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Brief communication

Effects of hold time after extracellular ice formation on intracellular freezing of mouse oocytes ☆

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Abstract

MII mouse oocytes in 1 and 1.5 M ethylene glycol(EG)/phosphate buffered saline have been subjected to rapid freezing at 50 °C/min to −70 °C. When this rapid freezing is preceded by a variable hold time of 0–3 min after the initial extracellular ice formation (EIF), the duration of the hold time has a substantial effect on the temperature at which the oocytes subsequently undergo intracellular ice formation (IIF). For example, in 1 M EG, the IIF temperatures are −23.7 and −39.2 °C with 0 and 2 min hold times; in 1.5 M EG, the corresponding IIF temperatures are −29.1 and −40.8 °C.

Keywords: Oocytes; Mouse; Freezing; Intracellular and extracellular

We are conducting research into factors affecting the temperatures at which mouse oocytes undergo intracellular ice formation (IIF). One series of experiments involved a determination of the IIF temperatures when oocytes were cooled at a continuous high rate (50 °C/min) from room temperature to <−50 °C. Unexpectedly, the IIF temperatures were considerably higher than we had observed in earlier experiments in which we had allowed a couple of minutes to elapse between the appearance of extracellular ice and the onset of rapid cooling [5]. The present report deals with a more systematic study of the relation between the length of that post-EIF interval and the subsequent IIF temperature of mouse oocytes.

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Source and handling of oocytes

MII oocytes were collected from superovulated ICR mice in Edashige's laboratory at Kochi University, Japan, suspended in EFAS 10/10 medium consisting primarily of 10%(v/v) ethylene glycol (EG), 24% (w/v) Ficoll, and 0.4 M sucrose, loaded into 0.25 ml straws, vitrified in liquid nitrogen, and express shipped in liquid nitrogen to Knoxville. For use in experiments, straws were placed for 7 s in water at 25–30 °C for thawing, and their contents expelled into PB1 medium containing 0.5 M sucrose, and ~10 min later transferred with washing to PB1 without sucrose. The oocytes were then transferred to and held for 2–3 h in droplets of M16 medium that had been previously equilibrated for ~16 h in 5% CO_2/air. About 10–20% of the oocytes became degenerate or necrotic during the incubation and were discarded; the other 80–90% had normal appearance and were used in the experiments. Details of these procedures are given by Mazur et al. [5]. That paper also summarizes eight pieces of evidence showing that oocytes subjected to the above procedures appear to behave like fresh oocytes with respect to permeability and osmotic properties, membrane intactness, and the temperatures at which they undergo intracellular ice formation.

Transfer to EG-containing media

For experiments, three (usually) oocytes were transferred with washing from the M16 medium to 1 or 1.5 M EG in Dulbecco's phosphate-buffered saline (PBS). The medium also contained 0.001% (w/v) SnoMax, a freeze-dried preparation of the ice nucleating bacterium *Pseudomonas syringii*. Fifteen minutes later, the oocytes were transferred to a 1.5-μl drop of the same medium centered in the opening of a 75-μm thick spacer placed in the quartz sample holder of a Linkam BCS 196 cryostage (Linkam Scientific Instruments, Waterfield, UK). A coverslip was applied, and the sample quickly inserted into the cryostage, and cooling initiated. The oocytes are highly permeable to EG, and the 15 min exposure is sufficient to allow nearly full permeation as evidenced by the fact that the oocytes are at nearly normal volume.

The Linkam cryostage uses liquid nitrogen vapor to provide cooling to desired subzero temperatures and electrical heaters for purposes of temperature control and warming. A software interface allows the user to specify cooling and warming rates, limit temperatures, and holding times at desired temperatures. The temperature, derived from a thermocouple in the cooling block of the stage, is displayed continuously to 0.1 °C. Various calibrations indicate it reflects the sample temperature to ±1 °C or better. The microscope image is also visually displayed continuously in real time via a digital camera, and images can be captured to the computer hard drive as frequently as one per 10 s. In these experiments, samples were cooled to −70 °C and then thawed. The criterion of intracellular ice formation is the abrupt darkening of the oocyte, commonly referred to as "flashing."

Table 1 shows the thermal ramps (cooling and warming rates, temperature limits, and hold times) to which the oocytes were subjected. The chief variable was the holding time after EIF (0–3 min) in Ramp 3. EIF occurred during the initial cooling in Ramp 2; namely, at a mean of −7.2 °C in 1 M EG and ~−8.9 °C in 1.5 M EG (Table 2, column 2).

Table 1
Linkam cryostage cooling and warming ramps for oocytes frozen in 1 m[a]ethylene glycol

Ramp	Rate (°C/min)[b]	Limit (°C)	Hold	Comment
1	−50	−6.5	0 min	
2	−5	−8.5	See Ramp 3	EIF occurs above −8.5 °C
3	0	−8.5	0, 1, 2, or 3 min	Isothermal hold
4	−50	−70	0	IIF occurs in this ramp
5	+10	+20	0	Warming and thawing

[a] In 1.5 M EG, the limits for Ramps 1 and 2 were −8 and −10 °C.
[b] Cooling is indicated by negative rates; warming by positive rates. EIF and IIF refer to extra- and intracellular ice formation, respectively.

Table 2
EIF temperatures, post-EIF hold times, and subsequent IIF temperatures of mouse oocytes frozen in 1 or 1.5 M EG

Solution	EIF (°C)	Post-EIF hold		Flash temperature (°C)			*n*
		°C	Time (min)	Mean	Median	Range	
1 M EG	−7.0	−8.5	0	−23.7 ± 2.2	−24.8	−14.7 to −30.0	8
	−7.2	−8.5	1	−31.3 ± 2.3	−32.6	−21.3 to −39.9	10
	−7.4	−8.5	2	−39.2 ± 2.2	−39.9	−27.3 to −47.4	10
	−7.2	−8.5	3	−33.4 ± 2.5	−34.2	−20.0 to −41.7	10
1.5 M EG	−8.6	−10	0	−29.1 ± 2.3	−29.8	−19.3 to −38.9	9
	−9.2	−10	2	−40.8 ± 2.1	−43.5	−27.9 to −47.8	12

Errors are standard errors of the mean.

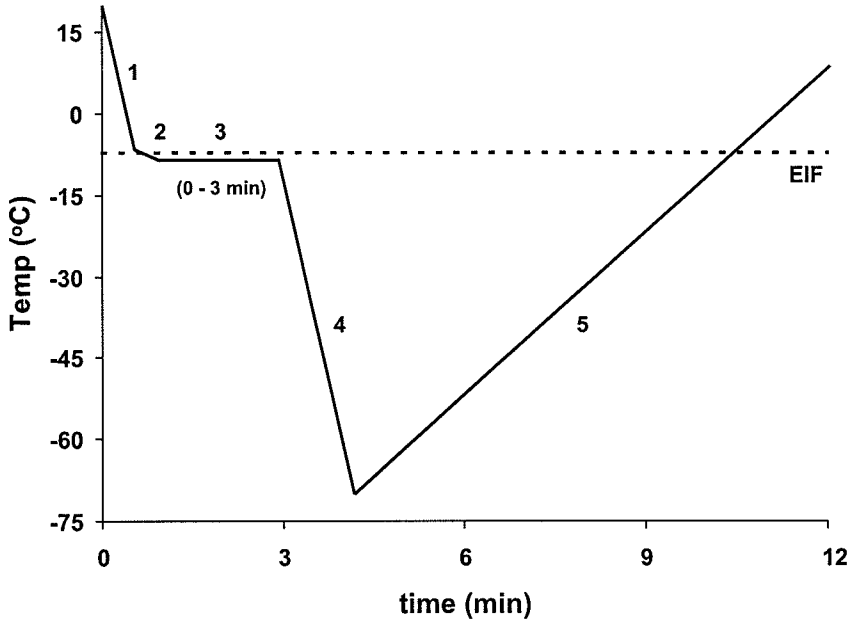

Fig. 1. Schematic of experimental temperature/time protocols for mouse oocytes in 1 M EG. The numbers 1, 2, 3, 4, and 5 refer to the Linkam cryostage temperature/time ramps described in Table 1.

Cooling at 5 °C/min continued to the hold temperature (−8.5 °C in 1 M EG; −10 °C in 1.5 M EG). After being held at those temperatures for 0–3 min, the oocytes were next cooled rapidly at 50 °C/min to −70 °C (Ramp 4). IIF, as evidenced by flashing, occurred in that interval at the temperatures indicated in column 5 of Table 2. The thermal/temporal events in 1 M EG are depicted schematically in Fig. 1.

The post-EIF holding time had a substantial effect on the flash temperature. In 1 M EG, in the absence of any hold, the mean flash temperature was −23.7 °C; after a 2-min hold, it dropped to −39.2 °C (The difference is highly significant [$p < 0.001$; tests of significance were based on ANOVA and two-tailed Student's t test as calculated by Graph Pad Software's Instat v. 3.02]). A hold time of 1 min produced an intermediate flash temperature (−31.3 °C) that was significantly lower ($p = 0.05$) than with a 0-min hold and significantly higher than with the 2-min hold ($p < 0.05$). The nucleation temperature appears to rise to −33.4 °C (median −34.2 °C) after a 3-min. hold, but the difference between the 3- and 2-min holds is of questionable significance ($p = 0.09$).

The differences in IIF temperature between a 0- and 2-min hold were similar in 1.5 M EG. In the absence of a post-EIF hold, IIF occurred at

−29.1 °C; after a 2-min hold, it dropped to −40.8 °C, a highly significant difference ($p = 0.001$).

To be precise, the total time the oocytes were exposed to extracellular ice before beginning the very rapid cool in Ramp 4 should include not only the hold time but also the time required for them to cool at 5 °C/min from the instant of EIF to the hold temperature. In 1 M EG that latter time was an average of 0.26 min. In 1.5 M EG, the time was 0.22 min.

The mean IIF temperatures in 1 and 1.5 M EG with the 2-min hold are nearly identical with those recently reported by Mazur et al. [5] for experiments using a different type of post-EIF treatment. In those latter experiments, after EIF occurred, the samples were warmed at 2 °C/min to just below the melting point before being recooled at a high rate to, in that case, −50 °C. The time spent during this post-EIF warming ramp was about 2.5 min. The mean IIF temperatures were −37.2 °C (vs −39.2 °C for the 2-min hold here) and −40.8 °C (vs −40.8 °C for the 2-min hold here) in 1 and 1.5 M EG, respectively.

Phase diagrams and interpolating equations for EG/NaCl solutions published by Woods et al. [9] allow one to calculate the mass fraction of water $(1 - U)$ and the mass fraction of the solution $(1 - L)$ that are frozen when compositional equilibrium has been attained at the hold temperature (see equations in Mazur et al. [5]). At the hold temperature of −8.5 °C in 1 M EG/PBS, U and L are 0.57 and 0.60, respectively; i.e., 43 and 40% of the water and solution are frozen. At the hold temperature of −10 °C in 1.5 M EG, U and L are 0.71 and 0.74; i.e., 29 and 26% of the water and solution are frozen.

Kinetics of the EIF process

Three events come into play after EIF. First, it will take a finite time for the external medium to attain the equilibrium state specified by the phase diagrams with respect to the amount of ice. Second, as the external ice progresses towards equilibrium, the solutes in the medium concentrate and the intracellular water becomes increasingly supercooled. Third, as a consequence, the oocyte will begin to lose water in response to the osmotic gradient that develops. Modeling calculations using $L_p = 0.4$ μm/min/atm, P_s for EG = 0.1 cm/min (at 20 °C), and activation energies of 12 and 16 kcal/mol, respectively (see, for example, Leibo [3]; Paynter et al. [6]; Mazur [4]) indicate that the percentage of cell water effluxing during a 1, 2, or 3-min. hold at −8.5 °C in 1 M EG is only 7, 10, and 13%, respectively. The corresponding figures for oocytes in 1.5 M EG at −10 °C are 6, 8, and 10%. These values assume that water is effluxing and EG is simultaneously influxing during the hold. These water losses, we believe, are too small to be of significance. Rather, we believe it is the first event that is especially relevant to the temperature at which IIF subsequently occurs. During the EIF equilibration, the oocytes have to accommodate shape-wise to the narrowing liquid channels between the growing ice crystals. We know that the amount of ice forming after EIF and the consequent reduction in the size of the unfrozen channels is sufficient to produce substantial deformation of the oocytes (Mazur et al. [5], Figure 4). In the presence of a 2-min hold, that shape accommodation can occur isothermally and gradually. In the absence of any hold, however, the accommodation has to take place during the rapidly lowering temperatures in Ramp 4. Perhaps the membranes become more brittle at these lower temperatures and become more readily damaged when attempting to change shape rapidly. The plasma membranes of erythrocytes, for instance, do undergo major physical changes with lowered temperature. Hochmuth et al. [2] have shown that when red cells are mechanically elongated, the time constant for their recovery to normal shape increases 4.5-fold when the temperature is lowered from 37 to 6 °C. This translates to a 6-fold increase in the coefficient of membrane surface viscosity. Thom et al. [8] have shown by the application of high frequency electric fields that the plasma membranes of red cells become more resistant to deformation at subzero temperatures. At −20 °C, they report that the deformability of red cells is zero. Fournier and Joós [1] indicate that the consensus is that cell membrane rupture is preceded by the formation of small transient pores of nanometer to micrometer size. If that were to

occur in the present context, these transient pores could represent pathways for the growth of external ice into the supercooled cytoplasm with consequent IIF.

It should be noted that the ~−40 °C flash temperature observed with a 2-min post-EIF hold is at the homogeneous nucleation temperature for the volume of water in an oocyte [7]. The homogeneous nucleation temperature is the temperature at which supercooled water nucleates spontaneously in the absence of a heterogeneous nucleating agent. Since, in our case, ice is present outside the oocyte, the absence of heterogeneous intracellular nucleation with the 2-min post-EIF hold must mean that the plasma membrane has remained sufficiently normal and intact to act as an absolute barrier to the movement of external ice into the cell interior. Conversely, the much higher nucleation temperature in the absence of a hold period suggests that the membrane of such oocytes has become sufficiently deformed to produce defects that cause it to partly lose its barrier properties. Regardless of the correct explanation, it is clear that there are strong interactions between the temporal events after EIF and the temperature at which IIF occurs.

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