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Effect of maturation on the expression of aquaporin 3 in mouse oocyte

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Summary

This study aimed to investigate whether aquaporin 3 (*Aqp3*) mRNAs are expressed in immature oocytes and altered during *in vitro* maturation process. Five- to 6-week-old female ICR mice were primed by gonadotropin for 24 and 48 h. Immature oocytes obtained 48 h after priming were also matured *in vitro* for 17 to 18 h. *In vivo* matured oocytes were obtained after 48 h priming followed by hCG injection. Total RNAs were extracted from 80 to 150 oocytes in each experimental group, and the levels of *Aqp3* mRNA were quantified by real-time reverse transcriptase polymerase chain reaction. The experiments were repeated twice using different oocytes. The *Aqp3* mRNA was expressed in immature oocytes, as well as in *in vitro* and *in vivo* matured oocytes. The expression level was higher in immature oocytes obtained 48 h after priming (17.2 ± 8.6, mean ± SD) than those with no priming (5.7 ± 0.8) or obtained 24 h after priming (2.5 ± 0.8). The expression of *Aqp3* mRNA decreased after *in vitro* maturation (1.2 ± 0.5), which was similar to *in vivo* matured oocytes (1.0 ± 0.0). Our work demonstrated that *Aqp3* mRNA expression increased during the development of immature oocyte but decreased after completion of *in vitro* maturation. The results indicate that AQP3 is certainly needed for the acquisition of immature oocytes' full growing potential within antral follicles.

Keywords: Aquaporin 3, Immature oocyte, *In vitro* maturation

Introduction

Water is a major component of cells and tissues, and its movement across cell plasma membranes is a fundamental property of life. The movement and proper regulation of fluids across cell membranes are also important aspects of reproduction. There are two mechanisms by which water moves across cell membranes: simple diffusion across lipid bilayer and facilitated process by use of various channels (Verkman *et al*., 1996). The transport of water primarily occurs by simple diffusion through lipid bilayer membrane. However, in the cells like RBC and renal tubular epithelial cells, high water permeability cannot be explained by simple diffusion because the lipid bilayer is hydrophobic (Preston *et al*., 1993).

Aquaporins (AQPs) are a family of small membrane-spanning monomer and hydrophobic integral membrane channel proteins that are expressed at plasma membranes of many cell types involving fluid transport (Agre *et al*., 2002). Being among the water channels, AQPs have recently been discovered and characterized in animals, humans, and plants (Preston *et al*., 1993; Li *et al*., 1994; Ishibashi *et al*., 1997; Frigeri *et al*., 1998; Page *et al*., 1998; Beitz *et al*., 1999; Shanahan *et al*., 1999).

To date, 13 isoforms (AQP0–AQP12) have been identified in mammals (Agre *et al*., 2002; Agre and Kozono, 2003; Huang *et al*., 2006) and 11 isoforms

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(AQP0–AQP10) have been identified in male and female reproductive systems (Huang *et al*., 2006).

Several mammalian AQPs (such as AQP0, 1, 2, 4, 5, 6, and 8) appear to be highly selective for passage of water, whereas AQP3, 7, 9, and 10 (recently termed as aquaglyceroporins) transport not only water but also neutral solutes, including glycerol, urea, and other small non-electrolytes (Ishibashi *et al*., 1994). Several studies have shown that AQPs play pathophysiological roles in mammalian reproductive systems by mediating fluid transport (Huang *et al*., 2006). The *Aqp3* genes are mainly distributed in the uterus, ovary, placenta, fetal membrane, and embryo (Damiano *et al*., 2001; Huang *et al*., 2006; Mann *et al*., 2002). Aquaporin 7 is also present in the ovary, testis, and embryo (Huang *et al*., 2006).

Edashige *et al*. (2000) found that mRNAs of *Aqp3* and *Aqp7* are expressed in unfertilized mouse oocytes and embryos at all stages from 1-cell up to the blastocyst stage, but *Aqp8* and *Aqp9* are expressed only in blastocysts. Richard *et al*. (2003) observed expression of *Aqp8* mRNAs in the inner cell mass and *Aqp9* mRNAs in the mural trophectoderm of the implanting blastocysts. In addition, AQP7, 8, and 9 were found in rat granulosa cells which might help in moving water and/or glycerol into antral follicles (McConnell *et al*., 2002). It was suggested that water permeability mediated by AQPs can control the rate of apoptosis in granulosa cells (Jablonski *et al*., 2004).

In the cryopreservation of mouse oocyte, controlled expression of AQP3 might improve the permeability of water, glycerol, and various cryoprotectants (Edashige *et al*., 2007). It might be important that the change of AQP3 expression level could influence oocyte quality and subsequent embryo development in mice. In immature rat oocytes, *Aqp9* transcripts were found to be present at the germinal vesicle stage but not in mature metaphase II oocytes (Ford *et al*., 2000).

To date, there have been no reports with regard to AQP expressions in immature and *in vitro* matured oocytes. Therefore, our aim in this study was to investigate whether *Aqp3* mRNAs are expressed in immature oocytes and altered during the oocyte maturation process. Furthermore, variable expressions of *Aqp3* mRNAs were investigated according to developmental stage of immature oocytes.

Materials and methods

Retrieval of immature oocyte and *in vitro* maturation

Five- to 6-week-old female ICR mice (Orient Co, Seoul, Korea) were used in this experiment. Animal care and use were in accordance with the institutional guidelines established by the Animal Care and Use Committee of Seoul National University Bundang Hospital. Sixty mice were randomly allocated into five experimental groups.

Immature oocytes were obtained without priming and after priming for 24 h or 48 h with 7.5 IU equine chorionic gonadotropin (eCG; Sigma). The mice were sacrificed by cervical dislocation, and both ovaries were excised and placed in 500 μl of washing medium (modified mouse tubal fluid, mMTF) supplemented with 0.4% human serum albumin (HSA; Sigma). The cumulus-enclosed oocytes (CEOs) covered with compact cumulus cells were collected by puncture of antral follicles. Immature oocytes were then retrieved as denuded state by treating with 85 IU/ml hyaluronidase (Cook).

A part of the immature oocytes was obtained 48 h after eCG priming and then matured *in vitro* using maturation medium for 17 to 18 h. Maturation medium consisted of a commercial TCM199 (Invitrogen) supplemented with 20% fetal bovine serum (FBS; Invitrogen) and recombinant FSH/hCG (75 mIU/ml and 0.5 IU/ml) (Serono, Geneva, Switzerland). In all cultures, groups of up to 10 oocytes were placed in 50 μl microdrops of medium under mineral oil (Sigma) in 35 × 10 mm Petri dishes (Falcon; Becton Dickinson) and were placed at 37°C in humidified 5% CO_2 in air for 17 to 18 h. At the end of IVM, all CEOs were denuded completely by treating with 85 IU/ml hyaluronidase, and nuclear maturation of oocytes was assessed. The first polar body extrusion was used as the maturation criterion under an inverted microscope (×200).

In vitro fertilization of *in vitro* matured oocytes

Parts of immature oocytes obtained without priming and after priming for 24 h or 48 h with eCG were subjected to IVM and subsequently IVF to clarify their developmental competence. Only matured oocytes with the first polar body were transferred to fertilization medium (mMTF supplemented with 0.8% HSA). The epididymal spermatozoa were retrieved from the cauda epididymis of 8- to 10-week-old ICR mice, and the sperm suspensions were preincubated for 1.5 h in capacitation medium (mMTF supplemented with 0.8% HSA). The matured oocytes were then inseminated by sperms at a final dilution of 2×10^6/ml and incubated at 37°C in humidified 5% CO_2 in air. Inseminated oocytes were washed away from sperms by gently pipetting 6 h later and then placed in embryo maintenance medium (mMTF supplemented with 0.4% HSA). Fertilization was assessed by the formation of two cells on day 1 (the day after insemination). The cleaved embryos were transferred to new embryo maintenance medium and development to blastocyst was recorded on day 5 after insemination.

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Collection of *in vivo* matured oocytes

The mice were treated with 7.5 IU eCG and 7.5 IU hCG (Sigma) given 48 h apart. The mice were sacrificed by cervical dislocation 17 to 18 h later and the oviducts were collected. The oviducts were dissected and placed into a Petri dish containing washing medium. The CEOs were released by tearing the ampulla of the oviducts. The cumulus cells were removed enzymatically using 85 IU/ml hyaluronidase and by mechanical dissociation using a glass pipette. The denuded oocytes were then washed three times in washing medium. Only morphologically normal mature MII oocytes, as judged by the presence of a first polar body, were used in our study.

RT-PCR analysis

Total RNAs were extracted from 80 to 150 oocytes in each experiment group using the RNeasy Mini Kit (Qiagen), and cDNAs were synthesized by the Reverse Transcription System (Promega) according to the manufacturer's instructions.

One microgram of total RNA was reverse transcribed using AMV-reverse transcriptase (Promega) and oligo dT primers. We used nested PCR amplifications; in the first PCR amplification, 1.5 µl of reverse-transcription product were amplified by PCR with primers specific for *Aqp3* (outer set; sense primer 5′-GGCTTCCTCACCATCAACTT-3′ and anti-sense primer 5′-GATCTGCTCCTTGTGCTTCA-3′) (GenBank accession no. D17695). In the second round, one out of 20 of the first PCR products was used as template. One microlitre of the first PCR product was amplified by PCR with primers specific for *Aqp3* (inner set; sense primer 5′-ATCAAGCTGCCCATCTACAC-3′ and anti-sense primer 5′- GGGCCAGCTTCACATTCTC-3′) (GenBank accession no. D17695). We used mRNA for actin as a housekeeping gene (sense primer 5′-GTACTTGCGCTCAGGAGGAGC-3′ and anti-sense primer 5′-CGGGGTCACCCACACTGTGCC-3′). One microgram of total RNA obtained from the mouse kidney was used as a positive control. Cumulus cells from immature CEOs were considered as negative control because our preliminary experiments showed that *Aqp3* mRNA was not detected by RT-PCR.

The PCR was carried out in a 20 µl reaction mixture containing 2 µl 10× PCR buffer (containing 2.5 mM Mg^{2+}), 2 µl dNTP (each 2.5 mM), 1 µl sense and anti-sense primer (10 µM), 0.2 µl Nova *Taq* (Nova clean *Taq*, Genenmed), and 10 µl cDNA sample. The PCR was conducted in the following profile: 94°C for 30 s, 60°C for 60 s, and 72°C for 30 s (40 cycles). For the positive control, the condition for PCR reaction was set as follows: 94°C for 15 s, 55°C for 30 s, and 72°C for 60 s (35 cycles). The PCR products were analysed by 2% agarose gel electrophoresis and stained with ethidium bromide.

Quantitative real-time PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen), and cDNAs were synthesized by the Reverse Transcription System (Promega) according to the manufacturer's instructions. Real-time PCR was performed with a 7500 Real Time PCR system with SYBR Green I (Applied Biosystems). Real-time PCR was carried out in a 30 µl reaction volume containing 15 µl 2× Universal master mix, 1.5 µl 20 × TaqMan probe (TaqMan gene expression probe: Mm00507977_g1 for *Aqp3*, Mm02619580_g1 for *Actb*), 10 µl cDNA, and 3.5 µl distilled water. Data were analysed by the comparative threshold cycle method in all experiments (Schefe *et al*., 2006). When the standard quantity of the *Aqp3* mRNA in *in vivo* matured oocytes was considered as 1.0, the level of *Aqp3* mRNA in kidney was 2758.9 ± 1047.4. Real-time PCR was repeated twice using entirely different sets of oocytes obtained from different mice, and the values were averaged.

Statistical methodology

Data were analysed with MedCalc Software (V6.1). The mean values and standard deviations were calculated in each group, but we did not perform a statistical comparison because the experiment was repeated only twice.

Results

A site of 559 bp, which represents the *Aqp3* band marker, was detected in mouse kidney (positive control), in *in vivo* as well as *in vitro* matured oocytes; *Aqp3* bands were also detected in immature oocytes obtained with no eCG priming and 24 h or 48 h after priming (Fig. 1). *Aqp3* mRNA was not detected by RT-PCR in cumulus cells obtained from immature CEOs.

The relative expression levels of *Aqp3* mRNA were different in three groups of immature oocytes according to eCG priming. Quantitative real-time PCR revealed that the level of *Aqp3* mRNA expression was lowest in immature oocytes obtained 24 h after priming but highest in immature oocytes obtained 48 h after priming (Fig. 2). The expression level in the 48-h-primed group was approximately seven times higher than in the 24-h-primed group. However, the expression level was dramatically decreased after maturation *in vitro*, which is similar to *in vivo* matured oocytes. Both *in vitro* and *in vivo* matured oocytes showed reduced levels of *Aqp3* mRNA compared with all of the immature oocyte groups.

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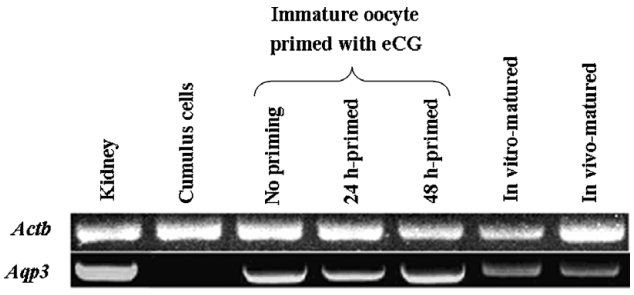

Figure 1 The mRNAs encoding actin (upper part, band size 500 bp) and AQP3 (lower part, band size 559 bp) were analysed by RT-PCR followed by agarose gel electrophoresis in various developmental stages of denuded mouse oocytes. The *Aqp3* mRNA was detected in kidney (positive control), immature oocytes obtained with no priming, 24-h- and 48-h-primed by equine chorionic gonadotropin (eCG). The *Aqp3* mRNA was also detected in *in vitro* and *in vivo* matured oocytes, but not in cumulus cells obtained from immature cumulus–oocyte complexes (negative control).

As expected, the *in vitro* developmental experiment demonstrated that 48-h-primed immature oocytes had a higher maturation (49.6%) and fertilization rate (61.0%) compared with no priming (33.6%, 47.2%) or the 24-h-primed group (44.4%, 50.0%).

Discussion

Expression of AQP3 was previously demonstrated in mature mouse oocyte (Edashige *et al*., 2000; McConnell *et al*., 2002; Jablonski *et al*., 2003; Meng *et al*., 2008). In the present study, we demonstrated for the first time that *Aqp3* mRNA is also present in immature and *in vitro* matured mouse oocytes. In addition, we confirmed that *Aqp3* mRNA is present in *in vivo* matured mouse oocytes but not in cumulus cells, which is in good agreement with previous studies (Edashige *et al*., 2000; Meng *et al*., 2008).

We employed a mouse model designed specifically to approximate the cytoplasmic maturity. Follicular development in mice is more synchronized than in humans; therefore, to generate a model of cytoplasmic maturity, we retrieved immature oocytes from mice that were not primed and that were primed with eCG for 24 and 48 h. It can be assumed that immature oocytes retrieved 48 h after eCG priming are developmentally more competent compared with those obtained 24 h after priming or without priming. This was evident in that a higher maturation and fertilization rate was noted in immature oocytes retrieved 48 h after eCG.

Three types of immature oocytes according to priming showed quite different levels of *Aqp3* mRNA expression; the level of *Aqp3* mRNA was much higher in immature oocytes obtained 48 h primed compared with no priming or 24 h of priming. This result suggests that levels of *Aqp3* mRNA are altered during development of immature oocytes and AQP3 is certainly needed for acquisition of immature oocytes' full growing potential within antral follicles. Under gonadotropin stimulation, antral expansion

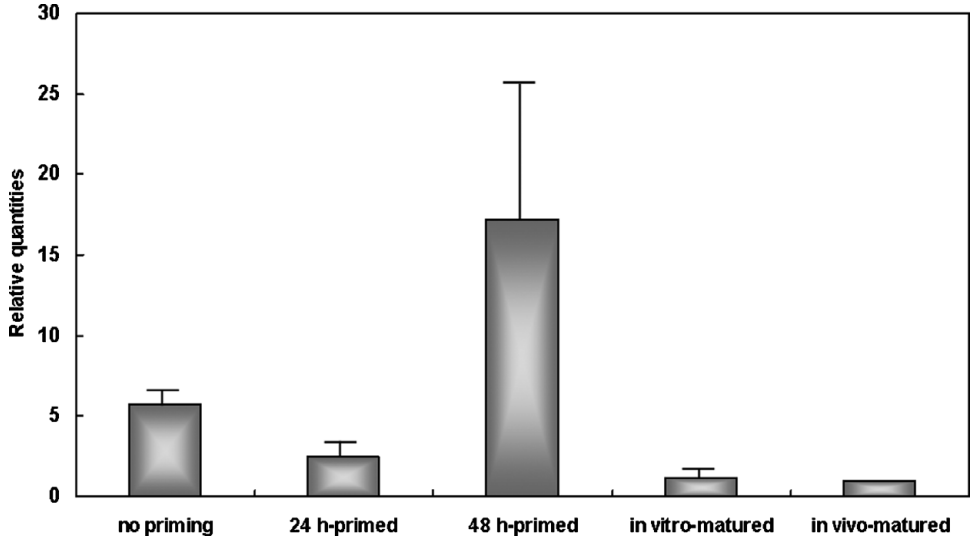

Figure 2 Expression levels of *Aqp3* mRNA in three types of immature oocytes, as well as in *in vitro* and *in vivo* matured mouse oocytes. Quantitative real-time PCR analysis revealed that a higher *Aqp3* expression level was detected in immature oocytes obtained 48 h after eCG than those with no priming and those obtained 24 h after eCG priming. The standard quantity of the *Aqp3* mRNA in *in vivo* matured oocytes was considered 1.0 and the experiment was repeated two times (no priming group; 5.7 ± 0.8, 24-h-primed group; 2.5 ± 0.8, 48-h-primed group; 17.2 ± 8.6, *in vitro* matured group; 1.2 ± 0.5, *in vivo* matured group; 1.0 ± 0.0, mean ± SD).

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occurs rapidly and follicles as well as oocytes may require a quick and massive transport of water. Thus, the increase of AQP3 expression during follicular development might represent cytoplasmic maturity in more competent immature oocytes.

Most interestingly, *Aqp3* mRNA expression dramatically decreased during the maturation process in the present study. This finding indicates that AQP3 expression is closely related to the oocyte maturation process; however, the reason for the inverse relation between *Aqp3* expression and nuclear maturity is unclear. Aquaporin 3 might no longer be required in matured oocytes; otherwise, it might be replaced by other types of water channel. Further study will be needed to clarify this topic.

In the previous rat study, *Aqp9* mRNA disappeared in mature oocytes and at the same time the water permeability was reduced (Ford *et al*., 2000). These findings demonstrated that immature oocytes had higher water and mannitol permeabilities than do mature ones. The presence of a broad selectivity to water and neutral solute during oocyte maturation may have important implications in their adaptation to osmotic stress; hence, future experiments will be warranted to clarify the link between expression and the function of oocyte water channels.

Another interesting finding was the similar expression of *Aqp3* mRNA between *in vitro* and *in vivo* matured oocytes. In general, *in vivo* matured oocytes have better embryonic developmental potential and higher efficiency after cryopreservation than do *in vitro* matured oocytes. Therefore, our finding supports the insignificant role of AQP3 in the process of *in vitro* fertilization and cryopreservation of oocytes. A definite conclusion, however, cannot be drawn because the possibility exists that *in vitro* culture condition might change the expression of *Aqp3* mRNA and we did not examine AQP3 expression in subsequent embryos or cryopreserved–thawed oocytes.

It has been reported that AQP3 expression is regulated by magnesium in Caco-2 cells, a colonic cell line (Okahira *et al*., 2008), retinoic acid in human skin (Bellemère *et al*., 2008), nickel in lung epithelial cells (Zelenina *et al*., 2003), copper in human bronchial epithelial cell line (Zelenina *et al*., 2004), and insulin in Caco-2 cells (Higuchi *et al*., 2007). Further studies on the regulation of AQP3 and other types of AQP in reproductive systems will be necessary for the development of efficient *in vitro* maturation and *in vitro* fertilization methods.

Further studies on AQP3-null mice would clarify the role of AQP3 within reproductive systems. Previous studies demonstrated that the growth and phenotype of AQP3-null mice are grossly normal but have impairment of urinary-concentrating ability (Ma *et al*., 2000), as well as impairment of skin hydration, elasticity, and wound healing (Hara *et al*., 2002). The reproductive phenotypes in these AQP3-null mice are currently unknown. A recent study revealed that AQP4-null mice display subfertility as evidenced by a lower rate of pregnancy and decreased litter size (Sun *et al*., 2009).

In conclusion, we demonstrate for the first time that *Aqp3* mRNA is expressed in immature oocytes as well as *in vitro* matured oocytes in mice. The expression of *Aqp3* mRNA increased during development of immature oocytes, but decreased after *in vitro* maturation. The AQP3 is certainly needed for the acquisition of immature oocytes' full growing potential within antral follicles.

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