1. Introduction

Medaka (*Oryzias latipes*) is a useful vertebrate model for the study of various life processes. This teleost has relatively large embryos (~1.2 mm in diameter), which develop and hatch easily, facilitating observation and examination of developmental processes. Various strains and genetic variants of medaka have been established, which makes this fish a valuable model in genetics. However, they have been maintained by mating because it is not possible to cryopreserve fish embryos at ultra-low temperatures. Therefore, cryopreservation of teleost embryos would have a great impact not only on the preservation of laboratory species, but also on aquaculture and the conservation of diverse aquatic species.

To develop a method of cryopreserving fish embryos, a number of obstacles must be overcome. One limiting factor is the susceptibility to chilling injury, in which cells are injured by cooling to temperatures above freezing, i.e., around 0 °C. In zebrafish, another small model fish, embryos at some stages are quite sensitive to chilling [1,2]. In other teleost species, including rainbow trout [3], red sea bream [4], olive flounder [4], multicolorfin rainbowfish [4], fathead minnow [5], goldfish [6], red drum [7], and carp [8], it has also been reported that embryos at some stages are sensitive to chilling. In medaka, however, the susceptibility of the embryo to chilling has not been reported. In the present study, we examined the sensitivity to chilling of medaka embryos at various developmental stages.

2. Materials and methods

2.1. Embryos

Adult wild-type medaka were purchased from a local fish breeder. Up to 100 mature male and female medaka were kept together in an aquarium of about 60 L under a 14-h light and 10-h dark cycle in aerated and filtered water at 25 °C. The fish were fed with powdered dry flakes (TetraMin, TetraWelke, Melle, Germany) thrice daily.

To collect embryos at the 2–4 cell stage, a female medaka bearing an embryo cluster at the abdominal wall was netted at the beginning of the light period, presumably 1–2 h after ovulation. The medaka was held on its back and the embryo cluster was collected gently using the surface of the net. Embryo clusters from several females were placed in Hanks solution containing 0.05 g/L gentamycin at pH 7.0 with the osmolality of 300 mOsm/kg [9], further supplemented with ~0.003 mg/L methylene blue, in a petri dish (90 mm × 10 mm). In an embryo cluster, eight to 10 embryos are usually included and attached to each other with long hairs on the chorion at the vegetal pole area. To separate the embryos, the attaching hairs were held with fine watchmaker forceps and were excised with microsurgery scissors (with the aid of a dissecting microscope). Compared with a previous report in which attached embryos were rolled between moistened finger tips [10], our method for embryo separation minimized embryo damage and microbial contamination.

The separated 2–4 cell embryos were placed in 4 mL of Hanks solution in a culture dish (35 mm × 10 mm, no. 153066, Nunc, Roskilde, Denmark). The embryos were used as 2–4 cell embryos or were incubated in the solution at 26 °C for 1, 4, 5–8, or 9–11 h, to promote

development to the 8–16 cell, morula, blastula, or early gastrula stage, respectively. The stages were identified using the figures described by Iwamatsu [11]. We had previously confirmed that the Hanks solution supported the development of 2–4 cell medaka embryos. Methylene blue was included to inhibit bacterial and fungal growth and to aid in the visualization of damaged embryos.

After chilling treatments, embryos were incubated in 4 mL of Hanks solution at 26 °C in a culture dish for up to 14 d. The solution was changed every 3 d until the ninth day, and daily thereafter. The survival of the embryos was assessed by their ability to hatch to live fries during culture. As a control, 2–4 cell embryos were cultured without any treatment.

2.2. *Chilling sensitivity of embryos at various developmental stages*

One milliliter of Hank's solution was placed in 1.5 mL microcentrifuge tubes (no. 616201, Greiner Bio-One, Kremsmunster, Austria) and the tubes were in ice water for 10 min. A Pasteur pipette and mouth piece was used to place embryos at the 2–4 cell, 8–16 cell, morula, blastula, and early gastrula stages into the tubes, which were then placed in ice water at 0 °C. After 20 min, the tubes were placed into a water bath at 25 °C for 5 min. To assess survival, embryos were recovered from the bottom of the tube (with a pipette) and cultured in 4 mL of Hanks solution at 26 °C in a culture dish. For each treatment, 115–141 embryos were used, except for the control in which 164 embryos were treated. The experiment was replicated 9 or 10 times.

2.3. *Chilling sensitivity of 2–4 cell embryos*

To examine the effect of the duration of chilling, embryos at the 2–4 cell stage were placed in 1 mL of Hanks solution (pre-cooled at 0 °C in a microcentrifuge tube), After 2, 10, 20, or 40 min, the sample was warmed (25 °C) and then the embryos were recovered and cultured in Hanks solution at 26 °C. For each treatment, 150–170 embryos were used, except for the control in which 197 embryos were treated. The experiment was replicated 12 times.

To examine the effect of the chilling temperature, embryos at the 2–4 cell stage were suspended in 1 mL of Hanks solution in a microcentrifuge tube at −5, 0, 5, or 10 °C for 20 min. The solution in a tube at −5 °C had been placed in water in an incubator at −5 °C for 1 h, and solutions at 0, 5, and 10 °C had been kept in water at each temperature for 10 min beforehand. The temperature of each solution after equilibration (±0.5 °C) was confirmed by a small portable digital thermometer. As a control, embryos were kept at 25 °C for 20 min. After the treatment, embryos were recovered and cultured in Hanks solution at 26 °C. For each treatment, 117–139 embryos were used. The experiment was replicated 13 times.

To examine the effect of repeated rapid cooling and warming, 2–4 cell embryos in Hanks solution were loaded in a 0.25 mL insemination straw (IMV, L' Aigle, France) and sealed. The straw was immersed in ice water, shaken for 10 s, and then kept there for an additional 50 s (total time at 0 °C, 1 min). Thereafter, the straw was immersed in water at 25 °C for 1 min (shaken for 10 s and retained for an additional 50 s). This cooling/warming treatment was repeated five times, thus the total time chilled at 0 °C was 5 min. The

survival of the embryos was compared with that of those kept in Hanks solution at 0 °C for 20 min in a microcentrifuge tube. Control embryos were kept in Hanks solution at 25 °C for 20 min. Embryos were recovered and cultured in Hanks solution at 26 °C. For each treatment, 180 embryos were used and the experiment was replicated 10 times.

2.4. Preservation of early gastrula embryos by refrigeration

To examine the tolerance to prolonged chilling, embryos at the early gastrula stage were placed in 1 mL of Hanks solution in a microcentrifuge tube and kept at 0 or 5 °C for 12 h, 24 h, 3 d, 5 d, or 10 d. Then the tubes were placed in water at 25 °C for 5 min, and the embryos were recovered and cultured in Hanks solution at 26 °C. For each treatment, 132–172 embryos were used. The experiment was replicated eight or nine times.

2.5. Statistics

The proportion of embryos that hatched was compared with a χ^2-test ($P < 0.05$ was considered significant).

3. Results

3.1. Chilling sensitivity of embryos at various developmental stages

Embryos at the 2–4 cell stage were sensitive to chilling (Fig. 1); the hatching rate after 20 min of chilling at 0 °C (28%) was markedly lower ($P < 0.01$) than the rate for the untreated control (85%). The hatching rate increased as development proceeded to the

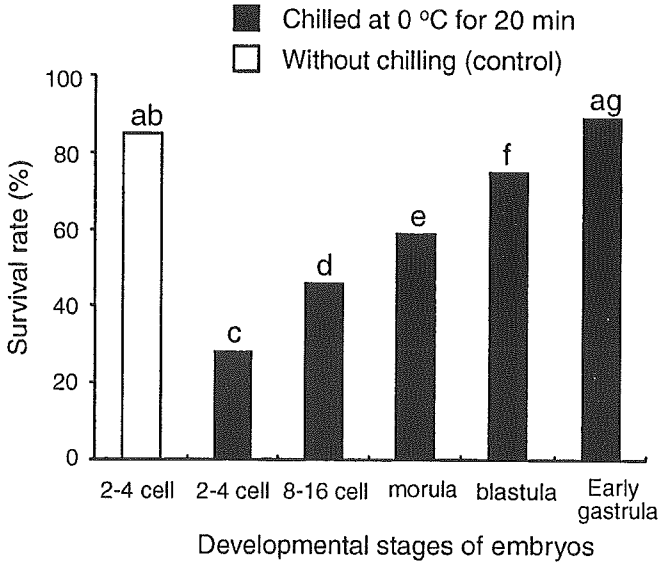

Fig. 1. Survival of medaka embryos at various developmental stages after chilling at 0 °C for 20 min. Survival was assessed as the ability to hatch into live fries during 14 d of culture. For each treatment, 115–141 embryos were used, except for the control in which 164 embryos were treated. The experiment was replicated 9–10 times. Values with different superscripts are different ($P < 0.01$; b–f and d–e, $P < 0.05$).

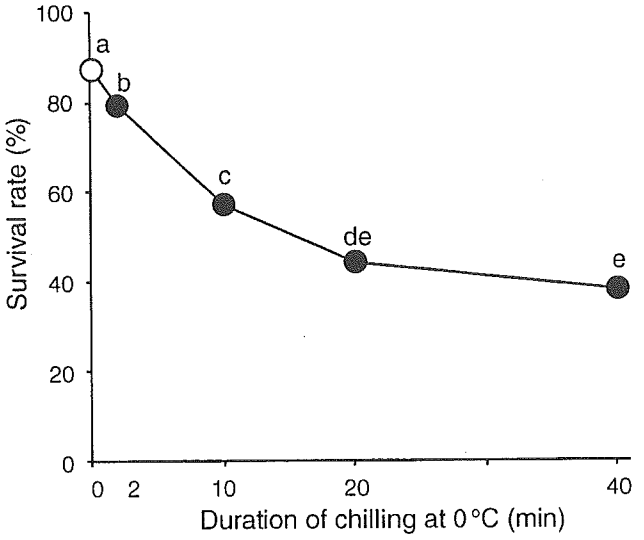

Fig. 2. Survival of medaka embryos at the 2–4 cell stage after chilling at 0 °C for various periods. Survival was assessed as the ability to hatch into live fries during 14 d of culture. For each treatment, 150–170 embryos were used, except for the control in which 197 embryos were treated. The experiment was replicated 12 times. Values with different superscripts are different ($P < 0.01$; a–b and c–d, $P < 0.05$).

8–16 cell (46%; $P < 0.01$), morula (59%; $P< 0.05$), and blastula (75%; $P < 0.01$) stages, and the rate for early gastrula embryos reached 89%.

3.2. *Chilling sensitivity of 2–4 cell embryos*

Based on the results of the first series of experiments, in which embryos at the 2–4 cell stage were found to be the most sensitive to chilling, the nature of the chilling sensitivity was examined in these embryos. As shown in Fig. 2, the hatching rate of 2–4 cell embryos was lower than controls after only 2 min of chilling at 0 °C (79% versus 87%, $P < 0.05$).

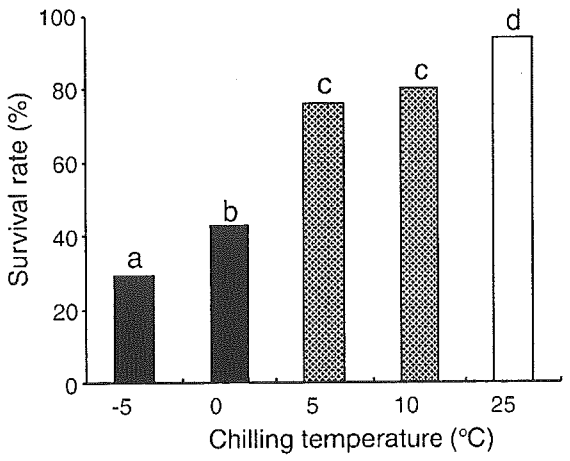

Fig. 3. Survival of medaka embryos at the 2–4 cell stage after chilling at various temperatures for 20 min. Survival was assessed as the ability to hatch into live fries during 14 d of culture. For each treatment, 117–139 embryos were used. The experiment was replicated 13 times. Values with different superscripts are different ($P < 0.01$; a–b, $P < 0.05$).

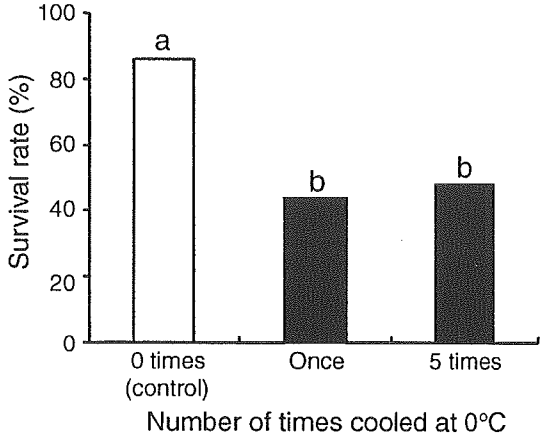

Fig. 4. Survival of medaka embryos at the 2–4 cell stage after 5 rounds of rapid cooling between 25 and 0 °C. The survival of the embryos, assessed as the ability to hatch into live fries during 14 d of culture, was compared with that of those kept chilled at 0 °C. For each treatment, 180 embryos were used and the experiment was replicated 10 times. Values with different superscripts are different ($P < 0.01$).

The hatching rate further decreased as the chilling time increased to 10 min (57%; $P < 0.01$) and 20 min (44%; $P < 0.05$). However, 38% of embryos still retained the ability to hatch after 40 min of chilling.

Fig. 3 shows the effect of the chilling temperature on the survival of 2–4 cell embryos. They were apparently sensitive to 20 min of chilling at 0 or −5 °C, because the hatching rates (43 and 29%, respectively) were considerably lower than the control rate (94%; $P < 0.01$). On the other hand, the decrease in the hatching rate after chilling to 5 or 10 °C (76–80%) was small but significant ($P < 0.05$).

To examine the nature of the chilling sensitivity of 2–4 cell embryos, they were chilled and warmed rapidly (0 and 25 °C) five times. The survival rate of the embryos (48%) was not different from that of embryos kept at 0 °C for 20 min (44%; Fig. 4).

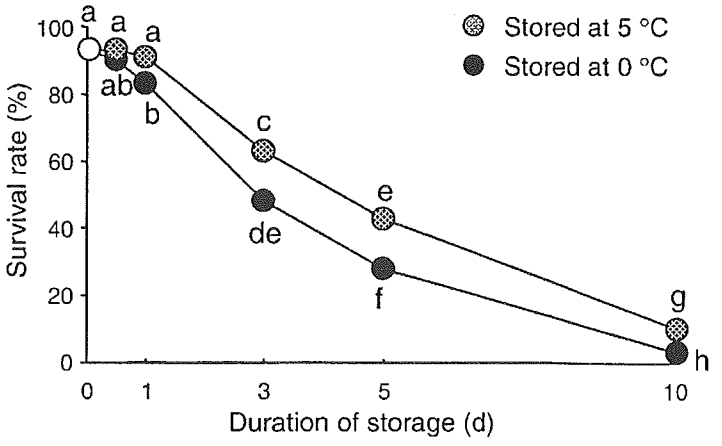

Fig. 5. Survival of medaka embryos at the early gastrula stage after storage at 5 or 0 °C for prolonged periods. Survival was assessed based on the ability to hatch into live fries during 14 d of culture. For each treatment, 132–172 embryos were used. The experiment was replicated eight to nine times. Values with different superscripts are different ($P < 0.01$; a–b, c–d and g–h, $P < 0.05$).

3.3. Preservation of early gastrula embryos by refrigeration

From the results of the first series of experiments, where embryos at the early gastrula stage were found to be quite less sensitive to chilling, embryos were preserved at 0 or 5 °C for prolonged periods. As shown in Fig. 5, the hatching rate did not decrease after 12 and 24 h of chilling at 0 and 5 °C, respectively (91–93%). However, the rate decreased to 48–63% as the duration of storage increased to 3 d ($P < 0.01$). Nevertheless, 3–10% of the embryos hatched after 10 d of storage. Therefore, embryos at the early gastrula stage would be nearly insensitive to chilling. After storage for >1 d, higher proportions of embryos survived at 5 °C versus 0 °C ($P < 0.05$).

4. Discussion

In mammals, embryo cryopreservation techniques have been established and used for the preservation of genetic variants [12]. However, cryopreservation of fish embryos has not been possible. For successful cryopreservation, major obstacles such as intracellular ice formation, cryoprotectant toxicity, and osmotic overswelling need to be overcome. In fish embryos, these obstacles are related to the large size of the embryo, low permeability of membranes surrounding the embryo, and presence of a large amount of yolk, a different compartment from embryonic cells. Another primary obstacle is chilling injury, in which certain types of cells are damaged just by cooling to supra-freezing temperatures, because cells must pass through chilling temperatures before being cooled at ultra-low temperatures.

In the present study, medaka embryos at the 2–4 cell stage were the most sensitive to chilling at 0 °C. However, sensitivity decreased as the development proceeded and early gastrula embryos were considered insensitive to chilling. In supplementary experiments, embryos at the 4-somite stage were also insensitive to chilling at 0 °C (data not shown). A study on the impregnation of cryoprotectant suggests that 20 min of exposure of medaka eyed embryos at 0–5 °C does not affect the survival [14]. Although 2–4 cell embryos were sensitive to chilling at 0 °C, more than 30% retained the ability to hatch even after 40 min of chilling (Fig. 2). In contrast, all zebrafish embryos at early cell division stages (8–64 cell stage) lost their ability to develop after 40 min of chilling at 0 °C [2]. It appears that medaka embryos are less susceptible than zebrafish embryos to chilling injury. If slower developing embryos recover from chilling stress during the subsequent culture [8], this difference between species may be related to rate of development. In that regard, medaka embryos take 9–14 d to hatch whereas zebrafish embryos develop to the hatching stage in only 3 d.

Zebrafish embryos at early cell division stages are sensitive to chilling, but this sensitivity decreases as development proceeds [1,2]. Furthermore, early stage embryos are more sensitive to chilling than later-stage embryos in some fish, including rainbow trout [3], fathead minnow [5], and olive flounder [4], whereas in other species like red seabream [4], multicolorfin rainbowfish [4], goldfish [6], red drum [7], and carp [8], later-stage embryos are more sensitive to chilling than some earlier stage embryos. Generally, however, embryos at early cleavage stages are the most sensitive to chilling, with the

exception of red drum embryos in which those at the 8-cell to morula stage are more sensitive to chilling than 2-cell embryos [7]. It is interesting that earlier stage embryos are also more sensitive to chilling in *Drosophila* embryos [13].

The sensitivity of fish embryos to chilling is attributed to the amounts of lipid components [15]. In fish embryos, a majority of lipid components are present in the yolk and the rest in the membranes. In zebrafish embryos, partial removal of yolk reduced sensitivity to chilling [15,16]. In porcine embryos, the amount of lipid is related to chilling sensitivity [17]. Perhaps medaka embryos at the 2–4 cell stage contain more lipids than later-stage embryos.

In the present study, the survival rate of 2–4 cell embryos chilled at −5 °C (29%) was lower ($P < 0.05$) than that at 0 °C (43%). At 5 °C, on the other hand, the chill sensitivity of 2–4 cell embryos was marginal, because the survival rate at this temperature was relatively high (76%). Similarly, the survival of chilled zebrafish embryos is temperature-dependent; the survival rate is higher at 0 than at −5 °C [1,16]. The chilling sensitive temperature will be determined by the lipid profile of the membrane [18–20]. In mammalian gametes, the change in composition of membrane lipids alters the phase transition temperature, and thus reduces the chilling sensitivity [21]. However, it is not known if this theory applies to non-membrane lipids like those in teleost yolk.

In the present study, the survival of 2–4 cell medaka embryos was affected not by repeated rapid cooling but by the duration of chilling. Liu et al. [16] reported that zebrafish embryos at the 64-cell stage are also more sensitive to duration of exposure than the cooling rate. The nature of chilling injury is classified into two categories; one which depends more on the cooling rate and the other which depends more on the duration of chilling [22]. For instance, chilling injury in pig spermatozoa is dependent on cooling rate [19], whereas injury to *Drosophila* embryos is more dependent on the duration of chilling [13]. Therefore, the type of chilling injury in medaka embryos may be more like that in zebrafish embryos and even *Drosophila* embryos. Since *Drosophila* embryos have been successfully cryopreserved, information on them might be valuable in the development of protocols for cryopreservation of medaka embryos.

The mechanism of chilling injury has been studied mostly in the membrane system. In mammalian sperm [19,20] and oocytes [22], lipid phase transitions in cell membranes are responsible for the injury. At temperatures around phase transition, chilled membranes lose fluidity and become leaky, which causes damage to cells [22,23]. Because the fluidity of lipids is determined by fatty acids constituting the lipid bilayer, membrane lipid profiles determine the lipid phase transition temperature [20,24]. Membranes with higher levels of cholesterol or polyunsaturated fatty acids are more fluid and stable at low temperatures; therefore, they are more resistant to chilling [18–20]. As described above, Zeron et al. [21] showed that it is possible to change the lipid phase transition temperature, and thus reduce chilling injury, by altering the membrane profile using liposomes. On the other hand, the mechanism of chilling injury in non-membrane lipid components such as those in teleost yolk is not well understood. However, it is clear that yolk lipids are associated with chilling injury; Liu et al. [15,16] showed that a partial removal of yolk from zebrafish embryos reduced the chilling sensitivity. Perhaps similar mechanisms are also involved in yolky lipids. As development proceeds in fish embryos, it is expected that the composition of yolky lipids changes. Therefore, the phase transition temperature of the yolky lipids would

change with the development of embryos, which could be related to the stage-dependent differences in the chilling sensitivity of fish embryos.

In the present study, we did not further characterize chilling sensitivity for 8–16 cell, morula and blastula embryos, because we assumed that the characteristics of these embryos would qualitatively be the same as the characteristics of 2–4 cell embryos, and quantitatively decrease gradually as the stage proceeds.

In our first series of experiments, embryos at the early gastrula stage were much less sensitive to chilling at 0 °C for 20 min. In the experiment on prolonged refrigeration, survival of early gastrula embryos (assessed by the hatching rate) did not decrease after storage for 12 h at 0 °C and for 24 h at 5 °C. Therefore, embryos at this stage were totally insensitive to chilling. That it is possible to arrest embryonic development without causing adverse effects should be useful for experiments that use embryos at this stage; many embryos in the same batch could be used for time-consuming experiments. Furthermore, higher proportions (48–63%) of early gastrula embryos could develop to the hatching stage after storage for 3 d. Therefore, it should be possible to transport medaka colonies by sending refrigerated embryos.

For the cryopreservation of medaka embryos, chilling injury seems to be overcome by using embryos at less- or non-sensitive stages. On the other hand, it may be possible to use even 2–4 cell embryos, because the chill sensitivity in medaka embryos seems to be relatively low even at earlier stages. In bovine oocytes or porcine embryos, which are considerably sensitive to chilling, the ultrarapid vitrification approach is effective in which cells are vitrified and warmed with a minimal volume of the solution, using minute tools [25,26]. Although it might be possible to circumvent the chilling injury of medaka embryos by an ultrarapid approach, the larger size of medaka embryos should be considered.

Blastomeres of medaka embryos have been cryopreserved at approximately −79 °C [27], and medaka whole embryos have been successfully frozen, but only to −40 °C [28]. For the successful cryopreservation of teleost embryos at ultra-low temperatures, various problems must be solved in addition to chilling injury. The biggest obstacle would be the formation of intracellular ice. An essential limiting factor that relates to preventing intracellular ice from forming is the permeability of the cell membrane to water and cryoprotectant. To overcome the permeability barrier, Janik et al. [29] tried to microinject cryoprotectants into zebrafish embryos. Although this elevated the concentration of the cryoprotectant in the yolk, it did not improve the post-thaw morphology of the embryo. As a strategy to increase membrane permeability, Hagedorn et al. [30] showed that the artificial expression for mRNA of a water- and cryoprotectant-channel (aquaporin 3) increased the permeability of zebrafish embryos. Therefore, this strategy might also be effective for medaka embryos.

In the present study, the criterion of survival was hatching. Although precise data on abnormalities of chilled fish embryos after hatching have not been reported, a more extensive examination of normality [2,16] would have been preferable.

The survival rate of 2–4 cell embryos chilled for 20 min at 0 °C seemed lower in Experiment 1 versus Experiment 2 (28% versus 44%). Although the same protocol was used in the two experiments, they were conducted consecutively and not contemporaneously. The apparent difference in survival rates might have been due to difference in the colony of medaka with regard to the season of introduction, age, etc.

In conclusion, medaka embryos in the earlier stages of cell division were more sensitive to chilling at 0 to −5 °C; the sensitivity was related to the duration of exposure to low temperatures, and not to repeated rapid cooling and warming. The sensitivity decreased as development proceeded; early gastrula embryos were totally insensitive (they tolerated 12 h of refrigeration at 0 °C or 24 h at 5 °C with no decrease in viability). From the viewpoint of chilling sensitivity, therefore, later-stage embryos would be preferable for cryopreservation, although chilling injury was not highly deleterious even in earlier stage embryos.

Acknowledgment

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Water- and cryoprotectant-permeability of mature and immature oocytes in the medaka (*Oryzias latipes*)☆

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Abstract

The permeability of the plasma membrane plays a crucial role in the successful cryopreservation of oocytes/embryos. To identify a stage feasible for the cryopreservation of teleost oocytes, we investigated the permeability to water and various cryoprotectants of medaka (*Oryzias latipes*) oocytes at the germinal vesicle (GV) and metaphase II (MII) stages. In sucrose solutions, the volume changes were greater in GV oocytes than MII oocytes. Estimated values for osmotically inactive volume were 0.41 for GV oocytes and 0.74 for MII oocytes. Water-permeability (μm/min/atm) at 25 °C was higher in GV oocytes (0.13 ± 0.01) than MII oocytes (0.06 ± 0.01). The permeability of MII oocytes to various cryoprotectants (glycerol, propylene glycol, ethylene glycol, and DMSO) was quite low because the oocytes remained shrunken during 2 h of exposure in the cryoprotectant solutions at 25 °C. When the chorion of MII oocytes was removed, the volume change was not affected, except in DMSO solution, where dechorionated oocytes shrunk and then regained their volume slowly; the P_{DMSO} value was estimated to be $0.14 \pm 0.01 \times 10^{-3}$ cm/min. On the other hand, the permeability of GV oocytes to cryoprotectants were markedly high, the P_s values ($\times 10^{-3}$ cm/min) for propylene glycol, ethylene glycol, and DMSO being 2.21 ± 0.29, 1.36 ± 0.18, and 1.19 ± 0.01, respectively. However, the permeability to glycerol was too low to be estimated, because GV oocytes remained shrunken after 2 h of exposure in glycerol solution. These results suggest that, during maturation, medaka oocytes become less permeable to water and to small neutral solutes, probably by acquiring resistance to hypotonic conditions before being spawned in fresh water. Since such changes would make it difficult to cryopreserve mature oocytes, immature oocytes would be more suitable for the cryopreservation of teleosts.

Keywords: Medaka; Oocyte; Cryoprotectant; Water; Permeability

Long-term storage of embryos is useful for the management of various stocks of model organisms. Such storage has been used for several mammalian embryos. However, the cryopreservation of

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fish embryos has not succeeded because several factors are suspected to complicate the process [21]. Fish embryos have a large volume, a large amount of egg-yolk, and a thick chorion, form a complex structure during development, and are susceptible to chilling. Furthermore, fish embryos have several compartments with differing osmotic properties [7]. For instance, it is known in zebrafish embryos that, soon after the 50–70% epiboly stage, the yolk is surrounded by the multinucleated yolk syncytial layer, which is a permeability barrier to cryoprotectants [4,7]. For successful cryopreservation, the rapid movement of water and cryoprotectants through the membrane is essential.

The medaka (*Oryzias latipes*) is an important experimental fish for developmental biology, genetics, and physiology [2]. Strüssmann et al. [23] reported the successful cryopreservation of blastomeres of medaka embryos, but as with embryos of other teleosts, it is not possible to cryopreserve the whole embryo. Fortunately, however, medaka sperm can be easily cryopreserved [1]. Therefore, the long-term preservation of various stocks of medaka could be realized if the oocytes could be cryopreserved.

Unfortunately, fish oocytes have similar features to embryos that complicate cryopreservation, i.e., a large size and the presence of a yolk and chorion. However, oocytes are constituted from only a single compartment and do not have permeability barriers like the multinucleated yolk syncytial layer found in embryos. These features would be advantages for cryopreservation. The permeability of matured fish oocytes has been estimated in salmon [17,20], trout [9], and medaka [22]. In medaka, it has been shown that the cryoprotectant-permeability of the oocyte is lower than that of embryos [22]. Moreover, it is known that, in early stages of medaka embryos, the cryoprotectant-permeability of the plasma membrane increases as development proceeds [22]. Therefore, it seems that fish oocytes have the disadvantage of a lower cryoprotectant-permeability. However, the oocytes used in the studies mentioned above were ovulated, and were in the stage where they were just about to be exposed to non-isotonic conditions, that is, fresh water (hypotonic). Therefore, it would be reasonable to assume that such matured oocytes have already acquired the ability to tolerate non-isotonic conditions. On the other hand, fish oocytes at earlier stages might not have acquired this tolerance and might have different permeability properties from matured ones. However, no information is available on the permeability of immature oocytes in fish.

In this study, we examined the water- and cryoprotectant-permeability of medaka oocytes at the germinal vesicle (GV) stage and the metaphase II (MII) stage in order to identify a stage feasible for oocyte cryopreservation.

Materials and methods

Collection of oocytes

About 20–100 matured orange-red type medaka, purchased from a local fish dealer, were maintained in 60-liter aquariums under 14-h light and 10-h dark periods at 25 °C. Newly ovulated MII oocytes were obtained from ovarian cavities of actively spawning females humanely killed 1 h before the start of the light period and kept in saline formulated for medaka oocytes (SMO medium) [13] at room temperature prior to use. The composition of the solution is as follows; NaCl, 6.50 g; KCl, 0.40 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; $MgSO_4 \cdot 7H_2O$, 0.15 g; $NaHCO_3$, 1.00 g; phenol red, 0.015 g; in 1 L of distilled water with 5 mM Hepes–HCl, pH 7.0 [13]. The oocytes were used for experiments within 2 h of collection. Fully grown GV oocytes were obtained from ovaries of actively spawning females humanely killed 1–3 h before the start of the dark period, and cultured in 90% tissue culture medium (TCM)-199 with Earle's salts (Cat. No. 31100-035, Gibco Invitrogen, Carlsbad, CA, USA) [12] at 25 °C for 2–4 h prior to use.

In some experiments, the chorion of MII oocytes was removed using microsurgical scissors (6 mm) and tweezers (0.1 × 0.06 mm tip, 11 cm length) [13]. After being kept in SMO medium at room temperature for 30 min, dechorionated oocytes were examined and the non-activated ones (with small oil droplets dispersed in the cytoplasm)

selected and used for experiments within 1.5 h. We did not measure volume changes of dechorionated GV oocytes in sucrose or cryoprotectant solutions because we could not surgically or enzymatically remove the chorion of GV oocytes without causing damages.

Osmotically inactive fractions

Intact MII oocytes were placed in SMO medium containing 0.26 and 1.26 Osm/kg sucrose (0.24 and 0.91 M, respectively), excluding the 0.24 Osm/kg contributed by the SMO medium, or in SMO medium diluted with distilled water (×0.50 or ×0.63, 0.12 or 0.15 Osm/kg, respectively) at 25 ± 1 °C until the oocytes were equilibrated with the solutions (for 3 h). GV oocytes were placed in 90% (v/v) TCM-199 containing 0.23 and 1.23 Osm/kg sucrose (0.21 and 0.89 M, respectively), excluding the 0.27 Osm/kg contributed by the 90% TCM-199 medium, or in 90% TCM-199 diluted with distilled water (×0.44 or ×0.56, 0.12 or 0.15 Osm/kg, respectively), at 25 °C for 2 h. The osmolality of sucrose was calculated from published data on the colligative properties of sucrose in aqueous solutions [27]. The osmolality of the basic media and diluted basic media was measured with an osmometer (OM801; Vogel, Giessen, Germany). Total osmolality of the solutions is shown in Table 1. We assumed that the osmolality of oocyte cytoplasm was equilibrated with that of basic media (GV oocytes, 0.27 Osm/kg; MII oocytes, 0.24 Osm/kg) because, until use for experiments, GV oocytes were incubated in 90% TCM-199 medium for 2–3 h and MII oocytes were incubated in SMO medium for 1–2 h; those periods were sufficient for the oocytes to be equilibrated with the basic media. The microscopic images of the oocytes were recorded using a time-lapse video recorder (ETV-820, Sony, Tokyo, Japan) for 2–3 h during exposure. The cross-sectional area of the oocyte was measured using an image analyzer (VM-50, Olympus, Tokyo, Japan). Relative cross-sectional area, S, is expressed by

Table 1
The osmolality of solutions used

Diluent			Solute		Total osmolality (Osm/kg)
Basic medium	Tonicity	Osmolality[a] (Osm/kg)	Name	Osmolality (Osm/kg)	
SMO	×0.50	0.12	—	—	0.12
	×0.63	0.15	—	—	0.15
	×1.00	0.24	—	—	0.24
	×1.00	0.24	0.24 M sucrose	0.26[b]	0.50
	×1.00	0.24	0.91 M sucrose	1.26[b]	1.50
	×1.00	0.24	8% ethylene glycol	1.61[b]	1.85
	×1.00	0.24	10% glycerol	1.59[b]	1.83
	×1.00	0.24	10% propylene glycol	1.56[b]	1.80
	×1.00	0.24	9.5% DMSO	1.55[c]	1.79
90% TCM-199	×0.44	0.12	—	—	0.12
	×0.56	0.15	—	—	0.15
	×1.00	0.27	—	—	0.27
	×1.00	0.27	0.21 M sucrose	0.23[b]	0.50
	×1.00	0.27	0.89 M sucrose	1.23[b]	1.50
	×1.00	0.27	8% ethylene glycol	1.61[b]	1.88
	×1.00	0.27	10% glycerol	1.59[b]	1.86
	×1.00	0.27	10% propylene glycol	1.56[b]	1.83
	×1.00	0.27	9.5% DMSO	1.55[c]	1.82

[a] Osmolality measured with a freezing point depression osmometer.
[b] Osmolality calculated from published data on the colligative properties of the solutes in aqueous solutions [27].
[c] Osmolality measured with a vapor pressure osmometer.

dividing it by the initial area of the same oocyte. The relative volume was obtained from $V = S^{3/2}$. Osmotically inactive fractions (v_b) were obtained from Boyle–van't Hoff plots.

Water- and cryoprotectant-permeability of oocytes

Oocytes placed in isotonic basic medium (SMO medium or 90% TCM-199 medium) were introduced into basic media containing sucrose or cryoprotectants (200 μl) covered with mineral oil in a Petri dish (35 × 10 mm) at 25 ± 1 °C for 1 h (GV oocytes) or 2 h (MII oocytes), with a minimal amount of isotonic solution using a pipette. The microscopic images of the oocytes were recorded using a time-lapse video recorder for 1 or 2 h during exposure. The cross-sectional area of the oocyte was measured and the relative volume at various time points of exposure was obtained as described above. Hydraulic conductivity (L_P) and cryoprotectant-permeability (P_s) were determined by fitting water and solute movements using a two-parameter formalism as described elsewhere [3]. In principle, the use of the Kedem–Katchalsky formalism [14], which incorporates a solvent–solute interaction parameter (sigma) for calculating water- and cryoprotectant-permeability, would be appropriate. However, determination of a reliable sigma value is quite difficult [15]. Since we require only a phenomenological description of the oocyte behavior, we used the two-parameter formalism for the calculation of water- and cryoprotectant-permeability. L_P values of intact and dechorionated MII oocytes were calculated from volumetric changes in SMO medium containing 1.26 Osm/kg sucrose, excluding the 0.24 Osm/kg contributed by the SMO medium, and that of GV oocytes was calculated from volumetric changes in 90% TCM-199 medium containing 1.23 Osm/kg sucrose, excluding the 0.27 Osm/kg contributed by the 90% TCM-199 medium. The P_s for each cryoprotectant was calculated from volumetric changes in a medium containing 10% (v/v) glycerol, 10% (v/v) propylene glycol, 8% (v/v) ethylene glycol, or 9.5% (v/v) DMSO. The volume percentage was varied to prepare cryoprotectant solutions with similar osmolality (Table 1). The osmolality contributed by glycerol, propylene glycol, ethylene glycol, and DMSO was 1.59, 1.56, 1.61, and 1.55 Osm/kg, respectively, excluding the osmolality contributed by the media (Table 1). Total osmolality of the solutions is shown in Table 1. The osmolality of glycerol, propylene glycol, and ethylene glycol was calculated from published data on the colligative properties of each cryoprotectant in aqueous solutions [27]. The osmolality of 9.5% DMSO in an aqueous solution was measured with a vapor pressure osmometer (Vapro 5520; Wescor, Logan, UT, USA). Other various constants and parameters are listed in Table 2.

Results

Osmotically inactive fractions of oocytes at the GV and MII stages

Fig. 1 shows the Boyle–van't Hoff plots of oocytes at the GV and MII stages. Oocytes at both stages shrunk in hypertonic solutions and swelled

Table 2
Constants and parameters used for fitting permeability parameters

Symbol	Meaning	Values
R	Gas constant (liter atm K^{-1} mol^{-1})	8.206×10^{-2}
T	Absolute temperature	298 K
$\overline{V}_w$	Partial molar volume of water	0.018 L/mol
$\overline{V}_{EG}$	Partial molar volume of ethylene glycol[a]	0.054 L/mol
$\overline{V}_{GLY}$	Partial molar volume of glycerol[a]	0.071 L/mol
$\overline{V}_{PG}$	Partial molar volume of propylene glycol[a]	0.070 L/mol
$\overline{V}_{DMSO}$	Partial molar volume of DMSO[b]	0.069 L/mol

[a] Partial molar volumes of cryoprotectants from Wolf et al. [27].
[b] Partial molar volume of DMSO from Mazur [19].

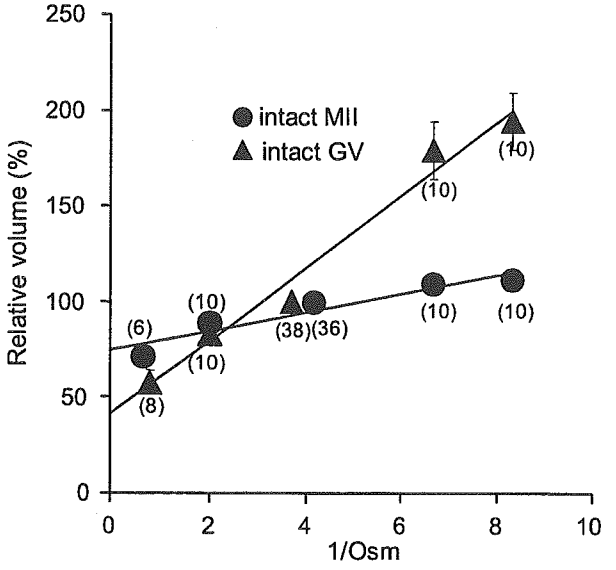

Fig. 1. Boyle–van't Hoff plots of the relative volume of intact medaka oocytes at the germinal vesicle (closed triangles) and metaphase II stages (closed circles). Oocytes at the germinal vesicle stage were equilibrated in 90% TCM-199 medium containing different concentrations of sucrose or in 90% TCM-199 diluted with distilled water at 25 °C for 2 h. Intact oocytes at the metaphase II stage were equilibrated in SMO medium containing different concentrations of sucrose or in SMO medium diluted with distilled water at 25 °C for 3 h. Data are indicated as means of relative volumes ± SD. Numbers in parentheses indicate the number of oocytes used.

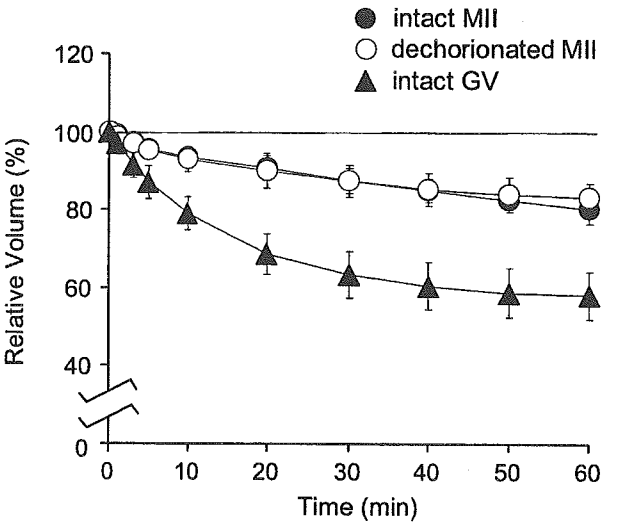

Fig. 2. Change in cell volume of intact (closed circles) and dechorionated (open circles) medaka oocytes at the metaphase II stage and intact oocytes at the germinal vesicle stage (closed triangles) in a hypertonic sucrose solution. Intact and dechorionated MII oocytes incubated in SMO medium (0.24 Osm/kg) at 25 °C were exposed to SMO medium containing 1.26 Osm/kg sucrose at 25 °C for 1 h. Intact GV oocytes cultured in 90% TCM-199 medium (0.27 Osm/kg) were exposed to 90% TCM-199 medium containing 1.23 Osm/kg sucrose at 25 °C for 1 h. Data are indicated as means of relative volumes ± SD. Data of intact MII oocytes and GV oocytes were from 10 oocytes and those of dechorionated MII oocytes were from six oocytes.

in hypotonic solutions. However, changes in equilibrated volume of MII oocytes in solutions of various osmolalities were much smaller than those of GV oocytes, suggesting that the v_b value of GV oocytes is smaller than that of MII oocytes. Actually, the calculated value for v_b of GV oocytes was 0.41 and the value of r^2 of the regression line was 0.98, whereas that of MII oocytes was 0.74 and the value of r^2 of the regression line was 0.90.

We used these v_b values for calculation of the water- and cryoprotectant-permeability of the oocytes.

Permeability to water of oocytes at the GV and MII stages

Fig. 2 shows changes in the volume of oocytes at the GV and MII stages in media containing sucrose (the osmolality of both solutions was 1.50 Osm/kg). MII oocytes shrunk slowly and the change in volume did not differ between intact and dechorionated oocytes. This suggests that the chorion does not affect the water-permeability of MII oocytes. On the other hand, GV oocytes shrunk more rapidly, perhaps due to differences in the osmotically inactive volume and water-permeability. As described above, the value for v_b of MII oocytes was much higher than that of GV oocytes (Fig. 1). L_P values of intact and dechorionated MII oocytes were the same (0.06 ± 0.01 μm/min/atm). This value was much lower than the value for GV oocytes (0.13 ± 0.01 μm/min/atm) (Table 3). These results show that water permeates GV oocytes much faster than MII oocytes.

Permeability to cryoprotectants of oocytes at the MII stage

Fig. 3 shows volume changes of intact and dechorionated oocytes at the MII stage in SMO medium containing glycerol, propylene glycol, ethylene glycol, or DMSO at 25 °C. In glycerol

Table 3
Water-permeability of medaka oocytes

Oocytes	L_P (μm/min/atm)
Intact MII oocytes	0.06 ± 0.01
Dechor MII oocytes[a]	0.06 ± 0.01
GV oocytes	0.13 ± 0.01*

[a] Dechorionated MII oocytes.
* Significantly different from intact and dechorionated MII oocytes (Student's t test, $P < 0.01$).

solution, both types of oocytes shrunk slowly and did not regain their volume during 2-h exposure, suggesting that glycerol permeates both oocytes quite slowly (Fig. 3A). Since we did not observe a reswelling phase on permeation of the glycerol during 2-h exposure, we could not calculate glycerol-permeability for the two types of oocytes. MII oocytes in propylene glycol and ethylene glycol solutions also shrunk slowly and did not regain their volume regardless of the presence or absence of the chorion (Figs. 3B and C), suggesting that propylene glycol and ethylene glycol also permeate MII oocytes quite slowly. Thus, we could not calculate the cryoprotectant-permeability of MII oocytes from these data. In DMSO solution, intact oocytes also remained shrunken during 2-h exposure whereas dechorionated oocytes shrunk but regained their volume thereafter (Fig. 3D), suggesting that DMSO permeated dechorionated oocytes. The value for P_{DMSO} of the dechorionated oocytes was calculated to be $0.14 \pm 0.01 \times 10^{-3}$ cm/min (Table 4).

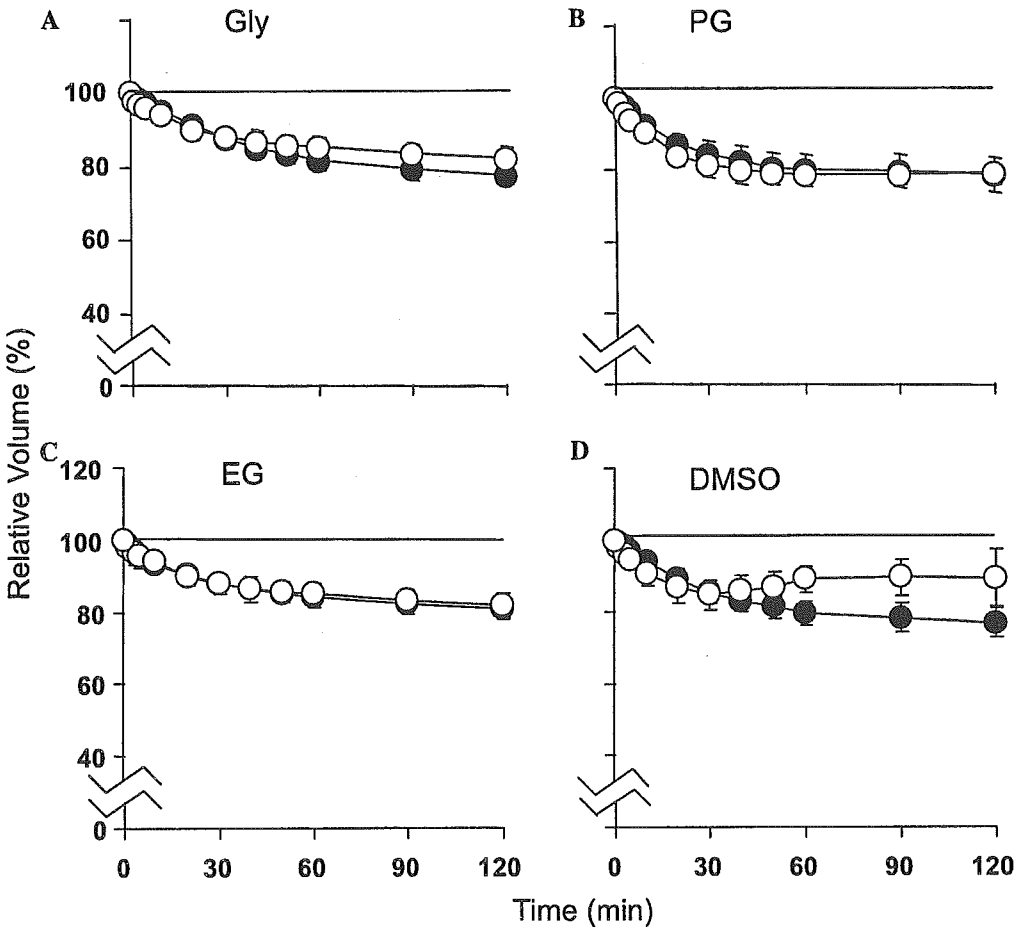

Fig. 3. Change in cell volume of intact (closed circles) and dechorionated (open circles) medaka oocytes at the metaphase II stage in 10% glycerol (A), 10% propylene glycol (B), 8% ethylene glycol (C), and 9.5% DMSO (D) solutions. Oocytes were exposed to SMO medium containing various cryoprotectants at 25 °C for 2 h. Data are indicated as means of relative volumes ± SD. Data of intact MII oocytes were from 10 oocytes and those of dechorionated MII oocytes were from six oocytes.

Table 4
Permeability ($\times 10^{-3}$ cm/min) of medaka oocytes to cryoprotectants

Oocytes	P_{GLY}[a]	P_{PG}[b]	P_{EG}[c]	P_{DMSO}[d]
Intact MII oocytes	nd[e]	nd	nd	nd
Dechor MII oocytes[f]	nd	nd	nd	0.14 ± 0.01
GV oocytes	nd	$2.21 \pm 0.29^*$	$1.36 \pm 0.18^*$	$1.19 \pm 0.01^*$

[a] Glycerol-permeability.
[b] Propylene glycol-permeability.
[c] Ethylene glycol-permeability.
[d] DMSO-permeability.
[e] Not determined.
[f] Dechorionated MII oocytes.
* Significantly different from intact and dechorionated MII oocytes (Student's t test, $P < 0.01$).

These results suggest that the polyol-permeability of MII oocytes is very low whereas the DMSO-permeability is relatively high but that the chorion hinders the permeation of DMSO.

Permeability to cryoprotectants of oocytes at the GV stage

Fig. 4 shows volume changes of GV oocytes in 90% TCM-199 containing glycerol, propylene glycol, ethylene glycol, or DMSO at 25 °C. In glycerol solution, GV oocytes shrunk slowly and did not regain their volume during 2-h exposure (Fig. 4A) similar to MII oocytes, suggesting that glycerol permeates GV oocytes quite slowly. On the other hand, in propylene glycol (Fig. 4B), ethylene glycol (Fig. 4C), and DMSO (Fig. 4D) solutions, GV oocytes shrunk and regained their volume, suggesting that these cryoprotectants permeate GV oocytes much faster than MII oocytes. Values for propylene glycol-, ethylene glycol-, and DMSO-permeability of GV oocytes were 2.21 ± 0.29, 1.36 ± 0.18, and $1.19 \pm 0.01 \times 10^{-3}$ cm/min, respectively (Table 4).

These results show that GV oocytes are permeable to various cryoprotectants.

Discussion

As an essential step for obtaining L_P and P_S for medaka oocytes, we first measured v_b for GV oocytes and MII oocytes. From the data shown in Fig. 1, the v_b value of GV oocytes was estimated to be 0.41. This value would be reliable, because the volumes of GV oocytes equilibrated in both hypertonic and hypotonic solutions were linearly arranged in the Boyle–van't Hoff plots ($r^2 = 0.98$). On the other hand, the v_b value for MII oocytes was estimated to be 0.74, which was remarkably higher than the value for GV oocytes (0.41). This value for MII oocytes was larger than that of matured zebrafish oocytes (0.64) [28]. Since it is unlikely that the amount of materials in medaka oocytes actually increases that much during a short period (10 h) of final maturation, it may show the physiological changes of medaka oocytes just before spawning. Moreover, in the Boyle–van't Hoff plots (Fig. 1), equilibrated volumes of MII oocytes in hypotonic solutions were smaller than those expected from equilibrated volumes in hypertonic solutions. This suggests that, during the final maturation stage, medaka oocytes acquire the ability to resist osmotic changes, especially in hypotonic conditions. This seems reasonable because medaka oocytes are exposed to fresh water at spawning, and thus oocytes would be ruptured if they could not acquire such tolerance. If so, such a change in the membrane permeability in maturing oocytes is expected to occur in other fresh water fish also. In zebrafish, it has been shown that embryos do not swell in hypotonic solutions whereas they shrink in hypertonic solutions [8]. However, the mechanism by which oocytes and embryos resist osmotic swelling in fresh water remains to be elucidated. In any case, the observations and speculation made here strongly suggest that the v_b value of medaka MII oocytes was actually overestimated. In this case, the water- and cryoprotectant-permeability of the MII oocytes would be

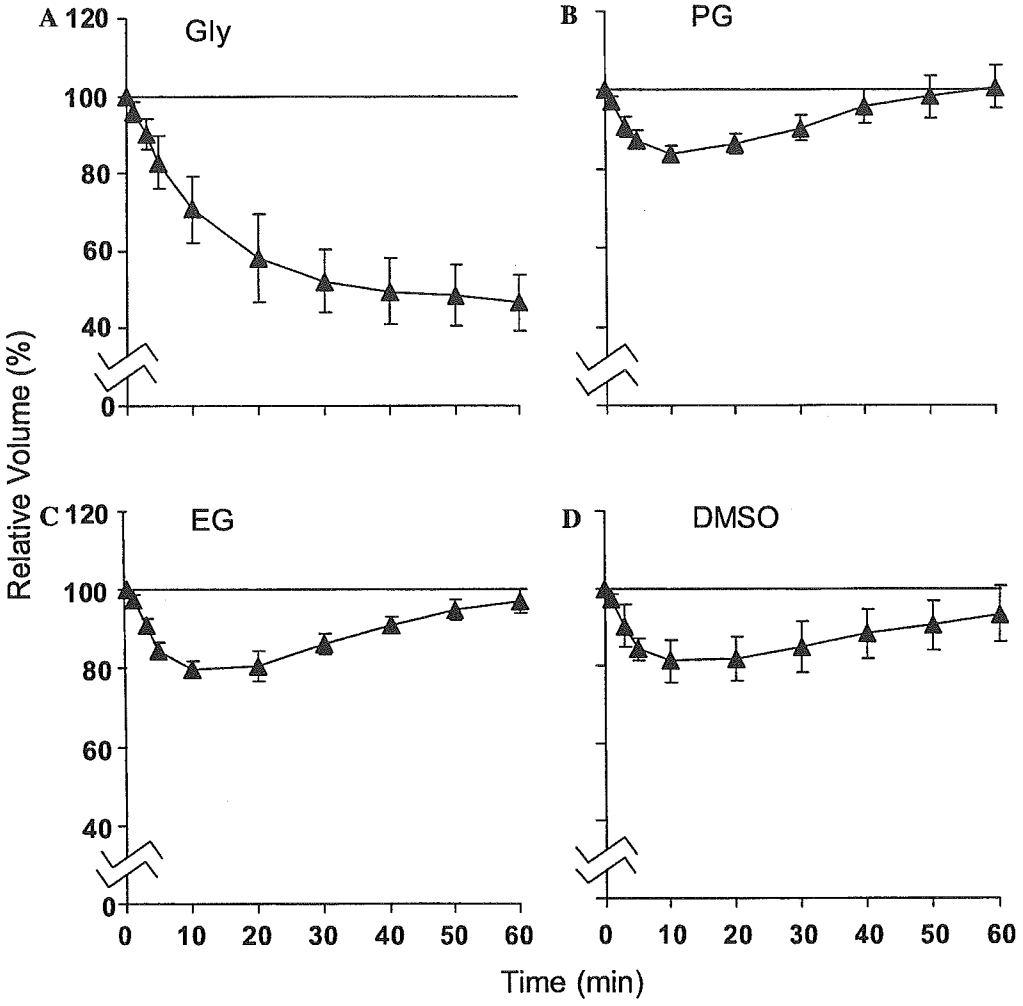

Fig. 4. Change in cell volume of intact medaka oocytes at the germinal vesicle stage in 10% glycerol (A), 10% propylene glycol (B), 8% ethylene glycol (C), and 9.5% DMSO (D) solutions. Oocytes were exposed to 90% TCM-199 medium containing various cryoprotectants at 25 °C for 1 h. Data are indicated as means of relative volumes ± SD. Data of GV oocytes in glycerol, propylene glycol, and DMSO solutions were from 10 oocytes, and those in ethylene glycol solutions were from nine oocytes.

slightly overestimated. However, the permeability values obtained for medaka MII oocytes were low.

The L_P of medaka MII oocytes obtained in this study was 0.06 ± 0.01 μm/min/atm, and the removal of the chorion from the oocytes did not affect the water-permeability (Fig. 2, Table 3). This shows that the chorion does not hinder water movement. Harvey and Chamberlain [10] also reported that the chorion of zebrafish embryos was freely permeable to water. The L_P value obtained (0.06 ± 0.01 μm/min/atm) is comparable to that for pacific salmon oocytes (0.08 μm/min/atm; a value converted using an equation reported by Leibo [16]; 1 μm/min/atm = 22.4 μm/s) [20] or zebrafish embryos from the 75% epiboly to three-somite stage (0.05 ± 0.01 μm/min/atm) [6]. On the other hand, the L_P of medaka GV oocytes obtained in this study (0.13 ± 0.01 μm/min/atm) (Fig. 2 and Table 3) was more than twice that of MII oocytes. This higher water-permeability of medaka GV oocytes would be an advantage over MII oocytes for cryopreservation.

The cryoprotectant-permeability of MII oocytes was quite low. Except for the DMSO-permeability of dechorionated MII oocytes, we could not calculate P_s values because oocytes did not regain their volumes during 2-h exposure in cryoprotectant solutions. The shrinkage curves of intact

and dechorionated MII oocytes in glycerol, propylene, glycol, and ethylene glycol solutions were substantially the same. As an exception, dechorionated MII oocytes in DMSO solution shrunk and regained their volume although intact MII oocytes remained shrunken. This shows that DMSO permeates MII oocytes but the chorion hinders the permeation. Similar results have already been reported for zebrafish embryos [11]. However, the DMSO-permeability of dechorionated medaka MII oocytes was not high ($0.14 \pm 0.01 \times 10^{-3}$ cm/min) (Table 4). Thus, it will be difficult to introduce an amount of cryoprotectant into medaka MII oocytes sufficient for cryopreservation regardless of the absence of their chorion.

Compared with MII oocytes, GV oocytes, with the chorion, had a much higher cryoprotectant-permeability; the P_s values ($\times 10^{-3}$ cm/min) for propylene glycol, ethylene glycol, and DMSO being 2.21 ± 0.29, 1.36 ± 0.18, and 1.19 ± 0.01, respectively (Fig. 4, Table 4). It has been reported that the DMSO-permeability of medaka oocytes and embryos increases as development proceeds (from MII oocytes to eight-cell and eyed embryos) [22], and the value for P_{DMSO} at the eyed-embryo stage was 0.82×10^{-3} cm/min [24]. Considering that the P_s values of GV oocytes are $1.19–2.21 \times 10^{-3}$ cm/min (Table 4), GV oocytes have an advantage in cryopreservation at least in medaka. However, glycerol is not suitable for cryopreservation because the permeability of GV oocytes to glycerol was too low to be calculated, as in the case of MII oocytes.

In MII oocytes, it was shown that the chorion hinders the permeation of DMSO. However, the P_{DMSO} of intact GV oocytes ($1.19 \pm 0.01 \times 10^{-3}$ cm/min) was much higher than that of dechorionated MII oocytes ($0.14 \pm 0.01 \times 10^{-3}$ cm/min) (Table 4). This suggests that the permeability to DMSO of the plasma membrane and the chorion of GV oocytes is markedly higher than that of MII oocytes and that the permeability decreases during the final maturation period. Thus, not only the higher permeability of the plasma membrane but also the higher permeability of the chorion is also an advantage of GV oocytes for cryopreservation.

Zhang and Rawson showed much larger values for water-permeability of zebrafish embryos at the one-cell (0.34 ± 0.16 μm/min/atm) and six-somite (0.35 ± 0.15 μm/min/atm) stages [28] compared with those in other studies (0.04 0–0.100 μm/min/atm) [5,6] and those of medaka MII oocytes (0.06 ± 0.01 μm/min/atm) in this study. This large difference may be due to the presence or absence of cryoprotectants in solutions for measuring permeability to water. We, and Hagedorn et al. [6], calculated water-permeability of oocytes and embryos in solutions containing cell non-permeating solutes. On the other hand, Zhang and Rawson [28] obtained the values of L_P in the presence of methanol. In the presence of cryoprotectants, an increase in the membrane permeability parameter has been noted [18].

In this study, we have demonstrated that immature medaka oocytes have relatively high water- and cryoprotectant-permeability. But the permeability may not be sufficiently high for cryopreserving them because fish oocytes have a huge volume compared with mammalian oocytes and embryos. During the last decade, small intrinsic membrane proteins that act as water channels have been discovered and characterized [26]. These proteins, called aquaporins, occur in two groups: one subgroup is highly selective for water and the other subgroup transports not only water but also neutral solutes with a small molecular weight, such as cell-permeating cryoprotectants. We have already demonstrated that the artificial expression of aquaporin-3, which acts as a water/cryoprotectant channel, through injection of its cRNA, increases the water- and cryoprotectant-permeability of mouse oocytes [3]. We have also demonstrated that the artificial expression improves the tolerance of mouse oocytes for cryopreservation [3]. The artificial expression of aquaporin-1 also improves tolerance in yeast [25]. Aquaporin-3 can be artificially expressed in zebrafish embryos and the expression improves water- and cryoprotectant-permeability [8]. Thus, the expression of such water channels in immature fish oocytes might be effective for the cryopreservation of fish oocytes.

In conclusion, we have shown that immature oocytes are relatively permeable by water and cryoprotectant, whereas matured oocytes have quite low membrane permeability. These results suggest that, with maturation, medaka oocytes become less permeable to water and to small neutral solutes, probably by acquiring resistance to hypo-

tonic conditions before being spawned in fresh water. Since such changes would make it difficult to cryopreserve matured oocytes (and maybe embryos), immature oocytes would be more suitable for cryopreservation.

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