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Formation of extracellular and intracellular ice during warming of vitrified mouse morulae and its effect on embryo survival ☆

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ABSTRACT

In vitrified solutions, ice can form during warming if the concentration of the cryoprotectant is insufficient. For the cryopreservation of cells, ice is innocuous when it remains outside the cell, but intracellular ice (ICI) is lethal. We tried to estimate the conditions in which ICI forms in vitrified mouse morulae during warming. The solutions for the experiments (EFS10–EFS50) contained 10–50% ethylene glycol plus Ficoll plus sucrose. When vitrified EFS20, EFS30, and EFS40 were kept at −80 °C, they remained transparent after 3 min, but turned opaque after 60 min (EFS20, EFS30) or 24 h (EFS40). Morulae were vitrified with EFS solutions after exposure for 30–120 s at 25 °C. They were warmed by various methods and survival was assessed in culture. After rapid warming (control), survival was high with EFS30 (79–93%) and EFS40 (96–99%). After slow warming, survival decreased with both EFS30 (48–62%) and EFS40 (44–64%). This must be from the formation of ICI. To examine the temperature at which ICI formed during slow warming, vitrified embryos were kept at various sub-zero temperatures during warming. Survival with EFS30 and EFS40 decreased on keeping samples for 3 min at −80 (25–75%), −60 (7–49%), −40 (0–41%), or −20 °C (26–60%). When samples were kept at −80 °C for 24 h, the survival decreased to 0–14%. These results suggest that ICI forms at a wide range of temperatures including −80 and −20 °C, more likely between −60 and −40 °C, and the ice forms not only quickly but also slowly.

Embryo transfer technology in mammals has been widely used for the production and preservation of laboratory animals, the improvement and production of domestic animals, and the treatment of infertility in humans. The technology is also expected to contribute to the conservation of wild animal species. For the practical use of embryo transfer technology, the long-term preservation of embryos is essential. In addition, the handy transportation of cryopreserved embryos would increase the availability of this technology.

It is now possible to cryopreserve embryos of various mammalian species. In order to preserve embryos without reducing their viability with storage, they must be placed at a temperature below the glass transition temperature of the cytoplasm, which is around −130 °C. To maintain such a temperature, liquid nitrogen (LN_2) (−196 °C) is routinely used. For transportation, cryopreserved embryos are placed in LN_2. However, this needs special equipment, dry nitrogen shippers, in which cryopreserved embryos are placed in a gas, because one is not usually allowed to send LN_2 in a liquid state [23]. Unfortunately, however, dry shippers are large and expensive. If cryopreserved embryos could be transported without using LN_2 at higher sub-zero temperatures, for instance at −79 °C using dry ice, the availability of cryopreserved embryos would increase, especially for short-range transportation because dry ice decreases with time.

Mammalian embryos have been cryopreserved either by slow freezing [39,41], or by vitrification [31,32]. Embryos which had been cooled slowly to −50 °C or below before preservation in LN_2 can be placed at the temperature of dry ice (−79 °C) for at least 7 days without any decrease in survival [Dr. Minesuke Yokoyama, personal communication], probably because the embryos are nearly in equilibrium with the extracellular unfrozen fraction.

Recently, the use of the vitrification method is increasing because the protocol is simple and it can achieve higher survival rates than slow freezing in some cases [5,6,8,10,11]. However, when vitrified embryos were kept at −80 °C, survival decreased significantly (unpublished preliminary observation). The most probable reason for this decrease would be the formation of intracellular ice (ICI), because the vitrification solution must have been vitrified in a state of substantial supercooling.

Another possible reason for the decrease in survival is the toxicity of the vitrification solution, mainly of the permeating

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cryoprotectant. A strategy to reduce the toxicity of the vitrification solution is to use a low toxicity cryoprotectant. The first vitrification solution (VS1) contained Me_2SO, acetamide, and propylene glycol as permeating cryoprotectants [31], all of which are highly toxic [7]. However, since it was found that ethylene glycol is much less toxic than other agents [7], and is effective for vitrification [5], ethylene glycol is now widely used as the principal cryoprotectant for vitrification of mammalian embryos [10]. Among vitrification solutions, EFS solution (EFS40) [5], based on ethylene glycol (cell-permeating cryoprotectant), supplemented with Ficoll (macromolecule) and sucrose (small saccharide), has proven effective for a variety of mammalian embryos [10].

Another way to reduce the toxicity of the solution is to reduce the concentration of the permeating cryoprotectant. However, this increases the probability of ICI forming.

Since the pioneering work on the formation of ICI by Mazur [18], the conditions under which ICI develops in embryos/oocytes have been studied during cooling by slow freezing [14,19–21,29,30,35,36], but not vitrification.

Information about the formation of ICI during warming is important for the handling of vitrified embryos. In addition, it might lead to the development of new vitrification methods in which vitrified embryos can be transiently preserved at a higher subzero temperature, e.g., the temperature of dry ice (−79 °C), without a decrease in survival. This would enable the transportation of vitrified embryos without using LN_2.

In the present study, we try to specify the conditions in which ICI forms during warming. To assess the formation of ice in solutions, we observed the appearance of each solution visually [5]. We used mouse morulae vitrified in EFS solutions, because it has been confirmed that mouse morulae survive vitrification with an EFS solution at high rates [5,8,33].

Materials and methods

Vitrification solutions

Ethylene glycol was diluted to 10%, 20%, 30%, 40%, and 50% (v/v) with FS solution, which is PB1 medium [37] containing 30% (w/v) Ficoll PM-70 (average molecular weight 70,000; GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) plus 0.5 M sucrose, to make EFS10, EFS20, EFS30, EFS40, and EFS50, respectively [5,42].

Unless otherwise noted, chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and water was prepared by a Milli-Q Labo system (Nihon Millipore Ltd, Tokyo, Japan) after distillation and deionization.

Vitrification and warming of solutions

Each EFS solution was loaded into a 0.25 ml plastic straw (I.M.V., L'Aigle, France), sealed with a heat-sealer, and cooled with LN_2 moderately or rapidly. For moderate cooling, the straw was placed horizontally on a Styrofoam boat (thickness being 15 mm) floated on LN_2 in a Dewar flask (inner diameter being 140 mm, and the temperature on the boat being ~ −150 °C), and left there for 3 min or more before the straw was immersed in LN_2. The average cooling rate between 25 and −100 °C was ~300 °C/min. For rapid cooling, the straw was directly plunged into LN_2 in a Dewar flask. The average cooling rate between 25 and −150 °C was ~2000 °C/min [9].

Straw samples cooled in LN_2 were warmed by three methods, as follows. (1) The straw was taken out of LN_2, kept in air at room temperature for 10 s to pass the glass transition temperature moderately (the temperature of the sample being ~ −100 °C) [8], and then immersed in water at 25 °C (the average warming rate between −100 and 0 °C being ~1200 °C). This method was named "rapid warming" and was considered the control. (2) The straw was taken out of LN_2, and kept in air at room temperature for 120 s, when S-PB1 medium in the straw began to melt, before being immersed in water at 25 °C. This method was named "slow warming" (the average warming rate between −100 and 0 °C being ~170 °C/min). (3) The straw was taken out of LN_2, kept in air at room temperature for 10 s, and then immersed in ethanol at various sub-zero temperatures (−80, −60, −40, or −20 °C). Ethanol had been put in a Dewar flask and placed in a freezer set at each temperature for 24 h or more. After being kept at each temperature for 3 min, 60 min, or 24 h, the straw was immersed in water at 25 °C. This method was named "interrupted rapid warming".

During cooling with LN_2 and during warming, the appearance of the solution was examined. Solutions that remained transparent were considered not to have crystallized whereas those that turned opaque were considered to have formed ice crystals.

Animals and embryos

ICR mice, females about 8 weeks old and adult males (CLEA Japan Inc., Tokyo, Japan), kept on a 14 h light–10 h dark cycle were used. Female mice were superovulated by an intraperitoneal (i.p.) injection of 5 IU of equine chorionic gonadotrophin (Serotropin, Teikokuzoki, Tokyo, Japan), followed by an i.p. injection of human chorionic gonadotropin (hCG) (Puberogen, Sankyozoki, Tokyo, Japan) 48 h later. After the hCG was injected, the females were housed with males of the same strain overnight, and checked for mating by the presence of a vaginal plug the following morning. The females were euthanized by cervical dislocation 76–78 h after the hCG injection, and embryos were collected from uterine horns and from a small portion of the oviduct near the uterine horn using PB1 medium. Embryos judged to be morphologically normal compacted morulae were washed twice in PB1 medium. Morulae from the same cohort were randomly distributed to experimental groups. The room temperature for embryos was adjusted so that the temperature on the bench was at 25 ± 0.5 °C.

All experiments were approved by the Animal Ethics Committee of the College of Agriculture, Kochi University.

Vitrification of embryos

Embryos were vitrified by the protocol reported by Kasai et al. (1990) [5], with some modifications [11,33]. An insemination straw (0.25 ml) was loaded with a large column (~55 mm) of PB1 medium containing 0.5 M sucrose (S-PB1 medium), and two columns of an EFS solution (~5 and ~12 mm) separated by air columns (~25 and ~5 mm). When the embryos were exposed to the EFS solution for 30 or 60 s at 25 °C, they were directly transferred to the solution (~12 mm) in the straw with a minimum volume of PB1 medium. When embryos were exposed to the EFS solution for 120 s, they were first suspended in the EFS solution in a culture dish, washed twice, and transferred into the EFS solution (~12 mm) in the straw. Quickly, by aspiration of the syringe, the cotton plug end was sealed with the first larger column of the S-PB1 medium. Finally, the open end of the straw was sealed with a heat-sealer. After the embryos were exposed for 30, 60, or 120 s in the EFS solution at 25 °C, the straw was placed horizontally on a Styrofoam boat floated on the surface of LN_2 in a Dewar flask for at least 3 min before being immersed in LN_2.

Warming and recovery of vitrified embryos

Vitrified embryos were warmed by a rapid method, a slow method, or an interrupted rapid method as described for the warming of solutions. In all the warming methods, straws were