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Channel-Dependent Permeation of Water and Glycerol in Mouse Morulae[1]

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ABSTRACT

The cryosensitivity of mammalian embryos depends on the stage of development. Because permeability to water and cryoprotectants plays an important role in cryopreservation, it is plausible that the permeability is involved in the difference in the tolerance to cryopreservation among embryos at different developmental stages. In this study, we examined the permeability to water and glycerol of mouse oocytes and embryos, and tried to deduce the pathway for the movement of water and glycerol. The water permeability (L_P, μm min^{-1} atm^{-1}) of oocytes and four-cell embryos at 25°C was low (0.63–0.70) and its Arrhenius activation energy (E_a, kcal/mol) was high (11.6–12.3), which implies that the water permeates through the plasma membrane by simple diffusion. On the other hand, the L_p of morulae and blastocysts was quite high (3.6–4.5) and its E_a was quite low (5.1–6.3), which implies that the water moves through water channels. Aquaporin inhibitors, phloretin and *p*-(chloromercuri) benzene-sulfonate, reduced the L_p of morulae significantly but not that of oocytes. By immunocytochemical analysis, aquaporin 3, which transports not only water but also glycerol, was detected in the morulae but not in the oocytes. Accordingly, the glycerol permeability (P_{GLY}, $\times 10^{-3}$ cm/min) of oocytes was also low (0.01) and its E_a was remarkably high (41.6), whereas P_{GLY} of morulae was quite high (4.63) and its E_a was low (10.0). Aquaporin inhibitors reduced the P_{GLY} of morulae significantly. In conclusion, water and glycerol appear to move across the plasma membrane mainly by simple diffusion in oocytes but by facilitated diffusion through water channel(s) including aquaporin 3 in morulae.

embryo, developmental biology, ovum

INTRODUCTION

Since the first successful cryopreservation of mouse embryos in 1972 [1], various protocols have been developed to cryopreserve oocytes and embryos of many mammalian species. However, it is difficult to obtain high survival rates of embryos at different stages with a single cryopreservation protocol. For example, mouse morulae can be cryopreserved without appreciable loss of viability by a simple one-step vitrification method using an ethylene glycol-based solution [2], but pretreatment with a lower concentration of cryoprotectant is needed to vitrify mouse embryos at early stages [3]. This indicates that cryobiological properties differ among embryos at different developmental stages.

There are many factors that affect the survival of cells after cryopreservation [4]. Plasma membrane permeability is one factor determining the tolerance of cells to cryopreservation because the permeability modulates several major forms of cell injury caused by cryopreservation, i.e., injuries by intracellular ice formation, cryoprotectant toxicity, and osmotic swelling. Thus, it is likely that the plasma membrane permeability markedly differs among embryos at different developmental stages and with different cryosensitivities.

Water was long believed to move across the plasma membrane only by simple diffusion. In the 1990s, however, small intrinsic membrane proteins that act as water channels, called aquaporins (AQPs), were discovered and characterized [5]. We have already demonstrated that mRNAs of *Aqp3* and *Aqp7* are present in mouse oocytes at the metaphase II stage and embryos at the four-cell, morula, and blastocyst stages, and that mRNAs of *Aqp8* and *Aqp9* are expressed in embryos at the blastocyst stage [6]. Other researchers have also detected mRNAs of *Aqps* [7, 8] and AQP proteins [9] in mouse embryos. Thus, it is plausible that these AQPs play a role in the water permeability of mouse oocytes and embryos. However, it has not been shown whether such a channel pathway is a significant one in mouse oocytes and embryos.

The involvement of water channels in water movement across the plasma membrane can be deduced from the membrane's properties. In general, an osmotic water permeability higher than 4.5 μm min^{-1} atm^{-1} (or $P_f \geq 0.01$ cm/sec) and an Arrhenius activation energy (E_a) lower than 6 kcal/mol are suggestive of water movement principally through water channels, whereas an E_a higher than 10 kcal/mol is suggestive of movement principally via channel-independent diffusion [10]. Thus, it would be possible to deduce the pathway of movement from the water permeability and its E_a value.

If this criterion is applied to mature mouse oocytes, water channels may not be the major pathway for water movement across the plasma membrane because many studies have already shown that mouse oocytes have low water permeability (0.41–0.61 μm min^{-1} atm^{-1}) [11–16] and high E_a values (9.84–13.3 kcal/mol) [11–13, 17], although mouse oocytes have mRNAs of *Aqp3* and *Aqp7* [6]. They could be maternal stores of mRNA to be expressed later. Thus, most water molecules are assumed to move across the plasma membrane of oocytes by simple diffusion. In mouse embryos after cleavage, on the other hand, only a small number of studies have been available about the permeability of the plasma membrane. Pfaff et al. [16] estimated the water permeability of oocytes and embryos from the one-cell to eight-cell stage and demonstrated that eight-cell embryos are slightly more permeable to water than oocytes and one- to four-cell embryos, suggesting that water channels do not play a major role in

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water movement in embryos until the eight-cell stage. However, the water permeability of embryos at later stages has not been studied.

In this study, we first examined the water permeability of mouse oocytes and embryos and temperature dependence to deduce the involvement of water channels in water movement. Because the results of this experiment suggested the involvement of a water channel that can transport not only water but also glycerol in embryos at later developmental stages, we also examined the glycerol permeability of oocytes and morulae and temperature dependence to deduce the involvement of channels in glycerol movement.

MATERIALS AND METHODS

Collection of Oocytes and Embryos

Female ICR mice (CLEA Japan, Inc., Tokyo, Japan) were induced to superovulate with intraperitoneal injections of 5 IU of eCG and 5 IU of hCG given 48 h apart, and were mated with male ICR mice. Ovulated unfertilized oocytes were collected from the ampullar portion of the oviducts at 13 h after hCG injection without mating and were freed from cumulus cells by suspending them in modified phosphate-buffered saline (PB1) containing 145 U/ml hyaluronidase followed by washing with fresh PB1 medium. Four-cell embryos were flushed from the oviducts of mated animals with PB1 medium 55 h after hCG injection. Morulae were collected from the uteri by flushing them with PB1 medium 76 h after hCG injection, and only compacted morulae were used. To obtain early blastocysts, morulae collected 76 h after hCG injection were cultured in modified M16 medium supplemented with 10 μM EDTA, 1 mM glutamine, and 10 μM β-mercaptoethanol (modified M16 medium) [18], covered with paraffin oil for 6–8 h in a humidified CO_2 incubator equilibrated with 5% CO_2 in air at 37°C. Only early blastocysts, with a blastocoele larger than the inner cell mass and an unexpanded zona pellucida, were collected. To measure total cell volume of blastocysts, the blastocysts were pipetted repeatedly with a narrow-bore pipette to shrink their blastocoele. After being pipetted, early blastocysts with a shrunken blastocoele were incubated in modified M16 medium in a CO_2 incubator for 4–5 min and those without a re-expanded blastocoele were used for experiments. All experiments were approved by the Animal Ethics Committee of Kochi University.

Measurement of Water Permeability and Glycerol Permeability of Mouse Oocytes and Embryos

Water permeability was measured as described previously [19, 20]. Each oocyte or embryo was placed in a 100-μl drop of PB1 medium covered with paraffin oil in a Petri dish (90 × 10 mm) at 25°C and was held by a holding pipette (outer diameter being 80–120 μm) connected to a micromanipulator on an inverted microscope. The inner diameter of the holding pipette was small enough not to distort the oocyte or embryo. The temperature of the paraffin oil covering the various solutions was considered as the temperature of the solutions and was kept at 25 ± 1°C by controlling the temperature of the room. An oocyte or embryo held by a holding pipette was then covered with a covering pipette with a larger inner diameter (~200 μm) connected to another micromanipulator. Then, by sliding the dish, the oocyte or embryo was introduced into a drop of hypertonic PB1 medium containing sucrose (100 μl) at 25°C. By removing the covering pipette, the oocyte or embryo was abruptly exposed to the solution. The microscopic image of the oocytes and embryos during exposure to the solution was recorded with a time-lapse video tape recorder (ETV-820; Sony, Tokyo, Japan) every 0.5 sec. The cross-sectional area of oocytes and embryos was measured using an image analyzer (VM-50; Olympus, Tokyo, Japan). In the case of four-cell embryos, the cross-sectional area of only one blastomere in each embryo was measured. The cross-sectional area was expressed as a relative cross-sectional area, S, by dividing it by the area of the same oocyte and embryo in isotonic PB1 medium. The relative volume was obtained from $V = S^{3/2}$.

The osmotically inactive portion (V_b) of oocytes and embryos is required for the permeability analysis. To determine V_b, they were exposed to PB1 medium (0.295 Osm/kg) containing 0.110, 0.310, or 0.505 Osm/kg sucrose at 25°C for 60 min; the total osmolalities of the solutions were 0.405, 0.605, and 0.800 Osm/kg, respectively. Values for V_b were obtained from Boyle-van't Hoff plots.

The water permeability of oocytes and embryos was determined by measuring the shrinkage of oocytes and embryos after their transfer from isotonic PB1 medium to PB1 medium containing 0.505 Osm/kg sucrose (total osmolality, 0.800 Osm/kg) (sucrose/PB1) for 5 min at 25°C. In this study, we expressed water permeability as hydraulic conductivity (L_p). L_p values of the oocytes and embryos were determined by fitting water movement using a two-parameter formalism as described previously [19, 21]. The related constants and parameters used are listed in Table 1.

TABLE 1. Constant and parameters used for fitting permeability parameters.

Symbol	Meaning	Values
R	Gas constant	8.206×10^{-2} L atm $K^{-1}mol^{-1}$
T	Absolute temperatures	288 K and 298 K
$\overline{V_w}$	Partial molar volume of water	0.018 L/mol
$\overline{V_s}$	Partial molar volume of glycerol	0.071 L/mol[a]
V_b	Osmotically inactive fraction	—

[a] Value from Wolf et al. [22].

The L_p of oocytes and embryos was also measured at 15°C for 5 min, and the Arrhenius activation energy, E_a, or temperature dependence of L_p at each stage was obtained from Arrhenius plots.

In one experiment, oocytes and morulae were treated with phloretin or *p*-(chloromercuri) benzene-sulfonate (*p*-CMBS), which are AQP inhibitors, before water permeability was measured to study whether channels sensitive to these inhibitors were involved in water movement in oocytes and morulae. For this experiment, the water permeability of untreated oocytes and morulae was first measured at 25°C as described above, then the oocytes and morulae were transferred to PB1 medium and equilibrated for 10 min at 25°C. Next, they were treated with a water-channel inhibitor, 0.7 mM phloretin in PB1 medium for 2.5 min or 0.5 mM *p*-CMBS in PB1 medium for 30 min at 25°C. Finally, they were washed with PB1 medium, and water permeability was remeasured for 5 min at 25°C as described above.

The glycerol-permeability (P_{GLY}) of oocytes and embryos was determined by measuring the shrinkage and swelling of oocytes and embryos after transfer from isotonic PB1 medium to PB1 medium containing 10% (vol/vol) glycerol (1.565 Osm/kg) (total osmolality, 1.860 Osm/kg) (glycerol/PB1) for 20 min at 25°C. Values for S and V were obtained, and the P_{GLY} of the oocytes and embryos was determined by fitting water and solute movement using a two-parameter formalism as described above.

The P_{GLY} of oocytes and morulae was also measured at 15 and 25°C for 180 min and 5 min, respectively, and the Arrhenius activation energy of the P_{GLY} for oocytes and morulae was obtained, as for the water permeability.

For the study of effects of AQP inhibitors on the glycerol permeability of morulae, the glycerol permeability of untreated morulae was first measured in glycerol/PB1 for 5 min at 25°C as described above, then the morulae were diluted with PB1 medium containing 0.5 M sucrose for 20 min at 25°C, and equilibrated with fresh PB1 medium for 10 min at 25°C. Next, they were treated with a water-channel inhibitor in PB1 medium using the same conditions as for water permeability, washed with PB1 medium, and the permeability remeasured in glycerol/PB1 for 5 min at 25°C.

The osmolality of sucrose and glycerol (the osmolality of extracellular permeating solute) was calculated from published data about colligative properties of sucrose and glycerol in aqueous solutions [22]. The osmolality of PB1 medium (isotonic buffer) was measured with an osmometer (OM801; Vogel, Giessen, Germany).

In this report, we limited data collection to cells in the following volume ranges (before treatment): oocytes, 1.9–2.7 × 10E5 μm^3 (95% of all oocytes used); blastomeres of four-cell embryos, 0.5–0.7 × 10E5 μm^3 (92% of all four-cell embryos used); and morulae, 1.7–2.5 × 10E5 μm^3 (82% of all morulae used) because the equilibrated relative volume of extraordinarily larger or smaller oocytes and embryos in hypertonic sucrose solutions had smaller or larger V_b values than that of those with an ordinary size, suggesting that such extraordinarily large or small oocytes and embryos have different V_b values from ordinary ones (data not shown). For early blastocysts, however, we used all of the data because the size of blastocysts with a shrunken blastocoele varied greatly, probably due to the variable size of the blastocoele.

Expression of AQP3 and AQP7 in Oocytes and Morulae

For detection of AQP3, we used commercially available anti-human AQP3 goat antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), which cross-reacted with mouse AQP3. For AQP7, rabbit anti-mouse AQP7 serum was raised against a synthetic peptide (Asahi Techno Glass; Chiba, Japan) corresponding to the 15 C-terminal amino acids of mouse AQP7 (KNAASA-NISGSVPLE) (GenBank accession number AB010100). Using an ECL

Western Blotting Kit (Amersham Biosciences Corp., Piscataway, NJ), we confirmed that the antiserum detected a 21-kDa protein in the membrane fraction of mouse kidney, which was consistent with the molecular weight of mouse AQP7, on a polyvinylidene fluoride membrane after electric transfer from an SDS-PAGE gel.

The zona pellucida of oocytes and morulae was removed by brief exposure to an acidic Tyrode solution (pH 2.5) [23]. The zona-free oocytes and morulae were fixed with a 2% paraformaldehyde solution containing 0.01 M sodium metaperiodate, 0.075 M lysine. and 0.075 M phosphate buffer (pH 7.4) at 4°C for 60 min. After being washed with PBS containing 5 mg/ml bovine serum albumin, they were permeabilized with PBS containing 0.25% Triton X-100. Then they were incubated with blocking solution; PBS containing 10% nonimmune donkey serum (Santa Cruz Biotechnology, Inc.) and 5 mg/ml bovine serum albumin (for AQP3) or 10% nonimmune goat serum (Santa Cruz Biotechnology, Inc.) and 5 mg/ml bovine serum albumin (for AQP7) at 25°C for 60 min. After being rinsed, they were incubated with diluted anti-human AQP3 goat antibody (1/400) or anti-mouse AQP7 rabbit antiserum with blocking solution at 25°C for 60 min. As a control, instead of the primary antibodies, anti-human AQP3 goat antibody or anti-mouse AQP7 rabbit antiserum preincubated with a blocking peptide, which was the same peptide used for an antigen, at room temperature for 1 h, was used for the experiments. After being rinsed, the oocytes and embryos were incubated with diluted fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG donkey antibody (1/1600) (Santa Cruz Biotechnology, Inc.) (for AQP3) or diluted FITC-conjugated anti-rabbit IgG goat antibody (Chemicon International, Temecula, CA) (for AQP7) with blocking solution at 25°C for 30 min. They were observed under a fluorescence microscope. No staining was observed when primary antibodies preincubated with blocking peptides were used (data not shown). When cross sections of mouse kidney and testis that were fixed with paraformaldehyde, dehydrated with ethanol, embedded in paraffin, and cut at a thickness of 7 μm were stained with the same antibodies at the same dilution, the collecting duct or seminiferous epithelium was stained with anti-AQP3 antibody or anti-AQP7 antibody, respectively, where the presence of AQP3 and AQP7 has been elucidated (data not shown) [24, 25].

RESULTS

Fraction of Osmotically Inactive Cell Content of Oocytes and Embryos

Figure 1 shows Boyle-van't Hoff plots of the relative volume of oocytes and embryos at various stages. The intercept of the regression line indicates osmotically inactive volumes for oocytes, four-cell embryos, morulae, and early blastocysts to be 15%, 16%, 15%, and 14%, respectively. We used these values to estimate the L_P and P_{GLY} of oocytes and embryos.

Water Permeability and Arrhenius Activation Energy of Oocytes and Embryos

In sucrose/PB1, oocytes and four-cell embryos shrank relatively slowly at 25°C, and shrank more slowly at 15°C than at 25°C (Fig. 2). On the other hand, morulae and early blastocysts shrank very rapidly at 15 and 25°C, the difference of the volume changes between the two temperatures being small (Fig. 2).

In oocytes and four-cell embryos, the value for L_P at 25°C was low (0.70 ± 0.12 and 0.63 ± 0.11 μm min^{-1} atm^{-1}) (Table 2) and the value for E_a of the L_P was high (12.3 and 11.6 kcal/mol) (Table 2), in which case water is expected to permeate through the plasma membrane predominately by simple diffusion [10]. In morulae, on the other hand, the average value for L_P at 25°C was quite high (4.45 ± 1.83 μm min^{-1} atm^{-1}), although the L_P value varied quite widely among embryos, from 2.04 to 9.35 μm min^{-1} atm^{-1} (data not shown). The value for E_a of the L_P was quite low (6.3 kcal/mol) (Table 2), in which case water is expected to move predominately through water channels [10]. In early blastocysts, L_P was also high (3.61 ± 1.72 μm min^{-1} atm^{-1}) and the value for E_a of the L_P was also quite low (5.1 kcal/mol) (Table 2). These results strongly suggest that most water molecules move across the plasma membrane by simple diffusion in oocytes and four-cell embryos but by facilitated diffusion through water channels in morulae and early blastocysts.

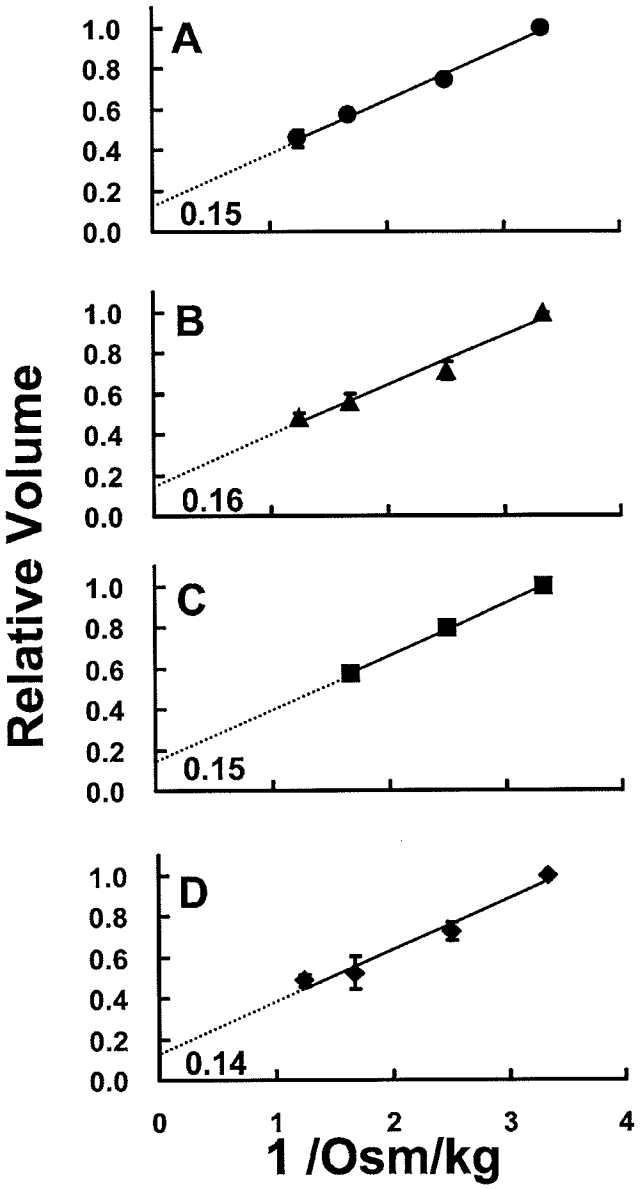


FIG. 1. Boyle-van't Hoff plots of mouse oocytes (**A**) and embryos at the four-cell (**B**), morula (**C**), and early blastocyst (**D**) stages. Oocytes (n = 5) and embryos at the four-cell (n = 5), morula (n = 4), and early blastocyst (n = 5) stages were exposed to various concentrations of sucrose in PB1 medium (0.405, 0.605, and 0.800 Osm/kg) at 25°C for 60 min, and relative volumes at 60 min were measured. Data are indicated as means of relative volume ± SD.

Effects of Water-Channel Inhibitors on Water Permeability of Oocytes and Morulae

To examine whether water permeates through water channels, we also examined the effect of water-channel inhibitors on the L_P of oocytes and morulae. As shown in Figure 3, neither phloretin nor *p*-CMBS affected the L_P of oocytes, whereas both inhibitors suppressed the L_P of morulae;

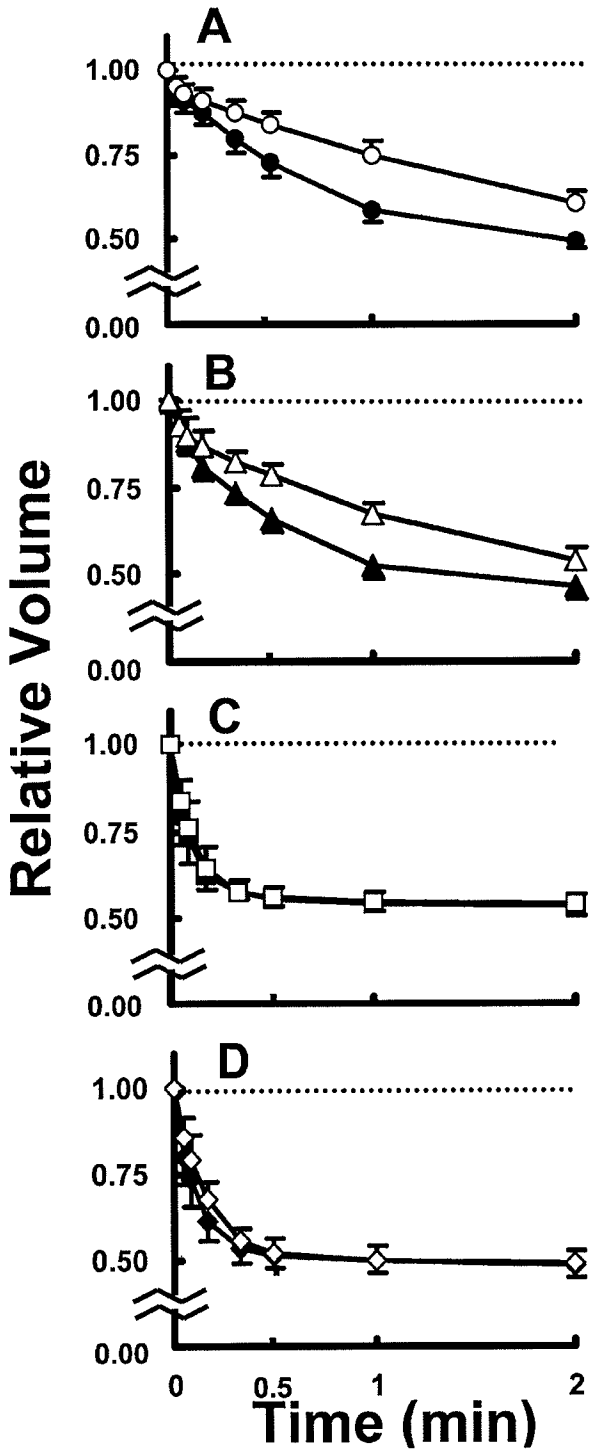


FIG. 2. Change in volume of mouse oocytes (**A**) and embryos at the four-cell (**B**), morula (**C**), and early blastocyst (**D**) stages in a hypertonic sucrose solution at 15°C (open symbols) and 25°C (closed symbols). Oocytes and embryos were exposed to sucrose in PB1 medium (0.800 Osm/kg) at 15 or 25°C for 5 min. Graphs show the volume changes during the first 2 min. Data are indicated as means of relative volume ± SD. Curves of oocytes and embryos at the four-cell, morula, and early blastocyst stages at 15°C are from 20 oocytes and 13, 26, and 22 embryos, respectively, and those at 25°C are from 27 oocytes and 17, 13, and 26 embryos, respectively.

TABLE 2. Hydraulic conductivity (L_P) and Arrhenius activation energy (E_a) of mouse oocytes and embryos.[a]

	L_P (μm min^{-1}atm^{-1})[b]		
Oocytes/Embryos	15°C	25°C	E_a (kcal/mol)
Oocyte	0.34 ± 0.07	0.70 ± 0.12[c]	12.3
4-cell embryo	0.32 ± 0.08	0.63 ± 0.11[c]	11.6
Morula	3.07 ± 1.39	4.45 ± 1.83[d]	6.3
Blastocyst	2.68 ± 0.98	3.61 ± 1.72[d]	5.1

[a] Values are calculated from data in Figure 2.
[b] Values are expressed as means ± SD.
[c,d] Values with different superscripts within the same column are significantly different (Student *t*-test; $P < 0.01$).

treatment with phloretin and *p*-CMBS reduced the L_P value of morulae significantly from 4.24 ± 1.76 to 2.42 ± 0.94 μm min^{-1} atm^{-1} and from 3.31 ± 0.88 to 1.94 ± 0.75 μm min^{-1} atm^{-1}, respectively (Table 3). These results strongly suggest that phloretin- and *p*-CMBS-sensitive water channels are abundantly expressed in morulae and contribute to the permeation of water.

Immunofluorescence Staining of AQP3 and AQP7 in Oocytes and Morulae

Among mRNAs of the *Aqp* family, those of *Aqp3* and *Aqp7* have been detected in morulae in ICR mice [6]. So, it is plausible that AQP3 and/or AQP7 are abundantly expressed in morulae and that water permeates through these AQPs. Thus, we examined the expression of AQP3 and AQP7 in oocytes and morulae by an immunofluorescence technique (Fig. 4). The anti-AQP antibodies did not detect AQP3 and AQP7 in oocytes but detected the marked expression of AQP3 in morulae.

These results strongly suggest that rapid water movement in morulae relies on AQP3.

Glycerol Permeability of Oocytes and Embryos

Because AQP3 transports not only water but also glycerol [24], it is plausible that mouse morulae are highly permeable to glycerol. In glycerol/PB1 at 25°C, oocytes and four-cell embryos shrank relatively slowly and reached their minimal volume (35–42% of their isotonic volume) within 30 sec (Fig. 5), but regained a little of their volume after 20 min of exposure. On the other hand, morulae and early blastocysts shrank rapidly to 64% of their isotonic volume and then regained their volume after 3 min of exposure. The values for P_{GLY} of oocytes and four-cell embryos were low (0.01 ± 0.00 and 0.06 ± 0.01 × 10^{-3} cm/min, respectively), whereas the values of morulae and early blastocysts were remarkably high (4.63 ± 0.94 and 4.10 ± 0.73 × 10^{-3} cm/min, respectively), as in the case of L_P (Table 2).

These results support the hypothesis that AQP3 plays an important role in rapid water movement in morulae and suggest that AQP3 is also involved in rapid glycerol movement in morulae. The results also suggest that channels like AQP3 are involved in glycerol movement in blastocysts.

Arrhenius Activation Energy for Glycerol Permeability of Oocytes and Morulae

To elucidate the involvement of a channel process in glycerol movement in morulae, we examined the E_a for the P_{GLY} of oocytes and morulae. At 15°C, oocytes shrank and regained their volumes markedly more slowly than at 25°C, and they regained their volumes only slightly even after 180

TABLE 3. Hydraulic conductivity (L_p) of mouse oocytes and morulae treated with water-channel inhibitors.[a]

Inhibitors	L_p (μm min^{-1}atm^{-1})[b] Before treatment	After treatment
Oocyte		
Control	0.67 ± 0.14	0.63 ± 0.17
Phloretin	0.77 ± 0.15	0.86 ± 0.20
p-CMBS	0.70 ± 0.16	0.71 ± 0.16
Morula		
Control	3.28 ± 1.32	3.06 ± 0.90
Phloretin	4.24 ± 1.76	2.42 ± 0.94*
p-CMBS	3.31 ± 0.88	1.94 ± 0.75*

[a] Values for control are calculated from 10 oocytes and 10 morulae, and other values are calculated from the data in Figure 3.
[b] Values are expressed as means ± SD.
* Significantly different from the L_p before treatment (Student *t*-test; $P < 0.01$).

min of exposure (Fig. 6A). On the other hand, morulae shrank and regained their original volumes only after 5 min of exposure at 15°C (Fig. 6B). The value of E_a for the P_{GLY} of oocytes was quite high (41.6 kcal/mol), whereas that of morulae was relatively low (10.0 kcal/mol) (Table 4), suggesting that glycerol permeates into oocytes mainly by simple diffusion whereas it permeates into morulae by facilitated diffusion through the plasma membrane.

Effects of Water-Channel Inhibitors on Glycerol Permeability of Morulae

To examine whether glycerol permeates through water channels in morulae, we examined the effect of water-channel inhibitors on P_{GLY} of morulae. As shown in Figure 7 and Table 5, both inhibitors suppressed the permeability of morulae; pretreatment with phloretin and *p*-CMBS reduced the P_{GLY} value of morulae significantly from 5.17 ± 1.56 to 3.15 ± 1.19 × 10^{-3} cm/min and from 5.11 ± 0.80 to 2.92 ± 1.13 × 10^{-3} cm/min, respectively (Table 5). In control morulae, no difference was observed between the P_{GLY} values of first exposure and second exposure to glycerol/PB1.

These results suggest that phloretin- and *p*-CMBS-sensitive water channels and/or glycerol channels are abundantly expressed in mouse morulae and that the channels contribute to the permeation of the embryos by glycerol.

DISCUSSION

In this study, we show that the movement of water in mouse embryos at the morula stage relies on channel processes and suggest that the movement of glycerol in morulae also relies on channel processes.

TABLE 4. Glycerol permeability (P_{GLY}) at 15 and 25°C and Arrhenius activation energy (E_a) of mouse oocytes and morulae.[a]

Oocyte/Embryo	P_{GLY} (× 10^{-3} cm/min)[b] 15°C	25°C	E_a (kcal/mol)
Oocyte	0.00 ± 0.00	0.02 ± 0.02[c]	41.6
Morula	2.61 ± 0.88	4.68 ± 1.50[d]	10.0

[a] Values are calculated from the data in Figure 6.
[b] Values are expressed as means ± SD.
[c,d] Values with different superscripts within the same column are significantly different (Student *t*-test; $P < 0.01$).

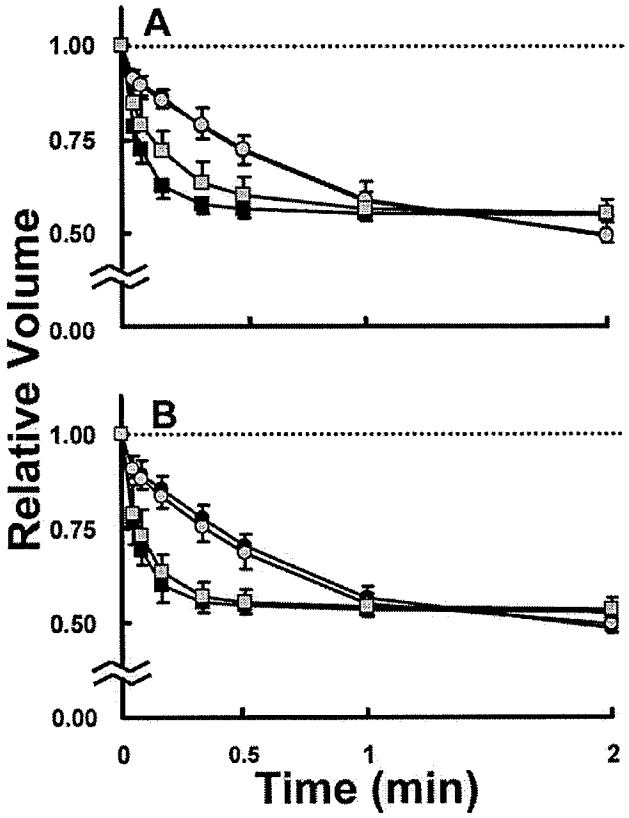
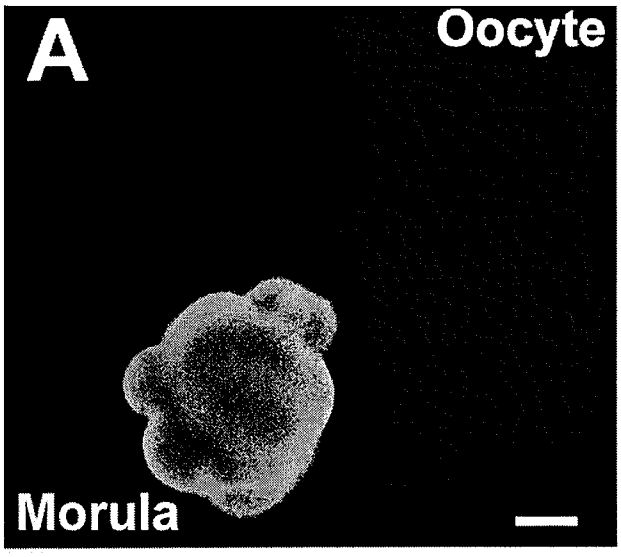


FIG. 3. Inhibition of water permeability of mouse oocytes and morulae by water-channel inhibitors. Oocytes (circles) and morulae (squares) were exposed to 0.7 mM phloretin in PB1 medium at 25°C for 2.5 min (**A**) (n = 10 and n = 10, respectively) or 0.5 mM *p*-(chloromercuri) benzenesulfonate in PB1 medium at 25°C for 30 min (**B**) (n = 12 and n = 11, respectively). Hydraulic conductivity was calculated from volume changes of oocytes and morulae in sucrose in PB1 medium (0.800 Osm/kg) at 25°C for 5 min before (closed symbols) and after (shaded symbols) treatment with the inhibitor. Graphs show the volume changes during the first 2 min. Data are indicated as means of relative volume ± SD. In **A**, the untreated and treated oocyte data points fall on top of each other.

Mature oocytes had low L_P and high E_a values (0.70 μm min^{-1} atm^{-1} and 12.3 kcal/mol, respectively) (Table 2). These values are similar to those of other studies [11–17] and suggestive of water movement by simple diffusion across the plasma membrane [10]. Four-cell embryos also had low L_P and high E_a values (0.63 μm min^{-1} atm^{-1} and 11.6 kcal/mol, respectively), similar to those of oocytes (Table 2). This L_P value is similar to that reported in another study (0.73 μm min^{-1} atm^{-1}) [16]. Thus, it appears that water also permeates through the plasma membrane of four-cell embryos primarily by simple diffusion.

On the other hand, morulae and early blastocysts had very high L_P and very low E_a values (4.45 and 3.61 μm min^{-1} atm^{-1} and 6.3 and 5.1 kcal/mol, respectively) (Table 2). The L_P value is close to 4.5 μm min^{-1} atm^{-1} and the E_a value is also close to 6 kcal/mol, suggesting the dependence of water movement on a channel pathway [10]. Thus, most water molecules would move across the plasma membrane through channel processes in morulae and blastocysts. Moreover, values for the L_P of morulae varied quite widely from 2.04 to 9.35 μm min^{-1} atm^{-1} among embryos (data not shown). The high variation in L_P can be explained by the different levels of channel expression



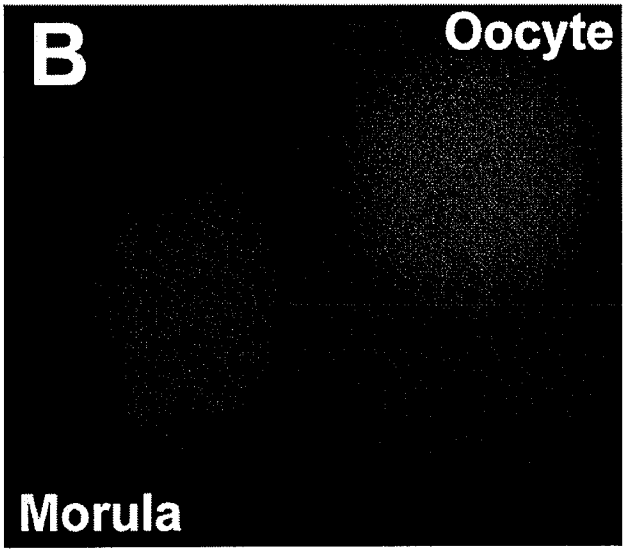


FIG. 4. Expression of aquaporin 3 (A) and aquaporin 7 (B) in a mouse oocyte and a morula. Expression of aquaporin proteins was detected by an immunofluorescence technique with anti-aquaporin 3 antibody (A) or anti-aquaporin 7 antiserum (B). Bar = 20 μm.

among morulae but not by simple diffusion across the plasma membrane. Furthermore, AQP inhibitors did not affect the L_P of oocytes but significantly suppressed that of morulae (Table 3). All of these results strongly suggest that water channels are the major contributor to water movement in morulae and early blastocysts.

There has been no report that suggests that experimentally water-permeable channels other than AQPs expressed in the plasma membrane at physiological levels contribute to total plasma membrane water permeability [26]. Thus, AQPs may be the major contributor to the permeation of mouse morulae and early blastocysts by water.

We have already shown that mRNAs of *Aqp3* and *Aqp7* are present in mouse oocytes at the metaphase II stage and embryos from the four-cell to morula stages but those of other *Aqps* were not in ICR mice [6]. Thus, these two AQPs may be

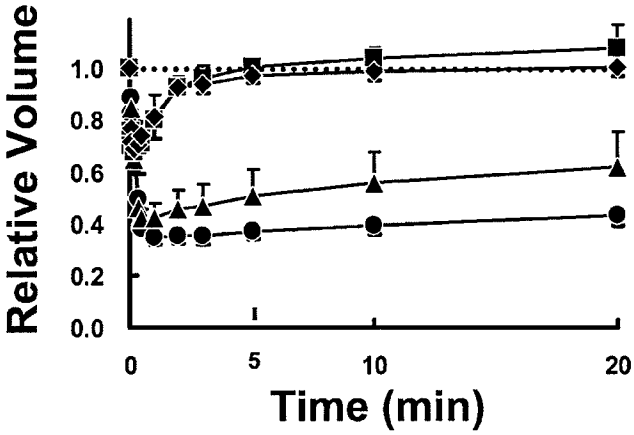


FIG. 5. Change in volume of mouse oocytes and embryos in 10% glycerol solution at 25°C. Mouse oocytes (circles) (n = 18) and embryos at the four-cell (triangles) (n = 7), morula (squares) (n = 14), and early blastocyst (diamonds) (n = 8) stages were exposed to 10% (vol/vol) glycerol in PB1 medium at 25°C for 20 min. Data are indicated as means of relative volume ± SD.

involved in the marked increase in the L_P of the embryos at the morula stage. Offenberg et al. [7] and Offenberg and Thomsen [8] reported the expression of mRNAs of other *Aqps*, including *Aqp1*, *Aqp5*, and *Aqp6*, in CD-1 X CB6F1/J embryos at various stages. The reason for this discrepancy is not known, but there may be mouse strain-specific differences in *Aqp* mRNA subtypes present.

It is known that mercuric compounds, such as $HgCl_2$ and *p*-CMBS, inhibit various AQPs, including AQP1, AQP2, and AQP3, by binding to a critical sulfhydryl [27], but not AQP7 because it does not have a critical sulfhydryl [25]. Phloretin is known as a urea-transporter inhibitor and inhibits the transport of water by AQP3 [24, 28], although its effect on AQP7 is not known. Thus, it was expected that phloretin and *p*-CMBS would decrease the L_P of morulae if AQP3 was dominantly expressed in morulae, whereas the inhibitors might not affect the L_P if AQP7 was dominantly expressed. Both phloretin and *p*-CMBS significantly reduced the L_P of morulae (Fig. 3 and Table 3), supporting the former assumption that AQP3 was expressed dominantly in morulae.

This hypothesis was confirmed by detection of the marked expression of AQP3 protein on the apical side of blastomeres of morulae but not in oocytes by an immunofluorescence technique (Fig. 4A). On the other hand, little expression of AQP7 was detected in oocytes or morulae (Fig. 4B). Thus, AQP3 must be the major contributor to water transport in morulae.

TABLE 5. Glycerol permeability (P_{GLY}) of mouse morulae treated with water-channel inhibitors.[a]

	P_{GLY} ($\times 10^{-3}$ cm/min)[b]	
Inhibitors	Before treatment	After treatment
Control	4.62 ± 0.92	4.72 ± 0.83
Phloretin	5.17 ± 1.56	3.15 ± 1.19*
p-CMBS	5.11 ± 0.80	2.92 ± 1.13*

[a] Values for control are calculated from 10 morulae, and other values are calculated from the data in Figure 7.
[b] Values are expressed as means ± SD.
* Significantly different from the P_{GLY} before treatment (Student *t*-test; $P < 0.01$).

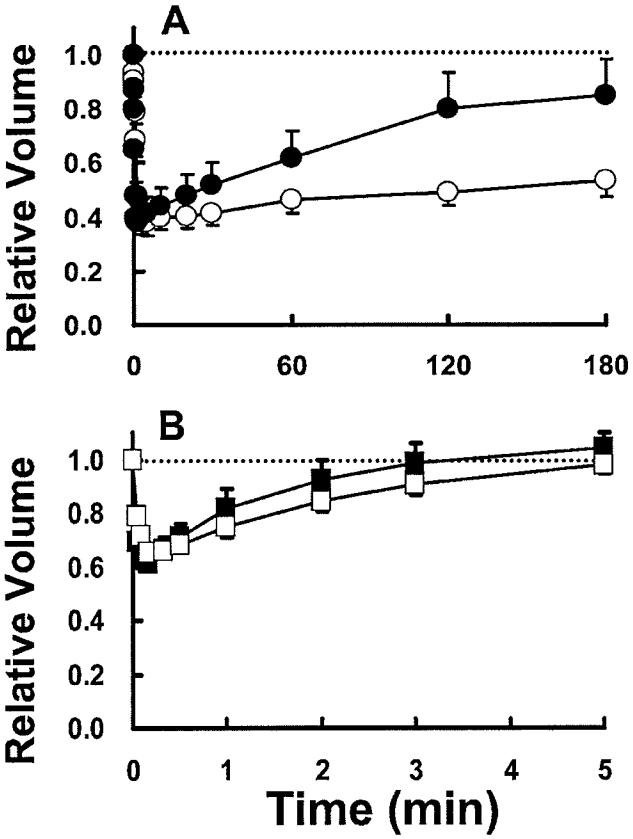


FIG. 6. Change in volume of mouse oocytes (**A**) and morulae (**B**) in 10% glycerol solution at 15°C (open symbols) and 25°C (closed symbols). Oocytes were exposed to 10% (vol/vol) glycerol in PB1 medium (1.89 Osm/kg) at 15 and 25°C for 180 min, and morulae were exposed to the solutions for 5 min. Data are indicated as means of relative volume ± SD. Each curve is from six oocytes or six morulae.

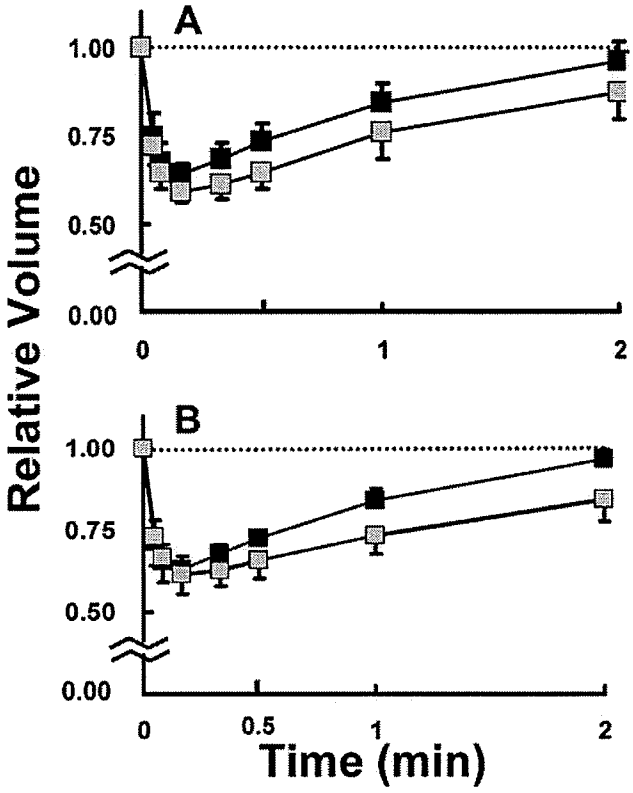


FIG. 7. Inhibition of glycerol permeability of mouse morulae by water-channel inhibitors. Morulae were exposed to 0.7 mM phloretin (**A**) or 0.5 mM *p*-(chloromercuri) benzensulfonate (**B**) in PB1 medium. Glycerol permeability was calculated from volume changes of morulae in 10% (vol/vol) glycerol in PB1 medium at 25°C for 5 min before (closed symbols) and after (shaded symbols) treatment with the inhibitor. Data are indicated as means of relative volume ± SD. Each curve is from 10 oocytes or 10 morulae.

Barcroft et al. [9] also reported that AQP3 was detected markedly on the apical side of blastomeres of morulae of CD-1 X CB6F1/J mice. Moreover, they did not detect other AQPs on the apical side of blastomeres of the morulae, although they detected other AQPs in the region of cell-cell contact. Thus, in morulae, the major water pathway must be AQP3 in the mouse.

In early blastocysts, the additional expression of mRNAs of *Aqp8* and *Aqp9*, in addition to those of *Aqp3* and *Aqp7*, has also been observed in ICR mice [6]. Barcroft et al. [9] showed in CD-1 X CB6F1/J embryos that expression of AQP3 became restricted in the inner cell mass and the basolateral cell margins of the trophectoderm and that AQP9 was expressed in the inner cell mass and the apical membrane of the trophectoderm. If the AQPs are distributed in blastocysts of ICR mice similarly, AQPs other than AQP3 could play a role in water movement in the blastocysts. Because the blastocysts we used were pipetted repeatedly to shrink their blastocoele and thus may have had small rips in their trophectoderm from the pipetting, it is possible that water moved not only across the apical side but also across the blastocoele-side of the plasma membrane in hypertonic sucrose solution. Thus, AQPs expressed in the inner cell mass and the basolateral cell margins of the trophectoderm might partially contribute to the water movement in our experiments. Further studies are needed to clarify which type of AQP is involved in water movement in mouse blastocysts.

AQP3 is an aqua glyceroporin, which can transport not only water but also neutral solutes with a low molecular weight, such as glycerol [24]. Thus, we examined the P_{GLY} of mouse oocytes and embryos (Fig. 5). The P_{GLY} of oocytes and four-cell embryos was low (0.01–0.06 $\times$ 10^{-3} cm/min), whereas that of morulae was high (4.63 $\times$ 10^{-3} cm/min), being more than 100 times higher than that of oocytes. This result suggests that AQP3 plays a role in the marked increase in the permeability of morulae to glycerol.

The pioneering studies by Mazur and his group [29, 30] demonstrated that the permeability of mouse embryos to glycerol increased from oocytes and one-cell zygotes to eight-cell embryos. The P_{GLY} values of oocytes and four-cell embryos in their studies (0.01 and 0.05 $\times$ 10^{-3} cm/min, respectively, at 20–22°C) are almost the same as those in the present study (0.01 and 0.06 $\times$ 10^{-3} cm/min, respectively, at 25°C). They also reported that a marked increase in the P_{GLY} of embryos was observed at the eight-cell stage (0.34 $\times$ 10^{-3} cm/min). In the present study, we observed further remarkable increase in the P_{GLY} at the morula stage (4.63 $\times$ 10^{-3} cm/min at 25°C). Thus, the marked increase in the expression of glycerol-permeable channels appears to begin at around the stage when mouse embryos develop to compacted morulae.

Thus, we studied the E_a for P_{GLY} and the sensitivity of P_{GLY} to AQP inhibitors in oocytes and morulae. The value of E_a for the P_{GLY} of oocytes was quite high (41.6 kcal/mol), whereas that of morulae was much lower (10.0 kcal/mol) (Table 4). The marked decrease in E_a suggests that channel processes play a role in rapid glycerol movement in morulae. Moreover,

phloretin and *p*-CMBS suppressed the P_{GLY} of morulae significantly (Table 5), suggesting that channels sensitive to these inhibitors were involved in glycerol movement in morulae. It has been shown that *p*-CMBS also suppresses the P_{GLY} of AQP3 [24, 28]. On the other hand, phloretin suppresses the L_p [24, 28] but not P_{GLY} of AQP3 [28]. Thus, rapid glycerol movement in morulae should rely partly on AQP3, but other glycerol channels might be involved in the process.

Early blastocysts also had remarkably high glycerol permeability, like the morulae (Fig. 5), suggesting that glycerol movement in blastocysts also relies on channel processes. It has been reported that the permeation by glycerol into CD-1 X CB6F1/J blastocysts is suppressed by *p*-CMBS [9]. Thus, mouse blastocysts also have mercury-sensitive channel-dependent glycerol pathways, the same as morulae. Further studies are needed to clarify which channels are involved in glycerol transport in blastocysts.

We have already examined changes in volume 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 embryos at the two-cell stage, but permeability to glycerol and ethylene glycol drastically increases at around the morula stage [20]. In bovine oocytes and embryos, we have also observed similar changes; the pattern of cryoprotectant permeation does not change from matured oocytes up to embryos at the 16-cell stage, but permeability to ethylene glycol and glycerol drastically increases at the morula stage [31]. Thus, the marked increase in aqua glyceroporins, such as AQP3, in the later stages of development may occur not only in the mouse but also in other mammalian species. Such increased permeability of mammalian embryos might play a role in the uptake of small molecules from tubal or uterine fluid or in the excretion of metabolites from embryos. However, the role appears not to be vital for the development of mouse embryos because *Aqp3* knockout mice can develop to term and are grossly normal except for polyurea [32]. However, because aqua glyceroporins are permeable to various cryoprotectants, the expression may also affect the tolerance of embryos at different developmental stages to cryopreservation. Thus, the present study provides important information for understanding the cryobiological properties of mammalian oocytes and embryos and for formulating cryopreservation protocols for mammalian oocytes and embryos.

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