

Artificial Expression of Aquaporin-3 Improves the Survival of Mouse Oocytes after Cryopreservation¹

Keisuke Edashige,^{2,3} Yohei Yamaji,³ F.W. Kleinhans,⁴ and Magosaburo Kasai³

Laboratory of Animal Science,³ College of Agriculture, Kochi University, Nankoku, Kochi 783-8502, Japan
Department of Physics,⁴ Indianapolis University and Purdue University Indianapolis, Indianapolis, Indiana 46202

ABSTRACT

Successful cryopreservation of mammalian cells requires rapid transport of water and cryoprotective solutes across the plasma membrane. Aquaporin-3 is known as a water/solute channel that can transport water and neutral solutes such as glycerol. In this study we examined whether artificial expression of aquaporin-3 in mouse oocytes can improve water and glycerol permeability and oocyte survival after cryopreservation. Immature mouse oocytes were injected with aquaporin-3 cRNA and were cultured for 12 h. Then the hydraulic conductivity (L_p) and glycerol permeability (P_{GLY}) of matured oocytes were determined from the relative volume changes in 10% glycerol in PB1 medium at 25°C. Mean \pm SD values of L_p and P_{GLY} of cRNA-injected oocytes ($3.09 \pm 1.22 \mu\text{m min}^{-1} \text{atm}^{-1}$ and $3.69 \pm 1.47 \times 10^{-3} \text{cm/min}$, respectively; numbers of oocytes = 25) were significantly higher than those of noninjected oocytes ($0.83 \pm 0.02 \mu\text{m min}^{-1} \text{atm}^{-1}$ and $0.07 \pm 0.02 \times 10^{-3} \text{cm/min}$, respectively; $n = 13$) and water-injected oocytes ($0.87 \pm 0.10 \mu\text{m min}^{-1} \text{atm}^{-1}$ and $0.08 \pm 0.02 \times 10^{-3} \text{cm/min}$, respectively; $n = 20$). After cryopreservation in a glycerol-based solution, 74% of cRNA-injected oocytes ($n = 27$) survived as assessed by their morphological appearance, whereas none of the water-injected oocytes survived ($n = 10$). When cRNA-injected oocytes that survived cryopreservation were inseminated in vitro, the penetration rate was 40% ($n = 48$) and the cleavage rate was 31% ($n = 70$), showing that oocytes retain their ability to be fertilized. This is the first report to show that artificial expression of a water/solute channel in a cell improves its survival after cryopreservation. This approach may enable cryopreservation of cells that have been difficult to cryopreserve.

oocyte development

INTRODUCTION

Cryopreservation of mammalian embryos has been used for various purposes (i.e., for preservation of genetic variants in laboratory animals, for breeding and reproduction of farm animals, and for treatment of infertility in humans) [1]. Successful cryopreservation of oocytes would also greatly assist the application of several reproductive biotechnologies, such as in vitro production of embryos, treatment of infertility, cloning, and gene banking [1]. However,

¹This study was supported by grants-in-aid for scientific research from the Japanese Society for the Promotion of Science (RFTF Program); the Ministry of Education, Sports, Culture, and Science of Japan; and the Inamori Foundation.

²Correspondence. FAX: 81 88 864 5219;
e-mail: keisuke@cc.kochi-u.ac.jp

Received: 7 December 2001.
First decision: 2 January 2002.
Accepted: 15 July 2002.

© 2003 by the Society for the Study of Reproduction, Inc.
ISSN: 0006-3363. <http://www.biolreprod.org>

oocyte cryopreservation has been achieved only in a limited number of mammalian species.

For successful cell cryopreservation to occur, it is important that water and cryoprotective solutes be transported across the plasma membrane. There are two mechanisms by which water and cryoprotectants move across the plasma membrane: by simple diffusion across the membrane and by a channel-mediated process. During the last decade, small intrinsic membrane proteins that act as water channels were discovered and have been characterized [2]. These proteins, called aquaporins (AQPs), occur in two types in mammals: one subgroup, including AQP1, AQP2, AQP4, and AQP5, is highly selective for the passage of water, and the other subgroup, including AQP3, AQP7, and AQP9, transports water as well as neutral solutes with a small molecular weight, such as glycerol. Because glycerol is a representative cryoprotectant, expression of the latter subgroup of AQPs might be relevant to the survival of cryopreserved cells. Specifically, if a channel that transports water and neutral solutes such as AQP3 can be artificially expressed in cells, it may be possible to improve the survival of the cells that presently suffer high mortality during cryopreservation.

Vitrification, an innovative, rapid method for cryopreserving mammalian embryos and oocytes, has been extensively studied by many cryobiologists because mammalian embryos and oocytes can be cryopreserved quickly and simply by this method [3]. However, for successful vitrification to occur, high concentrations of cryoprotectants are required, which may damage the cells, probably by cryoprotectant toxicity. On the other hand, insufficient exposure can cause intracellular ice formation. To minimize damaging exposure times to cryoprotectants, rapid movement of water and cryoprotectants through the plasma membranes is essential. Mouse oocytes have been cryopreserved successfully and effectively by slow-freezing methods, mostly using DMSO as the cryoprotectant [4], and also by vitrification using DMSO [5] or ethylene glycol [6]. However, using a glycerol-based solution, mouse oocytes have not been successfully cryopreserved by vitrification, because the permeability of mouse oocytes to glycerol is low [7, 8]. Therefore, vitrification of mouse oocytes coupled with glycerol is a good model for examining changes in the permeability and survival after cryopreservation through artificial expression of water/cryoprotectant channels. In this study we examined whether artificial expression of AQP3 in mouse oocytes can improve their water and glycerol permeability, and their survival after vitrification with glycerol.

MATERIALS AND METHODS

Preparation of AQP3 cRNA

AQP3 cDNA was cloned from rat kidney cDNA by polymerase chain reaction (PCR). The sense strand was 5'-CGGGATCCCATGGGTCGA-

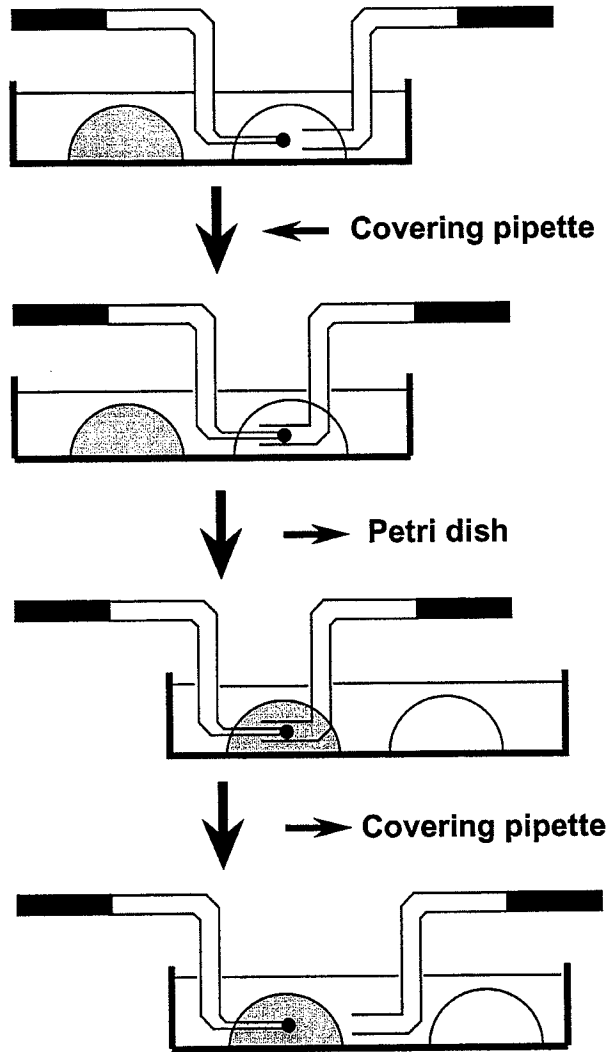


FIG. 1. Schematic representation of the device and the procedure for direct transfer of an oocyte (solid circle) to a cryoprotectant solution (shaded hemisphere) from isotonic PB1 medium (open hemisphere) using a micromanipulator system.

CAGAAGGAGTT-3', and the antisense strand was 5'-GCTCTAGAGGGTTTTATGGGGTGTCC-3' (underlined sequences indicate inserted *Bam*HI and *Xba*I sites, respectively). These primers were derived from the rat AQP3 sequence [9] (GenBank accession number D17695). The PCR cycle consisted of 30 cycles at 94°C for 1 min, 58°C for 2.5 min, and 72°C for 2.5 min. The PCR product contained the open reading frame for AQP3. The *Bam*HI/*Xba*I fragment of the PCR product was subcloned into the *Bgl*III/*Xba*I site of a pSP64T (a generous gift from Dr. Paul A. Krieg, University of Texas at Austin), a *Xenopus* expression plasmid. After digestion of the construct by *Eco*RI (Toyobo, Osaka, Japan), capped cRNA of AQP3 was synthesized using SP6 polymerase (Takara Shuzo, Otsu, Japan). *Bam*HI, *Xba*I, and *Bgl*III were obtained from New England Biolabs (Beverly, MA).

Microinjection of AQP3 cRNA into Mouse Oocytes

Mature female ICR mice (8–15 wk old) were injected with 5 IU eCG, and were killed by cervical dislocation 48 h after eCG injection. Oocytes at the germinal vesicle (GV) stage were collected from the ovaries bathed in PBS supplemented with glucose, pyruvate, penicillin, and BSA (PB1 medium) [10] by puncturing antrum follicles with a needle. About 20–40 oocytes obtained from 3–4 mice were pooled and used in each experiment. Cumulus cells surrounding oocytes were removed by repeatedly pipetting

them in PB1 medium, and oocytes with normal size and color and having a GV were then placed in a 200- μ l drop of PB1 medium covered with paraffin oil in a Petri dish (90 \times 10 mm). Then an oocyte was held with a holding pipette connected to a micromanipulator on an inverted microscope and injected with 2–5 μ l of water (a control) or AQP3 cRNA solution (1 pg/ μ l) with an injection needle connected to another micromanipulator. As another control, noninjected, intact oocytes were used. Both noninjected and injected oocytes were cultured at 37°C in a humidified CO₂ incubator (5% CO₂ in air) for 12–14 h in modified Eagle medium (11090-081; Gibco BRL, Rockville, MD) supplemented with 10% fetal calf serum (26140-079; Gibco BRL), 50 μ g/ml sodium pyruvate, 2 mM glutamine, 60 μ g/ml penicillin G, and 50 μ g/ml streptomycin (maturation medium). Oocytes without the GV and having a polar body after culture were considered matured and were used as noninjected and water- or AQP3 cRNA-injected oocytes. Only a limited number of matured oocytes were used in each experiment because the culture period for maturation was limited to 12–14 h in order not to use matured but aged oocytes.

Expression of AQP3 in AQP3 cRNA-Injected Oocytes

Noninjected and AQP3 cRNA-injected oocytes were cultured for 12 h in maturation medium as described above. The zona pellucida of the oocytes was then removed by brief exposure to acidic Tyrode solution (pH 2.5) [11]. The zona-free oocytes were fixed with 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 BSA, the oocytes were incubated with PBS containing 0.25% Triton X-100. Then, the oocytes were incubated with PBS containing 10% nonimmune goat serum, 5 mg/ml BSA, and 0.01% Tween-20 (blocking solution) at 25°C for 60 min. After being rinsed, the oocytes were incubated with diluted anti-rat AQP3 rabbit antibody (1:100; Alomone Labs, Jerusalem, Israel) with blocking solution at 25°C for 60 min. After being rinsed, the oocytes were incubated with diluted fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G goat antibody (1:200; Chemicon International, Temecula, CA) with blocking solution at 25°C for 30 min. Oocytes were observed under a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Measurement of Water and Cryoprotectant Permeability

An oocyte cultured for 12–14 h without injection or after water or cRNA injection was placed in a 200- μ l drop of PB1 medium covered with paraffin oil in a Petri dish (90 \times 10 mm), and was held by a holding pipette (outer diameter 150–200 μ m) connected to a micromanipulator on an inverted microscope. The inner diameter of the holding pipette was small enough not to distort the oocyte. The oocyte was then covered with a covering pipette with a narrow inner diameter (\approx 400 μ m) connected to another micromanipulator (Fig. 1). Then, by sliding the dish, the oocyte was introduced into a drop of 10% (v/v) glycerol in PB1 medium (10% glycerol/PB1). By removing the covering pipette, the oocyte was abruptly exposed to the 10% glycerol/PB1. The osmolality of glycerol (the osmolality of extracellular permeating solute) was 1.559 Osm/kg (calculated from published data on the colligative properties of glycerol in aqueous solutions) [12] excluding the 0.298 Osm/kg contributed by the PB1 medium (the initial osmolality of intracellular and extracellular nonpermeating solutes) (measured with an osmometer [OM801; Vogel, Giessen, Germany]). The temperature of the solution was monitored, and room temperature was adjusted so that the solution temperature was kept at 25.0 \pm 0.5°C.

The hydraulic conductivity (L_p) and glycerol permeability (P_{GLY}) of oocytes were measured by the shrinkage and swelling of noninjected and water- or cRNA-injected oocytes after transfer from isotonic PB1 medium to 10% glycerol/PB1 at 25°C for 10 min. The microscopic image of the oocytes during exposure to the solution was recorded by a time-lapse videotape recorder (ETV-820; Sony, Tokyo, Japan) every 0.5 sec for 10 min. The cross-sectional area of oocytes was measured using an image analyzer (VM-50; Olympus). This was expressed as a relative cross-sectional area, S , by dividing it by the area of the same oocyte in isotonic PB1 medium. The relative volume was obtained from $V = S^{3/2}$. L_p and P_{GLY} were determined by fitting water and solute movements using a two-parameter formalism [13]. In principle, the Kedem-Katchalsky formalism, which incorporates a solvent-solute interaction parameter (σ) for channel transport, would be appropriate. In practice, however, a reliable determination of σ is quite difficult—and unwarranted—because we require only a phenomenological description of the oocyte behavior [13].

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

Sym- bol	Meaning	Values
R	Gas constant (liter atm K ⁻¹ mol ⁻¹)	8.206×10^{-2}
T	Absolute temperature	298 K
\bar{V}_w	Partial molar volume of water	0.018 L/mol
\bar{V}_s	Partial molar volume of glycerol ^a	0.071 L/mol
ν_b	Fraction of osmotically inactive cell contents ^b	0.18
A	Surface area in isotonic medium ^b	$1.82 \times 10^4 \mu\text{m}^2$
V_{co}	Total volume in isotonic medium ^b	$2.30 \times 10^5 \mu\text{m}^3$

^a A partial molar volume of glycerol from Wolf et al. [12].

^b Published values from Leibo [22].

The volume change of oocytes is simply the sum of the volume changes due to water and solute fluxes:

$$dV_c/dt = dV_w/dt + dV_s/dt \quad (1)$$

where V_c , V_w , and V_s are the actual oocyte volume, intracellular water volume, and intracellular solute volume, respectively. In terms of the hydraulic conductivity (L_p) and solute (cryoprotectant) permeability (P_s), this equation may be expressed as:

$$dV_c/dt = -L_p A R T (M^e - M^i) + P_s A \bar{V}_s (M_s^e - M_s^i) \quad (2)$$

where R is the gas constant, T is the absolute temperature, A is the oocyte surface area, and \bar{V}_s is the partial molar volume of the solute. The quantities M^i and M^e are the total intracellular and extracellular osmolalities, respectively, and M_s^i and M_s^e are the osmolality of the intracellular and extracellular permeating solutes, respectively. In principle, the activity, a , of glycerol rather than its osmolality, M , should be used to find the solute flux. But considering the noise level found in volumetric assays, there is no practical difference between a and M . The total osmolalities may be expressed as:

$$M^e = M_n^e + M_o^e \quad (3)$$

and (assuming a linear Boyle-van't Hoff cell response)

$$M^i = M_{no}^i (V_{wo}/V_w) + M_s^i \quad (4)$$

where the subscripts n , o , and w refer to nonpermeating solute, initial condition, and water, respectively.

The solute flux is described by:

$$dN_s/dt = P_s A (M_s^e - M_s^i) \quad (5)$$

where N_s is the number of osmoles of cryoprotectant in the cell. Because $N_s = V_w M_s^i$, this may be reexpressed in terms of concentration as:

$$dM_s^i/dt = P_s (A/V_w) (M_s^e - M_s^i) + L_p (A/V_w) M_s^i R T (M^e - M^i) \quad (6)$$

Finally, the absolute cell volume at any time is given by the sum of the water, solute, and solids absolute volumes:

$$V_c = V_w + V_s + V_b = V_w + M_s^i V_w \bar{V}_s + \nu_b V_{co} \quad (7)$$

where V_b is the fractional volume of the osmotically inactive cell contents and V_{co} is the isotonic volume of the oocyte. Solving for the water volume yields:

$$V_w = (V_c - \nu_b V_{co}) / (1 + M_s^i \bar{V}_s) \quad (8)$$

Equations (2) and (6) along with the subsidiary equations (3, 4, and 8) were numerically integrated using the Stiff Episode integrator in the Scientist program from MicroMath (Salt Lake City, UT), and the results were normalized to the isotonic cell volume (V_{co}). The various constants and parameters appearing in the equations are listed in Table 1.

Cryopreservation of Water- or AQP3 cRNA-Injected Oocytes

Water- or AQP3 cRNA-injected oocytes were cryopreserved in liquid nitrogen by vitrification after a three-step treatment. Briefly, each matured oocyte was suspended first in 10% glycerol/PB1 for 2 min, then in GFS20 (a mixture of 20% [v/v] glycerol and 80% [v/v] Ficoll-sucrose [FS] solution) [14] for 1 min, and then in GFS40 (a mixture of 40% [v/v] glycerol and 60% [v/v] FS solution) [15] for 30 sec at 25°C. The components of the FS solution were 30% (w/v) Ficoll 70 (Amersham Pharmacia Biotech,

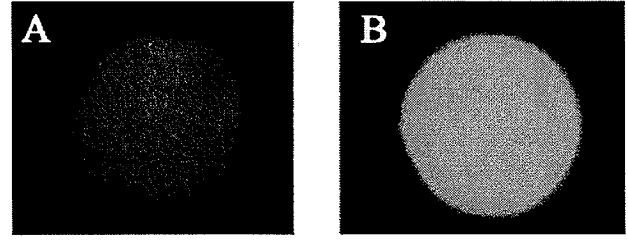


FIG. 2. Expression of AQP3 in an intact (noninjected) oocyte (A) and an AQP3 cRNA-injected oocyte (B). Immunostaining was carried out 12 h after oocyte culture.

Buckinghamshire, U.K.) and 0.5 M sucrose in PB1 medium. The configuration of the straw is described elsewhere [16]. In the first series of experiments, in which survival of cryopreserved oocytes was assessed by their appearance, the microscopic image of each oocyte was video-recorded for 2 min during the exposure to 10% glycerol/PB1 in order to calculate L_p and P_{GLY} of the oocyte. The oocyte was exposed to GFS20 in a watch glass for 1 min, and then introduced into a small column of GFS40 in a 0.25-ml plastic insemination straw with a finely drawn Pasteur pipette. Thirty seconds after exposure of the oocyte to GFS40, the straw was cooled in liquid nitrogen vapor for 10–30 min, plunged into liquid nitrogen, and stored for 5 min to 2 wk. In the second series of experiments, in which survival of cryopreserved oocytes was assessed by their susceptibility to be fertilized by spermatozoa, the oocytes were vitrified in the first series of experiments, except that 6–10 oocytes were vitrified in a straw without recording their microscopic images during the exposure to 10% glycerol/PB1.

The straws were warmed by holding them in air at 25°C for 5 sec and then suspending them in water at 25°C for about 5 sec, and the contents of the straw were expelled into 0.8 ml of PB1 medium containing 2.0 M sucrose in a watch glass. After 30 sec, the oocytes were resuspended in fresh PB1 medium containing 0.5 M sucrose for 5 min, and then recovered in fresh PB1 medium. All procedures were carried out at 25°C. In the first series of experiments, oocytes were cultured in modified M16 medium [17] at 37°C in a CO₂ incubator for 3 h, and then their survival was assessed by examining the appearance of the cytoplasm and the plasma membrane under a stereomicroscope. Oocytes having a clear outline of the plasma membrane and normal size and color were considered as surviving. In the second series of experiments, oocytes were cultured for 1 h, and oocytes having a “surviving appearance” were subject to *in vitro* fertilization.

In Vitro Fertilization of AQP3 cRNA-Injected Oocytes after Cryopreservation

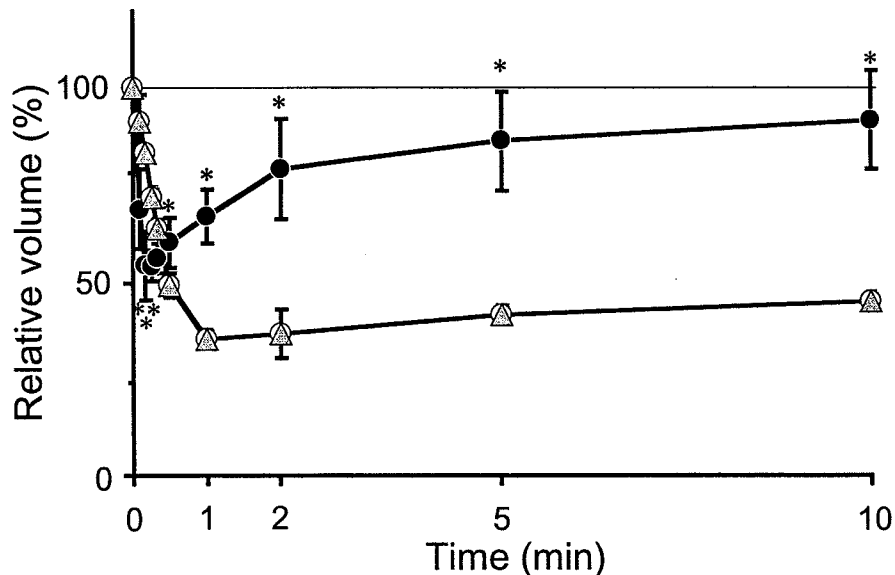
The zona pellucida of AQP3 cRNA-injected oocytes that survived cryopreservation was removed by brief exposure of the oocytes to acidic Tyrode solution. Zona-free oocytes were inseminated with 2.5×10^5 cells/ml of cauda epididymal spermatozoa obtained from ICR male mice 3–6 mo old. Spermatozoa had been preincubated in modified M16 medium at 37°C in a CO₂ incubator for 1 h as described previously [17]. Oocytes were cultured in modified M16 medium in a CO₂ incubator for 24 h, and the ability of the oocytes to cleave to the 2-cell stage was examined. The cleavage of water-injected oocytes after cryopreservation was not examined because none of these oocytes survived after cryopreservation. In some experiments, oocytes cultured for 6 h after insemination were fixed overnight with 10% formalin in PBS. The oocytes were rinsed with distilled water, dehydrated with 95% ethanol, and stained with 0.25% lacmoid in 45% acetic acid [18]. Oocytes having two pronuclei and a sperm tail within them were considered as fertilized.

RESULTS

Expression of AQP3 in AQP3 cRNA-Injected Oocytes

Figure 2 shows the immunofluorescence staining of non-injected and AQP3 cRNA-injected mouse oocytes 12 h after culture. The AQP3 cRNA-injected oocyte was intensely stained, whereas the noninjected one was not stained, indicating that AQP3 cRNA was translated and expressed in mouse oocytes during culture.

FIG. 3. Change in cell volume of noninjected (shaded triangles, $n = 13$) and water-injected (open circles, $n = 20$) or AQP3 cRNA-injected (closed circles, $n = 25$) mouse oocytes in 10% glycerol/PB1. Noninjected and water-injected or AQP3 cRNA-injected oocytes were cultured for 12–14 h and transferred to isotonic PB1 medium at 25°C. Oocytes were then exposed to 10% glycerol/PB1 at 25°C for 10 min. Data are indicated as means of relative volumes \pm SD. Data are the sum of nine experiments. The oocytes were obtained from ~ 35 mice in total. Only 3–8 matured oocytes were used in each experiment because the period of culture for maturation was limited to 12–14 h. *Significantly different from water-injected oocytes (Student *t*-test, $P < 0.05$).



L_p and P_{GLY} of AQP3 cRNA-Injected Oocytes

Figure 3 shows changes in the cell volume of noninjected and water- or AQP3 cRNA-injected mouse oocytes during 10 min of suspension in 10% glycerol/PB1 at 25°C. The cRNA-injected oocytes shrank rapidly to 54% of their isotonic volume and then regained 92% of their isotonic volume. On the other hand, the noninjected and water-injected oocytes shrank relatively slowly to 35% of their isotonic volume and regained little volume, and there was no appreciable difference in volumes between noninjected oocytes and water-injected ones. These results suggest that noninjected and water-injected oocytes have low permeability to water and glycerol, and that cRNA-injected oocytes have high water and glycerol permeability.

Table 2 shows the L_p and P_{GLY} of the noninjected and water- or cRNA-injected oocytes calculated from the volume change of each oocyte shown in Figure 3. Most AQP3 cRNA-injected oocytes exhibited higher L_p and P_{GLY} than noninjected and water-injected oocytes did, but there was substantial variability (values of L_p and P_{GLY} ranged from 1.65 to 5.20 $\mu\text{m min}^{-1} \text{atm}^{-1}$ and from 1.19 to 5.27 $\times 10^{-3}$ cm/min, respectively). The mean L_p value \pm SD of cRNA-injected oocytes was $3.09 \pm 1.22 \mu\text{m min}^{-1} \text{atm}^{-1}$, which was about three times higher than that of noninjected and water-injected oocytes (0.83 ± 0.02 and $0.87 \pm 0.10 \mu\text{m min}^{-1} \text{atm}^{-1}$, respectively). The mean P_{GLY} value \pm SD of cRNA-injected oocytes was $3.69 \pm 1.47 \times 10^{-3}$ cm/min, whereas those of noninjected and water-injected oocytes was low (0.07 ± 0.02 and $0.08 \pm 0.02 \times 10^{-3}$ cm/min,

TABLE 2. Hydraulic conductivity and glycerol permeability of AQP3 cRNA-injected oocytes.

Oocytes	No. of oocytes	Hydraulic conductivity ^a ($\mu\text{m min}^{-1} \text{atm}^{-1}$)	Glycerol permeability ^a (10^{-3} cm/min)
Noninjected	13	0.83 ± 0.02	0.07 ± 0.02
Water-injected	20	0.87 ± 0.10	0.08 ± 0.02
AQP3 cRNA-injected	25	$3.09 \pm 1.22^{**}$	$3.69 \pm 1.47^{**}$

^a Data are indicated as mean values \pm SD.

** Significantly different from water-injected oocytes (Student *t*-test, $P < 0.01$).

respectively). These results indicate that AQP3 cRNA-injected mouse oocytes expressed AQP3, and it functioned as a water and neutral solute channel in the oocyte plasma membrane. Because L_p and P_{GLY} values for water-injected oocytes were quite similar to those of noninjected oocytes, we used only water-injected oocytes as controls in the following experiments.

Survival of AQP3 cRNA-Injected Oocytes after Cryopreservation

The survival of oocytes was assessed by examining the appearance of the cytoplasm and the plasma membrane under a stereomicroscope. After cryopreservation of 27 cRNA-injected oocytes, 20 oocytes (74%) survived, but the survival rate was dependent on the values of L_p and P_{GLY} for each oocyte (Figs. 4 and 5). Figure 4 shows the correlation between water and glycerol permeability of water- or AQP3 cRNA-injected oocytes. Water-injected oocytes had low L_p and P_{GLY} and no oocytes survived after vitrification. On the other hand, most AQP3 cRNA-injected oocytes had higher L_p and P_{GLY} values than water-injected ones, and higher survival rates were obtained as the values of L_p and P_{GLY} increased.

When cRNA-injected oocytes had a higher value for L_p ($\geq 4.4 \mu\text{m min}^{-1} \text{atm}^{-1}$; Fig. 5A) or P_{GLY} ($\geq 4.0 \times 10^{-3}$ cm/min; Fig. 5B), the survival rates of the oocytes after cryopreservation were very high (100% for oocytes with high L_p , 93% for oocytes with high P_{GLY}). However, when cRNA-injected oocytes had a low value for L_p ($\leq 4.2 \mu\text{m min}^{-1} \text{atm}^{-1}$; Fig. 5A) or P_{GLY} ($< 4.0 \times 10^{-3}$ cm/min; Fig. 5B), the survival rates were significantly lower (53% for oocytes with low L_p , 50% for oocytes with low P_{GLY}). On the other hand, none of the water-injected oocytes survived after cryopreservation (Fig. 5, A and B). There was a positive correlation between water and glycerol permeabilities in AQP3 cRNA-injected oocytes ($n = 27$, $r^2 = 0.58$).

When cRNA-injected oocytes that survived cryopreservation were inseminated in vitro, 31% (22 of 70) cleaved to the 2-cell stage (Fig. 6B). This result was not significantly different from that of water- or cRNA-injected oocytes without cryopreservation (45%, 46 of 103 or 39%, 42 of 109, respectively). The fertilization rate of water-in-

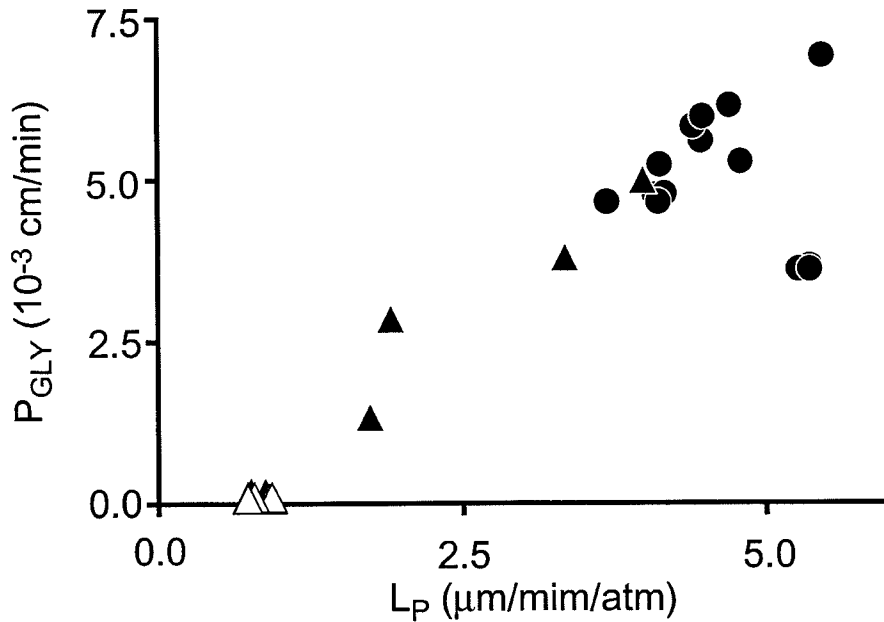


FIG. 4. Survival of cryopreserved water-injected or AQP3 cRNA-injected mouse oocytes assessed by appearance, shown as a function of L_p and P_{GLY} . Open symbols indicate water-injected oocytes ($n = 10$) and closed symbols indicate AQP3 cRNA-injected oocytes ($n = 27$). Circles are surviving oocytes ($n = 20$) and triangles are dead oocytes ($n = 17$) after vitrification. Data are the sum of five experiments. Oocytes were obtained from ~ 20 mice in total. Only 7–8 matured oocytes were used in each experiment because the period of culture before cryopreservation was limited to 12–14 h.

jected but not vitrified oocytes at 6 h after insemination was 56% (33 of 59, the sum of 3 experiments; data not shown in figures), which was not significantly different from the cleaving rate at 24 h after insemination (45%; Fig. 6A). Moreover, the fertilization rate of AQP3 cRNA-injected and vitrified oocytes was 40% (19 of 48, the sum of 3 experiments), which also was not significantly different from the cleaving rate at 24 h after insemination (31%; Fig. 6B).

DISCUSSION

To our knowledge, this is the first report to show that artificial expression of a water/solute channel in a cell improves its survival after cryopreservation.

We have already demonstrated the expression of mRNA of AQP3 and AQP7 in mouse oocytes by reverse transcriptase-PCR [19]. Thus, it is possible that a channel process exists in mouse oocytes by which water and cryoprotective solutes move across the plasma membrane. Osmotic water permeability, L_p , higher than $4.5 \mu\text{m min}^{-1} \text{atm}^{-1}$ (or $P_f > 0.01 \text{ cm/sec}$) and an Arrhenius activation energy (E_a) lower than 6 kcal/mol are generally suggestive of water movement through water channels [20]. On the other hand, E_a higher than 10 kcal/mol is suggestive of water movement through channel-independent diffusion [20]. In this study, the mean values of L_p of noninjected and water-injected mouse oocytes were 0.83 ± 0.02 and $0.87 \pm 0.10 \mu\text{m min}^{-1} \text{atm}^{-1}$ at 25°C , respectively (Table 2). There have been many studies of the water permeability of mouse oocytes [21]. The value of L_p obtained in our study is similar to those found in other studies, such as 0.44 ± 0.03 [22], 0.48 ± 0.20 [23], and $0.64 \pm 0.15 \mu\text{m min}^{-1} \text{atm}^{-1}$ at 20°C [24]; 0.40 ± 0.1 [25] and $0.47 \pm 0.05 \mu\text{m min}^{-1} \text{atm}^{-1}$ at 22°C [26]; and $0.98 \pm 0.70 \mu\text{m min}^{-1} \text{atm}^{-1}$ at 24°C [8]. These values are all substantially less than the high value ($\geq 4.5 \mu\text{m min}^{-1} \text{atm}^{-1}$) expected when there is significant water channel transport. Moreover, the water permeability E_a of mouse oocytes was reported to be 14.5 [22] or $11.38 \pm 2.21 \text{ kcal/mol}$ [25], which is higher than 10 kcal/mol. These results strongly suggest that most water molecules

permeate mouse oocytes by simple diffusion across the plasma membrane, and that mouse oocytes express only small amounts of AQP3 and AQP7. In addition, the low P_{GLY} (0.07 ± 0.02 and $0.08 \pm 0.02 \times 10^{-3} \text{ cm/min}$ at 25°C) observed in the control oocytes also indicates minimal expression of the solute transporters, AQP3 and AQP7.

Osmotically induced water transport pathways are associated with other transporters, including glucose transporters [27], the cystic fibrosis transmembrane conductance regulator [28], urea transporter 3 [29], and multiple sodium-solute cotransporters [30]. However, it is unclear whether these transporters contribute significantly to total plasma membrane water permeability [2]. The low water permeability of mouse oocytes suggests that water-permeable transporters other than those in the AQP family are not involved in the water permeability of mouse oocytes.

The values of P_{GLY} of the control oocytes in this study (noninjected and water-injected oocytes, 0.07 ± 0.02 and $0.08 \pm 0.02 \times 10^{-3} \text{ cm/min}$ at 25°C , respectively) are higher than those reported in other studies ($0.01 \times 10^{-3} \text{ cm/min}$ at 20°C [7] and $0.02 \pm 0.01 \times 10^{-3} \text{ cm/min}$ at 24°C [8]). This may reflect biological variability among mice, different concentrations of glycerol, or both.

There was substantial variability in the L_p and P_{GLY} values of cRNA-injected oocytes (Table 2). This may be caused by the difference of ability to translate injected cRNA among the oocytes. Also, some of the cRNA solution might leak out after injection in some cases.

Mouse oocytes can survive after cryopreservation by vitrification using a DMSO-based or ethylene glycol-based solution [5, 6]. However, there is no report of mouse oocyte survival after vitrification with a glycerol-based solution because, as described above, the permeability of mouse oocytes to glycerol is low. Survival of cRNA-injected oocytes after vitrification in a glycerol-based solution depended on their values of L_p and P_{GLY} (Figs. 4 and 5). Thus, the greater survival of cRNA-injected oocytes after vitrification must be due to an increase in the expression of AQP3.

We have not examined how long exogenous expression of AQP3 lasts. However, as a preliminary experiment, we

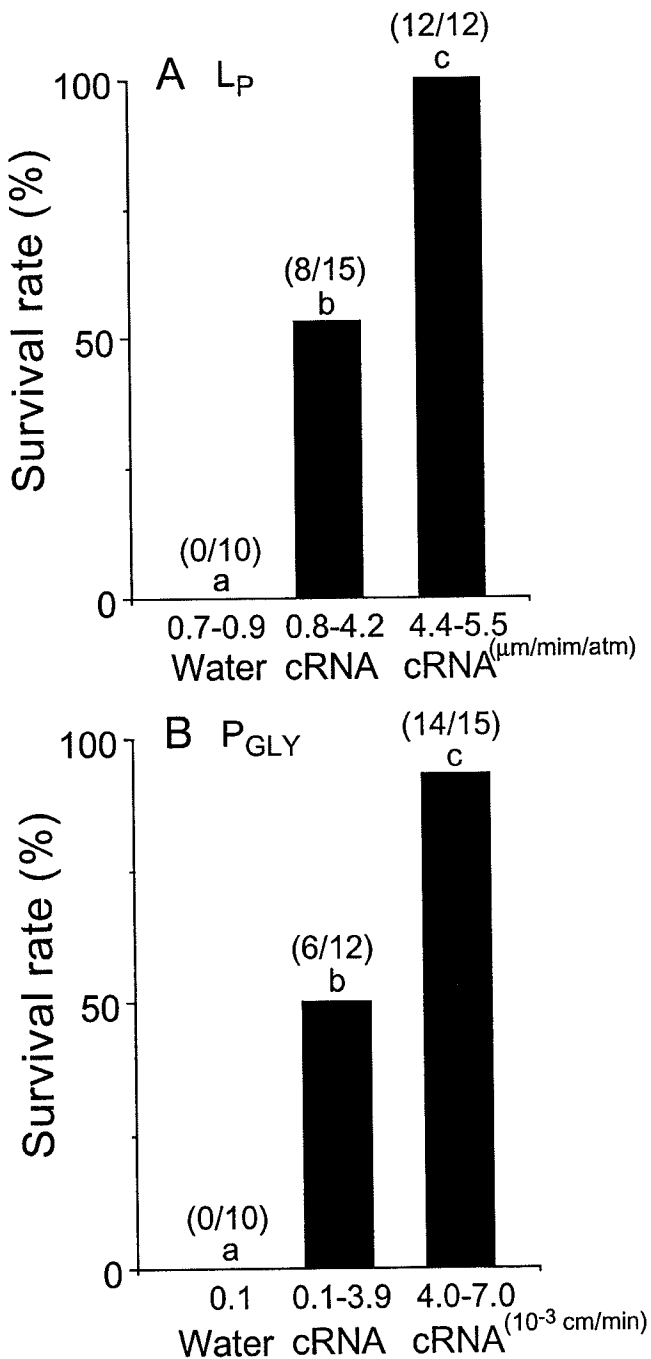


FIG. 5. Survival rates of cryopreserved water-injected (Water, $n = 10$) and AQP3 cRNA-injected (cRNA, $n = 27$) mouse oocytes having different L_p (A) and P_{GLY} (B). Data were taken from Figure 4. Numbers in parentheses indicate surviving oocytes/cryopreserved oocytes. Columns with different letters are significantly different (Fisher exact probability test, $P < 0.05$).

tried to measure L_p of some cleaved zona-free oocytes that had been injected with AQP3 cRNA, matured, and vitrified, by enclosing them in empty zona pellucidae of other oocytes. However, the values of L_p were as low as those of noninjected oocytes (data not shown), suggesting that AQP3 cRNA and expressed AQP3 were degraded after a relatively short period.

We have shown that injection of AQP3 cRNA into oo-

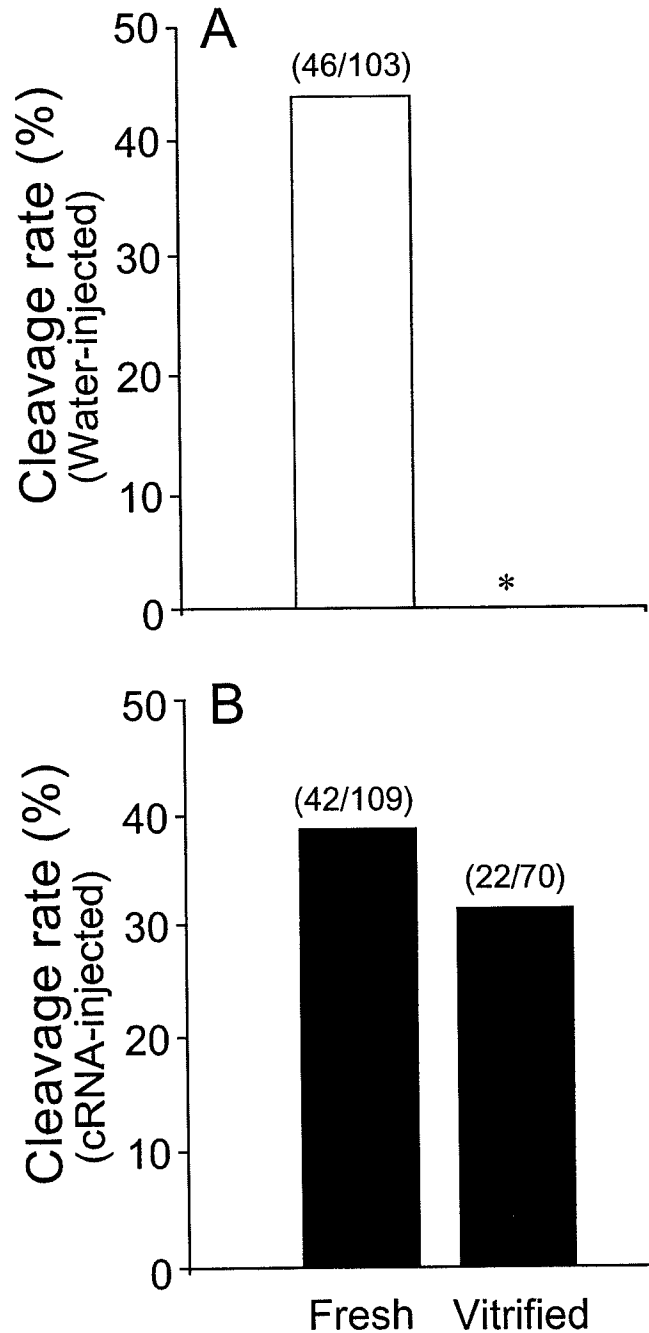


FIG. 6. Ability of water-injected (A) and AQP3 cRNA-injected (B) mouse oocytes to be fertilized before (fresh) or after (vitrified) vitrification. AQP3 cRNA-injected oocytes were inseminated with mouse sperm after cryopreservation. Data are the sum of five experiments. Oocytes and sperm were obtained from 20 female mice and 5 male mice in total. Numbers in parentheses indicate cleaved oocytes/inseminated oocytes. *Not examined because none of the oocytes survived after cryopreservation. Significant differences were not observed among columns (chi-square test, $P < 0.05$).

cytes markedly increased both water and glycerol permeability. However, mouse oocytes have been successfully cryopreserved by vitrification using DMSO [5] or ethylene glycol [6] as the cryoprotectant, suggesting that intact mouse oocytes have sufficiently high water permeability to survive vitrification. Thus, the marked increase in water

permeability in AQP3 cRNA-injected oocytes may not play a vital role in survival of the oocytes after vitrification, but the increase in glycerol permeability of the oocytes plays an essential role in survival after vitrification. However, in other types of cells, an AQP-mediated increase in water permeability may contribute to successful cryopreservation.

It would be reasonable to assume that a substantial increase in glycerol permeability was effective for cryopreservation by preventing injury from intracellular ice formation. However, there is a possibility that vitrified non-injected oocytes were injured by osmotic overswelling during removal of permeated glycerol, rather than by cryopreservation. To examine this possibility, we performed a supplementary experiment in which noninjected oocytes were treated for vitrification and recovered without cooling. All the recovered oocytes were morphologically normal ($n = 40$), indicating that vitrified, noninjected oocytes were injured by vitrification and not by osmotic swelling damage, probably because the amount of permeated glycerol was quite small.

In the *in vitro* fertilization experiment, it was shown that AQP3 cRNA-injected oocytes retained the ability to be fertilized after vitrification (Fig. 6). When we cultured some of the cleaved oocytes for a longer period, most of the cRNA-injected and vitrified oocytes did not develop beyond the 2-cell stage and only a few oocytes developed to the blastocyst stage. However, because many of the water- or AQP3 cRNA-injected, nonvitrified oocytes developed to the blastocyst stage (73%, 29 of 40; and 58%, 23 of 40, respectively; data not shown), the exposure to the vitrification solution, the cooling/warming process, or both may damage the cRNA-injected oocytes during vitrification procedures. Further studies are needed to clarify where and how the oocytes are damaged.

In this study we carried out direct, real-time observations of the change in cell volume of mammalian oocytes using micromanipulators in order to measure L_p and P_{GLY} of the oocytes. In these experiments, the shrinkage of the oocytes occurred in the first 30 sec of exposure to 10% glycerol/PB1. A major advantage of the technique reported here is that it permitted the collection of images at several time points within that period, and this in turn permitted the calculation of L_p from the shrinkage curve. It also permitted image collection following the full re-expansion curve, which allowed a more accurate fitting of the permeability parameters to the experimental data than is the case using methods that miss the early points. As described in *Materials and Methods*, this was achieved by the combination of holding an oocyte on a micropipette and covering it with a second pipette with a narrow inner diameter. The removal of the latter pipette allowed the oocyte to be abruptly placed in contact with 10% glycerol/PB1. Moreover, when the covering pipette was removed, isotonic PB1 medium remaining around the oocyte should be quickly floated up because of its lower density compared with that of glycerol/PB1. Actually, when oocytes were exposed to 10% glycerol/PB1 by removing the covering pipette, they began to shrink within 0.5 sec, showing that the oocytes were mixed with 10% glycerol/PB1 very rapidly, probably because the amount of PB1 medium in the covering pipette is relatively small. Moreover, before pipette removal, the oocyte could be brought into precise focus so that no time was lost in achieving good focus after the onset of exposure. Thus, this method is suitable for measuring the change in volume of mammalian oocytes or embryos in hypertonic cryoprotectant solutions. Finally, the L_p value obtained in noninjected

oocytes was similar to the values found in other studies at 20–24°C as described above, suggesting that accurate volume changes can be obtained in our device.

In this study, we have demonstrated that artificial expression of channels for water and cryoprotectant improves the survival of cells with low water permeability, cryoprotectant permeability, or both after cryopreservation. Although we used mouse oocytes as a model, and they are relatively small compared with oocytes of other vertebrates, we expect that the artificial expression of channels can also improve the cryosurvival of cells with larger volumes, such as eggs and embryos of amphibians and fish.

ACKNOWLEDGMENTS

We thank Drs. Kazuya Matsumoto and Kazuhiro Saeki (Kinki University) for helping with the microinjection experiments. We are grateful for the generous gift of a pSP64T plasmid from Dr. Paul A. Krieg (University of Texas, Austin).

REFERENCES

- Rall WF. Cryopreservation of mammalian embryos, gametes, and ovarian tissues. In: Wolf DP, Zelinski-Wooten M (eds.), *Assisted Fertilization and Nuclear Transfer in Mammals*. Totowa, NJ: Humana Press; 2001: 173–187.
- Verkman AS, Mitra AK. Structure and function of aquaporin water channels. *Am J Physiol Renal Physiol* 2000; 278:F13–F28.
- Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature* 1985; 313:573–575.
- Schroeder AC, Champlin AK, Mobraaten LE, Eppig JJ. Developmental capacity of mouse oocytes cryopreserved before and after maturation *in vitro*. *J Reprod Fertil* 1990; 89:43–50.
- Nakagata N. High survival rate of unfertilized mouse oocytes after vitrification. *J Reprod Fertil* 1989; 87:479–483.
- Hotamisligil S, Toner M, Powers RD. Changes in membrane integrity, cytoskeletal structure, and developmental potential of murine oocytes after vitrification in ethylene glycol. *Biol Reprod* 1996; 55:161–168.
- Jackowski S, Leibo SP, Mazur P. Glycerol permeabilities of fertilized and unfertilized mouse ova. *J Exp Zool* 1980; 212:329–341.
- Paynter SJ, McGrath JJ, Fuller BJ, Shaw RW. A method for differentiating nonunique estimates of membrane transport properties: mature mouse oocytes exposed to glycerol. *Cryobiology* 1999; 39:205–214.
- Ishibashi K, Sasaki S, Fushimi K, Uchida S, Kuwahara M, Saito H, Furukawa T, Nakajima K, Yamaguchi Y, Gojobori T, Marumo F. Molecular cloning and expression of a member of the aquaporin family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collecting duct cells. *Proc Natl Acad Sci U S A* 1994; 91:6269–6273.
- Whittingham DG. Survival of mouse embryos after freezing and thawing. *Nature* 1971; 233:125–126.
- Hogan B, Beddington R, Costantini F, Lacy E. Removing the zona pellucida. In: Hogan B, Beddington R, Costantini F, Lacy E (eds.), *Manipulating the Mouse Embryo*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1994: 191.
- Wolf AV, Brown MG, Prentiss PG. Concentration properties of aqueous solutions: conversion tables. In: Weast RC (ed.), *Handbook of Chemistry and Physics*, 51st ed. Cleveland: CRC Press; 1970: D181–D226.
- Kleinhans FW. Membrane permeability modeling: Kedem-Katchalsky vs a two-parameter formalism. *Cryobiology* 1998; 37:271–289.
- Zhu SE, Sakurai T, Edashige K, Machida T, Kasai M. Cryopreservation of zona-hatched mouse blastocysts. *J Reprod Fertil* 1996; 107: 37–42.
- Zhu SE, Sakurai T, Edashige K, Machida T, Kasai M. Optimization of the procedures for the vitrification of expanded mouse blastocysts in glycerol-based solutions. *J Reprod Dev* 1994; 40:293–300.
- Kasai M, Komi JH, Takakamo A, Tsudera H, Sakurai T, Machida T. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *J Reprod Fertil* 1990; 89:91–97.
- An TZ, Wada S, Edashige K, Sakurai T, Kasai M. Viable spermatozoa can be recovered from refrigerated mice up to 7 days after death. *Cryobiology* 1999; 38:27–34.

18. Toyoda Y, Chang MC. Fertilization of rat eggs in vitro by epididymal spermatozoa and the development of eggs following transfer. *J Reprod Fertil* 1974; 36:9-22.
19. Edashige K, Sakamoto M, Kasai M. Expression of mRNAs of the aquaporin family in mouse oocytes and embryos. *Cryobiology* 2000; 40:171-175.
20. Verkman AS, van Hoek AN, Ma T, Frigeri A, Skach WR, Mitra A, Tamarappoo BK, Farinas J. Water transport across mammalian cell membranes. *Am J Physiol* 1996; 270:C12-C30.
21. Critser JK, Agca Y, Gunasena KT. The cryobiology of mammalian oocytes. In: Karow AM, Critser JK (eds.), *Reproductive Tissue Banking*. San Diego: Academic Press; 1997: 329-357.
22. Leibo SP. Water permeability and its activation energy of fertilized and unfertilized mouse ova. *J Membr Biol* 1980; 53:179-188.
23. Hunter JE, Bernard A, Fuller BJ, McGrath JJ, Shaw RW. Measurements of the membrane water permeability (L_p) and its temperature dependence (activation energy) in human fresh and failed-to-fertilize oocytes and mouse oocytes. *Cryobiology* 1992; 29:240-249.
24. Litkouhi B, Marlow D, McGrath JJ, Fuller B. The influence of cryopreservation on murine oocyte water permeability and osmotically inactive volume. *Cryobiology* 1997; 34:23-35.
25. Benson CT, Critser JK. Variation of water permeability (L_p) and its activation energy (E_a) among unfertilized golden hamster and ICR murine oocytes. *Cryobiology* 1994; 31:215-223.
26. Gao DY, Benson CT, Liu C, McGrath JJ, Critser ES, Critser JK. Development of a novel microperfusion chamber for determination of cell membrane transport properties. *Biophys J* 1996; 71:443-450.
27. Fischbarg J, Kuang K, Vera JC, Arant S, Silverstein SC, Loike J, Rosen OM. Glucose transporters serve as water channels. *Proc Natl Acad Sci U S A* 1990; 87:3244-3247.
28. Hasegawa H, Skach W, Baker O, Calayag MC, Lingappa V, Verkman AS. A multifunctional aqueous channel formed by CFTR. *Science* 1992; 258:1477-1479.
29. Yang B, Verkman AS. Urea transporter UT3 functions as an efficient water channel: direct evidence for a common water/urea pathway. *J Biol Chem* 1998; 273:9369-9372.
30. Loo DDF, Hirayama BA, Meinild AK, Chandy G, Zeuthen T, Wright EM. Passive water and ion transport by cotransporters. *J Physiol* 1999; 518:195-202.