Table 4
Permeability of immature medaka oocytes to cryoprotectants

Oocyte	P_{GLY}[a] ($\times 10^{-3}$ cm/min)	P_{EG}[b] ($\times 10^{-3}$ cm/min)	P_{PG}[c] ($\times 10^{-3}$ cm/min)	P_{DMSO}[d] ($\times 10^{-3}$ cm/min)
Intact	n.d.[e]	1.36 ± 0.34	1.97 ± 0.20	1.17 ± 0.52
AQP3 cRNA-injected	2.20 ± 1.29	2.98 ± 0.36**	3.93 ± 1.70**	3.11 ± 0.74**

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

Table 5
Developmental ability of immature medaka oocytes

Oocyte	No. of oocytes				No. of embryos
	Cultured	Matured (%)[a]	Artificially inseminated	Fertilized (%)[b]	Hatched (%)[c]
Intact	128	98 (77)	98	73 (74)	41 (42)
AQP3 cRNA-injected	67	34 (51)**	23	19 (83)	10 (43)

[a] % of matured oocytes.
[b] % of fertilized oocytes.
[c] % of hatched embryos after 10–14 days of culture.
** Significantly different from intact oocytes (χ^2 test, $P < 0.01$).

shrunk and regained their volume more rapidly in the cryoprotectant solutions, even in the glycerol solution, suggesting that they are highly permeable to cryoprotectants. The P_{GLY} value calculated from the volume changes was $2.20 \pm 1.29 \times 10^{-3}$ cm/min, and the P_{EG}, P_{PG}, and P_{DMSO} of cRNA-injected oocytes were 2.98 ± 0.36, 3.93 ± 1.70, and $3.11 \pm 0.74 \times 10^{-3}$ cm/min, respectively (Table 4). These values, except for the P_{GLY}, were about twice larger than those in intact oocytes. The results show that the artificial expression of AQP3 in immature medaka oocytes enhances the permeability of the plasma membrane to various cryoprotectants.

Developmental ability of aquaporin-3 cRNA-injected oocytes

Table 5 shows the developmental ability of intact and AQP3 cRNA-injected oocytes. Half of the cRNA-injected oocytes (51%) were able to mature *in vitro*, although this was significantly lower than the rate for intact oocytes (77%). After *in vitro* fertilization, 83% of matured AQP3 cRNA-injected oocytes were fertilized and 43% of inseminated oocytes developed to term, rates similar to those for intact oocytes (74% and 42%, respectively). These results suggest that many of the AQP3 cRNA-injected oocytes retained their ability to mature, to be fertilized, and to develop to term.

Discussion

Oocytes and embryos of freshwater fish have not been successfully cryopreserved. Previously, we suggested that medaka oocytes become less permeable to water and to cryoprotectants during maturation, probably by acquiring resistance to a hypotonic environment before being spawned in fresh water [23]. This would make it difficult to cryopreserve matured oocytes. Such a change has also been observed in zebrafish embryos; zebrafish embryos shrunk in hypertonic solutions, depending on the osmolality, but did not swell in hypotonic solutions [7]. On the other hand, immature oocytes have not yet acquired osmotic resistance [23]. They are therefore markedly more permeable to water and cryoprotectants than matured oocytes and behave as an ideal osmometer in hypertonic and hypotonic conditions. Immature zebrafish oocytes also appear to be highly permeable to cryoprotectants [31]. Thus, immature oocytes would be more suitable for the cryopreservation of freshwater fish oocytes than matured oocytes. However, they still may not be sufficiently permeable for cryopreservation because fish oocytes have a huge volume compared with mammalian oocytes and embryos.

In this study, we showed that immature medaka oocytes became more permeable to water (Fig. 1) and cryoprotectants (Fig. 2) when injected with

AQP3 cRNA. The L_P, P_{EG}, P_{PG}, and P_{DMSO} values of the cRNA-injected oocytes were about twice larger than those of intact oocytes (Table 4). The AQP3 cRNA-injected oocytes also had very high P_{GLY} values, although we could not calculate the P_{GLY} of intact oocytes because the permeability was too low.

In this study, we did not directly detect the marked expression of AQP3 in AQP3 cRNA-injected oocytes using immunological techniques because the cRNA did not contain reporter gene inserts (such as that of green fluorescent protein) and because immature oocytes of medaka have endogenous proteins of similar molecular weight to AQP3 which reacted to the commercially available anti rat AQP3 antibody we used (Santa Cruz Biotechnology, Inc., CA, USA) (data not shown). Therefore, we measured the L_P and the E_a for L_P of AQP3 cRNA-injected oocytes for deducing the expression of AQP3. Since the L_P value of AQP3 cRNA-injected oocytes (0.22 μm/min/atm) was significantly higher than that of intact oocytes (0.14 μm/min/atm) and the E_a value for L_P (5.5 kcal/mol) was markedly lower than that of intact oocytes (11.0 kcal/mol) (Table 3), AQP3 must have been expressed in AQP3 cRNA-injected medaka oocytes and increased the permeability of immature oocytes to water and cryoprotectants. However, the L_P value was not so high as those of cells predominantly expressing AQP3. Thus, AQP3 expressed in the oocytes might not be abundant. On the other hand, in intact immature oocytes, water should move principally through simple diffusion because a low L_P of cells with an E_a value higher than 10 kcal/mol is suggestive of simple diffusion across the plasma membrane [25].

It has been reported in *Xenopus* oocytes that AQP3 is permeable to various cryoprotectants, including glycerol [3,8,18,22,29,30], ethylene glycol [30], propylene glycol [30], and acetamide [18,22]. In the present study, we obtained similar results in AQP3-expressing medaka oocytes. Since the P_{PG}, P_{GLY}, P_{EG}, and P_{DMSO} values of AQP3 cRNA-injected oocytes were substantially the same, all the cryoprotectants used in this study would have a similar efficiency for the cryopreservation of AQP3 cRNA-injected oocytes from the viewpoint of permeability.

In zebrafish, Hagedorn et al. injected AQP3 cRNA into embryos at the 1–8 cell stage and succeeded in expressing AQP3 artificially, increasing the permeability to water and propylene glycol of their blastoderm tissues and yolk membrane at the 50% epiboly stage [7]. They reported that the L_P and P_{PG} values of AQP3-expressing embryos at the 50% epiboly stage (0.081 μm/min/atm and 0.05×10^{-3} cm/min, respectively) were markedly higher than those of intact embryos (0.004 μm/min/atm and 0.0084×10^{-3} cm/min, respectively). Our previous study showed that intact immature medaka oocytes are much more permeable to water (0.13 μm/min/atm) and propylene glycol (2.21×10^{-3} cm/min) [23]. These values are much higher than those of AQP3-expressing zebrafish embryos [7]. The present study shows that the permeability of immature medaka oocytes to water and propylene glycol was further increased by the artificial expression of AQP3 (0.22 μm/min/atm and 3.93×10^{-3} cm/min, respectively). Thus, AQP3-expressing immature medaka oocytes have remarkably greater permeability to water and propylene glycol than AQP3-expressing zebrafish embryos. Moreover, AQP3 cRNA-injected medaka oocytes retained the ability to develop to term (Table 5). These results also support our idea that the use of immature oocytes after the artificial expression of water channels is feasible for the cryopreservation of medaka. Although the maturation rate of AQP3 cRNA-injected oocytes was significantly lower than that of intact oocytes, the fertilization rate and hatching rate were not significantly different from those of intact oocytes. Hagedorn et al. showed in zebrafish that AQP3 cRNA-injected embryos expressed AQP3 for 96 h without abnormality. Thus, it seems that AQP3 cRNA-injected oocytes expressed AQP3 not only during their maturation but also until development to early stages after fertilization. If this is true for medaka oocytes, the decrease in maturation rates of the cRNA-injected oocytes might be caused by the toxicity of a large amount of RNA but the expression of AQP itself might not affect their abilities to fertilize and develop to early stages of embryos. However, in both intact and AQP3 cRNA-injected oocytes, hatching rates of oocytes matured and fertilized *in vitro* were low (42% for intact oocytes and 43% for AQP3 cRNA-injected oocytes) compared with naturally fertilized oocytes in our previous study (about 90%) [24]. Therefore, further improvements of methods for maturation and fertilization *in vitro* are required.

It has been shown that the membrane permeability of matured fish oocytes and embryos is low [7,23] and it has been suggested that the low permeability

is the most serious obstacle for cryopreservation of them [19]. Therefore, even a small change in membrane permeability of immature oocytes can have profound changes in the time needed for the cryoprotectant movement in oocytes in cryopreservation. Nevertheless, the increase in the permeability of immature oocytes on AQP3 expression might not be sufficient for cryopreservation because medaka oocytes are about 1000 times larger than mammalian oocytes and the artificial expression of AQP3 increased the permeability to water and cryoprotectants only about twofold. One way to increase the permeability of AQP3 cRNA-injected immature oocytes would be to elevate the level of AQP3 protein further. As a preliminary experiment, we prolonged the culture period of AQP3 cRNA-injected oocytes to 8–10 h in order for AQP3 protein to accumulate on the plasma membrane. However, the volume changes of the oocytes in cryoprotectant solutions were smaller than those of 6–7 h-cultured oocytes (data not shown). Since osmotic changes become suppressed in matured oocytes [23], the prolonged culture might cause the maturation of cRNA-injected oocytes and thus suppress changes in volume, even if more AQP3 were expressed in AQP3 cRNA-injected oocytes.

Therefore, improving the translation efficiency of AQP3 cRNA would be more suitable for increasing the permeability of AQP3 cRNA-injected immature oocytes. We used rat AQP3 cDNA inserted into the vector pSP64T for the synthesis of AQP3 cRNA. In this vector, 60 base pairs of the 5′ untranslated region of *Xenopus* β-globin cDNA exist at the 5′ of AQP3 cDNA. If the region is replaced with other proper sequences (such as the 5′ untranslated region of medaka β-globin cDNA), the expression of AQP3 might be increased. Further study is needed to improve the translation efficiency of AQP3 cRNA.

Another approach to enhance the permeability of AQP3 cRNA-injected immature oocytes would be to remove the chorion and follicular cell layer if they hinder the rapid movement of water and cryoprotectants. In general, an L_P value higher than 4.5 μm/min/atm and its E_a value lower than 6 kcal/mol are suggestive of the movement of water principally through water channels [25]. In this study, the L_P of AQP3 cRNA-injected immature oocytes (0.22 μm/min/atm) was about twice larger than that of intact immature oocytes (0.14 μm/min/atm) but was not so high, although the E_a value for L_P was quite low (5.5 kcal/mol) (Table 3). Therefore, it is plausible that the chorion and/or follicular cell layer of AQP3 cRNA-injected oocytes hindered the increased movement of water and cryoprotectants across the plasma membrane. However, it is difficult to remove the chorion and follicular cell layer of immature medaka oocytes without damaging them as previously described [23]. More studies are needed to develop a method for removing the chorion and follicular cell layer of immature oocytes without damaging them and to clarify if the chorion and/or follicular cell layer actually hinder the movement of water and cryoprotectants in the medaka.

In conclusion, the artificial expression of AQP3 in immature medaka oocytes would be a feasible way to increase permeability to water and cryoprotectants. With a further increase in permeability, the cryopreservation of teleost oocytes may be realized.

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Cryoprotectant permeability of aquaporin-3 expressed in Xenopus oocytes ☆

Yohei Yamaji [a], Delgado M. Valdez Jr. [a], Shinsuke Seki [a], Ken-ichi Yazawa [a], Chika Urakawa [a], Bo Jin [a], Magosaburo Kasai [a], F.W. Kleinhans [b], Keisuke Edashige [a,*]

[a] *Laboratory of Animal Science, College of Agriculture, Kochi University, Nankoku, Kochi 783 8502, Japan*
[b] *Department of Physics, Indianapolis University Purdue University Indianapolis, Indianapolis, IN 46202, USA*

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Abstract

It has been shown that aquaporin-3, a water channel, is expressed in mouse embryos. This type of aquaporin transports not only water but also neutral solutes, including cell-permeating cryoprotectants. Therefore, the expression of this channel may have significant influence on the survival of cryopreserved embryos. However, permeability coefficients of aquaporin-3 to cryoprotectants have not been determined except for glycerol. In addition, permeability coefficients under concentration gradients are important for developing and improving cryopreservation protocols. In this study, we examined the permeability of aquaporin-3 to various cryoprotectants using Xenopus oocytes. The permeability of aquaporin-3 to cryoprotectants was measured by the volume change of aquaporin-3 cRNA-injected oocytes in modified Barth's solution containing either 10% glycerol, 8% ethylene glycol, 10% propylene glycol, 1.5 M acetamide, or 9.5% DMSO (1.51–1.83 Osm/kg) at 25 °C. Permeability coefficients of aquaporin-3 for ethylene glycol and propylene glycol were 33.50 and 31.45×10^{-3} cm/min, respectively, which were as high as the value for glycerol (36.13×10^{-3} cm/min). These values were much higher than those for water-injected control oocytes ($0.04–0.11 \times 10^{-3}$ cm/min). On the other hand, the coefficients for acetamide and DMSO were not well determined because the volume data were poorly fitted by the two parameter model, possibly because of membrane damage. To avoid this, the permeability for these cryoprotectants was measured under a low concentration gradient by suspending oocytes in aqueous solutions containing low concentrations of acetamide or DMSO dissolved in water (0.20 Osm/kg). The coefficient for acetamide (24.60×10^{-3} cm/min) was as high as the coefficients for glycerol, ethylene glycol, and propylene glycol, and was significantly higher than the value for control (6.50×10^{-3} cm/min). The value for DMSO (6.33×10^{-3} cm/min) was relatively low, although higher than the value for control (0.79×10^{-3} cm/min). This is the first reported observation of DMSO transport by aquaporin-3.

Keywords: Aquaporin-3; Xenopus; Oocyte; Cryoprotectant; Permeability

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* Corresponding author. Fax: +81 88 864 5200.
E-mail address: keisuke@cc.kochi-u.ac.jp (K. Edashige).

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In most types of cells, water moves through the plasma membrane with limited permeability by simple diffusion. However, the plasma membrane of red blood cells and cells in renal proximal tubules are extremely permeable to water. From biophysical analysis of the water-transport pathway by measurements of temperature dependence and osmotic-to-diffusional water permeabilities, the presence of channels for facilitated transport of water had been expected in these cells. In 1990s, small membrane integral proteins that act as the water channel, called aquaporins (AQPs), were discovered [11]. The AQPs occur in two groups; one group, such as AQP1 and AQP2, is highly selective for the passage of water, and the other group, such as AQP3 and AQP7, transports not only water but also neutral solutes with a small molecular weight, including cell-permeating cryoprotectants.

It has been shown that mRNAs of AQPs, including AQP3, are expressed in mouse oocytes at the metaphase II stage and embryos at various developmental stages [4,16,17], and that AQP proteins, including AQP3, are also expressed in mouse embryos [1,6]. Thus, it is possible that AQP3 plays a significant role in the transport of water and cryoprotective solutes and thus in the tolerance for cryopreservation in oocytes and embryos. This possibility is supported by our recent study that artificial expression of AQP3 in mouse oocytes elevates the permeability to water and glycerol and that this actually improves the survival of mouse oocytes after cryopreservation [5].

Using Xenopus oocytes, many researchers have shown that AQP3 transports glycerol [3,8,15,22,24]. It has also been shown that AQP3 transports other cryoprotective solutes, i.e., ethylene glycol, propylene glycol and acetamide, in the Xenopus system [15,20,24]. However, the possibilities for exploiting these channels in cryobiology have been largely unexplored to date.

First, in many studies, glycerol-permeability was measured with very low concentrations of radio-labeled glycerol in isotonic salt solutions [8,22,24]. In this circumstance, the permeability of the plasma membrane to solutes, which can permeate through the plasma membrane rapidly, would be underestimated because of the presence of unstirred layer outside the plasma membrane. Indeed, it has been shown that, without osmotic gradients, the permeabilities of the plasma membrane to water and ions are limited during diffusion through the unstirred layer outside the cells [7]. For cryopreservation, cells have to be exposed to high concentrations of cryoprotectants. Thus, the permeability to cryoprotectants under high concentration gradients of the cryoprotectants is important for developing and improving cryopreservation protocols.

Second, many studies only report the reflection coefficient, sigma, for cryoprotectant permeation of AQP3 without specifying the cryoprotectant-permeability coefficient [15,20,24]. However, this only shows that cryoprotectants can move through AQP3-expressing Xenopus oocytes. To develop and improve cryopreservation protocols, it is very useful to know cryoprotectant movement in cells during exposure to cryopreservation solutions and during removal of cryoprotectants after warming. Thus, it is important to determine the permeability coefficients of AQP3-expressing cells to cryoprotectants in cryobiology studies.

Although we have tried to examine the glycerol-permeability of AQP3 using mouse oocytes [5], they are difficult to handle because of their small size, whereas Xenopus oocytes are large and commonly used for cloning and characterizing channel proteins. In this study, therefore, we examined the permeability of AQP3 to water and five cryoprotectants, i.e., glycerol, ethylene glycol, propylene glycol, acetamide, and DMSO, using Xenopus oocytes injected with AQP3 cRNA.

Materials and methods

Preparation of AQP3 cRNA

The cDNA of AQP3 was cloned from rat kidney cDNA by polymerase chain reaction (PCR) as described previously [5] by use of the rat AQP3 sequence [2] (Genbank™ accession No. L35108). The BamHI/XbaI fragment of the PCR product was subcloned into the BglII/XbaI site of a pSP64T (a generous gift from Dr. Paul A. Krieg), a Xenopus expression plasmid. BamHI, Bg1III, and XbaI were obtained from Takara Shuzo Co. Ltd. (Tokyo, Japan). After digestion of the construct by EcoRI (Takara Shuzo), capped cRNA of AQP3 was synthesized using SP6 polymerase (New England Biolabs, Beverly, MA, USA).

Preparation of intact oocytes and water- or AQP3 cRNA-injected oocytes

A mature female frog (*Xenopus laevis*) was anesthetized by leaving it in crushed ice for at least

30 min, and was killed by cutting off its cervical spinal cord. The ovaries were recovered and oocytes were defolliculated by suspending them in modified Barth's solution (MBS) with 0.2% collagenase without Ca^{2+} at 18 °C for 40–90 min. The composition of MBS is as follows: NaCl, 5.13 g; KCl, 0.075 g; $NaHCO_3$, 0.20 g; $MgSO_4$ $7H_2O$, 0.20 g; $Ca(NO_3)_2$ $4H_2O$, 0.08 g; $CaCl_2$ $2H_2O$, 0.06 g; HEPES, 2.38 g in 1 l aqueous solution.

Oocytes at the V–VI stage with normal shape and color were collected, washed with MBS without Ca^{2+} repeatedly, and incubated in MBS at 18 °C for 10–14 h. Then, oocytes with normal shape and color were selected. Some of them were used for measuring the osmotically inactive content of oocytes. Others were placed in about 15 ml of MBS in a Petri dish (90 × 10 mm) in which two-layers of gauze were spread, and injected with 40 nl of water or AQP3 cRNA solution (1 ng/nl) with an injection needle connected to an injector (Nanoject™, Drummond, Broomall, PA, USA). Oocytes injected with water- or AQP3 cRNA and oocytes without injection were cultured in MBS in an incubator at 18 °C for about 48 h with daily exchange of MBS, and oocytes having normal shape and color were considered 'survivors', and used as 'water-injected', 'AQP3 cRNA-injected', and 'intact' oocytes, respectively.

Detection of AQP3 in AQP3 cRNA-injected oocytes

Intact and AQP3 cRNA-injected oocytes were cultured for 48 h in MBS as described above. In each group, five oocytes were disrupted by sonication at 4 °C in 1 ml of 20 mM Tris–HCl (pH 8.0) containing 0.15 M NaCl, 1 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 0.2 μg/ml aprotinin, 10 μg/ml phenylmethylsulphonyl fluoride (homogenation buffer). The homogenate was centrifuged at 10,000*g* for 15 min at 4 °C, and the supernatant was recovered. Then, the supernatant was centrifuged at 100,000*g* for 1 h at 4 °C in order to separate the membrane fraction (the precipitate) from the cytosol fraction (the supernatant). The cytosol fraction was discarded, and the membrane fraction was washed with ice-cold homogenation buffer repeatedly. The membrane fraction was dissolved in 0.05 M Tris–HCl (pH 6.8) containing 0.3% sodium dodecyl sulfate (SDS), 35 μg/ml dithiothreitol, 0.16 mg/ml bromophenol blue, and 5% glycerol, and separated by SDS-polyacrylamide gel electrophoresis (12% gel). AQP3 on a polyvinyliden fluoride (PVDF) membrane after electric transfer from an SDS-PAGE gel was detected by immunoblotting. Briefly, the PVDF membrane was incubated at 4 °C overnight with 5% skim milk in 20 mM Tris–HCl (pH 8.0) containing 0.15 M NaCl and 0.1% Tween 20 (TBS-T). After being washed with TBS-T, the membrane was incubated with 1:200 dilution of rabbit anti rat AQP3 antibody (Calbiochem, Darmstadt, Germany) in 1% skim milk in TBS-T at room temperature for 1 h. After being washed with TBS-T, the membrane was further incubated with 1:2500 dilution of horseradish peroxidase-labeled donkey anti rabbit IgG antibody (GE Healthcare Bio-Science, Piscataway, NJ, USA) dissolved in 1% skim milk in TBS-T at room temperature for 1 h. Immunoreactive proteins were visualized by use of ECL Western blotting detection (GE Healthcare Bio-Science).

Measurement of the osmotically inactive fraction of oocytes

The osmotically inactive cell content (V_b) of oocytes was determined from the osmotic behavior of oocytes in hypotonic NaCl solutions only, because oocytes shrunk then re-swelled in hypertonic NaCl solutions (over 0.40 Osm/kg) at 25 °C during 1–2 h of exposure. An oocyte, 10–14 h after being defolliculated, was equilibrated in an isotonic NaCl solution (0.20 Osm/kg) at 25 °C for at least 60 min, and then transferred carefully under a stereoscopic microscope, with a minimal amount of MBS using a small Pasteur pipette aided with a mouth piece, into 3 ml of hypotonic NaCl solutions (0.08, 0.10, and 0.15 Osm/kg) in a culture dish (35 × 10 mm) covered with paraffin oil and incubated at 25 °C. The microscopic images of the oocyte during the exposure to the solution were recorded for 4 h by a time-lapse videotape recorder (ETV-820, Sony, Tokyo, Japan). The cross-sectional area of each oocyte was measured using an image analyzer (VM-50, Olympus, Tokyo, Japan). The area was expressed as a relative cross-sectional area, S, by dividing it by the area of the same oocyte at 0 h, and then converted into relative volume; the relative volume V was obtained from $V = S^{3/2}$. The V_b value of the oocytes was obtained from the Boyle–van't Hoff relationship using the relative volume of each oocyte at 3 h after exposure to NaCl solutions. The osmolality of NaCl solutions were measured with a freezing point depression osmometer (OM801; Vogel, Giessen, Germany).

To cross check the V_b value obtained from the volumetric experiments, we measured the ratio of dry weight/wet weight of oocytes. Briefly, 20 intact oocytes were placed on a small piece of aluminum foil, wiped with wiper tissue in order to remove MBS around oocytes, and measured the weight (a). Then, the piece of foil with oocytes was dried at 110 °C for 2 h. The piece of foil was cooled to room temperature, and measured the weight (b). After being removed dried oocytes, the weight of the piece of foil was measured (c). The ratio of dry weight/wet weight of oocytes was calculated from (b − c)/(a − c).

Measurement of water- and cryoprotectant-permeability of oocytes

An intact oocyte or a water- or AQP3 cRNA-injected oocyte was equilibrated with 3 ml of isotonic MBS (0.20 Osm/kg) in a culture dish at 25 °C for at least 60 min. For determining the hydraulic conductivity (L_P), an oocyte was transferred carefully with a minimal volume of MBS into 3 ml of MBS diluted with distilled water (0.2 × 0.04 Osm/kg) in a culture dish at 25 °C, and it was kept there for 2 (AQP3 cRNA-injected oocytes) or 10 (intact and water-injected oocytes) min. For determining the cryoprotectant permeability (P_s) and L_P in the presence of cryoprotectants, an oocyte was transferred into 3 ml of cryoprotectant solutions at 25 °C and was kept for 10 min. The cryoprotectant solutions were MBS containing 10% (vol/vol) glycerol, 8% (vol/vol) ethylene glycol, 10% (vol/vol) propylene glycol, 1.5 M (8.86% wt/vol) acetamide, or 9.5% DMSO (vol/vol), and the osmolalities of the cryoprotectant solutions measured with a vapor pressure osmometer (Wescor 5500; Wescor Inc., Logan, UT, USA) were 1.73, 1.76, 1.51, 1.83, and 1.83 Osm/kg, respectively. Identical osmolalities of all the cryoprotectants were intended, but not fully achieved. In one experiment, we also used isomotic acetamide and DMSO solutions (in distilled water) at 25 °C (0.20 Osm/kg, measured with a vapor pressure osmometer). The osmolality of MBS was measured with a freezing point depression osmometer.

The microscopic images of oocytes during the exposure to the solutions were recorded by a time-lapse videotape recorder. The cross-sectional area of each oocyte was measured for 2 or 10 min using an image analyzer. The area, S, was obtained and converted into V as described above. By fitting water- and solute-movement using a two-parameter formalism, L_P of oocytes in hypotonic MBS and L_P and P_s of oocytes in cryoprotectant solutions were determined [5,13]. Since folding of the plasma membrane of Xenopus oocytes increases surface area of the oocytes by a factor of 9 [23], their surface area, A, was obtained by multiplying apparent surface area calculated from the cross-sectional area of oocytes by 9.

The parameters used are listed in Table 1.

Table 1
Parameters used for fitting permeability parameters

Symbol	Meaning	Values
R	Gas constant (l 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
$\overline{V}_{Gly}$	Partial molar volume of glycerol[a]	0.071 l/mol
$\overline{V}_{EG}$	Partial molar volume of ethylene glycol[a]	0.054 l/mol
$\overline{V}_{PG}$	Partial molar volume of propylene glycol[a]	0.070 l/mol
$\overline{V}_{AA}$	Partial molar volume of acetamide[b]	0.056 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. [21].
[b] Partial molar volumes of acetamide and DMSO from Kiyohara et al. [12].

Results

The expression of AQP3 in AQP3 cRNA-injected oocytes

Fig. 1 shows the expression of AQP3 in intact and AQP3 cRNA-injected Xenopus oocytes 48 h after culture. The immunoreactive-band with about 35 kDa, which was coincident with the molecular weight of AQP3, was detected in membrane fraction of AQP3 cRNA-injected oocytes but not in that of intact oocytes. The result indicates that AQP3 cRNA was translated and expressed in the membrane of Xenopus oocytes 48 h after culture.

Osmotically inactive fraction of oocytes

As a preliminary experiment, we exposed oocytes to hypertonic NaCl solutions at 25 °C (0.40, 0.60, and 1.00 Osm/kg). However, the oocytes gradually shrunk for the first 0.5–1.0 h but gradually swelled thereafter (data not shown). The oocytes may be injured by high concentrations of NaCl, or might actively regulate their volume during exposure to the NaCl solutions. Thus, we could not calculate the V_b value of Xenopus oocytes from their volume

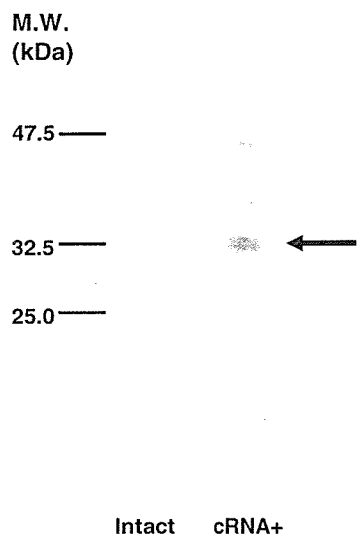

Fig. 1. Immunoblotting for the presence of AQP3 in Xenopus oocytes. Intact oocytes (intact) and AQP3 cRNA-injected oocytes (cRNA+) were cultured at 18 °C for 48 h. AQP3 protein (arrow) was detected in the membrane fraction of AQP3 cRNA-injected oocytes.

change in hypertonic NaCl solutions. On the other hand, when oocytes were suspended in hypotonic NaCl solutions with various osmolalities (0.08, 0.10, and 0.15 Osm/kg) for 4 h at 25 °C, the oocytes swelled for 1 h and then reached a steady state (data not shown).

Thus, we used the relative volumes of oocytes at 3 h after exposure to the hypotonic NaCl solutions to calculate V_b. Fig. 2 shows the Boyle–van't Hoff plot of relative volumes of oocytes, yielding a V_b of 0.68. This value is consistent with the wet-dry weight determination of V_b, which also yielded 0.68. This high value is consistent with large yolk stores in the oocyte.

We used this value for calculating L_P and P_s of oocytes.

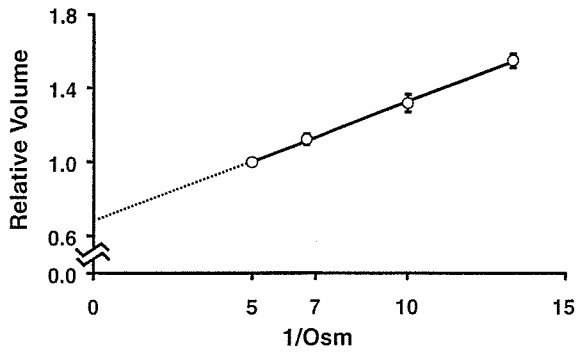

Fig. 2. Boyle–van't Hoff plot for Xenopus oocytes derived from equilibrium volumes after exposure to isotonic and hypotonic NaCl solutions having various osmolality (0.08, 0.10, 0.15, and 0.20 Osm/kg). Each point is the mean relative volume ± SD from 3 to 4 oocytes. The 'y' intercept indicates that the osmotically inactive volume, V_b, is 0.68 of the isotonic volume.

Water permeability of intact oocytes and water- or AQP3 cRNA-injected oocytes in hypotonic MBS solution

Fig. 3 shows relative volume changes of intact oocytes and water- or AQP3 cRNA-injected oocytes in hypotonic MBS solution diluted with distilled water at 25 °C for the first 2 min. Water-injected oocytes swelled very slowly whereas AQP3 cRNA-injected oocytes swelled very rapidly, indicating that water permeability of the oocyte increased markedly after injection of AQP3 cRNA. Intact oocytes behaved quite similarly to water-injected ones (Fig. 3). The L_P value of water-injected oocytes calculated from volume changes for 10 min was 0.06 ± 0.01 μm/min/atm (Table 2). On the other hand, the L_P value of AQP3 cRNA-injected oocytes calculated from the volume changes for 2 min was 1.09 ± 0.05 μm/min/atm, which was about 15 times higher than that of water-injected ones (Table 2). These results show that AQP3 cRNA-injected oocytes expressed AQP3 abundantly. Because the L_P value of intact oocytes (0.06 ± 0.01 μm/min/atm)

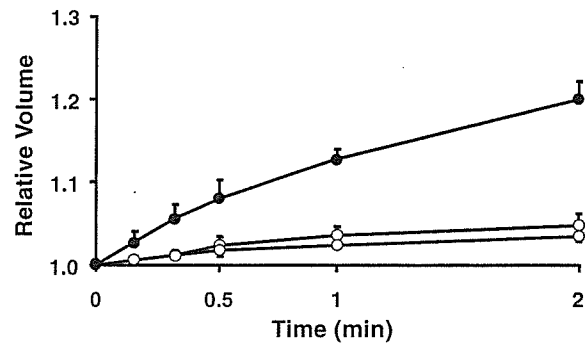

Fig. 3. Change in cell volume of intact (shaded circles) and water- (open circles) or AQP3 cRNA- (closed circles) injected Xenopus oocytes in hypotonic modified Barth's solution. Intact oocytes and water- or AQP3 cRNA-injected oocytes were equilibrated with isotonic modified Barth's solution (0.20 Osm/kg) at 25 °C. Then, the oocytes were exposed to 0.2 × MBS (0.04 Osm/kg) diluted with distilled water for 2 min (AQP3 cRNA-injected oocytes) to 10 min (intact and water-injected oocytes) at 25 °C. The graph shows the volume changes during the first 2 min. Data are means of relative volumes ± SD from 7 to 20 oocytes.

Table 2
Hydraulic conductivity (L_P) and cryoprotectant permeability (P_s) of AQP3 cRNA-injected Xenopus oocytes in hypotonic solution and cryoprotectant solutions

Permeability	AQP3[a]	0.2 × MBS[b]	10% Gly [c]	8% EG[d]	10% PG[e]	1.5 M AA[f]	9.5% DMSO
L_P (μm/min/atm)	–	0.06 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	nd[g]	nd
	+	1.09 ± 0.05*	1.18 ± 0.35*	1.14 ± 0.13*	1.04 ± 0.23*	nd	nd
P_s (10^{-3} cm/min)	–	–	0.04 ± 0.01	0.11 ± 0.03	0.10 ± 0.02	nd	nd
	+	–	36.13 ± 7.63*	33.50 ± 2.75*	31.45 ± 5.17*	nd	nd

[a] Oocytes injected with water (–) or AQP3 cRNA (+).
[b] 0.2 × modified Barth's solution diluted with distilled water.
[c] Glycerol.
[d] Ethylene glycol.
[e] Propylene glycol.
[f] Acetamide.
[g] Not determined.
* Significantly different from water-injected oocytes (Student's t-test, $P < 0.01$).

calculated from volume changes for 10 min was quite similar to that of water-injected ones (Table 2), we used only water-injected oocytes as the control in the following experiments.

The permeability of water- or AQP3 cRNA-injected oocytes to water and cryoprotectants in cryoprotectant solutions under high concentration gradients

Fig. 4A shows relative volume changes of oocytes in 10% glycerol in MBS at 25 °C for 10 min. Water-injected oocytes shrunk slowly but did not swell for 10 min, suggesting that water and glycerol permeated oocytes quite slowly. On the other hand, AQP3 cRNA-injected oocytes shrunk slightly and swelled rapidly, suggesting that glycerol permeated the oocytes very rapidly. The L_P value of cRNA-injected oocytes (1.18 ± 0.35 μm/min/atm) in 10% glycerol was much higher than that of water-injected ones (0.02 ± 0.00 μm/min/atm) (Table 2). The P_{Gly} value of cRNA-injected oocytes (36.13 ± 7.63 × 10^{-3} cm/min) was also substantially higher than that of water-injected ones (0.04 ± 0.01 × 10^{-3} cm/min) (Table 2). These results show that AQP3 transports water and glycerol efficiently in glycerol solution under a high concentration gradient of glycerol. Fig. 4B and C show relative volume changes of oocytes in 8% ethylene glycol and 10% propylene glycol in MBS at 25 °C, respectively. Water-injected oocytes shrunk slowly but did not swell for 10 min, suggesting that ethylene glycol and propylene glycol also permeate oocytes quite slowly. On the other hand, AQP3 cRNA-injected oocytes shrunk slightly and swelled rapidly in these solutions as in glycerol solution. Values for L_P and P_{EG} of cRNA-injected oocytes in ethylene glycol solution were 1.14 ± 0.13 μm/min/atm and 33.50 ± 2.75 × 10^{-3} cm/min, respectively, and those in propylene glycol solution were 1.04 ± 0.23 μm/min/atm and 31.45 ± 5.17 × 10^{-3} cm/min, respectively (Table 2). These values were substantially higher than those of water-injected oocytes in ethylene glycol solution (0.02 ± 0.00 μm/min/atm and 0.11 ± 0.03 × 10^{-3} cm/min) and in propylene glycol solution (0.03 ± 0.01 μm/min/atm, and 0.10 ± 0.02 × 10^{-3} cm/min) (Table 2). These results show that AQP3 transports not only water but also ethylene glycol and propylene glycol as efficiently as glycerol under high concentration gradients of the cryoprotectants.

In 1.5 M acetamide (Fig. 4D) or 9.5% (vol/vol) DMSO (Fig. 4E) in MBS, water-injected oocytes shrunk slowly and did not swell during exposure at 25 °C for 10 min. AQP3 cRNA-injected oocytes shrunk more slowly but swelled only a little during 10 min of exposure. However, these curves were poorly fitted by our two-parameter model for calculation of the L_P and P_s. Thus, we did not calculate the permeability of AQP3-exspressing oocytes to acetamide and DMSO under these conditions.

Cryoprotectant permeability of water- or AQP3 cRNA-injected oocytes to acetamide and DMSO solutions under low concentration gradients

Next, we tried to measure volume changes of oocytes in acetamide and DMSO solutions containing lower concentrations of the cryoprotectants (0.20 Osm/kg) for 10 min in order to determine the membrane permeability to acetamide and DMSO (Fig. 5). Oocytes injected with AQP3 cRNA swelled more rapidly than water-injected ones in isotonic

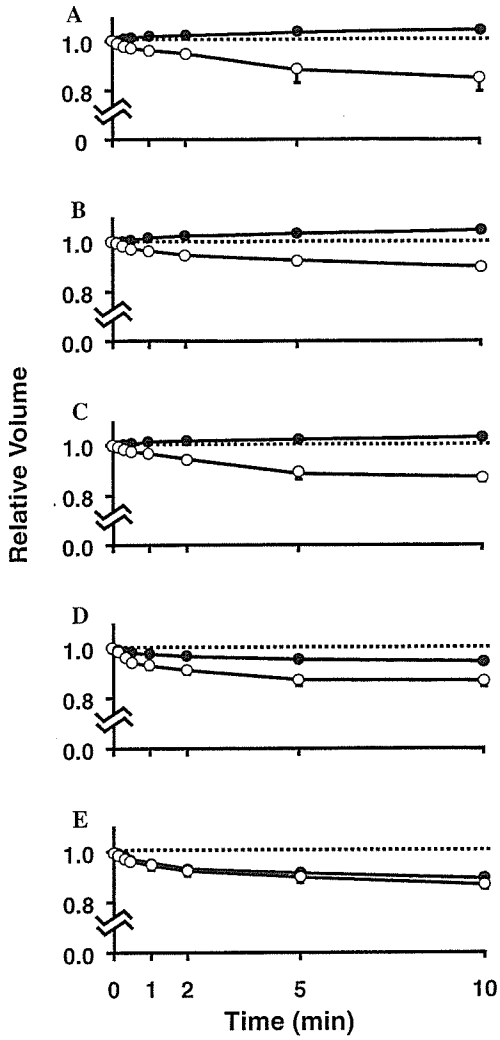

Fig. 4. Change in cell volume of water- (open circles) or AQP3 cRNA- (closed circles) injected Xenopus oocytes in 10% (vol/vol) glycerol (A), 8% (vol/vol) ethylene glycol (B), 10% (vol/vol) propylene glycol (C), 1.5 M (8.86% wt/vol) acetamide (D), and 9.5% (vol/vol) DMSO (E) in modified Barth's solution. Water- or AQP3 cRNA-injected oocytes were equilibrated with isotonic modified Barth's solution at 25 °C. Then, the oocytes were exposed to modified Barth's solutions containing various cryoprotectants at 25 °C for 10 min. Data are means of relative volumes ± SD from 5 oocytes.

acetamide (Fig. 5A) and DMSO (Fig. 5B) solutions, whereas water-injected oocytes swelled only slightly in these solutions, indicating that AQP3 transports acetamide and DMSO (Fig. 5). In acetamide solution, values for L_P and P_s of cRNA-injected oocytes were 0.09 ± 0.02 μm/min/atm and 24.60 ± 9.90 × 10^{-3} cm/min, respectively (Table 3), which were significantly higher than those for water-injected oocytes (0.02 ± 0.00 μm/min/atm and 6.50 ± 1.98 × 10^{-3} cm/min, respectively) (Table 3). In DMSO solution, values for L_P and P_s of cRNA-injected oocytes were 0.05 ± 0.01 μm/min/atm and 6.33 ± 2.76 × 10^{-3} cm/min, respectively, and they were also significantly higher than those of water-injected oocytes (0.02 ± 0.01 μm/min/atm and 0.79 ± 0.40 × 10^{-3} cm/min, respectively) (Table 3). Thus, it was found that AQP3 can also transport acetamide and DMSO under low concentration gradients, although the transport of DMSO is less efficient than that of other cryoprotectants.

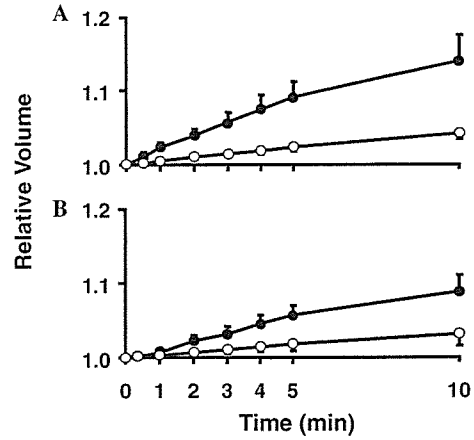

Fig. 5. Change in cell volume of water- (open circles) or AQP3 cRNA- (closed circles) injected Xenopus oocytes in isotonic acetamide (A) and DMSO (B) solutions. Water- or AQP3 cRNA-injected oocytes were equilibrated with isotonic modified Barth's solution at 25 °C. Then, the oocytes were exposed to an isotonic acetamide or DMSO solution (0.20 Osm/kg, dissolved in water) at 25 °C for 10 min. Data are means of relative volumes ± SD of 4–5 oocytes.

Table 3
Hydraulic conductivity (L_P) and cryoprotectant permeability (P_s) of AQP3 cRNA-injected Xenopus oocytes to acetamide (AA) and DMSO under low concentration gradients

Permeability	AQP3[a]	0.2 Osm/kg AA[b]	0.2 Osm/kg DMSO
L_P (μm/min/atm)	–	0.02 ± 0.00	0.02 ± 0.01
	+	0.09 ± 0.02*	0.05 ± 0.01*
P_s (10^{-3} cm/min)	–	6.50 ± 1.98	0.79 ± 0.40
	+	24.60 ± 9.90*	6.33 ± 2.76*

[a] Oocytes injected with water (–) or AQP3 cRNA (+).
[b] Acetamide.
* Significantly different from water-injected oocytes (Student's t-test, $P < 0.01$).

Discussion

In this study, we show that AQP3 transports ethylene glycol and propylene glycol as efficiently as

glycerol under high concentration gradients of the cryoprotectants. It has been reported that AQP3 transports glycerol [3,8,15,20,22,24] but, in many of the studies, values for P_{Gly} were measured with very low concentrations of radio-labeled glycerol in isotonic salt solutions [8,24] except for a report by Echevarria et al. [3], in which P_{Gly} was measured not only by a radio-isotopic method but also by a volumetric method. The P_{Gly} value measured with radio-labeled glycerol in their studies was ~0.05–0.17×10^{-3} cm/min at around room temperature [3,8,24] (P_{Gly} values that Ishibashi et al. [8] and Echevarria et al. [3] reported were recalculated, based on the assumption that the surface area of oocytes is nine times larger than that calculated from their diameter). On the other hand, in the present study, we measured P_{Gly} under high concentration gradients (1.73 Osm/kg) and obtained remarkably higher P_{Gly} value of AQP3 cRNA-injected oocytes (36.13×10^{-3} cm/min) (Table 2). There are two possibilities that may cause these differences in P_{Gly} values.

One is the presence or absence of concentration gradients of glycerol. We measured P_{Gly} under a very high concentration gradient (1.37 M) of glycerol whereas other researchers measured it under very low concentration gradients of glycerol. This is plausible because Echevarria et al. [3] reported that P_{Gly} obtained from a volumetric method (measured by swelling of oocytes in an isotonic solution containing relatively low concentration of glycerol (0.17 M)) was three times larger than that obtained from a radio-isotopic method (using a very low concentration of radio-labeled glycerol).

The other is the possible difference in the expression level of AQP3 in AQP3 cRNA-injected oocytes between this study and other studies. However, values for L_P of intact and water-injected oocytes (0.06 μm/min/atm) and of AQP3 cRNA-injected oocytes (1.09 μm/min/atm) in hypotonic salt solutions at 25 °C obtained in this study (Table 2) were similar to those in other studies using hypotonic salt solutions [3,8,15,24]. In the studies, L_P value was ~0.07–0.10 μm/min/atm for intact and water-injected oocytes and 0.84–1.85 μm/min/atm for AQP3 cRNA-injected oocytes (water-permeability, p_f, in Ishibashi et al. [8] and Echevarria et al. [3] was recalculated to L_P, based on the assumption that the surface area of oocytes is nine times larger than that calculated from their diameter). Thus, AQP3 cRNA-injected oocytes in the present study had similar expression level of AQP3 as those in the previous studies. Therefore, the remarkable difference between values for P_{Gly} in the present study and those in the previous studies may be caused by the presence or absence of high concentration gradient of glycerol.

On the other hand, P_{Gly} of water-injected oocytes (0.04×10^{-3} cm/min) obtained in the present study (under a high concentration gradient of glycerol) was similar to that obtained in previous studies ($0.01–0.03 \times 10^{-3}$ cm/min) (under very low concentration gradients) [3,8]. Since the P_{Gly} value of water-injected oocytes is quite low, concentration gradients may have little or no effect on the permeability of the oocytes to glycerol.

Thus, to predict water and cryoprotectant movements in cells during cryopreservation and to develop and improve cryopreservation protocols by using permeability coefficients of cells to water and cryoprotectants, it would be necessary to determine the coefficients under high concentration gradients of cryoprotectants if the cells abundantly express AQPs or other cryoprotectant-permeable channels.

In this study, we have determined the permeability coefficients of AQP3 to ethylene glycol and propylene glycol. These have not previously been measured, although both cryoprotectants have been shown to permeate AQP3 channels [20,24]. Since values for L_P and P_s of AQP3 cRNA-injected oocytes in ethylene glycol and propylene glycol solutions were also comparably high to those in glycerol (Table 2), these cryoprotectants may be as comparably effective as glycerol in cryopreserving AQP3-expressing cells, from the viewpoint of permeability of cells to cryoprotectants.

On the other hand, in solutions containing 1.5 M acetamide or 9.5% DMSO, AQP3 cRNA-injected oocytes shrunk more slowly than water-injected ones and re-swelled only slightly, and the volume changes were poorly fitted by the two-parameter model (Fig. 4). This poor fit suggests that the high acetamide and DMSO concentrations damaged the cell membrane, preventing them from acting as ideal osmometers. Thus, we failed to determine the membrane permeability to acetamide and DMSO under high concentration gradients. Since Tsukaguchi et al. [20] already showed that AQP3 cRNA-injected oocytes swelled in isotonic acetamide solution, acetamide (and possibly DMSO also) must move through AQP3-expressing oocytes. High concentrations of the cryoprotectants might affect the volume change of oocytes. Indeed, it has been shown that DMSO affects the cytoskeleton of cells

by polymerizing intracellular microtubules [10]. Thus, DMSO could reduce the volume change of AQP3 cRNA-injected oocytes by its effect on the cytoskeleton of oocytes. However, such an effect has not been demonstrated in acetamide. Further studies are needed to elucidate the reason why shrinkage and swelling of AQP3 cRNA-injected oocytes were suppressed in solutions containing high concentrations of acetamide.

Thus, using isotonic acetamide and DMSO solutions, we examined the permeability of AQP3 to acetamide and DMSO (under low concentration gradients of cryoprotectants) (Fig. 5). AQP3 cRNA-injected oocytes in these solutions swelled faster than water-injected ones, indicating that AQP3 transports acetamide and DMSO. To our knowledge, this is the first report to show that AQP3 transports DMSO.

Although L_P values of AQP3 cRNA-injected oocytes in isotonic acetamide and DMSO solutions were higher than those of water-injected ones (Table 3), the L_P of cRNA-injected oocytes in the isotonic solutions were much smaller than those in hypertonic glycerol, ethylene glycol, and propylene glycol solutions (Table 2). The presence or absence of osmotic gradients in the solutions may affect the L_P value of cRNA-injected oocytes.

The present study showed that AQP3 transports ethylene glycol and propylene glycol as efficiently as glycerol under conditions appropriate for cryopreservation of mammalian embryos. It has been shown that mRNA of AQP3 and AQP3 protein are expressed in mouse embryos [1,4,6,16,17], and the expression of AQP3 protein increases at later stages [1,6]. A few studies have demonstrated the changes in the permeability of mouse embryos to cryoprotectants. The pioneering studies by Mazur and his group demonstrated that the permeability of mouse embryos to glycerol slightly increased from oocytes and 1-cell zygotes to 8-cell embryos [9,14]. We reported that the permeability of mouse embryos to water and glycerol increased remarkably at around the morula stage, and that AQP3 was markedly expressed in that stage [6]. Barcroft et al. also reported that AQP3 was markedly expressed in the morula stage [1]. They also reported that the permeability of mouse embryos to glycerol was high at the blastocyst stage and various AQPs were expressed at that stage [1]. These studies strongly suggest that water and glycerol movement in mouse embryos at later stages depends on facilitated diffusion by AQP3. Moreover, we have also examined volume changes of mouse oocytes and embryos in various cryoprotectant solutions in preliminary experiments and showed that the pattern of cryoprotectant permeation does not change from matured oocytes up to cleavage stage embryos, but permeability of embryos to cryoprotectants, especially to glycerol and ethylene glycol, drastically increased at around the morula stage [19]. Similar changes were also observed in bovine embryos [18]. As shown in the present study, AQP3 can transport various cryoprotectants. Therefore, it is possible that marked increase in AQP3 in later stages of embryos occurs in various mammalian species, and that the expression markedly affects the permeability of the embryos to various cryoprotectants.

Since facilitated diffusion of cells by a channel process not only increases the membrane permeability but also markedly lowers the temperature-dependence of the membrane permeability, the expression of water- and/or cryoprotectant-permeable channels must substantially affect the optimum conditions for cryopreservation of the cells. Therefore, it is essential, for developing and improving cryopreservation protocols effectively, to measure the permeability of cells to water and cryoprotectants under concentration gradients of cryoprotectants and to know whether the movement of water and cryoprotectants depends on simple diffusion across the plasma membrane or on facilitated diffusion by channel processes.

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Japanese flounder (*Paralichthys olivaceus*) embryos are difficult to cryopreserve by vitrification ☆

Keisuke Edashige *, Delgado M. Valdez Jr., Takao Hara, Naoya Saida, Shinsuke Seki, Magosaburo Kasai

Laboratory of Animal Science, College of Agriculture, Kochi University, Nankoku, Kochi 783-8502, Japan

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Abstract

The first successful cryopreservation of fish embryos was reported in the Japanese flounder by vitrification [Chen and Tian, Theriogenology, 63, 1207–1219, 2005]. Since very high concentrations of cryoprotectants are needed for vitrification and fish embryos have a large volume, Japanese flounder embryos must have low sensitivity to cryoprotectant toxicity and high permeability to water and cryoprotectants. So, we investigated the sensitivity and the permeability of Japanese flounder embryos. In addition, we assessed the survival of flounder embryos after vitrification with solutions containing methanol and propylene glycol, following Chen and Tian's report. The embryos were relatively insensitive to the toxicity of individual cryoprotectants at lower concentrations, especially methanol and propylene glycol as their report. Although their permeability to water and cryoprotectants could not be measured from volume changes in cryoprotectant solutions, the embryos appeared to be permeable to methanol but less permeable to DMSO, ethylene glycol, and propylene glycol. Although vitrification solutions containing methanol and propylene glycol, which were used in Chen and Tian's report, were toxic to embryos, a small proportion of embryos did survived. However, when vitrified with the vitrification solutions, no embryos survived after warming. The embryos became opaque during cooling with liquid nitrogen, indicating the formation of intracellular ice during cooling. When embryos had been kept in vitrification solutions for 60 min after being treated with the vitrification solution, some remained transparent during cooling, but became opaque during warming. This suggests that dehydration and/or permeation by cryoprotectants were insufficient for vitrification of the embryos even after they had been over-treated with the vitrification solutions. Thus, Chen and Tian's cryopreservation method lacks general application to Japanese flounder embryos.

Keywords: Flounder; Embryo; Cryopreservation; Cryoprotectant; Permeability; Vitrification

The cryopreservation of embryos is useful for managing various strains of model animals, and has been used for several mammalian species. In some species of fish, such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), various strains and genetic variants have been established for laboratory use. However, they have been maintained by mating,

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* Corresponding author. Fax: +81 88 864 5200.
E-mail address: keisuke@cc.kochi-u.ac.jp (K. Edashige).

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because there has been no other option available. Thus, the cryopreservation of teleost embryos would have a great impact on the preservation of laboratory species. It would also have an impact on aquaculture and the conservation of various aquatic species. However, fish embryos have been quite difficult to cryopreserve. Several factors complicate the process [7], including a large volume, a large amount of egg-yolk, a thick chorion, and that a complex structure forms during development, and fish embryos are susceptible to chilling.

Very recently, Chen and Tian [1] reported the successful cryopreservation of Japanese flounder (*Paralichthys olivaceus*) embryos by vitrification [1]. Before their report, there had been only one article on the deep cryopreservation of fish embryos; in 1989, Zhang et al. [10] reported that common carp (*Cyprinus carpio*) embryos survived cryopreservation after slow-freezing [10], but this success has since to be reproduced.

Since fish embryos have a remarkably larger volume than mammalian embryos and thus have a very low surface/volume ratio, the movement of water and cryoprotectants across the plasma membrane takes a long time. Therefore, with any cryopreservation method, intracellular ice is likely to form. Moreover, with vitrification, the toxicity of cryoprotectants is a major obstacle to the survival of cryopreserved cells because high concentrations of cryoprotectants must be used. To circumvent the formation of intracellular ice and the toxicity of cryoprotectants, water and cryoprotectants need to move rapidly across the plasma membrane.

In this study, we examined the sensitivity to the toxicity of cryoprotectants and permeability to water and cryoprotectants of Japanese flounder embryos. We also examined the survival of flounder embryos after vitrification by Chen and Tian's method using solutions containing methanol and propylene glycol [1].

Materials and methods

Embryos

Japanese flounder embryos were obtained from Kochi Prefectual Sea Farming Center (Muroto, Kochi, Japan). About 50 mature flounders (4–5 years-old) were reared in seawater in a tank (30,000 L) at 15–18 °C. The cycle of light and dark periods (total 24 h) were changed every 1–2 months from 10 to 18 h and from 14 to 6 h, respectively, in a year. When we obtained embryos, flounders were reared under 15 h of light and 9 h of darkness. Embryos were collected every morning; the average fertilization rate of the eggs assessed by their cleavage was 68%. The embryos were transported to our laboratory in seawater at 16–17 °C within 2 h. Developmental stages of embryos were determined morphologically under a stereomicroscope. Transparent embryos floating in seawater with a diameter of 0.87–0.90 mm were selected, and were incubated at 15 °C in seawater filtered with 0.22-μm pore filters (filtered seawater). Embryos developed to the gastrula, somite, tail bud, and pre-hatching stages were used for experiments.

Sensitivity of embryos to the toxicity of individual cryoprotectants

The sensitivity of embryos to the toxicity of individual cryoprotectants was examined by a method similar to that reported by Chen and Tian [1]. Embryos at the gastrula, somite, tail bud, and pre-hatching stages were exposed to cryoprotectant solutions (filtered seawater containing 4.8–19% (vol/vol) DMSO, 4–16% (vol/vol) ethylene glycol, 5–20% (vol/vol) methanol, or 5–20% (vol/vol) propylene glycol) at 15 °C for 20 min. The embryos were then transferred to artificial seawater (BS2) [1] containing 0.125 M sucrose at 15 °C, kept for 10 min, transferred to filtered seawater, and incubated at 15 °C for up to 72 h. The survival of the embryos was assessed by their ability to hatch into live fry. The composition of BS2 medium is as follows; 0.423 M NaCl, 0.012 M KCl, 0.01 M $CaCl_2$ $2H_2O$, 0.024 M $MgCl_2$ $6H_2O$, and 0.002 M $NaHCO_3$ (pH 7.6) [1].

Assessment of permeation of embryos by water and cryoprotectants from the volume change

To examine the permeability of embryos to water, an embryo at the blastula, somite, or tail bud stage was exposed to BS2 medium (0.90 Osm/kg) containing 0.5 M sucrose (1.50 Osm/kg) or BS2 medium diluted with distilled water (0.18 Osm/kg), by essentially the same method as the one we used previously [2,8]. An embryo equilibrated with BS2 medium at 15 °C for 60 min was transferred with a minimal amount of BS2 medium using a Pasteur pipette to the hypertonic or hypotonic BS2 medium (200 μl) covered with paraffin oil at 25 ± 1 °C and kept there for 60 min. Microscopic images of the embryo were recorded using a time-lapse video

Table 1
The osmolality of solutions used in permeability experiments

Diluent			Solute		Total osmolality (Osm/kg)
Basic medium	Tonicity	Osmolality[a] (Osm/kg)	Name	Osmolality (Osm/kg)	
BS2[b]	0.20×	0.18	—	—	0.18
	1.00×	0.90	—	—	0.90
	1.00×	0.90	0.50 M Sucrose	0.60[c]	1.50
Filtered seawater	0.95×	0.96	—	—	0.96
	1.00×	1.01	—	—	1.01
	1.00×	1.01	10% DMSO	1.55[d]	2.56
	1.00×	1.01	8% Ethylene glycol	1.61[c]	2.62
	1.00×	1.01	5% Methanol	1.28[c]	2.29
	1.00×	1.01	10% Propylene glycol	1.56[c]	2.57

[a] Osmolality measured with a freezing point depression osmometer.
[b] Artificial sea water [1].
[c] Osmolality calculated from published data on the colligative properties of the solutes in aqueous solution [9].
[d] Osmolality measured with a vapor pressure osmometer.

recorder (ETV-820, Sony, Tokyo, Japan) during exposure. The cross-sectional area of the embryo was measured using an image analyzer (VM-50, Olympus, Tokyo, Japan). The relative cross-sectional area, S, was expressed by dividing the cross-sectional area by the initial area of the same embryo. The relative volume change of the embryo was calculated from $V = S^{3/2}$.

To examine the permeability of embryos to cryoprotectants, an embryo equilibrated with BS2 medium was exposed to BS2 medium containing 9.5% (vol/vol) DMSO, 8% (vol/vol) ethylene glycol, 5% (vol/vol) methanol, or 10% (vol/vol) propylene glycol at 25 ± 1 °C for 60 min. Microscopic images were recorded using the time-lapse video tape recorder. The cross-sectional area of the embryo was measured using the image analyzer. Relative volume changes of the embryos were calculated as described above.

The osmolality of solutions is shown in Table 1.

Assessment of permeation of embryos by cryoprotectants by density

To roughly assess whether the cryoprotectants permeate the embryos during exposure, we examined the change in density of embryos at the tail bud stage after exposure to BS2 medium containing 9.5% (vol/vol) DMSO, 8% (vol/vol) ethylene glycol, 5% (vol/vol) methanol, or 10% (vol/vol) propylene glycol. The density of embryos appears to be similar to that of 95% (vol/vol) filtered seawater diluted with distilled water (95% seawater) because most of the intact embryos used neither floated nor sank during their suspension in 95% seawater at 25 °C for 20 min. Methanol has a lower density than 95% seawater because it formed a layer on top of the seawater when poured gently onto it. On the other hand, ethylene glycol, propylene glycol, and DMSO have a higher density than 95% seawater because each of these cryoprotectants formed a layer at the bottom when poured gently onto 95% seawater. Thus, if embryos exposed to cryoprotectant solutions floated (as in the case of methanol) or sank (as in the case of ethylene glycol, propylene glycol, or DMSO) in 95% seawater, we supposed that a certain amount of the cryoprotectants permeated the flounder embryos.

We examined the position of the embryos, which had been exposed to cryoprotectant solutions, in 10 ml of 95% seawater in a plastic test tube ($\Phi = 15$ mm, $L = 100$ mm) 1 min after their transfer to the seawater at 25 °C. Briefly, 15–25 embryos were exposed to cryoprotectant solutions at 25 °C for 30 min and washed once with 95% seawater. Then, they were poured onto 10 ml of 95% seawater in a plastic test tube at 25 °C, mixed gently with the seawater by inverting the test tube once, and set on a table for 1 min. Then, the embryos which floated to the surface, sank to the bottom, and remained suspended in the middle of the solution were enumerated.

Sensitivity of embryos to the toxicity of vitrification solutions

Two vitrification solutions, FVS1 and FVS3 [1], were used. FVS1 and FVS3 consisted of methanol and propylene glycol (13% (vol/vol) + 20% (vol/vol)

and 18% (vol/vol) + 27% (vol/vol), respectively) in BS2 medium.

To examine the toxicity of FVS1 and FVS3 to embryos, embryos at the tail bud stage were treated stepwise with 0.25×, 0.33×, 0.50×, 0.67×, and 1.00× of FVS1 or FVS3 at 15 °C for 8 min at each step. In some experiments, embryos were exposed directly to the vitrification solutions at 15 °C for 30 or 60 min. After being treated, 5–10 embryos were diluted with 2 ml of BS2 medium containing 0.125 M sucrose at 15 °C. After 10 min, 8 ml of filtered seawater was slowly added to the solution, and the embryos were kept at 15 °C for 10 min. The embryos were incubated in fresh filtered seawater at 15 °C for up to 72 h. The survival of the embryos was assessed by their ability to hatch into live fry.

Survival of embryos after vitrification

As preliminary experiments, we examined the formation of ice in vitrification solutions during cooling and warming. About 200 μl of FVS1 or FVS3 was loaded into a 0.25-ml plastic straw (IMV, l'Aigle, France). The straw was then cooled with liquid nitrogen (LN_2). In some experiments, FVS1 or FVS3 was put on a small loop ($\Phi = \sim 0.9$ mm) made with fine platinum wire ($\Phi = 0.15$ mm) (a cryoloop), and cooled by directly plunging the cryoloop into LN_2. After 2–3 min, the straw or cryoloop was warmed in water at 40 °C or by being immersed quickly in 2 ml of BS2 medium at 15 °C, respectively. It was observed whether the vitrification solution became opaque during the cooling and/or warming.

Embryos were vitrified essentially as reported by Chen and Tian [1]. Briefly, embryos at the tail bud stage were exposed to FVS1 or FVS3 stepwise as described above. Then, 8–10 embryos were loaded into a 0.25-ml plastic straw containing ~200 μl of vitrification solution. The straw was heat-sealed, and directly plunged into LN_2. After being kept in the LN_2 for 5–20 min, embryos in the straw were warmed by immersing the straw in water at 40 °C for 7 s, expelled into 2 ml of BS2 medium containing 0.125 M sucrose, and kept at 15 °C for 10 min. Then, 8 ml of filtered seawater was slowly added to the solution, and the embryos were kept at 15 °C for 10 min. Finally, the embryos were transferred to fresh filtered seawater and incubated at 15 °C for 1 h. Survival of the embryos was assessed by their appearance under a stereomicroscope.

In some experiments, an embryo treated stepwise with FVS1 or FVS3 solution was put on a cryoloop, and directly plunged into LN_2. After being kept for 2–3 min in the LN_2, the embryo on the loop was warmed by being immersed quickly in 2 ml of BS2 medium containing 0.125 M sucrose at 15 °C and kept immersed for 10 min. Then, 8 ml of filtered seawater was slowly added to the solution, and the embryo was kept at 15 °C for 10 min. Finally, the embryo was transferred to fresh filtered seawater and incubated at 15 °C for 1 h. Survival of the embryos was assessed by their appearance under a stereomicroscope.

In some experiments, embryos had been kept in FVS1 or FVS3 at 15 °C for 60 min after the stepwise treatment. The embryos were vitrified and warmed, and survival was assessed as described above.

Statistics

Hatching rates of embryos exposed to cryoprotectant solutions were compared with Fisher's exact probably test and rates at which embryos were permeated by cryoprotectants assessed based on the density were compared with a Chi-square test ($P < 0.05$ was considered significant).

Results

Sensitivity of embryos to the toxicity of individual cryoprotectants

After exposure to 4–5% cryoprotectant solutions, the survival of embryos exposed to DMSO, methanol, or propylene glycol was quite high whereas the survival of embryos exposed to ethylene glycol decreased except for embryos at the tail bud stage (Fig. 1A). After exposure to 8–10% cryoprotectant solutions, the survival of embryos exposed to DMSO and ethylene glycol markedly decreased except for embryos at the tail bud stage whereas that of embryos exposed to methanol or propylene glycol remained high, except for embryos at the pre-hatching stage (Fig. 1B). In 16–20% cryoprotectant solutions, the survival of embryos exposed to DMSO or ethylene glycol was low at all the stages examined, especially pre-hatching embryos exposed to ethylene glycol, whereas the survival remained high after exposure to propylene glycol (Fig. 1C). The survival of embryos exposed to methanol also remained high but that of pre-hatching embryos decreased markedly (Fig. 1C). On the whole, Japanese flounder embryos were basically resistant to the toxicity of lower concentrations of cryoprotectants, especially

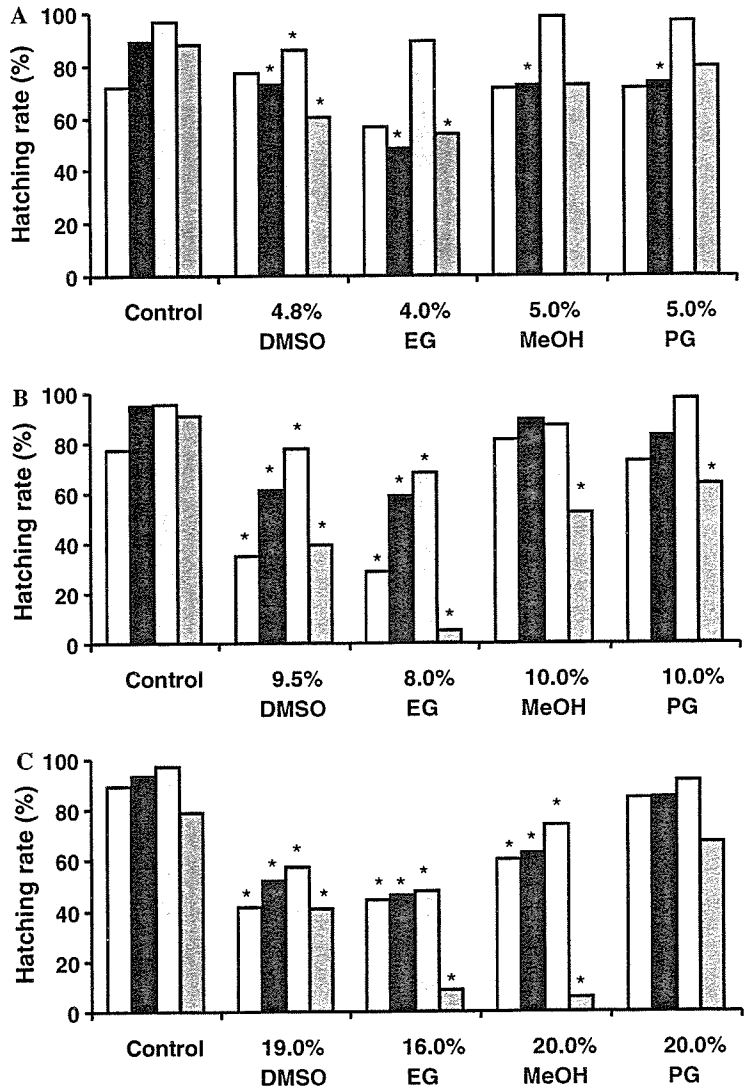

Fig. 1. The tolerance of Japanese flounder embryos at the blastula (open), somite (closed), tail bud (shaded lightly), and pre-hatching (shaded darkly) stages to the toxicity of DMSO, ethylene glycol (EG), methanol (MeOH), and propylene glycol (PG). Embryos were exposed to 4–5% (vol/vol) (A), 8–10% (vol/vol) (B), or 16–20% (vol/vol) (C) cryoprotectants in filtered seawater at 15 °C for 20 min. Survival of the embryos was assessed by their ability to hatch into live fry within 72 h at 15 °C. Data are indicated as the sum of triplicate determinants. For each treatment, a total of 60–110 embryos were used for each stage. Columns with asterisks are significantly different from the control ($P < 0.05$).

tail bud embryos, and methanol and propylene glycol were less toxic, except in pre-hatching embryos.

Assessment of permeation of embryos by water and cryoprotectants from the volume change

Fig. 2 shows the Boyle-van't Hoff plots of equilibrated volumes of embryos at the blastula, somite, and tail bud stages after exposure to the hypertonic or hypotonic solution at 25 °C for 60 min. Little or no change was observed in volume in either solution, suggesting that the embryos have very low permeability to water. Since the volume changes of embryos in hypertonic and hypotonic solutions were quite small, we could not calculate the osmotically inactive content and the water permeability of embryos. Minimal volume changes of embryos at all stages examined were also observed during exposure to cryoprotectant solutions at 25 °C for 60 min (Fig. 3). It was suggested that the permeability of Japanese flounder embryos to cryoprotectants should be quite low. However, we could not

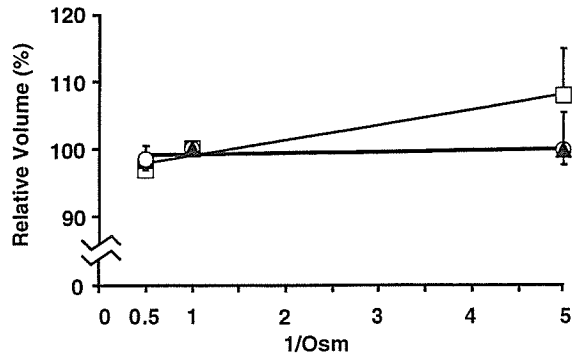

Fig. 2. Boyle-van't Hoff plots of the relative volume of Japanese flounder embryos at the blastula (open squares), somite (closed triangles), and tail bud (shaded circles) stages. Embryos were equilibrated with artificial seawater containing 0.5 M sucrose (1.50 Osm/kg) or artificial seawater diluted with distilled water (0.18 Osm/kg) at 25 °C for 60 min. Equilibrated volumes of embryos at the pre-hatching stage were not obtained because the embryos actively moved within their chorion. Each data point is the mean of the relative volume ±SD of five embryos.

calculate the permeability because volume changes of embryos in cryoprotectant solutions were quite small and because we could not calculate the osmotically inactive content of embryos as described above.

Assessment of permeation of embryos by cryoprotectants by density

By a volumetric method, it was suggested that the permeability of Japanese flounder embryos at the tail bud stage to water and cryoprotectants was quite low. However, it is possible that flounder embryos do not behave as an osmometer because they develop in non-physiological conditions, in seawater. In such conditions, they would not retain their shape if they changed their volume in response to extracellular osmolality. Thus, it is plausible that Japanese flounder embryos have the ability to resist extracellular non-physiological conditions and that they do not behave as an osmometer in hypertonic or hypotonic solutions and cryoprotectant solutions. If so, the movement of water and cryoprotectant across the plasma membrane can not be estimated from their volume changes. Thus, we examined briefly whether cryoprotectants can permeate Japanese flounder embryos from the

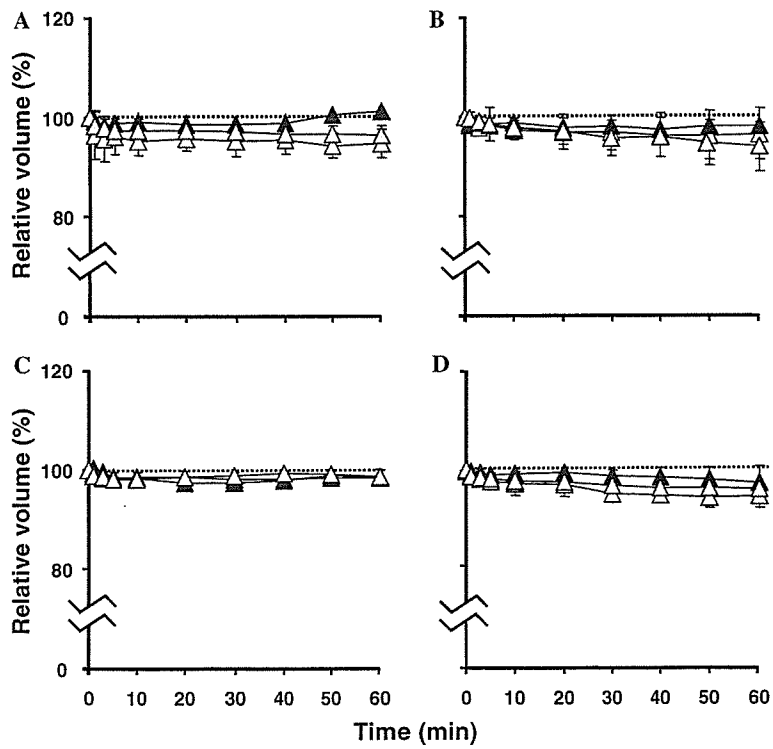

Fig. 3. The change in volume of Japanese flounder embryos at the blastula (open), somite (closed), and tail bud (shaded) stages during 60 min of exposure in artificial seawater containing 9.5% (vol/vol) DMSO (A), 8% (vol/vol) ethylene glycol (B), 5% (vol/vol) methanol (C), or 10% (vol/vol) propylene glycol (D) at 25 °C. The relative volume of embryos at the pre-hatching stage was not obtained because the embryos actively moved in their chorion. Each curve indicates means of the relative volume ±SD of five embryos.