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The movement of water and cryoprotectants across the plasma membrane of mammalian oocytes and embryos and its relevance to vitrification

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Abstract. The permeability of the plasma membrane to water and cryoprotectants is one of the most important factors for determining suitable conditions for vitrification of mammalian oocytes and embryos. In mouse oocytes and early stage embryos, water and cryoprotectants move slowly, principally by simple diffusion. In contrast, in morulae (and probably blastocysts), water, glycerol, and ethylene glycerol move rapidly, principally by facilitated diffusion via aquaporin 3, and DMSO moves rapidly via channels other than aquaporin 3. However, propylene glycol moves principally by simple diffusion. In cows and pigs, similar results were obtained. However, in bovine morulae, DMSO moves principally by simple diffusion. In pigs, permeability to water, glycerol, and ethylene glycol increases not at the morula stage but at the blastocyst stage, and increases further at the expanded blastocyst stage. Therefore, in general, the permeability of mammalian oocytes and early stage embryos to water and cryoprotectants is low. Then, at later stages, the permeability to water and some cryoprotectants markedly increases and occurs by facilitated diffusion via channels, although there are some species-specific differences.
Key words: Aquaporin, Cryoprotectant, Membrane-permeability, Vitrification, Water

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I first encountered studies on the cryopreservation of mammalian oocytes and embryos in 1983, when I was a fourth year college student. In the summer, the fifth World Conference on Animal Production was held in Kyoto, and one of the most famous cryobiologists, Dr Christopher Polge, visited our lab (Fig. 1). In 1949, he was the first to demonstrate that glycerol is protective for freezing fowl sperm [1]. This discovery laid the foundation for the field of cryobiology. After I graduated college, I got a job in the Institute for Laboratory Animals at Kochi Medical School where various mouse strains were maintained.

In 1993, I moved to the College of Agriculture at Kochi University, where I studied cryobiology of oocytes/embryos with my supervisor, Dr. Magosaburo Kasai (Fig. 1). In 1990 and 1991, he visited the laboratory of Dr. Peter Mazur (Fig. 2), who had succeeded in the cryopreservation of embryos for the first time [2], and he began to study the movement of water and cryoprotectants across the plasma membrane of oocytes/embryos for cryopreservation based on a theoretical approach.

During cryopreservation, several factors can injure the cells, including chilling, extracellular ice formation during cooling, intracellular ice formation during cooling and warming, the toxicity of the cryoprotectant, fracture damage, and osmotic swelling/shrinkage

during the removal of cryoprotectants after warming [3, 4]. For successful cryopreservation, the cell must avoid all of these potential damaging effects. The cryobiological properties of a cell affect its susceptibility to injury. Suitable conditions for the cryopreservation of mammalian oocytes/embryos differ according to developmental stage and species. This may be because the cryobiological properties of oocytes/embryos, including sensitivity to chilling, cryoprotectant toxicity, permeability of the plasma membrane to water and cryoprotectants, and tolerance to osmotic swelling/shrinkage, also differ according to developmental stage and species. Among these properties, the permeability of the plasma membrane to water and cryoprotectants is the most important, because this property is closely related to the major causes of cell injury, i.e., the formation of intracellular ice, chemical toxicity of cryoprotectants, and osmotic swelling of the cell.

Vitrification has been widely used for the cryopreservation of mammalian oocytes/embryos. Vitrification solutions contain a high concentration of cryoprotectant that is highly toxic to cells. Therefore, the permeability of the plasma membrane is more important during vitrification than during slow freezing, as the exposure time to the vitrification solution must be limited because of its toxicity. However, shorter exposure time can result in insufficient permeation, which leads to intracellular ice formation. Therefore, the permeability of the plasma membrane markedly affects the conditions suitable for vitrification.

Fig. 1. Dr. Polge in Kyoto (1983). This group photo was taken in Kyoto during the 5th World Conference on Animal Production. In the back row (from right to left) are Drs. Minami, Matsumoto, Higuchi, Maruyama, Polge, Nagai, and King. In the front row (from right to left) are Mr. Shinkai and Drs. Miyake, Edashige, and Kasai.
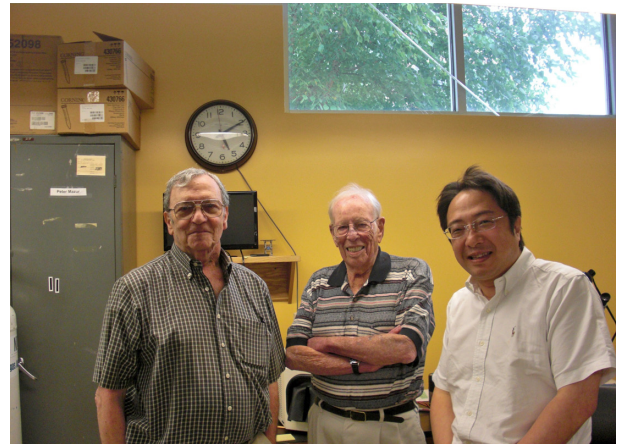

Fig. 2. Dr. Mazur in Knoxville (2013). This photo was taken in Dr. Mazur's lab at the University of Tennessee in Knoxville, TN. From right to left are Drs. Edashige, Mazur, and Leibo.

The Movement of Water Across the Plasma Membrane of Mouse Oocytes/embryos in a Solution Containing Sucrose

In most cell types, water moves slowly through the plasma membrane via simple diffusion through the lipid bilayer. However, in some cell types, such as red blood cells, water moves rapidly through the plasma membrane by facilitated diffusion through intrinsic membrane proteins called aquaporins that function as water channels [5]. Therefore, water moves across the plasma membrane by either simple diffusion or facilitated diffusion via aquaporins. We showed, for the first time, that aquaporins are expressed in mammalian oocytes and embryos [6].

The major pathway for the movement of water across the plasma membrane can be deduced from the permeability to water (L_P) and its temperature dependence (Arrhenius activation energy, E_a). In general, an L_P value higher than 4.5 μm/min/atm with an E_a value lower than 6 kcal/mol is suggestive of water movement by facilitated diffusion via aquaporins [7]. In contrast, a low L_P value with an E_a value higher than 10 kcal/mol is suggestive of simple diffusion across the plasma membrane [7]. However, there is no theoretical explanation for the values.

In mature mouse oocytes, the L_P value in a hypertonic solution containing sucrose at 20–25°C is low (0.4–1.0 μm/min/atm), and its E_a value is high (11–15 kcal/mol) [8–15]. This suggests that water moves through oocytes principally by simple diffusion (Fig. 3A). In early stage mouse embryos at (1–4 cell stage), the L_P value remains low (0.4–0.7 μm/min/atm), and the E_a value is high (12–13 kcal/mol) [8, 13, 15]. Therefore, water also moves through early embryos principally by simple diffusion (Fig. 3A). In contrast, in mouse morulae and blastocysts, the L_P value is high (3.1–4.5 μm/min/atm), and its E_a value is low (5.1–6.3 kcal/mol) [15], suggesting that water moves through these later stage embryos principally by facilitated diffusion through water channels. In mouse morulae and blastocysts,

aquaporin 3 expression is higher than that of other aquaporins [16, 17]. Furthermore, suppression of aquaporin 3 expression in morulae, by injecting a double stranded RNA targeting aquaporin 3 into 1-cell zygotes, markedly decreases the L_P value [18]. Therefore, water moves through mouse morulae/blastocysts principally by facilitated diffusion via aquaporin 3 (Fig. 3B). There are two groups of aquaporins; one is highly selective for water, and the other can transport not only water but also neutral solutes, including cryoprotectants [5]. Aquaporin 3 can transport both water and cryoprotectants [19].

The Movement of Cryoprotectants and Water Across the Plasma Membrane of Mouse Oocytes/embryos in a Solution Containing a Cryoprotectant

There are no quantitative values for evaluating the movement of cryoprotectants across the plasma membrane. However, it is reasonable to deduce that low permeability to cryoprotectant (P_S) with a high E_a value for permeability is suggestive of movement principally by simple diffusion and that a high P_S value with a low E_a value for permeability is suggestive of movement principally by facilitated diffusion through channels.

In mouse oocytes/early embryos, permeability to glycerol (P_{Gly}) is quite low (0.01–0.02 × 10^{-3} cm/min) at 20–25°C [15, 20, 21], and its E_a value is remarkably high (42 kcal/mol) [15,18]. Therefore, glycerol moves through oocytes and early embryos principally by simple diffusion. In contrast, the P_{Gly} value of morulae is high (4–5 mm/min/atm), and its E_a value is low (10 kcal/mol) [15, 18], suggesting that glycerol moves through morulae principally by facilitated diffusion. In addition, the high P_{Gly} value of morulae is markedly decreased by suppressing the expression of aquaporin 3 [18]. Therefore, glycerol moves through morulae (and probably blastocysts) principally via aquaporin 3 (Fig. 3C). In a solution containing glycerol, water movement through oocytes and embryos is similar to that of glycerol. In oocytes at 25°C, the L_P value is low (0.6 μm/min/atm), and its E_a value is quite high (14 kcal/mol)
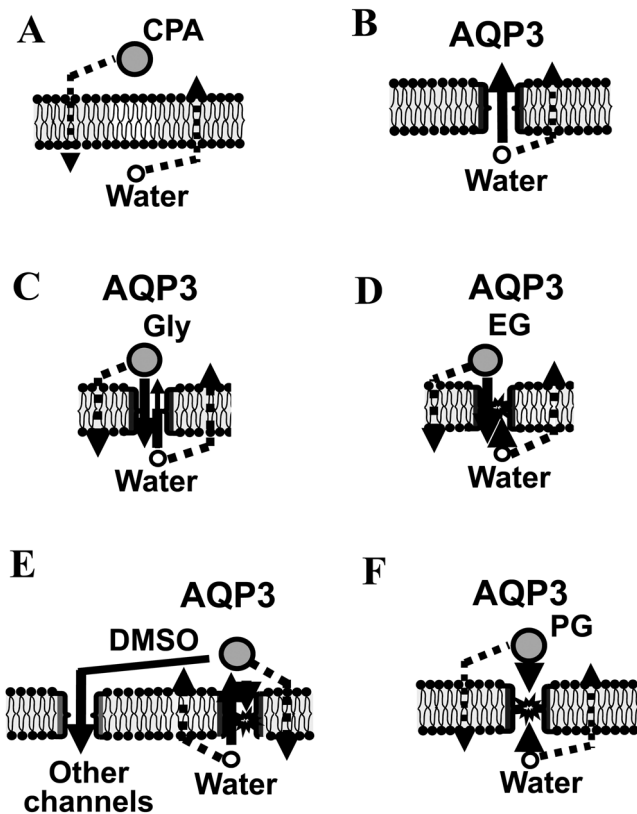
Fig. 3. Schematic representation of the pathways for the movement of water and cryoprotectants across the plasma membrane of mouse oocytes and morulae in the presence of cryoprotectants. Dotted lines indicate the movement of water and cryoprotectants by simple diffusion, and solid lines indicate the movement of water and cryoprotectants across the plasma membrane by facilitated diffusion through channel pathways. A, the movement of water and cryoprotectant in an oocyte; B, the movement of water in a morula in a solution containing sucrose; C, the movement of water and glycerol in a morula in a solution containing glycerol; D, the movement of water and ethylene glycol in a morula in a solution containing ethylene glycol; E, the movement of water and DMSO in a morula in a solution containing DMSO; F, the movement of water and propylene glycol in a morula in a solution containing propylene glycol. AQP3, aquaporin 3; Other channels, DMSO-permeable channels; Gly, glycerol; EG, ethylene glycol, PG, propylene glycol; open small circles, water molecules; shaded large circle, cryoprotectant molecules (CPA). Modified from Kasai & Edashige (2010) [27].

[15, 18], suggesting that, in the presence of glycerol, water moves through oocytes principally by simple diffusion. Conversely, in morulae, the L_P value is relatively high (2.2 μm/min/atm), and its E_a value is relatively low (9 kcal/mol) [15, 18]. Since suppression of aquaporin 3 expression in morulae decreases the L_P value [18], many water molecules move through morulae by facilitated diffusion via aquaporin 3 (Fig. 3C).

The permeability of oocytes to ethylene glycol (P_{EG}) is low (0.6 × 10^{-3} cm/min) at 25°C, and its E_a value is high (17 kcal/mol) [18]. Therefore, ethylene glycol moves through oocytes (and probably

early embryos) principally by simple diffusion. In contrast, in morulae, the P_{EG} value is quite high (10 × 10^{-3} cm/min), and its E_a value is low (9 kcal/mol) [18]. In addition, the high P_{EG} value of morulae is markedly decreased by suppressing the expression of aquaporin 3 [18]. Therefore, ethylene glycol moves through morulae (and probably blastocysts) principally by facilitated diffusion via aquaporin 3 (Fig. 3D).

In a solution containing ethylene glycol at 25°C, the L_P value of oocytes is low (0.4 μm/min/atm), and its E_a value is high (10 kcal/mol) [18], suggesting that water moves through oocytes principally by simple diffusion. Unexpectedly, the L_P value of morulae is low (0.5 μm/min/atm), and its E_a value is quite high (14 kcal/mol) [18]. In addition, suppression of aquaporin 3 expression in morulae does not affect the L_P value (0.5 μm/min/atm) [18]. Therefore, in the presence of ethylene glycol, water moves through morulae principally by simple diffusion, even though aquaporin 3 is expressed abundantly (Fig. 3D). This might be caused by the interaction between water and ethylene glycol molecules through aquaporin 3. Thus, although ethylene glycol moves through aquaporin 3, water does not.

The permeability of oocytes to DMSO (P_{DMSO}) at 25°C is low (1.0 × 10^{-3} cm/min), and its E_a value is high (18 kcal/mol) [18]. Therefore, DMSO moves through oocytes (and probably early embryos) principally by simple diffusion. However, the P_{DMSO} of morulae is higher (3.0 × 10^{-3} cm/min), and its E_a value is lower (12 kcal/mol) than that of oocytes [18]. Therefore, DMSO moves through morulae principally by facilitated diffusion via channels. However, suppression of aquaporin 3 expression in morulae does not decrease the P_{DMSO} value [18], suggesting that channels other than aquaporin 3 are involved in the facilitated diffusion of DMSO (Fig. 3E), even though aquaporin 3 expressed in *Xenopus* oocytes can transport DMSO [22].

In a solution containing DMSO at 25°C, the L_P value of oocytes is low (0.5 μm/min/atm), and its E_a value is high (12 kcal/mol) [18], suggesting that water moves through oocytes principally by simple diffusion. On the contrary, in morulae, the L_P value is high (2.0 μm/min/atm), and its E_a value is relatively low (9 kcal/mol) [18]. Since suppression of aquaporin 3 expression in morulae decreases the high L_P value (0.8 μm/min/atm) [18], water moves through morulae principally by facilitated diffusion via aquaporin 3 (Fig. 3E).

The permeability of oocytes to propylene glycol (P_{PG}) at 25°C is relatively low (1.7 × 10^{-3} cm/min), and its E_a is high (20 kcal/mol) [18]. Therefore, propylene glycol moves through oocytes (and probably early embryos) principally by simple diffusion. However, the P_{PG} value is higher than the permeability values of other cryoprotectants in oocytes. This may be because it is more hydrophobic than other cryoprotectants. In morulae, the P_{PG} value is more than twice as high as that in oocytes (3.8 × 10^{-3} cm/min) [18], and it is comparable to the P_{Gly} and P_{DMSO} values in morulae. Glycerol and DMSO are thought to move through morulae principally by facilitated diffusion; however, the E_a value for P_{PG} is high (20 kcal/mol) in morulae [18]. Therefore, propylene glycol moves through morulae principally by simple diffusion rather rapidly (Fig. 3F), although aquaporin 3 expressed in *Xenopus* oocytes can effectively transport propylene glycol [22–23]. In a solution containing propylene glycol at 25ºC, the L_P value of oocytes is low (0.5 μm/min/atm) , and its E_a value is quite high (13 kcal/mol) [18], suggesting that water moves through

oocytes principally by simple diffusion. In morulae, the L_P value is relatively low (1.0 µm/min/atm), its E_a value is high (15 kcal/mol), and suppression of aquaporin 3 expression in morulae does not affect the L_P value (0.7 µm/min/atm) [18]. Therefore, in the presence of propylene glycol, water moves through morulae principally by simple diffusion, even though aquaporin is expressed abundantly. This may be caused by interaction between water and propylene glycol molecules moving through aquaporin 3. As a result, neither water nor propylene glycol can move through morulae via aquaporin 3 (Fig. 3F).

The Movement of Water and Cryoprotectants Across the Plasma Membrane of Oocytes and Embryos in Mammals Other Than the Mouse

Bovine oocytes and embryos

In bovine oocytes, the L_P value at 25°C is low (1.8 µm/min/atm), and its E_a value is high (9 kcal/mol) [24]. Therefore, water moves through bovine oocytes principally by simple diffusion. However, the L_P value in bovine oocytes is higher than that in mouse oocytes (0.4–1.0 µm/min/atm), and its E_a value is slightly lower than 10 kcal/mol [24]. Moreover, the L_P value is higher than that of bovine morulae in which the expression of aquaporin 3 is suppressed (0.6 µm/min/atm) [24]. Therefore, in bovine oocytes, some water moves through water channels. In morulae and blastocysts, the L_P value at 25°C is high (3 µm/min/atm), and its E_a value is low (3 kcal/mol). Suppression of aquaporin 3 expression in morulae markedly decreases the L_P value [24]. Therefore, water moves through bovine morulae (and probably blastocysts) principally by facilitated diffusion via aquaporin 3, similar to in mouse morulae.

The P_{Gly}, P_{DMSO}, and P_{PG} values in bovine oocytes at 25°C are low, and its E_a values are high [24], which is essentially the same as in mouse oocytes. Therefore, these cryoprotectants move through bovine oocytes principally by simple diffusion. However, the P_{Gly} and P_{EG} values (0.5×10^{-3} cm/min and 3.5×10^{-3} cm/min, respectively) in bovine oocytes are higher than those in mouse oocytes (0.01–0.02×10^{-3} cm/min and 0.6×10^{-3} cm/min, respectively) [24]. Since bovine oocytes express water channels to some extent and the channels are partially involved in the movement of water as described above, glycerol and ethylene glycol would move through bovine oocytes primarily by simple diffusion and partially via water channels. In bovine morulae, the P_{Gly}, P_{EG}, and P_{PG} values at 25°C and the pathway for the movement are essentially the same as those in mouse morulae. Glycerol and ethylene glycol move rapidly through morulae principally by facilitated diffusion via aquaporin 3 because suppression of aquaporin 3 expression markedly decreases the P_{Gly} and P_{EG} values, and propylene glycol moves principally by simple diffusion, although the P_{PG} is higher than that of oocytes [24].

The P_{DMSO} of bovine oocytes at 25°C is relatively low (1.5×10^{-3} cm/min), and its E_a value is high (13 kcal/mol) [24], suggesting that DMSO moves through bovine oocytes principally by simple diffusion. However, in bovine morulae, the P_{DMSO} is low (1.7×10^{-3} cm/min), and its E_a value is higher (21 kcal/mol) than that of bovine oocytes [24]. Therefore, regardless of developmental stage, DMSO moves through bovine morulae principally by simple diffusion.

Pig oocytes and embryos

In pigs, the L_P value of oocytes at 25°C is low (1.0 µm/min/atm), and its E_a value is high (19 kcal/mol), similar to that of mouse oocytes [25]. However, unlike mouse and bovine morulae, in pig morulae, the L_P value remains low, [25], probably because pig embryos become compacted at the 4-cell stage. Therefore, water moves through pig oocytes and morulae principally by simple diffusion. In pig blastocysts, the L_P value increases significantly and increases further in expanded blastocysts (3.4 µm/min/atm) at 25°C [25]. The E_a value for the permeability of expanded blastocysts (7 kcal/mol) is close to 6 kcal/mol. Therefore, water moves through pig expanded blastocysts principally by facilitated diffusion via water channels. Since pig expanded blastocysts abundantly express aquaporin 3 mRNA, aquaporin 3 might be involved in the facilitated diffusion of water [25].

In pig oocytes, the P_{Gly}, P_{EG}, P_{DMSO}, and P_{PG} values are low at 25°C, and the E_a values are high, suggesting that glycerol, ethylene glycol, DMSO, and propylene glycol move through pig oocytes principally by simple diffusion, similar to in mouse oocytes [25]. In pig morulae, these permeabilities remain low, as does the L_P value. Therefore, these cryoprotectants move through morulae principally by simple diffusion. In blastocysts, the P_{Gly} and P_{EG} values increase, and they further increase in expanded blastocysts [25]. The E_a values for the permeabilities in expanded blastocysts are much lower than those in oocytes. Therefore, glycerol and ethylene glycol would move through pig expanded blastocysts principally by facilitated diffusion. Since aquaporin 3 can transport these cryoprotectants, aquaporin 3 might be involved in the facilitated diffusion of these cryoprotectants in pig expanded blastocysts. Compared to the P_{DMSO} values in oocytes and morulae, the P_{DMSO} value is slightly increased [25] and the E_a value is lower at the expanded blastocyst stage. Therefore, DMSO moves through pig expanded blastocysts partially by facilitated diffusion via channels. In contrast, the P_{PG} value remains low, and its E_a remains high at the blastocyst and expanded blastocyst stages [25]. Therefore, propylene glycol would move principally by simple diffusion, regardless of developmental stage, similar to mouse and bovine embryos.

Future Prospects

In general, it is easier to vitrify later stage embryos, such as morulae, than earlier stage embryos, because their membranes are highly permeable to water and cryoprotectants. Since high membrane permeability depends on the expression of aquaporins, artificial expression of aquaporins in oocytes/early embryos could improve their tolerance to vitrification. As described above, mouse oocytes have very low permeability to glycerol. Therefore, it is quite difficult to vitrify them with a glycerol-based solution. When immature mouse oocytes were injected with aquaporin 3 cRNA and cultured for 12 h to allow the oocytes to mature and express aquaporin 3 protein, permeability to glycerol and water increased markedly, and the oocytes survived vitrification with a glycerol-based solution [26]. Therefore, artificial expression of aquaporins might also improve the cryosurvival of cells with larger volumes, such as fish eggs and embryos.

Conclusion

Our assessment of the movement of water and cryoprotectants in oocytes and embryos shows that the movement patterns are more stage specific than species specific, although some species specificity exists. Therefore, based on cryoprotectant permeability, the protocols developed for the cryopreservation of oocytes/embryos of one species should generally be applicable to oocytes/embryos of other species at the same stage.

To design effective protocols for the vitrification of mammalian oocytes/embryos, it is important to consider the pathway for the movement of water and cryoprotectants at each stage. In vitrification, the exposure time and temperature of the vitrification solution are important, because the vitrification solution contains a high concentration of cryoprotectant, which is highly toxic to oocytes/embryos. When water and cryoprotectants move through oocytes/embryos principally by simple diffusion, the exposure time and temperature of the vitrification solution is important because the temperature affects permeability to water and cryoprotectants. When water and cryoprotectants move through embryos principally by facilitated diffusion via channels, the exposure time is more important because the permeability is less affected by temperature. However, exposure of oocytes/embryos to vitrification solutions at high temperatures should be avoided because cryoprotectants are more toxic at higher temperatures.

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