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

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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.

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

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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-