—Original Article—

A Trial to Cryopreserve Immature Medaka (*Oryzias latipes*) Oocytes after Enhancing Their Permeability by Exogenous Expression of Aquaporin 3

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ABSTRACT. Fish oocytes have not been cryopreserved successfully, probably because it is difficult to prevent intracellular ice from forming. Previously, we have shown in medaka that immature oocytes are more suitable for cryopreservation than mature oocytes or embryos, in terms of permeability. We have also shown in immature medaka oocytes that the exogenous expression of aquaporin 3 (AQP3), a water/cryoprotectant channel, promotes the movement of water and cryoprotectants through the plasma membrane. In the present study, we attempted to cryopreserve immature medaka oocytes expressing AQP3. We first examined effects of hypertonic stress and the chemical toxicity of cryoprotectants on the survival of the AQP3-expressing oocytes. Exposure to hypertonic solutions containing sucrose decreased the survival of oocytes, but the expression of AQP3 did not affect sensitivity to hypertonic stress. Also, AQP3 expression did not markedly increase sensitivity to the toxicity of cryoprotectants. Of the four cryoprotectants tested, propylene glycol was the least toxic. Using a propylene glycol-based solution, therefore, we tried to cryopreserve immature oocytes by vitrification. During cooling with liquid nitrogen, all intact oocytes became opaque, but many AQP3-expressing oocytes remained transparent. This indicates that the expression of AQP3 is effective in preventing intracellular ice from forming during cooling. During warming, however, all the AQP3-expressing oocytes became opaque, indicating that intracellular ice formed. Therefore, the dehydration and permeation by propylene glycol were still insufficient. Further studies are necessary to realize the cryopreservation of fish oocytes.
Key words: Aquaporin, Cryoprotectant, Fish, Medaka, Oocyte, Vitrification

In model animals, the cryopreservation of embryos plays an important role in the management of various genetic variants. In the mouse, most variants are maintained in cryopreserved embryos. In aquatic species, successful cryopreservation of embryos would also provide great benefits not only in model species but also in aquaculture and for the conservation of biodiversity.

There have been a few reports on the successful cryopreservation of fish embryos. In 1989, Zhang *et al.* [1] reported the survival of common carp (*Cyprinus carpio*) embryos after slow freezing, but this result has not been reproduced. In 2005, Chen and Tian [2] documented the survival of Japanese flounder (*Paralichthys olivaceus*) embryos after vitrification. However, it was not possible to cryopreserve Japanese flounder embryos using the protocol they proposed due to the low permeability of the plasma membrane to water and cryoprotectants for the large size [3]. Moreover, other attempts to cryopreserve embryos of fish have failed to achieve success, including in zebrafish [4], turbot [5], winter flounder (*Pseudopleuronectes americanus*) [6] and gilthead seabream [7].

Fish embryos have a large volume, a large amount of egg yolk, a thick chorion, high sensitivity to chilling [8] and low membrane permeability to water and cryoprotectants [9, 10]. In addition, they form a complex structure during development. These cryobiological properties make them difficult to cryopreserve. The low membrane permeability for the large size appears to be the greatest obstacle. Since fish embryos are large cell masses, they have a low surface/volume ratio. Therefore, dehydration and permeation by cryoprotectant(s) require a long period of time. Consequently, the embryos are likely to be damaged by the toxicity of the cryoprotectant or by the formation of intracellular ice.

An alternative to embryos for the preservation of fish variants would be oocytes, because sperm of fish including medaka have already been frozen successfully and oocytes can be easily fertilized with frozen sperm [11]. Therefore, the preservation of fish genetic resources could be realized if oocytes survive cryopreservation. We have shown in the medaka that immature oocytes have advantages for cryopreservation over mature oocytes and embryos, because the plasma membrane and chorion are more permeable to water and cryoprotectants [12]. Similar results have been obtained in zebrafish oocytes [13, 14]. However, we have also suggested that the permeability of immature oocytes is not sufficient for cryopreservation [12]. Recently, Guan *et al.* (2008) [15] attempted to cryopreserve immature zebrafish oocytes by slow freezing, but the oocytes did not survive after thawing. We tried to

increase the permeability to water and cryoprotectants of immature oocytes of medaka and zebrafish through the artificial expression of a water/cryoprotectant channel protein, aquaporin 3 (AQP3) [13, 16]. In those studies, we expressed AQP3 by injecting AQP3 cRNA into oocytes because, by that method, the protein can be exogenously expressed without any genetic modification. This strategy would be suitable for preservation of various strains of laboratory animals. The injection of cRNA of AQP3 into oocytes of medaka [16] and zebrafish [13] increased the permeability significantly, as in the case of mice [17] and *Xenopus laevis* [18].

In the present study, we tried to vitrify oocytes injected with AQP3 cRNA by two methods, conventional vitrification using semen straws and rapid vitrification using cryoloops. To design the vitrification solution and pretreatment solution and the protocol for vitrification, we modified a two-step vitrification method using EFS solution, a mixture of a cell-permeating cryoprotectant (30–40%, vol/vol) and FS solution (medium containing Ficoll PM70 and sucrose, 60–70%, vol/vol) that is effective for vitrification of embryos in various mammalian species [19]. We first examined the tolerance of immature medaka oocytes expressing AQP3 to hypertonic sucrose solutions, and then we examined the toxicity of various cryoprotectants at low/high concentrations and a vitrification solution. Based on the results, we finally tried to vitrify AQP3-expressing oocytes.

Materials and Methods

Collection of oocytes

About 20–50 mature orange-red type medaka, purchased from a local fish dealer, were maintained in 60-liter aquaria under 14-h light and 10-h dark periods at 26 C. Actively spawning females were decapitated under anesthesia with 0.2 mg/ml tricaine in distilled water 1–3 h before the start of the dark period (right after the LH surge). From the ovaries, fully grown immature oocytes at the germinal vesicle stage, 0.8–0.9 mm in diameter, were obtained. They were cultured in 90% TCM199 with Earle's salts (90% TCM199) (Invitrogen, Carlsbad, CA, USA) [20] at 26 C for 1–2 h prior to use. All experiments were approved by the Animal Care and Use Committee of Kochi University.

Preparation of AQP3 cRNA

Rat AQP3 cRNA was synthesized as described elsewhere [16, 17]. AQP3 cDNA was cloned from rat kidney cDNA by polymerase chain reaction (PCR): the sense strand was 5'-CGGGATCCCATGGGTCGACAGAAGGAGTT-3', and the antisense strand was 5'-GCTCTAGAGGGTTTTATGGGGTGTCC-3' (underlined sequences indicate inserted BamHI and XbaI sites, respectively). These primers were derived from the rat AQP3 sequence [21] (GenBank[TM] accession No. L35108). The PCR cycle had the following profile: 94 C for 1 min, 58 C for 0.5 min and 72 C for 1 min for 30 cycles. The PCR product contained the open reading frame of AQP3. The BamHI/XbaI fragment of the PCR product was subcloned into the BglII/XbaI site of pSP64T (a gift from Dr Paul A. Krieg), a *Xenopus* expression plasmid. After digestion of the construct by EcoRI (Takara Bio, Otsu, Japan), capped cRNA of AQP3 was synthesized using SP6 polymerase (Takara Bio).

Microinjection of immature oocytes with AQP3 cRNA

AQP3 cRNA was injected into immature oocytes as described previously [16]. Briefly, an oocyte at the germinal vesicle stage was held with a holding pipette connected to a micromanipulator on an inverted microscope and injected into the cytoplasm with ~60 nl of AQP3 cRNA solution (1 ng/nl water) using an injection needle connected to another micromanipulator. Injected and non-injected (intact) oocytes were cultured in 90% TCM199 at 26 C for 6–7 h until they reached the germinal vesicle breakdown stage and used as immature oocytes.

Since, in a previous study [16], the water permeability of water-injected oocytes did not differ from that of intact oocytes, we used intact oocytes as controls in the present study.

Permeability to water of oocytes

The permeability to water of oocytes was determined as described in a previous study [12]. Oocytes cultured in 90% TCM199 for 6–7 h were transferred with a minimal amount of 90% TCM199 to 90% TCM199 containing 0.89 M sucrose covered with paraffin oil in a Petri dish for 1 h at 25 ± 1 C. Microscopic images of the oocytes were recorded using a time-lapse video recorder (ETV-820, Sony, Tokyo, Japan) for 1 h. The cross-sectional area of oocytes was measured using an image analyzer (VM-50, Olympus, Tokyo, Japan). Relative cross-sectional area, S, was expressed by dividing by the initial area of the same oocyte. Relative volume was obtained from $V = S^{3/2}$. Permeability to water was determined by fitting water and solute movements using a two-parameter formalism [22] as described elsewhere [12, 16, 17]. The osmolality of sucrose was calculated from published data on colligative properties in aqueous solutions [23]. The osmolality of 90% TCM199 was measured with a freezing point depression osmometer (OM801; Vogel, Giessen, Germany). The total osmolality of the solution used is shown in Table 1. Other constants and parameters are listed in Table 2. We assumed that the osmolality of the oocyte cytoplasm was equilibrated with that of 90% TCM199 (0.27 Osm/kg) because oocytes were cultured in 90% TCM199 for 6–7 h before use, a period sufficient for the oocytes to be equilibrated with the medium.

Sensitivity of oocytes to hypertonic sucrose solutions

To examine the sensitivity to hypertonic sucrose solutions, intact and AQP3-expressing oocytes were suspended in 4 ml of 90% TCM199 (control) or 90% TCM199 containing 0.2, 0.3 or 0.4 M sucrose for 30 min at 25 C. The oocytes were then washed three times with fresh 90% TCM199 and kept in the medium at 25 C for 10 min.

Toxicity of cryoprotectants to oocytes

To evaluate the toxicity of cell-permeating cryoprotectants, intact and AQP3-expressing oocytes were suspended in 4 ml of 90% TCM199 (control) or 90% TCM199 containing 10% (v/v) glycerol, 8% (v/v) ethylene glycol, 10% (v/v) propylene glycol or 9.5% (v/v) Me$_2$SO at 25 C. The concentrations of the cryoprotectants were varied to prepare solutions with similar osmolalities (1.6–1.8 Osm/kg). After 60 min of suspension, oocytes were transferred to the same cryoprotectant solution diluted with the same volume of 90% TCM199 (1:1) at 25 C for 10 min, washed three times with fresh 90% TCM199, transferred to fresh 90% TCM199 at 25 C and

Table 1. The osmolality of solutions used for determining the permeability to water of immature medaka oocytes

Solution	Diluent		Sucrose		Total osmolality (Osm/kg)
	Basic medium	Osmolality [a] (Osm/kg)	Concentration (M)	Osmolality (Osm/kg)	
Basic medium	90% TCM199	0.27	0	0	0.27
Sucrose solution	90% TCM199	0.27	0.89	1.23[b]	1.5

[a] Osmolality measured with a freezing point depression osmometer. [b] Osmolality calculated from published data on the colligative properties of sucrose in aqueous solutions [23].

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
v_b	Osmotically inactive volume [a]	0.41

[a] From Valdez *et al.* [12].

kept there for 10 min. Intact oocytes and AQP3-expressing oocytes without any treatments were used as controls.

A toxicity test using 8–10% cryoprotectant showed that propylene glycol was the least toxic of the four cryoprotectants tested. To examine the toxicity of propylene glycol further, oocytes were suspended in 90% TCM199 containing a higher concentration (30% v/v) of propylene glycol at 25 C. After 3, 5 or 10 min of suspension, the solution was diluted stepwise with 90% TCM199 containing 15% propylene glycol for 20 min and then with 90% TCM199 containing 7.5% propylene glycol for 10 min at 25 C. The oocytes were washed three times with fresh 90% TCM199 and kept in fresh 90% TCM199 at 25 C for 10 min. Intact oocytes and AQP3-expressing oocytes without any treatments were used as controls.

Toxicity of the vitrification solution to oocytes

Based on the results of the toxicity test of cryoprotectants, we composed a propylene glycol-based vitrification solution by mixing propylene glycol (30% v/v) and FS solution (70% v/v). The FS solution was 90% TCM199 containing 10% (w/v) Ficoll PM70 (GE Healthcare, Uppsala, Sweden) plus 0.2 M sucrose.

To examine the toxicity of the propylene glycol-based vitrification solution, intact and AQP3-expressing oocytes were suspended in 4 ml of the vitrification solution for 3, 5 or 10 min at 25 C. To remove propylene glycol, the oocytes were suspended in a mixture of vitrification solution (50% v/v) and 90% TCM199 (50% v/v) for 20 min and then in a mixture of vitrification solution (25% v/v) and 90% TCM199 (75% v/v) for 10 min at 25 C. The oocytes were washed three times with fresh 90% TCM199 and kept in the medium at 25 C for 10 min. Intact oocytes and AQP3-expressing oocytes without any treatments were used as controls.

Vitrification of oocytes

As a preliminary experiment, we examined whether the vitrification solution crystallizes or not during cooling and warming. In one group,

~200 μl of vitrification solution was loaded into a 0.5-ml plastic straw (Fujihira, Tokyo, Japan). The straw was heat-sealed at the open end and then cooled by being directly immersed in liquid nitrogen (LN_2). The average cooling rate from 20 to –120 C was assumed to be ~1,800 C/min [24]. After 1–2 min, the straw was warmed by being immersed in water at 25 C. The average warming rate from –70 to –35 C was assumed to be ~3,000 C/min [24]. In another group, a small amount of the vitrification solution was put on a small loop (Φ= ~0.7 mm) made of a fine platinum wire (Φ= ~0.15 mm) (cryoloop) and cooled rapidly by direct plunging into LN_2. The cryoloop was warmed by being immersed quickly in 4 ml of 90% TCM199 containing 15% (v/v) propylene glycol (dilution solution) at 25 C. The cooling rate and warming rate of small aqueous samples in ultrarapid vitrification using minute devices were expected to be as high as 100,000 C/min [25]. However, since immature medaka oocytes and the vitrification solution around the oocytes on cryoloops would have much larger volumes (~300–400 μl) than that on cryoloops for vitrification of mammalian oocytes/embryos (~0.1 μl), the cooling/warming rate of immature medaka oocytes using cryoloops would be much lower than that of mammalian oocytes/embryos. As a preliminary experiment, we vitrified 5 intact immature medaka oocytes using cryoloops and warmed them by transferring the oocytes from LN_2 to the dilution solution at 25 C. For intracellular ice to begin to melt in the dilution solution at 25 C, 2.1 ± 0.1 sec was required. From the result, the calculated warming rate of vitrified oocytes on cryoloops was ~6,000 C/min; the rate was higher than that using straws but much lower than that for mammalian oocytes/embryos using minute devices.

During vitrification of immature medaka oocytes, the appearance of the vitrification solution was observed during cooling and warming to see whether the solution remained transparent (uncrystallized) or turned opaque (crystallized).

For vitrification with straws, oocytes were first suspended in a pretreatment solution (90% TCM199 containing 10% (v/v) propylene glycol) for 60 min and then exposed to the vitrification solution at 25 C. After pretreatment, 5 to 10 oocytes were loaded into a 0.5-ml plastic straw containing ~200 μl of vitrification solution using a fine pipette, and the open end of the straw was heat-sealed. After 3 min of exposure of the oocytes to the vitrification solution, the straw was immersed in LN_2. After being kept in LN_2 for 2–5 min, the sample was warmed by immersing the straw in water at 25 C for ~5 sec. The heat-sealed end was cut off, and the contents of the straw were expelled into 4 ml of the dilution solution at 25 C by pushing with a cotton plug. Oocytes were recovered, transferred to fresh dilution solution and kept there for 20 min at 25 C. The oocytes were washed with fresh 90% TCM199 three times and incubated in

the medium at 26 C for 1 h.

For vitrification with a cryoloop, an oocyte was first suspended in 90% TCM199 containing 10% (v/v) propylene glycol for 60 min and then suspended in the vitrification solution at 25 C. The oocyte was placed on a cryoloop with a minimal amount of the vitrification solution. After 3 min of exposure of the oocyte to the vitrification solution, the cryoloop was immersed in LN_2. After ~1 min, the oocyte was warmed by immersing the cryoloop quickly in 4 ml of the dilution solution at 25 C and kept there for 20 min. Then, the oocyte was washed three times with fresh 90% TCM199 and incubated in the medium at 26 C for 1 h.

During cooling and warming, the oocytes in the straw and the cryoloop were observed to determine whether intracellular ice had formed (opaque) or not (transparent).

Assessment of the survival of oocytes

The survival of intact and AQP3-expressing oocytes after exposure to a sucrose solution, cryoprotectant solutions or a vitrification solution was assessed by their ability to mature, to be fertilized and to develop till hatching as described previously [16]. First, oocytes were cultured in 90% TCM199 for 6–7 h at 26 C (total culture period being ~14 h from the beginning of culture), and their maturation was assessed from their appearance, that is, the occurrence of spontaneous ovulation, a translucent appearance of the cytoplasm and a large number of small oil droplets in the cytoplasm [26]. Mature oocytes were inseminated using a modified version of Yamamoto's method [27, 28]. Briefly, testes were obtained from two mature males and immersed in 1 ml of saline formulated for medaka oocytes (SMO medium) at room temperature. The composition of the SMO medium was as follows: 6.50 g NaCl, 0.40 g KCl, 0.15 g $CaCl_2 2H_2O$, 0.15 g $MgSO_4 7H_2O$, 1.00 g $NaHCO_3$ and 0.015 g phenol red, in a liter of distilled water containing 5 mM Hepes-HCl, pH 7.0. Sperm were released by tearing the testes with forceps to make a sperm suspension. Five to 20 mature oocytes were placed in a culture dish (Φ35×10 mm) with a minimal amount of 90% TCM199 using a pipette and inseminated by adding 200 μl of the sperm suspension. After 5 min, 4 ml of Hank's balanced solution was added to the dish. The composition of Hank's balanced solution was as follows: 8.0 g NaCl, 0.4025 g KCl, 0.2 g $MgSO_4$, 0.06 g KH_2PO_4, 0.6721 g $NaHCO_3$, 0.05 g Na_2HPO_4, 0.9 g HEPES, 0.05 g gentamicin and 0.05 g $CaCl_2$, in a liter of distilled water at pH 7.0 [29]. Then, the oocytes were incubated for 1 h at 26 C and observed under a stereomicroscope to distinguish fertilized oocytes (being cleaved and having several large droplets in the cytoplasm) from unfertilized oocytes (not cleaved and having a large number of small oil droplets in the cytoplasm). Fertilized oocytes were transferred to Hank's balanced solution containing 3 μg/l methylene blue and incubated for 14 days at 26 C. Hatching was the criterion for full development.

The survival of vitrified oocytes was assessed by their appearance under a stereomicroscope for 1 h.

Statistic analysis

The significance of the difference in the permeability of oocytes to water was analyzed with the Student's *t*-test. The significance of the difference in the rates of maturation, fertilization and hatching was analyzed with a one-way ANOVA using GraphPad Software's

Table 3. Hydraulic conductivity (L_p) of immature medaka oocytes at 25 C

Oocyte	L_p (μm/min/atm)
Intact	0.14 ± 0.02
AQP3-expressing	0.22 ± 0.04*

L_P was determined from the change in volume of oocytes in a sucrose solution at 25 C. Data were from 4 oocytes in each group. * Significantly different from intact oocytes.

InStat, V. 3.02, followed by the Tukey-Kramer multiple comparison test. The significance of the difference in the rate at which intracellular ice formed in oocytes during cooling and warming and survival of oocytes after cryopreservation was analyzed with the χ^2-test. A P-value less than 0.05 was considered significant.

Results

Functional expression of AQP3 in AQP3 cRNA-injected oocytes

In a previous study, we detected AQP3 protein immunologically in AQP3 cRNA-injected oocytes and functionally by the increase in the permeability of the oocytes to water [16]. In the present study, we injected AQP3 cRNA into oocytes by the same method and tried to confirm the expression functionally. Table 3 shows the permeability to water of intact oocytes and AQP3 cRNA-injected oocytes in a hypertonic sucrose solution. The permeability of AQP3 cRNA-injected oocytes (0.22 mm/min/atm) was larger than that of intact oocytes (0.14 mm/min/atm), and the values for the permeability were the same as those in the previous study (0.22 and 0.14 mm/min/atm, respectively) [16]. Therefore, AQP3 cRNA-injected oocytes would express AQP3 at the same level as in the previous study.

Sensitivity of oocytes to hypertonic sucrose solutions

To reduce the amount of intracellular cryoprotectant by promoting shrinkage of oocytes/embryos before cooling and to prevent overswelling during removal of intracellular cryoprotectants after warming, sucrose and other sugars are added to solutions for cryopreservation and for removal of intracellular cryoprotectants.

Figure 1 shows the viability of oocytes after exposure to hypertonic solutions containing 0.2–0.4 M sucrose. Viability was assessed as the proportions of oocytes that matured, were fertilized and developed till hatching. When intact oocytes were exposed to the solution containing 0.2 M sucrose, these rates were 66 ± 8, 49 ± 12 and 19 ± 13%, respectively, which were not significantly different from the rates for control oocytes (80 ± 10, 56 ± 15 and 37 ± 14%, respectively). However, when the concentration of sucrose was increased to 0.3–0.4 M, the viability decreased significantly. In AQP3-expressing oocytes, essentially the same results were obtained. This indicates that medaka oocytes are quite sensitive to hypertonic conditions and suggests that the increase in the permeability to water and cryoprotectants caused by the expression of AQP3 does not affect the sensitivity to hypertonic stress.

Considering the high sensitivity of oocytes to hypertonic stress, we added a low concentration (0.2 M) of sucrose in FS solution
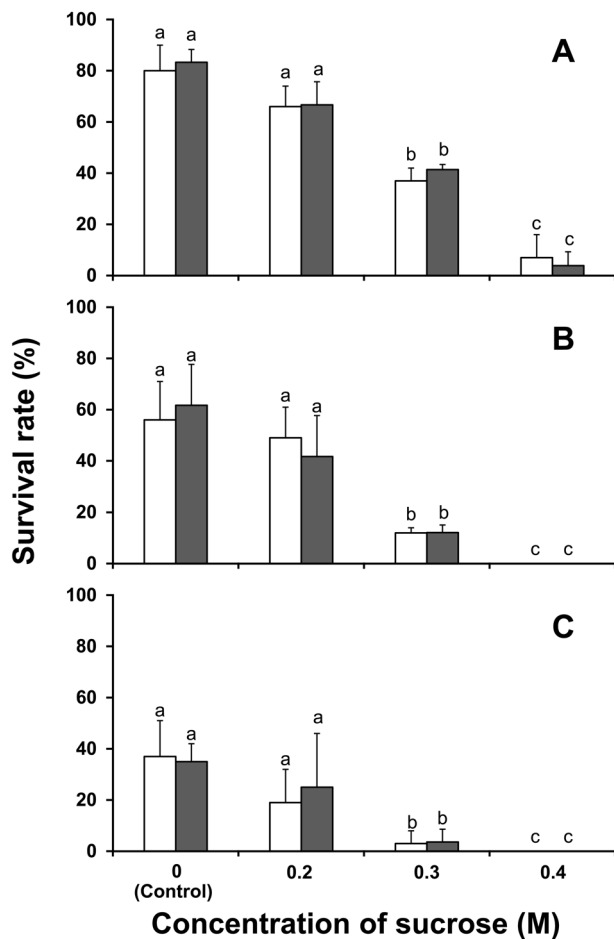
Fig. 1. The effect of hypertonic sucrose solutions on the viability of immature medaka oocytes. Intact (open) and aquaporin 3-expressing (shaded) oocytes were exposed to 90% TCM199 (control) and 90% TCM199 containing 0.2, 0.3 or 0.4 M sucrose for 30 min at 25 C. Their viability was assessed by the ability to mature (A), to be fertilized (B) and to develop till hatching within 14 days of culture (C) at 26 C. Data are indicated as means ± SD from triplicate determinations. For each treatment, 23–25 oocytes were used. Bars with different superscripts differ significantly within each criterion (P<0.05).
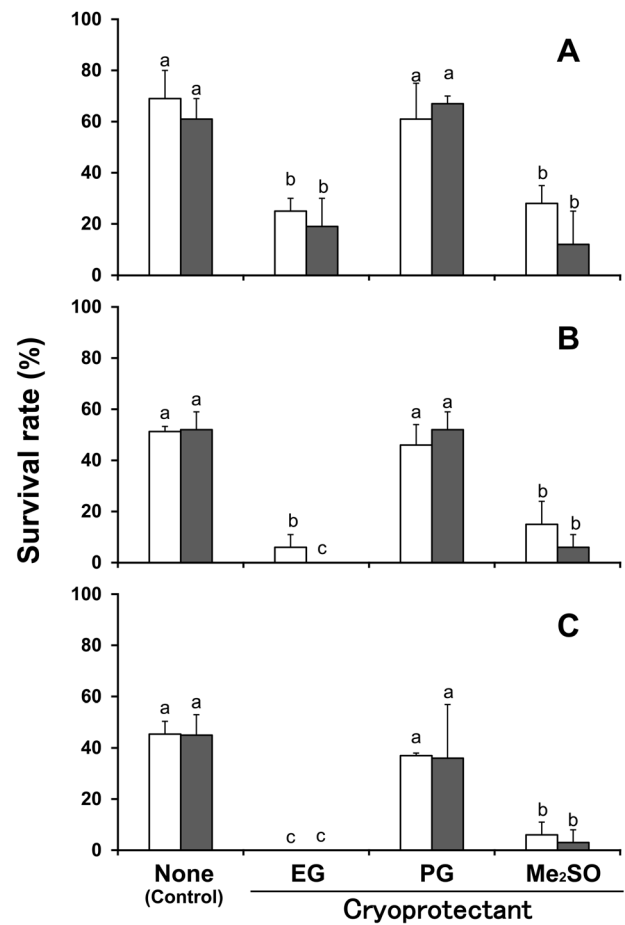
Fig. 2. The effect of cryoprotectants on the viability of immature medaka oocytes. Intact (open) and aquaporin 3-expressing (shaded) oocytes were exposed to 90% TCM199 (control) and 90% TCM199 containing 8% (v/v) ethylene glycol (EG), 10% (v/v) propylene glycol (PG) or 9.5% (v/v) Me$_2$SO (Me$_2$SO) for 60 min at 25 C. Their viability was assessed by the ability to mature (A), to be fertilized (B) and to develop till hatching within 14 days of culture (C) at 26 C. Data are indicated as means ± SD from triplicate determinations. For each treatment, 32–33 oocytes were used. Bars with different superscripts differ significantly within each criterion (P<0.05).

(the final concentration in the vitrification solution being 0.14 M) and did not add sucrose in the dilution solution for the removal of cryoprotectants in the subsequent experiments.

*Sensitivity of oocytes to the toxicity of cryoprotectants*

Figure 2 shows the viability of oocytes after exposure to solutions containing 8–10% cryoprotectant for 60 min at 25 C. When intact oocytes were exposed to 10% (v/v) propylene glycol, the proportions of oocytes that matured, were fertilized, and developed till hatching were 61 ± 14, 46 ± 8 and 37 ± 1%, respectively, which were not significantly different from the rates for control oocytes (69 ± 11, 51 ± 2 and 45 ± 8%, respectively). On the other hand, the viability of oocytes exposed to 8% (v/v) ethylene glycol or 9.5% (v/v) Me$_2$SO decreased considerably, and after exposure to 10% (v/v) glycerol,

no oocytes matured (data not shown). Similar results were obtained with AQP3-expressing oocytes. This suggests that the increase in permeability to water and cryoprotectants caused by the expression of AQP3 has virtually no effect on the sensitivity of oocytes to the toxicity of cryoprotectants.

Since propylene glycol was the least toxic of the cryoprotectants tested, it was selected for the vitrification of immature oocytes.

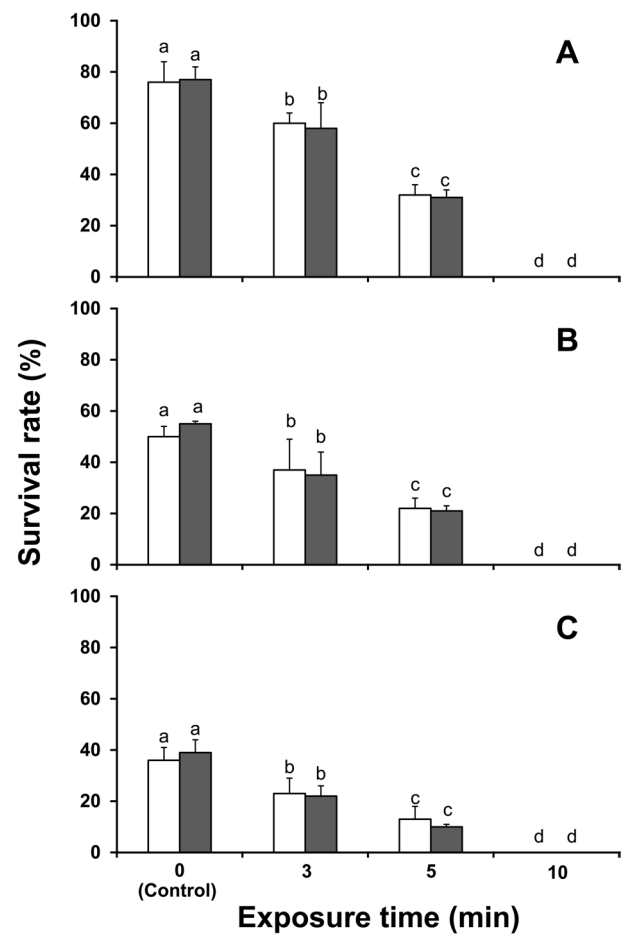*Sensitivity of oocytes to a high concentration of propylene glycol*

Figure 3 shows the viability of oocytes after exposure to a solution containing 30% (v/v) propylene glycol. When intact oocytes were exposed to the solution for 3 min, the proportions of oocytes that matured, were fertilized, and developed till hatching were 57 ± 15,
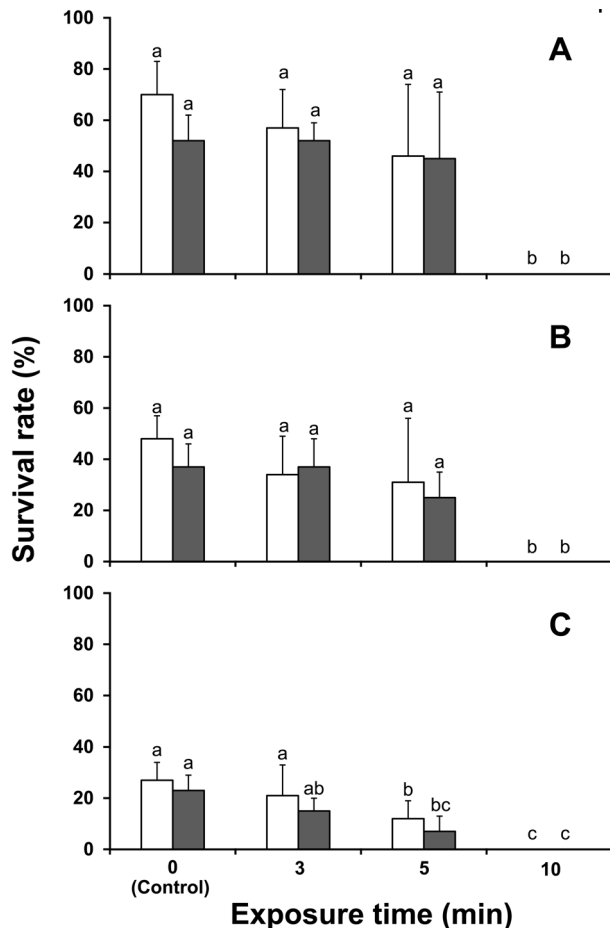
Fig. 3. The effect of a high concentration of propylene glycol on the viability of immature medaka oocytes. Intact (open) and aquaporin 3-expressing (shaded) oocytes were exposed to 90% TCM199 (control) and 90% TCM199 containing 30% (v/v) propylene glycol for 3, 5 or 10 min at 25 C. Their viability was assessed by the ability to mature (A), to be fertilized (B) and to develop till hatching within 14 days of culture (C) at 26 C. Data are indicated as means ± SD from triplicate determinations. For each treatment, 35–45 oocytes were used. Bars with different superscripts differ significantly within each criterion (P<0.05).

Fig. 4. The effect of a propylene glycol-based vitrification solution on the viability of immature medaka oocytes. Intact (open) and aquaporin 3-expressing (shaded) oocytes were exposed to the vitrification solution, a mixture of 30% (v/v) propylene glycol and 70% (v/v) FS solution, for 3, 5 or 10 min at 25 C. FS solution was 90% TCM199 medium containing 10% (w/v) Ficoll PM70 and 0.2 M sucrose. Their viability was assessed by the ability to mature (A), to be fertilized (B) and to develop till hatching within 14 days of culture (C) at 26 C. Data are indicated as means ± SD from triplicate determinations. For each treatment, 29–34 oocytes were used. Bars with different superscripts differ significantly within each criterion (P<0.05).

34 ± 15 and $21 \pm 12\%$, respectively, which were not significantly different from the rates for control oocytes (70 ± 13, 48 ± 9 and $27 \pm 7\%$, respectively). On exposure for 5 min, however, the hatching rate decreased significantly, and after exposure for 10 min, no oocytes matured. Similar results were obtained with AQP3-expressing oocytes. This indicates that oocytes can be exposed to 30% propylene glycol for 3 min at 25 C without a decrease in viability and suggests that the increase in permeability to propylene glycol caused by the expression of AQP3 little affects the sensitivity to propylene glycol.

Therefore, we composed a vitrification solution containing 30% (v/v) propylene glycol for following experiments.

Sensitivity of oocytes to a propylene glycol-based vitrification solution

Figure 4 shows the viability of oocytes after exposure to the vitrification solution consisting of 30% (v/v) propylene glycol and 70% (v/v) FS solution without cooling. When intact oocytes were exposed to the solution at 25 C for 3 min, the proportion that matured ($60 \pm 4\%$) was slightly but significantly lower than the rate for control oocytes ($76 \pm 8\%$). On exposure for 5 min, the rates at which oocytes matured, were fertilized and developed till hatching decreased significantly compared with those of control oocytes. After exposure for 10 min, no oocytes matured. Essentially the same results were obtained with AQP3-expressing oocytes. This indicates that oocytes can be exposed to the propylene glycol-based vitrification

Table 4. Formation of intracellular ice in immature medaka oocytes during cooling and warming and survival after warming

Container	Oocyte	No. of oocytes[a] vitrified	No. of oocytes with IIF[b] during		No. of oocytes survived[c] (%)
			cooling (%)*	warming (%)	
Straw	Intact	53	53 (100)[d]	53 (100)	0 (0)
	AQP3-expressing	38	8 (21)[e]	38 (100)	0 (0)
Cryoloop	Intact	29	29 (100)[d]	29 (100)	0 (0)
	AQP3-expressing	27	4 (15)[e]	27 (100)	0 (0)

[a] Oocytes were cooled with liquid nitrogen after exposure to a propylene glycol-based vitrification solution for 3 min at 25 C. [b] Intracellular ice formation; whitening of the oocyte was considered to indicate the formation of intracellular ice. [c] Survival was assessed based on the appearance of oocytes 1 h after warming. * Values with different superscripts differ significantly.

solution for 3 min with a minimal decrease in viability and suggests that the increase in permeability to propylene glycol caused by the expression of AQP has virtually no effect on the sensitivity of oocytes to the vitrification solution.

Therefore, we tried to vitrify oocytes with this solution.

Survival of oocytes after vitrification with a propylene glycol-based solution

The amount of time oocytes were kept in the pretreatment solution (90% TCM199 containing 10% (v/v) propylene glycol) was determined from the results of an experiment on the sensitivity of oocytes to the toxicity of the solution (Fig. 2); the viability of intact oocytes exposed to the pretreatment solution for 60 min at 25 C did not decrease significantly compared with that of untreated intact oocytes.

When a propylene glycol-based vitrification solution (without oocytes) was cooled with LN_2 in a straw, it remained transparent during cooling (data not shown). When the straw was warmed quickly in water at 25 C, however, it became opaque and then became transparent instantly. Since oocytes whitened by the formation of intracellular ice became transparent during warming later than the frozen extracellular vitrification solution became transparent in the straw, we could determine whether intracellular ice formed or not during warming. On a cryoloop, on the other hand, it was difficult to determine whether the vitrification solution around an oocyte became opaque or not. Considering that the cooling rate and warming rate of the vitrification solution on a cryoloop would be higher than those in a straw, ice crystals would not form in the vitrification solution around oocytes on a cryoloop during cooling. When an oocyte on a cryoloop became opaque during cooling and warming, we considered that intracellular ice had formed.

Table 4 shows the formation of intracellular ice in oocytes during cooling and warming and the survival of oocytes after vitrification with straws and cryoloops. In all intact oocytes, intracellular ice formed during the cooling process in both containers. In AQP3-expressing oocytes, however, ice did not form in 79–85% of the oocytes. This indicates that increasing the permeability of the plasma membrane to water and propylene glycol is effective in preventing the formation of intracellular ice during cooling. During warming, however, intracellular ice formed in all oocytes. Figure 5 shows the appearance of a vitrified AQP3 cRNA-injected oocyte after warming. Although all oocytes whitened during warming, they appeared to

be normal just after removal of the cryoprotectant. However, the oocytes swelled and finally ruptured after culture. In mammalian embryos, swelling suggests the formation of intracellular ice during cryopreservation [30]. As a result, no oocytes survived, regardless of the expression of AQP3. This suggests that dehydration and/or permeation by propylene glycol is insufficient to prevent intracellular ice from forming during warming even in AQP3-expressing oocytes.

Discussion

In this study, we tried to vitrify immature AQP3-expressing medaka oocytes. The expression of AQP3 was effective at preventing the formation of intracellular ice during cooling. However, no oocytes survived after vitrification.

Propylene glycol was much less toxic to intact immature medaka oocytes than ethylene glycol and Me_2SO (Fig. 2). Similar results have been reported for immature zebrafish oocytes [31]. Essentially the same results were obtained with AQP3-expressing oocytes (Figs. 2–4). We showed previously that the expression of AQP3 in immature medaka oocytes markedly increased permeability to water and cryoprotectants including propylene glycol [16]. This implies that AQP3-expressing oocytes would be more likely to be injured by the cryoprotectant. However, the results of the present study show that the expression of AQP3 does not enhance the toxic effect of cryoprotectants on immature oocytes (Figs. 2–4). One possible explanation is that exposure to a low concentration of the cryoprotectant for 60 min at 25 C is sufficient not only for AQP3-expressing oocytes but also for intact oocytes to equilibrate with the cryoprotectant solution.

At a high concentration (30% (v/v)) of propylene glycol (Fig. 3), on the other hand, the osmolality of the solution seems to have affected intact as well as AQP3-expressing oocytes, both of which were highly sensitive to hypertonic conditions (Fig. 1).

When intact oocytes were vitrified with a propylene glycol-based solution in a straw, all turned opaque during cooling, suggesting that dehydration and/or permeation by propylene glycol were insufficient for vitrification. Most AQP3-expressing oocytes, by contrast, remained transparent during cooling, suggesting that dehydration and permeation by propylene glycol were promoted by the expression of AQP3 as expected (Table 4). However, they became opaque during warming. To prevent intracellular ice from forming in AQP3-expressing oocytes
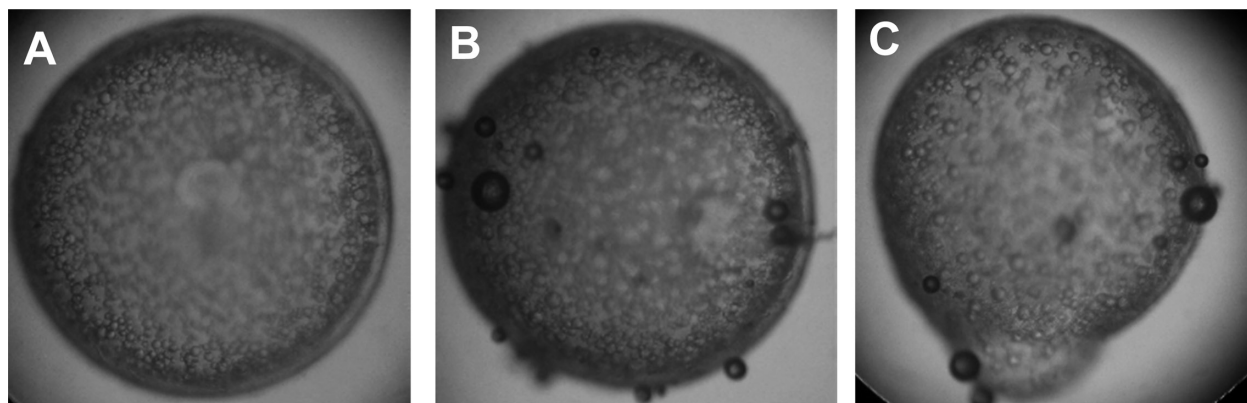
Fig. 5. The morphological change of immature medaka oocytes after vitrification with cryoloops. Aquaporin 3-expressing oocytes were exposed to the pretreatment solution (90% TCM199 medium containing 10% (v/v) propylene glycol) for 60 min at 25 C, suspended in the vitrification solution containing 30% (v/v) propylene glycol for 3 min at 25 C, and vitrified with liquid nitrogen. A, an aquaporin 3-expressing oocyte before exposure to the pretreatment solution; B, a vitrified aquaporin 3-expressing oocyte just after removal of cryoprotectants; C, a vitrified aquaporin 3-expressing oocyte after 1 h of culture at 26 C. Similar morphology was observed in vitrified intact oocytes.

during warming, we vitrified them using cryoloops. However, the oocytes became opaque during warming (Table 4). Since they became opaque quickly, it is possible that very small ice crystals had formed during cooling and recrystallized during warming [24]. To succeed in the cryopreservation of medaka oocytes, further enhancement of the permeability of the plasma membrane to water and cryoprotectants will be required.

It was found that immature medaka oocytes are highly sensitive to hypertonic conditions regardless of the expression of AQP3 (Fig. 1). In the present study, therefore, we reduced the concentration of sucrose in the vitrification solution. The mechanism by which oocytes are injured even under slightly hypertonic conditions is not known. However, the inclusion of a non-permeating small sugar like sucrose is effective in preventing excess swelling of oocytes during removal of the permeated cryoprotectant after warming [32]. Further study is necessary to clarify the mechanism behind and to find a way to overcome injury from hypertonic stress.

In the present study, we did not succeed in cryopreservation even with AQP3-expressing oocytes. Considering the large size of fish oocytes, however, our approach would be a step forward. To realize the cryopreservation of fish oocytes, it will be essential to further promote dehydration and permeation by cryoprotectants.

One way to increase permeability would be to increase the expression of AQP3 protein. In the present study, we cultured immature oocytes for 8-10 hours after AQP3 cRNA was injected. If the culture period could be extended further, AQP3 might be expressed more abundantly in the plasma membrane. However, we have previously reported that prolonged culture of immature oocytes collected after the LH surge makes them mature, which remarkably decreases the permeability of oocytes to water and cryoprotectants [16]. Therefore, the injection of AQP3 cRNA into oocytes at an earlier stage would be required. In zebrafish, we have recently developed such a system for zebrafish oocytes at stage III just before the LH surge [33]. In medaka, however, a reliable system of maturation *in vitro* for immature medaka oocytes before the LH surge has not been developed.

Another approach to increasing the dehydration and permeation by cryoprotectants of AQP-expressing oocytes would be exposure to cryoprotectants at low temperature. Generally, the lower the temperature is, the lower the toxicity is [34–36], and thus oocytes would better resist long-term exposure to cryoprotectants, which would increase their permeation. For simple diffusion through a lipid bilayer, however, the lower the temperature is, the slower the movement of water and solutes across the plasma membrane is. For facilitated diffusion through channels, on the other hand, movement is less dependent on temperature. Therefore, AQP3-expressing oocytes would resist exposure to cryoprotectants longer with less of a decrease in permeability.

In the present study, the exogenous expression of water/cryoprotectant channels in immature medaka oocytes was effective in preventing intracellular ice from forming during cooling. Although intracellular ice formed during warming, our strategy is a step forward for realizing the cryopreservation of fish oocytes.

Acknowledgments

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