curve is the calculated fractional volume of water in the oocytes during the various steps involved in cooling to and holding at −25 °C. A value of 1.82×10^{-3} μm³/μm² min atm for L_p (water permeability) was used to calculate the water loss during the hold [4].This value was arrived at from two facts. If the assumed value of L_p was doubled, the cell water content showed little dependence on the holding time at −25 °C; it was greatly reduced at all times. If the assumed value of L_p was halved, the water content of oocytes after even a 30 min hold was 36%, more than double the equilibrium value at −25 °C, and far too high to avoid IIF. After the indicated hold times at −25 °C, the oocytes were rapidly cooled (50 °C/min) to −70 °C.

ing during subsequent slow warming (∼2 °C/min) at ∼−90 °C followed by further blackening at ∼−55 °C. The first darkening they ascribe to devitrification of the cytoplasm of the dehydrated embryos; the second darkening they ascribe to recrystallization of the intracellular ice. It occurred at the same temperature we report here for **Class 4** oocytes, and we offer the same interpretation of it.

They also found [15] that the second darkening (recrystallization) was not observed with warming rates of 25, 50, 100 or 250 °C/min, but was observed with warming rates of 2 and 10 °C/min. We used a warming rate of 10 °C/min, and it also yielded blackening during the warming of **Class 4** oocytes beginning at about −55 °C.

Toner et al. [17] also found that that the sub-zero temperature of an intermediate hold and the duration of that hold was critical to avoiding IIF during a subsequent rapid cool of fertilized mouse ova (zygotes). In their case, the optimum time and temperature was 5 min at −10 °C. These values differ markedly from ours because (1) No CPA was present and (2) the zygotes were predehydrated in 1100 mOsm choline chloride/PBS, and (3) the water loss occurs much more rapidly at −10 °C than at −25 °C.

Consequences of a 30 min hold at −20 °C

Thirty minutes at −20 °C reduces the calculated cell water content to 23.1%, which again is 3% above the equilibrium value of 20.1%. Interestingly, the 23% attained after 30 min at −20 °C is identical with the water content attained after 20 min at −25 °C. This is especially interesting because both conditions yield substantially fewer **Class 5** surviving oocytes (11% and 38%, respectively) and correspondingly higher percentages of cells exhibiting IIF than those held for 30 or 40 min at −25 °C (Table 1). The water content of those held only 20 min at −25 °C is 6.5% above equilibrium (23% vs. 16.4%), too far above to avoid IIF during Ramp 4 cooling. Those held for 30 min at −20 °C, have dehydrated to within 3% of the equilibrium value of 20%, but that equilibrium water content is too high to preclude IIF on the subsequent Ramp 4 cooling.

Consequences of a 30 min hold at −30 °C

Holding at −30 °C for 30 min was considerably more damaging than holding for 30 min at −25 °C. Of the 11 oocytes held at −30 °C, none flashed during the initial rapid cooling to −30C, but 5/11 flashed 1–16 min into the hold. Four did not flash during cooling, but blackened during warming, and two did not flash during cooling or warming and looked normal after thaw. There seem to be two factors operating. One is that −30 °C lies on or near the boundary where some of the hydrated oocytes nucleate intracellularly during the hold. The other is that the calculated L_p is considerably lower at −30 °C (0.76×10^{-3} μm³/μm² min atm), so that the cell water content after 30 min (27%) is too far from equilibrium to avoid IIF.

Criteria of survival

We have defined **Class 5** oocytes as survivors. This is based in part on the absence of any IIF, in part on their

returning to normal volume and mophology upon their return to +20 °C, and in part because their plasma membranes are intact as evidenced by the fact that in a second rapid cool they remain supercooled to <−40 °C. Of course this provides no evidence of functional survival. In 1980 Wood and Farrant [18] conducted interrupted rapid cooling experiments in which 8-cell mouse embryos in 1.5 M DMSO were rapidly cooled to −25 °C and held for 8, 13, 18, and 23 min before rapid cooling to −196 °C. They did not take photographs, as here, but they did measure functional survivals based on *in vitro* delvelopment to blastocysts. They obtained survivals of 0, 0, 25, and 67%, values that are close to the values we obtained of 0, 0, 38, and 80% for holding times of 5, 10, 20, and 30 min (Table 1).

Interactions of time and temperature on the occurrence of IIF

In our previous experiments, which involved no holding time at an intermediate subzero temperature [9,10], rapidly cooled oocytes in 1.5 M EG flashed at a mean of −41 °C. None flashed at or above −25 °C and only 15% flashed at or above −30 °C. Here, we see from Table 1 that up to 25% flashed at or above −25 °C, and 45% flashed during a hold at −30 °C. Yet, when survivors of a first freeze were subjected to a second rapid freeze involving no hold time, they behaved like those previously subjected to a single rapid freeze [9]; i.e., eleven out of eleven flashed below −30 °C.

Thus, some time-dependent event(s) occurs during an isothermal hold at −25 or −30 °C that results in some of the oocytes undergoing IIF at a temperature that is 11–16 degrees above where it occurs on average in the absence of a hold. What might the time-dependant process(es) be? One possibility is that the time represents the time required for the external ice/solution to reach the equilibrium state called for by the phase diagram. Another possibility is that during the holding period, the cell membranes develop a defect that permits the entry of external ice and the consequent seeding of intracellular supercooled water.

Implications of the striking differences in morphology between oocytes dehydrated at −25 °C in a frozen solution and those dehydrated at −25 °C in the absence of external ice

As noted in Figs. 2 and 4, the former are extensively crenated immediately after thawing, whereas the latter remain smoothly spherical, although extensively shrunken. The solute concentrations in the liquid portions of the sample are nearly the same in the two situations and so the osmotic driving forces are nearly identical. The cooling and warming rates are the same. The only major physical difference between the two cases is the presence or absence of external ice.

This leads to the clear conclusion that the narrowing channels in the external ice are producing physical forces on the oocyte that are sufficient to cause severe distortion of their surfaces. We noted that IIF in **Class 1–3** oocytes occurs some 6–11 °C higher than in oocytes that are rapidly cooled (20–50 °C/min) to −50 °C or below without any holding period at −25 °C. Modeling demonstrates that oocytes cooled at these rates will undergo very little shrinkage, and probably relatively little distortion. All this suggests that the sharp bends and other distortions in the surface of the oocytes subjected to a hold at −20 to −30 °C, may produce entry points for the external ice and lead to IIF at the higher temperatures observed.

In prior papers, we have suggested that external ice itself is the nucleating agent causing the intracellular freezing of supercooled oocytes above ∼−40 °C. And we have suggested that to act in this capacity, the external ice would have to come in close contact with the oocyte surface. The extensive deformation of **Class 5** oocytes is still further evidence that such close contact is occurring. We should point out that alternative theories as to the intracellular nucleating agent have been proposed. They have been reviewed in some detail in [8].

Possible implications for cryopreservation of oocytes

The overall time expended at sub-zero temperatures for oocytes cooled by classical slow cooling is about the same as with an interrupted rapid cool procedure involving a 30-min hold at −25 °C. However, the distribution of time over the sub-zero temperature range differs sharply in the two methods (Fig. 6). If the slow freezing protocol involves cooling at 1 °C/min from −5 to −35 °C followed by a plunge to LN_2, that will expose the oocytes for 30 min to that −5 to −35 temperature range. The interrupted rapid cool procedure also exposes them for 30 min, but that full exposure occurs at −25° C. That difference could affect the resulting viability, especially if the oocytes are highly chill sensitive to temperatures near 0 °C, and much less so at say −25° C. Perhaps that is the case for human oocytes. The high chill sensitivity of various species of oocytes, whatever its cause, has led to an emphasis on high cooling rates and vitrification to "outrun" the chill sensitivity.

Further questions

Our study raises still other unanswered questions. Why does the blackening of **Class 4** oocytes during warming occur so consistently over the temperature range of −56 to −47 °C? We know that **Class 5** oocytes are "normal" with respect to membrane intactness and osmotic properties. Are they also functionally viable? Why is IIF so damaging? Is the damage a consequence of IIF or is IIF a consequence of prior membrane damage? If the latter, what is the nature and cause of the prior damage?

Acknowledgments

The oocytes used in this work were collected and vitrified by Dr. Shinsuke Seki and Professor Keisuke Edashige at Kochi University, Japan. We greatly appreciate their time and effort.

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