

—Mini Review—

# **The Movement of Water and Cryoprotectants in Mammalian Oocytes and Embryos and its Relevance to Cryopreservation**

Keisuke Edashige<sup>1\*</sup> and Magosaburo Kasai<sup>1</sup>

<sup>1</sup>Laboratory of Animal Science, College of Agriculture, Kochi University, Nankoku, Kochi 783-8502, Japan

**Abstract:** *The conditions suitable for cryopreservation differ among oocytes and embryos at different stages even in the same species. Differences in cryobiological properties would affect these conditions. Permeability to water and cryoprotectants is important because it modulates major forms of cell injury from cryopreservation. In the mouse, morulae and blastocysts tolerate cryopreservation better than oocytes or early stage embryos, because they are highly permeable to water and cryoprotectants. The permeability appears to stem from a substantial expression of water/cryoprotectant-permeable channels, such as aquaporin-3. Such facilitated diffusion by channel proteins is less dependent on temperature than the process of simple diffusion via the lipid bilayer. Therefore, it is important to know the expression level and permeation properties of water/cryoprotectant channels for efficient cryopreservation of oocytes and embryos.*

**Key words:** *Cryopreservation, Water, Cryoprotectant, Permeability, Oocyte*

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## **Introduction**

Since the first successful cryopreservation of mouse embryos in 1972 [1], various protocols have been developed for cryopreservation of oocytes and embryos in many mammalian species. However, it is difficult to obtain high survival rates in oocytes and embryos at different developmental stages with a single cryopreservation protocol even in the same species.

For example, mouse morulae can be cryopreserved without appreciable loss of viability by a simple one-step vitrification method [2], whereas mouse oocytes do not survive after vitrification by the same method [3], and pretreatment with a lower concentration of cryoprotectant is necessary to vitrify mouse embryos at early stages [4]. This indicates that cryobiological properties differ among oocytes and embryos at different developmental stages.

During the process of cryopreservation and recovery, mammalian oocytes/embryos can be damaged by chilling, the formation of extracellular ice, the formation of intracellular ice, the chemical toxicity of cryoprotectants, fracturing, osmotic swelling and osmotic shrinkage [5]. Among cryobiological properties, the permeability of the plasma membrane to water and cryoprotectants is the most important for determining the tolerance of cells to cryopreservation, because the permeability modulates major forms of cell injury related to cryopreservation: damage from intracellular ice formation, cryoprotectant toxicity and osmotic swelling. Thus, it is likely that the nature of the plasma membrane's permeability differs among oocytes and embryos at different developmental stages. To determine a suitable protocol for the cryopreservation of oocytes/embryos, it is necessary to know the nature of membrane permeability.

## **The Movement of Water in Oocytes and Embryos**

Water was long believed to move across the plasma membrane only by simple diffusion via the lipid bilayer. In the 1990s, however, small intrinsic membrane proteins that act as water channels, called aquaporins

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\*To whom correspondence should be addressed.

e-mail: keisuke@kochi-u.ac.jp

(AQPs), were discovered and characterized [6]. The channels occur in two groups: one is highly selective for water and the other transports not only water but also neutral solutes with a small molecular weight, such as cell-permeating cryoprotectants (Table 1).

We have shown in the ICR mouse that mRNAs of AQP3 and AQP7 are present in oocytes at the metaphase II stage and embryos at the 4-cell, morula and blastocyst stages, and that mRNAs of AQP8 and AQP9 are expressed in embryos at the blastocyst stage [7]. Offenberg and his group also detected mRNAs of AQPs [8] and AQP proteins [9] in mouse embryos of other strains. However, the detection of these mRNAs and proteins on oocytes and embryos does not imply that AQPs actually play a significant role in the movement of water and cryoprotectants. AQPs play a significant role when they are expressed substantially.

The significant involvement of channel processes in the movement of water across the plasma membrane can be deduced from the permeability to water and its dependency on temperature, because the movement of water through channels is much less dependent on temperature than that through the lipid bilayer. The temperature dependency of the permeability is expressed by the Arrhenius activation energy ( $E_a$ ). Verkman *et al.* (1996) reported that an osmotic water permeability higher than  $4.5 \mu\text{m}/\text{min}/\text{atm}$  and an  $E_a$  value lower than  $6 \text{ kcal}/\text{mol}$  are suggestive of movement principally through water channels, whereas an  $E_a$  value higher than  $10 \text{ kcal}/\text{mol}$  with a low water permeability is suggestive of movement principally via channel-independent diffusion [10]. If these criteria are applied to oocytes and embryos at early cleavage stages in mammals, water channels may not be the major pathway for the movement of water across the plasma membrane. It has been shown in the mouse that the permeability to water of oocytes and embryos at early cleavage stages is quite low ( $0.44\text{--}1.10 \mu\text{m}/\text{min}/\text{atm}$ ) [11–17]. The permeability of oocytes of cattle, Syrian hamsters, and humans is also quite low ( $0.40\text{--}0.54 \mu\text{m}/\text{min}/\text{atm}$ ) [12, 13, 18]. The  $E_a$  for water-permeability ( $9.5\text{--}14.5 \text{ kcal}/\text{mol}$ ) is around  $10 \text{ kcal}/\text{mol}$  or higher in mouse oocytes [11–13, 17, 19]. In oocytes of cattle, Syrian hamsters and humans,  $E_a$  values are also high ( $7.8\text{--}8.6 \text{ kcal}/\text{mol}$ ) [12, 13, 18]. Thus, in oocytes and embryos at early cleavage stages in mammals, water is thought to move across the plasma membrane predominantly by simple diffusion via the lipid bilayer. On the other hand, in mouse morulae and blastocysts, the permeability to water is quite high ( $3.6\text{--}4.5 \mu\text{m}/\text{min}/\text{atm}$ ) (Table 2) [17] and the  $E_a$  value is low ( $5.1\text{--}6.3 \text{ kcal}/$

**Table 1.** Permeability of aquaporin (AQP) s

Type of AQP	Permeable to	
	Water	Neutral Solutes
AQP0	+/-	-
AQP1	+	-
AQP2	+	-
AQP3	+	+
AQP4	+	-
AQP5	+	-
AQP6	+/-	-
AQP7	+	+
AQP8	+	-
AQP9	+	+
AQP11	?	?
AQP12	?	?

mol) (Table 2) [17]. In mouse morulae and blastocysts, therefore, most water is considered to move across the plasma membrane predominantly by facilitated diffusion through channels.

Since AQP3 is expressed abundantly in mouse morulae (Fig. 1) [9, 17] and mouse blastocysts [9], and its mRNA is much more abundant in mouse blastocysts than is the mRNA of any other AQP [20], AQP3 appears to be the major contributor to the rapid movement of water in mouse morulae and blastocysts.

### The Movement of Cryoprotectants in Oocytes and Embryos

It would also be possible to deduce the pathway of a cryoprotectant's movement from the permeability to the cryoprotectant and its  $E_a$  value for the permeability, as in the case of permeability to water, although few reports are available on the quantitative evaluation of this.

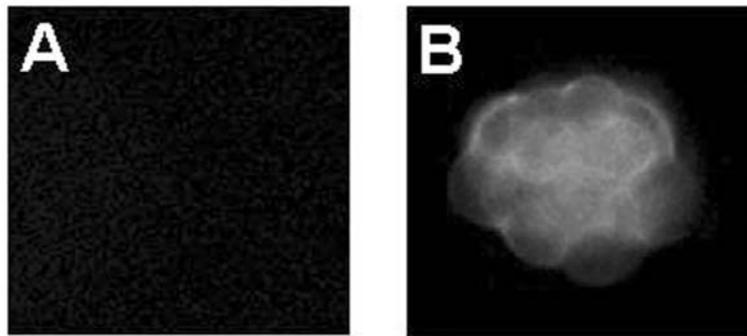
In mice, the permeability of oocytes and embryos to glycerol and its  $E_a$  at different developmental stages vary as in the case of water (Table 2). The permeability to glycerol of oocytes and embryos at early cleavage stages is quite low ( $0.01\text{--}0.06 \times 10^{-3} \text{ cm}/\text{min}$ ) [17, 21, 22] and the  $E_a$  value of oocytes is remarkably high ( $41.6 \text{ kcal}/\text{mol}$ ) (Table 2) [17]. On the other hand, the permeability to glycerol of morulae and blastocysts in mice is substantially high ( $4.1\text{--}4.7 \times 10^{-3} \text{ cm}/\text{min}$ ) [17], and the  $E_a$  value in mouse morulae ( $10 \text{ kcal}/\text{mol}$ ) is much lower than that in oocytes (Table 2) [17].

Since AQP3 is an aqua-glyceroporin [6, 23], glycerol must permeate mouse morulae and blastocysts via channel processes. It has been shown that AQP3 is permeable by various neutral solutes with a low

**Table 2.** Permeability to water ( $L_p$ ) and glycerol ( $P_{Gly}$ ) of oocytes at the MII stage and morulae at 15°C and 25°C and the Arrhenius activation energy ( $E_a$ ) in the mouse

Cell	Parameter	15°C	25°C	$E_a$ (kcal/mol)
Oocyte	$L_p$ ( $\mu\text{m}/\text{min}/\text{atm}$ )	$0.34 \pm 0.07$	$0.70 \pm 0.12$	12.3
	$P_{Gly}$ ( $\times 10^{-3}$ cm/min)	$0.00 \pm 0.00$	$0.02 \pm 0.02$	41.6
Morula	$L_p$ ( $\mu\text{m}/\text{min}/\text{atm}$ )	$3.07 \pm 1.39$	$4.45 \pm 1.83$	6.3
	$P_{Gly}$ ( $\times 10^{-3}$ cm/min)	$2.61 \pm 0.88$	$4.68 \pm 1.50$	10.0

Modified from Edashige *et al.* (2006) [17].



**Fig. 1.** The expression of aquaporin-3 in a mouse oocyte and morula, detected by an immunofluorescence technique with anti-aquaporin-3 antibody. A, Oocyte. B, Morula.

molecular weight including cryoprotectants such as glycerol, ethylene glycol, propylene glycol, acetamide and dimethylsulfoxide [23–29]. Therefore, AQP3 expressed in mouse morulae and blastocysts must contribute to the permeation of various cryoprotectants.

This speculation is supported by our previous finding that artificial expression of AQP3 in mouse oocytes achieved by injecting AQP3 cRNA markedly increased the permeability to water and glycerol of the oocytes, and that the artificial expression improved their survival after vitrification with a glycerol-based solution [27]. Therefore, AQP3 actually plays an important role in the movement of water and the cryoprotectant and the tolerance to cryopreservation in mouse morulae. Moreover, changes in the pattern of the permeability of embryos to various cryoprotectants were observed not only in mice [30] but also in cattle [31] and rabbits [unpublished observation], suggesting that the expression of water/cryoprotectant-permeable channels increases markedly in the later stages of embryogenesis in various mammalian species. Since substantial expression will affect the permeability of the embryos to various cryoprotectants, it must markedly affect tolerance to cryopreservation in various mammalian embryos.

### The Pathway for the Movement of Water and Cryoprotectants and Conditions for the Cryopreservation of Oocytes/Embryos

Since facilitated diffusion of cells by a channel process not only increases the permeability of the plasma membrane but also decreases the temperature dependency of the permeability markedly, the significant expression of water/cryoprotectant-permeable channels must affect the conditions suitable for the cryopreservation of cells. For the cryopreservation of mouse morulae, especially by vitrification, the period of exposure to the cryoprotectant solution should be shorter than that for oocytes and embryos at earlier stages, because morulae are dehydrated and permeated by cryoprotectants during a brief exposure period via water/cryoprotectant channel processes, and excess exposure to the solution would result in the embryos being damaged by the chemical toxicity of the cryoprotectant. From the viewpoint of membrane permeability, the temperature for exposure of morulae to the cryoprotectant solution before cryopreservation and for the removal of cryoprotectants after warming is not very important because the channel processes are less affected by temperature; the

movement of water and cryoprotectants should be fast and constant. Blastocysts are as permeable to water and cryoprotectants as morulae, but another factor should be considered, the presence of the blastocoel. In mouse blastocysts, it has been shown that the distribution of AQPs in the plasma membrane is not homogeneous among the apical and basolateral sides of the trophectoderm and the inner cell mass [9], suggesting that blastocysts have compartments differing in membrane permeability, although overall they are highly permeable to water and cryoprotectants. Moreover, expanded blastocysts have a large volume of water in the blastocoel, which should be removed to minimize the formation of ice crystals in the blastocoel and to prevent the toxic effect of cryoprotectants. One strategy for the cryopreservation of expanded blastocysts, especially by vitrification, is to puncture the blastocoel with a microneedle [32].

In oocytes and embryos at early cleavage stages in mice, a long exposure to the cryoprotectant solution is necessary for dehydration and permeation by the cryoprotectant, because the movement of water and cryoprotectants by simple diffusion via the lipid bilayer is much slower than that by facilitated diffusion through channels. In addition, the temperature at which oocytes/embryos are exposed to the cryoprotectant solution and the intracellular cryoprotectant is removed after warming is very important, because the movement of water and cryoprotectants by simple diffusion is greatly affected by temperature.

### Conclusion

The expression of water/cryoprotectant permeable channels in mammalian oocytes/embryos affects their membrane permeability and the conditions suitable for cryopreservation. Therefore, it is important to know the nature and expression levels of water/cryoprotectant-permeable channels for efficient cryopreservation of oocytes/embryos.

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