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Effects of Osmotic Shrinkage on the Survival of Mouse Oocytes and Embryos at Various Developmental Stages

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Abstract: *To examine the sensitivity of mammalian oocytes and embryos to osmotic shrinkage, which can occur during the process of removal of cryoprotectant from cryopreserved cells, the effect of shrinkage on the survival of fresh and vitrified mouse oocytes at metaphase II and embryos at 1-cell to blastocyst was examined. Oocytes and embryos were suspended in PBS media containing various concentrations of sucrose for 30 min at 25°C. They were then returned to isotonic PBS, and the survival was assessed in vitro. Fresh oocytes and embryos were almost entirely insensitive to shrinkage in a solution with 0.75 M sucrose, but in solutions with 1.0–1.5 M sucrose, oocytes and 8-cell embryos were more sensitive to the hypertonic stresses, whereas 2-cell embryos and expanded blastocysts were less sensitive. Vitrified embryos were more sensitive to hypertonic stresses than were fresh ones, but the sensitivity was reduced when the embryos had been cultured for a short period before subjecting them to the hypertonic stress.*

Key words: *Hypertonic stress, Sucrose, Mouse embryo, Developmental stage, Vitrification.*

For successful cryopreservation of mammalian oocytes and embryos in liquid nitrogen, permeation of a cryoprotectant into the cells before cooling is essential. The permeated cryoprotectant must therefore be removed after warming. If cryopreserved oocytes/embryos just after warming are directly returned to an isotonic solution, water will flow into the cell more rapidly than the cryoprotectant diffuses out, and the cells will swell and the membrane may be irreversibly damaged. To prevent this osmotic damage, sucrose is widely used in the process of diluting the cryoprotectant [1–3], because the inclusion of sucrose increases extracellular osmolality, as a non-permeating agent, and thus promotes shrinkage of cells [4]. During suspension of the cells in a sucrose solution, however, the cells shrink as the intracellular cryoprotectant diffuses out.

In 1981, Kasai *et al.* [5] showed that a sucrose solution is harmful to embryos. They exposed mouse morulae to PB1 medium containing 0.75 M sucrose for up to 24 h at 20°C, and found that the survival rate decreased more rapidly than the rate of embryos held in isotonic PB1. It was then shown that the harmful effect is dependent on the concentration of sucrose and on the exposure temperature, and that only 10–20 min of exposure can reduce survival [3, 6, 7]. Mazur and Schneider [7] reported that mouse blastocysts are more resistant to hypertonic solutions than 8-cell embryos, but they prepared the solutions by adding sodium chloride, which can have ionic effects in addition to an osmotic effect. And it is not known whether oocytes and embryos at other stages can survive under various hypertonic conditions. Furthermore, hypertonic effects have been investigated in fresh embryos, although the response of cryopreserved cells to shrinkage may be different from that of fresh cells. The present study examines the effect of hypertonic shrinkage in sucrose solutions on the survival of fresh and vitrified mouse oocytes and embryos at various stages.

Materials and Methods

Fresh oocytes and embryos

Female ICR mice (6–12 weeks old, CLEA Japan, Inc., Tokyo) were induced to superovulate with ip injections of 5 IU of equine chorionic gonadotrophin (eCG) (Serotropin, Teikokuzoki, Tokyo) and 5 IU of human chorionic gonadotrophin (hCG) (Puberogen, Sankyozoki,

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Tokyo) given 48 h apart and were mated with ICR or BDF1 male mice. Ovulated unfertilized oocytes were collected from the ampullar portion of the oviducts at 14 h after hCG injection (without mating) and were freed from cumulus cells by suspending them in modified phosphate-buffered saline (PB1) [8] containing 37 units/ml hyaluronidase instead of bovine serum albumin, followed by washing in fresh PB1 medium. One-cell zygotes, 2-cell embryos and 8-cell embryos were flushed from the oviducts with PB1 medium, and compacted morulae were collected from the uteri of mated animals at 25, 44 to 48, 67 to 68 and 78 to 80 h, respectively, after hCG administration. To obtain expanded blastocysts, morulae and early blastocysts collected at 78–80 h were cultured in a modified Krebs-Ringer-bicarbonate (KRB) medium [9] for 18–27 h under paraffin oil in a culture dish in a humidified CO_2 incubator at 37°C (5% CO_2 in air). They were used when they developed to blastocysts which had an apparently expanded but intact zona pellucida. Oocytes and embryos at each stage were washed and pooled in fresh PB1 medium before use.

Vitrified oocytes and embryos

Oocytes and embryos were vitrified essentially by a simple method reported elsewhere [10, 11], but in order to recover normal oocytes/embryos at maximal rates, they were treated in several solutions and with various procedures depending on the stage.

Vitrification solutions were made by mixing permeating cryoprotectants with FS solution. FS solution was PB1 medium containing 30% (w/v) Ficoll 70 (average molecular weight 70000; Pharmacia, Uppsala) + 0.5 M sucrose. For oocytes, a solution with 10.7% (w/v) acetamide + 10% (v/v) ethylene glycol diluted with FS solution was prepared (designated EAFS10/10). For embryos, ethylene glycol was diluted to 20%, 30% or 40% (v/v) with FS solution to make solutions designated EFS20, EFS30 and EFS40, respectively [12].

The room temperature for treatment of oocytes and embryos was maintained at 25 ± 0.5°C. The oocytes and embryos were suspended in a vitrification solution directly (1-step method) or after pretreatment in a solution containing a lower concentration of ethylene glycol (2-step method), and kept there for a fixed time before vitrification.

Oocytes were suspended in EAFS10/10 in the 1-step method and held there for 2 min [13]. Zygotes were directly suspended in EFS40 in a straw and held for 30 sec. Two-cell embryos were treated by the 2-step method [14]: they were first suspended in EFS20 for 2 min followed by 1 min of exposure to EFS40. Eight-cell embryos and compacted morulae were directly suspended in EFS30 and held for 2 min. Expanded blastocysts were treated by a 2-step method: they were pretreated in PB1 medium containing 10% (v/v) ethylene glycol for 5 min and then suspended in EFS40 for 30 sec [12].

About 10 oocytes/embryos were loaded in each straw, the configuration of which was described elsewhere [10]. After exposure of the oocytes/embryos to a vitrification solution at 25°C for a fixed time, the straws were plunged into a Dewar vessel (inner diameter 55 mm × inside depth 155 mm) containing 5–7 cm depth of liquid nitrogen, so as to immerse about half of the straw, which included the oocytes or embryos, in liquid nitrogen; the rest of the straw loaded with PB1 medium containing 0.5 M sucrose (S-PB1), being in the cold vapor. After 3 min or more, the straws were stored in liquid nitrogen.

The straws were taken out of liquid nitrogen and immediately plunged into water at 25°C. As soon as the crystallized S-PB1 medium in the straw began to melt (after about 5 sec), the straws were removed from the water, quickly wiped dry, and the contents of the straw were expelled into a watch glass containing 0.8 ml of S-PB1 medium, by pushing the cotton plug with a steel rod. The oocytes/embryos were then pipetted into fresh S-PB1 medium. At about 5 min after being flushed out, the oocytes/embryos were transferred to fresh PB1 medium. At recovery, more than 90% of vitrified oocytes and embryos were judged morphologically normal under a dissecting microscope. Some of the morphologically normal oocytes/embryos were then subjected to hypertonic treatments without culture, and others were cultured in a modified KRB medium for 1.5–2 h before hypertonic treatments.

Hypertonic treatments

Hypertonic solutions were prepared by adding sucrose to PB1 medium at concentrations of 0.25, 0.5, 0.75, 1.0 and 1.5 M. As the control, isotonic PB1 medium (0 M) was used. The solutions were placed in 0.2 ml microdrops in a culture dish covered with paraffin oil and equilibrated at 25°C.

Three groups of oocytes/embryos were subjected to hypertonic treatments; 1) fresh oocytes/embryos, 2) morphologically normal oocytes/embryos just after recovery in PB1 medium from liquid nitrogen, and 3) morphologically normal oocytes/embryos after vitrification and further culture for 1.5–2 h. Oocytes and embryos pooled in PB1 medium were directly suspended in PB1 media of various hypertonicities at 25°C. After 30 min of suspension, they were returned to isotonic PB1 medium.

Assessment of survival of oocytes and embryos

Oocytes/embryos recovered after hypertonic treatment were washed and cultured in 0.2 ml of a modified KRB medium [9]. For 2-cell embryos, the medium was further modified by removal of glucose and supplementation of 10 μM-EDTA, 1 mM-glutamine and 10 μM-β-mercaptoethanol. After 0.5–1 h of culture, oocytes, zygotes, 2-cell embryos and 8-cell embryos were examined for the apparent integrity of each blastomere under a dissecting microscope. Morphological scores for 2-cell and 8-cell embryos were calculated from the total number of morphologically normal blastomeres per total number of treated blastomeres. Survival of zygotes was further assessed by the ability to cleave to the 2-cell stage, and that of 2-cell embryos, 8-cell embryos and morulae was assessed by the ability to develop to expanded blastocysts in culture. Survival of expanded blastocysts was assessed by the reexpansion of the blastocoel during 48 h of culture.

Statistical analysis

The survival rate after each treatment was compared with χ^2 tests unless the expected frequency was less than 5, in which case Fisher's exact probability test was used.

Results

The survival rates of oocytes, zygotes, 2-cell embryos and 8-cell embryos assessed by the apparent integrity of the blastomere are shown in Fig. 1, and the survival rates of zygotes, 2-cell embryos, 8-cell embryos, morulae and expanded blastocysts assessed by the development or reexpansion of the blastocoel are shown in Fig. 2. The developmental potential of zygotes, 2-cell embryos and 8-cell embryos (Fig. 2) was in accordance with the morphological appearance (Fig. 1).

When fresh oocytes were treated in solutions containing 0.5 M or 0.75 M sucrose for 30 min at 25°C, no decrease in the proportion of normal morphology was observed. Similar results were obtained when morphologically normal oocytes after vitrification were subjected to the hypertonic treatments immediately after recovery or after a short period of culture, but when fresh oocytes were treated in solutions with 1.0 M and 1.5 M sucrose, the proportions of normal morphology dropped to 14% and 0%, respectively (Fig. 1-A).

High proportions of 1-cell zygotes also retained normal morphology after treatments with 0.5 M and 0.75 M sucrose (Fig. 1-B). After treatment with 1.0 M sucrose, 71% of fresh zygotes were morphologically normal (Fig. 1-B), but when normal zygotes after vitrification were immediately treated with 1.0 M sucrose, the proportion of zygotes which retained normal morphology (30%) was lower than that of fresh oocytes (71%), but when vitrified zygotes were cultured for a short period before the hypertonic treatment, the survival rate increased to 75%.

High proportions of the blastomeres of 2-cell embryos retained normal morphology even after treatment with 1.0 M and 1.5 M sucrose (94% and 71%, respectively) (Fig. 1-C). When vitrified 2-cell embryos were treated with the hypertonic solutions, their survival rates assessed by morphology were significantly lower (34–21%, P<0.01) than those of fresh embryos, but when vitrified embryos were cultured before the hypertonic treatment, their morphological scores (94–86%) were

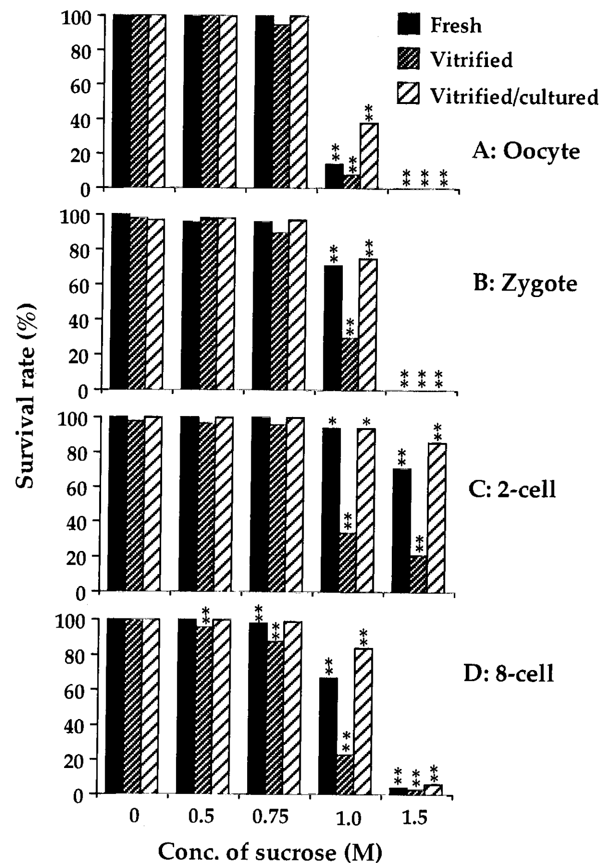

Fig. 1. Survival of mouse oocytes (A) and embryos at 1-cell (B), 2-cell (C), and 8-cell (D) stages, assessed by the apparent integrity of each blastomere, after treatment with PB1 media containing various concentrations of sucrose at 25°C for 30 min. Forty-five to 105 oocytes or embryos were treated for each treatment. Significantly different from the control (0 M sucrose); *P<0.05, **P<0.01.

as high as those of fresh embryos.

Eight-cell embryos were more sensitive to hypertonic stresses than were 2-cell embryos, the morphological scores of fresh embryos being 67% and 4% after treatments in 1.0 M and 1.5 M sucrose, respectively (Fig. 1-D). In fresh morulae, more embryos survived after hypertonic treatments with 1.0 M sucrose than 8-cell embryos (75% vs 49%, P<0.01) (Fig. 2-D). Expanded blastocysts were less sensitive to hypertonic stresses than morulae: 98% of fresh blastocysts regained their large blastocoels in culture after treatment with 1.0 M sucrose, and the proportion was not significantly different from that of control embryos (Fig. 2-E). In 8-cell embryos, morulae and expanded blastocysts, vitrification significantly increased the sensitivity to the hypertonic stress. But, as was typically observed in 2-cell embryos, a short period of culture after vitrification made the embryos less sensitive to hypertonic stresses.

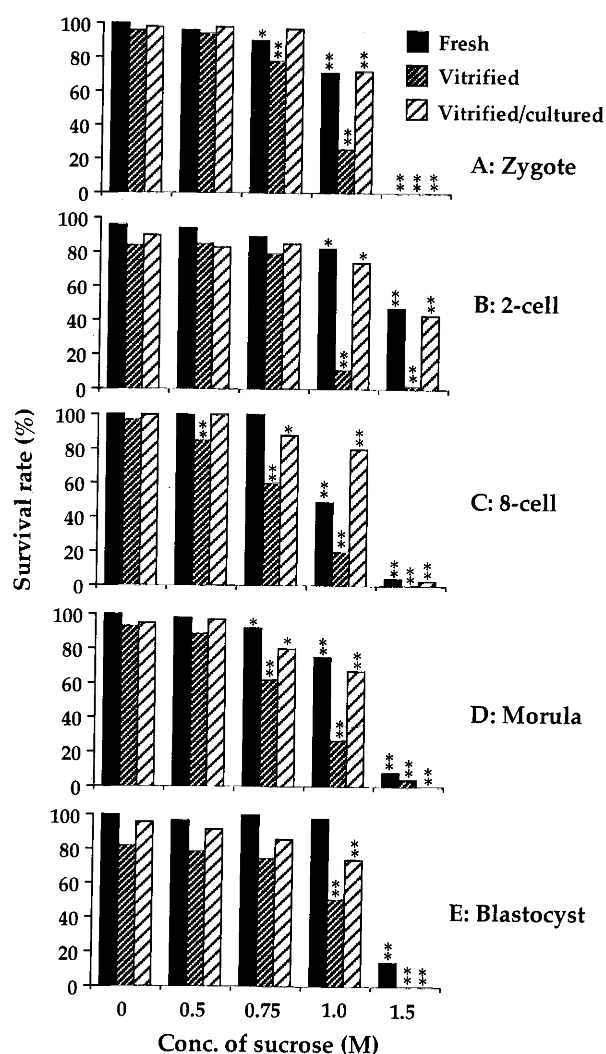

Fig. 2. Survival of mouse embryos at 1-cell (A), 2-cell (B), 8-cell (C), morula (D) and expanded blastocyst (E) stages, assessed by the cleavage to the 2-cell stage (A), by the development to expanded blastocysts (B, C and D), or by the reexpansion of the blastocoel (E), after treatment with PB1 media containing various concentrations of sucrose at 25°C for 30 min. Forty-five to 105 embryos were subjected to each treatment. Significantly different from the control (0 M sucrose); *P<0.05, **P<0.01.

Discussion

Because Kasai *et al.* [5, 15] found that hypertonic shrinkage in PB1 medium containing 0.5 to 0.75 M sucrose is not harmful but rather beneficial for the survival of mouse morulae if they are refrigerated at 0°C, shrinkage of the cells to a certain extent must not be harmful, but the results of the present study showed that 30 min of exposure to hypertonic solutions, especially those with 1.0 to 1.5 M sucrose, can reduce the survival in all the stages of oocytes/embryos examined. In our study, the survival rates for stressed embryos assessed by the integrity of each blastomere (Fig. 1) were close to those assessed by the developmental ability (Fig. 2), not only in 1-cell zygotes but also 2-cell and 8-cell embryos. This suggests that the hypertonic injury is related to the physical damage to the plasma membrane. Mazur and Schneider [7] observed the relative volume of mouse 8-cell embryos in hypertonic solutions with various concentrations of sodium chloride and showed that they behave as ideal osmometers over a wide range of tonicities but they cannot shrink in response to the osmolalities in highly anisotonic solutions (4X or more). On the other hand, Yang *et al.* [16] suspended rabbit morulae in hypertonic solutions containing various concentrations of sucrose and showed that exposure to 1.5 M sucrose was harmful. In addition, they measured the change in the volume of the morulae during suspension; following the initial rapid shrinkage, the volume of the embryos remained constant in the presence of 0.5 M and 1.0 M sucrose but increased gradually in 1.5 M sucrose during 60 min of suspension. They also observed that the embryos suspended in 1.5 M sucrose expanded and were damaged after returning to an isotonic solution. These observations strongly suggest that the harmful effect of sucrose is caused by membrane damage due to excessive shrinkage, permitting sucrose to enter the cells.

The present results show that the sensitivity of the cells to the hypertonic solutions varies according to the

stage of development. Most fresh oocytes/embryos were resistant in 0.75 M sucrose, but in 1.0 M sucrose unfertilized oocytes are the most sensitive, and 8-cell embryos were also sensitive, whereas expanded blastocysts were more resistant to the solution. These results are in agreement with the report of Mazur and Schneider [7] that blastocysts are more resistant to the hypertonic stress than 8-cell embryos, although they used solutions containing various concentrations of sodium chloride. Furthermore, 2-cell embryos seems to be the most resistant to osmotic shrinkage, being able to survive even in the presence of 1.5 M sucrose. These results are consistent with the report on rabbit embryos indicating that 2–4 cell embryos are more resistant to 1.5 M sucrose solution than morulae [16], but the reason for the resistance, probably of the cell membrane, of 2-cell embryos is not known.

After cryopreservation, the permeated cryoprotectant is usually removed at room temperature, because cryoprotectants will diffuse out of the cells more rapidly at a higher temperature since the permeability of the cell membrane increases as the temperature rises [17, 18]. Oocytes and embryos are therefore at a risk of shrinkage at room temperature just after warming from cryopreservation, so vitrified oocytes/embryos in the present study were then subjected to hypertonic stresses. The results show that embryos at all stages become more sensitive to the stresses after cryopreservation, although the decrease in survival observed in oocytes was not significant. In 8-cell embryos, even suspension in a solution with 0.5 M sucrose reduced the survival significantly, but the sensitivity to the hypertonicity which is derived from the cryopreservation, as is typically observed in a solution with 1.0 M sucrose, is reduced or abolished when they are cultured for a short period before subjecting them to the stress (Figs. 1 and 2). Kasai *et al.* [15] showed that mouse embryos refrigerated in a sucrose solution are more sensitive to freeze-thawing than are fresh ones, but that incorporation of a short period of culture between the refrigeration and the freezing decreases the sensitivity significantly. Although the nature of the positive effects of the culture is not yet known, the mechanism seems to be the same as was observed for cryopreserved embryos in the present study. One speculation is that some of the proteins responsible for the flexibility of the membrane are damaged by the cryopreservation but their synthesis is resumed during the culture.

Oocytes and embryos can shrink not only during the process of removal of permeated cryoprotectant, but also during the suspension in a cryoprotectant solution before cryopreservation. Sucrose is sometimes included in the freezing medium or vitrification medium [10, 19–21], and the concentration of the cryoprotectant can be extremely high in vitrification. On the other hand, oocytes and embryos are often suspended in a hypertonic solution to enlarge the perivitelline space for micromanipulation, but the present results suggest that exposure of fresh oocytes and embryos to hypertonic solutions is less harmful than exposure after cryopreservation.

In the present study, sucrose was adopted as a non-permeating molecule which has a considerable osmotic effect, but the effect will not be specific to sucrose, because other mono- and di-saccharides, such as glucose, galactose and fructose, have been shown to have the same protective effect as sucrose when added at 0°C [22]. It is shown in rabbit zygotes that sodium chloride is more toxic than sucrose, probably because of the ionic strength [16].

In conclusion, the sensitivity of mouse fresh oocytes and embryos to the hypertonic stress in sucrose solutions differs among developmental stages: 2-cell embryos and expanded blastocysts are more resistant, but unfertilized oocytes and 8-cell embryos are more sensitive. Cryopreserved cells just after warming are more sensitive to osmotic swelling than are fresh ones, and even shrinkage corresponding to suspension in solutions with 0.5 to 0.75 M sucrose may decrease survival in some stages.

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