

Altered aquaporin expression in women with polycystic ovary syndrome: hyperandrogenism in follicular fluid inhibits aquaporin-9 in granulosa cells through the phosphatidylinositol 3-kinase pathway

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BACKGROUND: The present study was designed to evaluate whether the alteration of aquaporin-9 (AQP-9) expression in granulosa cells (GCs) of patients with polycystic ovary syndrome (PCOS) was associated with the hyperandrogenism in follicular fluid (FF).

METHODS: We recruited infertile women with PCOS ($n = 14$) and infertile women with tubal blockage (controls, $n = 31$) for this study. We examined total testosterone (TT), free androgen index (FAI), sex hormone-binding globulin (SHBG), FSH, LH and estradiol in FF. Real-time PCR and western blotting were performed to assess AQP-9 expression in GCs, including effects of dihydrotestosterone (DHT) *in vitro*.

RESULTS: AQP-9 protein was localized in the nucleus, cytoplasm and cell membrane of the human GCs. The TT, FAI and LH levels were all higher, and SHBG levels lower, in the FF of women with PCOS versus controls ($P = 0.0145, 0.0001, 0.0191, 0.0001$, respectively). AQP-9 mRNA level in GCs of patients with PCOS was tightly correlated with the TT, SHBG levels and FAI in FF ($P = 0.0020, 0.0001, 0.0020$, respectively). *In vitro*, DHT (10^{-9} mol/l) decreased AQP-9 mRNA (lowest at 12 h) and protein levels in control GCs ($P = 0.0005, 0.0247$, respectively). The inhibitory effect of DHT on AQP-9 mRNA was attenuated by LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor ($P = 0.0013$). Fifty micromolar 4-(hydroxymercuri) benzoic acid sodium salt (PMB) and 10^{-9} mol/l DHT blunted the swelling of GCs in hypotonic medium, respectively ($P = 0.0350, 0.0027$).

CONCLUSION: Hyperandrogenism in FF of women with PCOS inhibited AQP-9 in GCs through the PI3K pathway.

Key words: aquaporin-9 / granulosa cells / polycystic ovary syndrome / hyperandrogenism

Introduction

Polycystic ovary syndrome (PCOS), with a prevalence of 5–10%, is the most common endocrinopathy in women of reproductive age (Knochenhauer *et al.*, 1998). PCOS is characterized by chronic anovulation and hyperandrogenism (Knochenhauer *et al.*, 1998), but the

pathogenesis is still unclear. Previous studies suggested that the survival and proliferation of granulosa cells (GCs) were altered in patients with PCOS and one of the mechanisms was an intrinsic abnormality of follicle development in the ovary (Stubbbs *et al.*, 2007; Das *et al.*, 2008). DNA fragmentation and apoptosis in GCs from FSH-primed ovaries have been found to be associated with PCOS (Onalan *et al.*, 2005).

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As pre-antral (primordial, primary and secondary follicles) and antral (early antral, antral and pre-ovulatory follicles) follicle development are two successive periods of the growth and development of ovarian follicles, to some degree, folliculogenesis is characterized by the formation and expansion of the fluid-filled antrum (Gosden et al., 1988). The increased proliferation of GCs in the early-growing follicles causes an increased density of primary follicles in the ovaries of women with PCOS (Stubbs et al., 2007).

Hyperandrogenism has been recommended as one of the three diagnostic criteria for PCOS in the Rotterdam consensus 2003 (2004), and presents as hirsutism, acne or male pattern alopecia (Franks, 1995). Hyperandrogenism in PCOS may proceed via dysregulated paracrine/endocrine control of androgen synthesis (Nelson et al., 2001), or result from adrenal androgen excess (Loughlin et al., 1986; Kumar et al., 2005). Intraovarian androgens have been found to promote GC proliferation and inhibit GC apoptosis in the PCOS patients, especially in small follicles whose GCs were rich in androgen receptors (Sir-Petermann et al., 2006), indicating that androgens may have a crucial effect on GC development, and intraovarian androgen may play an important role in the pathogenesis of PCOS via regulating early follicle growth.

The aquaporin (AQP) family are water-specific channel proteins which are present in certain membranes (Preston et al., 1992; King and Agre, 1996). It has been recognized that two functional groups of AQP proteins exist in mammals. The first group is permeable only to water, as classically defined, whereas the second group is not only highly permeable to water, but also to glycerol and other small molecules (Agre et al., 1998). The presence of AQPs in GCs suggests that water permeability of antral follicles occurs primarily through transcellular mechanisms, which may be mediated by AQP-7, AQP-8 and AQP-9 in GCs (McConnell et al., 2002). Overexpression of AQP has been found to enhance the rate of apoptosis of GCs (Jablonski et al., 2004). Development into an antral follicle is marked by the formation of the antrum, a fluid-filled cavity adjacent to the oocyte within the follicle, and the further increase in size of the follicle results mainly from an increase in size of the antral cavity (Hirshfield, 1991). Antral expansion, which occurs rapidly under gonadotrophin stimulation, requires a quick and massive transport of water. Three isoforms of AQPs (AQP-7, AQP-8 and AQP-9) expressed in GCs can meet this kind of need (Huang et al., 2006). On the other hand, the expression of AQP-9 in GCs suggests that rapid transport of small neutral molecules might be important in the follicle development (Huang et al., 2006).

Few studies have been conducted on the association between AQP and PCOS. As AQP-9 expression has been found to be regulated by androgens (Pastor-Soler et al., 2002; Wang et al., 2008) and the expression of AQP-9 in GCs may be involved in follicle development (Huang et al., 2006), it was then hypothesized that the alteration of AQP-9 expression in GCs of the patients with PCOS might be associated with the hyperandrogenism in follicular fluids (FF) of the patients. The present research was designed to explore an association between AQP-9 expression in GCs of the patients with PCOS and the hyperandrogenism in their FF. As mercury is an effective inhibitor of most AQP isoforms and widely used to evaluate functions of mercury-sensitive AQPs (McConnell et al., 2002), 4-(hydroxymercuri) benzoic acid sodium salt (PMB), which contains more than 95%

mercury, was used to evaluate the contribution of AQP-9 to water transport in GCs in the present study.

Materials and Methods

Subjects

The protocol was approved by the Institutional Review Board of School of Medicine, Zhejiang University, and informed consents were obtained from all of the participants. Subjects included 14 infertile women with PCOS, and 31 infertile women with tubal blockage who served as controls in this study. The GCs were collected from an additional 37 infertile women with tubal blockage for the *in vitro* study. All the women were referred to our department for IVF.

PCOS was diagnosed according to the Rotterdam Consensus (European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine criteria), i.e. the presence of two of the following three criteria: oligo- or anovulation, signs of clinical hyperandrogenism and/or biochemical signs of hyperandrogenism and polycystic ovaries on ultrasonography after exclusion of specific identifiable disorders (congenital adrenal hyperplasia, androgen-secreting tumors and Cushing's syndrome) (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004). Women with possible ovarian tumors and congenital adrenal hyperplasia were excluded. The 31 infertile women with tubal blockage, who served as controls in this study, were otherwise healthy women with regular menstrual cycles and sex hormone levels in the normal range. No structural abnormalities of uterus and ovaries were found by vaginal ultrasound or laparoscopy in all the women. All the partners of the women had normal spermograms and sperm morphology.

Collection of FF and GCs

The long agonist protocol for controlled ovarian hyperstimulation (COH) was used as previously described (Nikolettos et al., 2000). Briefly, COH was performed by administration of recombinant FSH (rFSH, Gonal-F; Serono International S.A., Geneva, Switzerland) after pituitary suppression with triptorelin (Serono) started in the midluteal phase of the preceding cycle. The dosages of gonadotrophins were individualized according to serum estradiol (E₂) levels and transvaginal ultrasonic measurements of the follicles. When at least three follicles reached diameters of 16–18 mm, ovulation was induced by the administration of 10 000 IU HCG (Libao Biochemistry Co, Zhuhai, China). Transvaginal oocyte aspiration was performed with ultrasound guidance under general anesthesia 36 h after injection of HCG. FF was sampled by transvaginal ultrasound-guided puncture. The FF samples were carefully collected from the first aspiration follicle of each ovary as previously described (Wu et al., 2007), and only FF samples which did not contain any visible blood contamination were used in this study. The FF samples were immediately centrifuged for 10 min at 550g, and the supernatants were stored at –80°C until further analysis. The GCs were obtained by follicular aspiration from the women undergoing oocyte retrieval for IVF as described above. Follicular aspirates were transported on ice to the laboratory and then centrifuged at 550g for 5 min. The GCs were isolated from the blood cells and cellular debris using Percoll gradient centrifugation. The GCs were frozen and stored at –80°C until the time of the assay.

Indirect immunofluorescence detection of AQP-9 in the human GCs

The GCs were collected from pre-ovulatory follicles of the infertile women with tubal blockage (controls), and isolated, cultured for 24 h

as described above. The GCs were washed three times with $1 \times$ phosphate-buffered saline (PBS) and fixed at room temperature for 30 min in 4% paraformaldehyde (in $1 \times$ PBS) for indirect immunofluorescence analysis. Fixed cells were washed three times with PBS and permeabilized with $1 \times$ PBS containing 1% Tween-20 and 0.1% bovine serum albumin (BSA) at 4°C for 1 h and washed three times with PBS. The cells were blocked in 2% BSA and 30% inactivated goat serum in $1 \times$ PBS for 30 min, followed by incubation with mouse monoclonal primary antibody of human AQP-9 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a 1:50 dilution in antibody dilution/wash buffer (2% BSA + 30% inactivated goat serum in $1 \times$ PBS) overnight at 4°C. The cells were rinsed three times with the wash buffer. The cells were incubated for 30 min in the dark with anti-mouse immunoglobulin G/DyLight594 secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at 1:100 in the dilution buffer and then washed three times. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich, Inc., St Louis, MO, USA). Fluorescent images were taken with an Inversion Fluorescence Microscope (Olympus IX71SIF-3, Japan). Negative control experiments were performed by omitting primary antibodies to AQP-9.

Measurement of hormones in FF

The levels of total testosterone (TT), FSH and LH in FF of the patients were measured with chemiluminescent assays (Diagnostic Products Corporation, USA). The concentrations of sex hormone-binding globulin (SHBG) in FF were measured with commercial enzyme-linked immunosorbent assay kits (Adlitteram Diagnostic Laboratories, USA). The levels of estradiol (E_2) in FF were detected with radioimmunoassay (RIA, provided by Jiuding Co, Ltd China). Free androgen index (FAI) was calculated as TT (nmol/l) divided by SHBG (nmol/l) \times 100 (Doi *et al.*, 2006). All the measurements were carried out in duplicate and were conducted according to the manufacturer's instruction.

Detection of AQP-9 mRNA expression in GCs by real-time PCR

Total RNA was isolated from GCs using the RNAiso™ Reagent (TAKARA, Dalian, China), according to the manufacturer's instructions. The purity and concentration of RNA were determined by NanoDrop®ND-100 Spectrophotometer (Thermo Fisher Scientific, Inc., USA). The cDNA was then prepared from 500 ng of total RNA by reverse transcription, using the PrimeScript™ RT reagent Kit (Perfect Real-time, TAKARA, Dalian, China). The cDNA samples were diluted in DNase- and RNase-free water at a proportion of 1:3 before further analysis. Quantitative real-time PCR was performed by using the iCycler iQ Real-Time Detection System (Bio-Rad). The human AQP-9 specific primers were provided by Sangon, Shanghai, China. PCR reactions were carried out using 2 μ l of cDNA, 10 μ M of each primer, and 2 \times SYBR® Premix Ex Taq™ (TAKARA) in 25- μ l reactions. Thermal cycling conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 20 s and 58.0°C for 1 min. A final melting curve verified formation of a single product. Gene starting quantity was based on the cycle threshold (C_t) method. A control cDNA serial dilution series of known concentration was constructed for each gene to establish a standard curve, plotting the logarithm of the standard concentration against the C_t -values. The samples were quantified from the measured C_t -values by interpolation, using the regression equation. Each value was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, to control for the amount of the input cDNA. The threshold cycle value for GAPDH mRNA was subtracted from that of the target gene, and the mRNA levels of the target gene were expressed as $2^{-\Delta C_t}$.

Detection of AQP-9 protein in GCs by western blotting

The GCs were lysed in the lysis buffer ($1 \times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 100 μ g/ml phenylmethylsulphonyl fluoride and 100 μ g/ml Leupeptin) and the cell lysates were centrifuged at 11 000 g for 15 min at 4°C. Bicinchoninic acid protein assay (Santa Cruz, CA, USA) was used to determine the protein concentration. Twenty micrograms of protein in the loading buffer (final buffer composition: 50 mM Tris-HCl, 100 mM dithiothreitol, 2% SDS (w/v), 10% glycerol (v/v) and a trace amount of Bromophenol blue) were incubated at 95°C for 5 min, cooled and then loaded per lane. Gel electrophoresis was performed on a Protean III mini-gel apparatus (Bio-Rad, Hercules, CA, USA) using 8% gel with 0.1% (w/v) SDS under a constant current of 35 mA and then transferred to nitrocellulose membranes (Dingguo Biotechnology Company, Beijing, China) for 1.5 h. The membranes were blocked for 2 h at room temperature with 5% dried milk in Tris-Buffered Saline Tween (10 mM Tris, pH 7.6, 150 mM NaCl and 0.05% Tween-20) and incubated with primary antibody (AQP-9 monoclonal antibody from Santa Cruz, 1:200; β -actin antibody from Sigma, 1:2000) overnight at 4°C. After washing, the membranes were incubated with their corresponding secondary antibody at room temperature for 1.5 h. The proteins were detected with the enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ, USA). Densitometric intensity was measured with a GS-800 densitometer (Bio-Rad) and normalized against a β -actin internal control.

Effects of dihydrotestosterone on AQP-9 expression in GCs

Thirty-seven infertile women with tubal blockage were enrolled for the *in vitro* study. The GCs were obtained from pre-ovulatory follicles. After the GCs were isolated from the lymphocytes using lymphocyte separation media (provided by Hengxin chemical Ltd, Shanghai, China), and cultured in RPMI 1640 medium containing 10% fetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂. The cells were plated in a six-well plate (2 ml per well) in duplicate and cultured for 24 h before the various treatments, as below. RPMI 1640, dihydrotestosterone (DHT), H-89 [a cAMP-dependent protein kinase (PKA) inhibitor], LY294002 [phosphatidylinositol 3-kinase (PI3K) inhibitor], forskolin and 4-(Hydroxymercuri) benzoic acid sodium salt (PMB, AQP inhibitor) were obtained from Sigma Chemical Co. Each treatment was repeated for at least three times.

- (1) The GCs were treated with DHT at concentration of 0, 10^{-9} , 10^{-10} , 10^{-11} or 10^{-12} mol/l for 24 h.
- (2) The GCs were treated with DHT at 10^{-9} mol/l for 0, 2, 6, 12 and 24 h.
- (3) The GCs were treated with 10 μ M H-89, 10 μ M forskolin or 25 μ M LY294002 in the presence of DHT at 10^{-9} mol/l for 24 h (Roberts *et al.*, 2004; Sayasith *et al.*, 2008). After the treatment, the adherent GCs were washed with PBS and then harvested.
- (4) A swelling assay was performed as described before (McConnell *et al.*, 2002). Briefly, the GCs were randomized into three groups. The first group was pre-treated with DHT at 10^{-9} mol/l for 24 h. At the beginning of the assay, all the GCs were resuspended and the initial photographs were taken. The second and third groups (both without the treatment with DHT) were incubated for 20 min in the presence (+PMB) or absence (-PMB) of 50 μ M PMB. Then all the GCs were washed three times with PBS and exposed to a hypotonic medium (H₂O: rPMI 1640 3:4, osmotic pressure: 161 mosm) for 30 s. Photographs (\times 40) were taken again from 0 to 30 s, at intervals of 3 s. Image-Pro Plus version 6.0 (Media Cybernetics, Inc. Silver

Spring, MD, USA) was used to measure the volume of the cells. Data are presented as mean \pm SEM of 12 separate measurements from three separate experiments performed on different days.

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS 15.0 for Windows). Comparisons between different groups were performed with one-way analysis of variance. Pearson's correlation coefficient was applied to investigate the correlation between AQP-9 mRNA expression and TT and SHBG levels, and the FAI in FF. Significance level was set at $\alpha = 0.05$ and two-tailed tests were used for all hypothesis tests.

Results

The baseline characteristics of the participants

There were no significant differences between the controls and patients with PCOS for age, BMI, years of infertility, the TT levels on the third day of spontaneous menstrual cycle, dosages of rFSH administered, induction length or numbers of oocytes (Table 1). However, there were significant differences in the LH, FSH and E_2 levels and the ratio of LH/FSH on the third day of spontaneous menstrual cycle, cycle length and numbers of follicles >14 mm ($P = 0.0172, 0.0155, 0.0026, 0.0399, 0.0001, 0.0026$, respectively) between two groups (Table 1).

Table 1 Baseline characteristics of participants in the study of aquaporin-9 expression in granulosa cells of patients with PCOS versus controls.

| | Control | PCOS | P-value |
|------------------------------|----------------------|----------------------|---------|
| <i>n</i> | 31 | 14 | |
| Age (years) | 30.45 \pm 0.748 | 30.36 \pm 0.94 | 1.0000 |
| BMI (kg/m ²) | 22.08 \pm 0.69 | 22.90 \pm 0.60 | 0.4643 |
| Years of infertility | 4.25 \pm 0.45 | 5.07 \pm 0.92 | 0.3767 |
| Day-3 LH (IU/l) | 4.56 \pm 0.46 | 6.90 \pm 0.97 | 0.0172 |
| Day-3 FSH (IU/l) | 6.68 \pm 0.32 | 5.21 \pm 0.50 | 0.0155 |
| LH/FSH | 0.68 \pm 0.25 | 1.66 \pm 0.43 | 0.0026 |
| Day-3 TT (nmol/l) | 1.09 \pm 0.10 | 1.52 \pm 0.27 | 0.1264 |
| Day-3 E_2 (pmol/l) | 111.13 \pm 8.42 | 196.07 \pm 57.43 | 0.0399 |
| Cycle length (days) | 31.9 \pm 1.57 | 69.1 \pm 14.7 | 0.0001 |
| rFSH administered (IU) | 2400.83 \pm 222.46 | 1769.64 \pm 100.07 | 0.0704 |
| Induction length (days) | 9.61 \pm 0.33 | 8.79 \pm 0.53 | 0.1813 |
| Number of follicles >14 mm | 12.23 \pm 1.29 | 19.93 \pm 2.19 | 0.0026 |
| Number of oocytes | 14.16 \pm 1.52 | 19.14 \pm 2.42 | 0.0806 |

Data are mean \pm SEM. The Mann-Whitney *U*-test was carried out. Day-3, the third day of spontaneous menstrual cycle; rFSH, recombinant FSH; TT, total testosterone. PCOS, polycystic ovary syndrome; E_2 , estradiol.

The presence and localization of AQP-9 protein in the human GCs

Indirect immunofluorescence analysis showed that AQP-9 protein was present and was localized in the nucleus, cytoplasm and cell membrane of the human GCs (Fig. 1).

Hormone levels in FF

As shown in Fig. 2A–F, the TT and LH levels and FAI were all significantly higher and the SHBG level was significantly lower in FF of the women with PCOS than controls ($P = 0.0145, 0.0001, 0.0191, 0.0001$, respectively). There were no significant differences between the two groups in the FF levels of FSH and E_2 ($P = 0.0859, 0.6673$, respectively).

AQP-9 mRNA expression in GCs

As shown in Fig. 2G, AQP-9 mRNA expression was significantly lower in GCs of the women with PCOS than controls ($P = 0.0257$).

Correlation between AQP-9 mRNA levels in GCs and hormone levels in FF of the patients with PCOS

As shown in Fig. 3, AQP-9 mRNA levels in GCs of the patients with PCOS were significantly correlated with the TT, SHBG and FAI levels in FF (Pearson $r = -0.725, 0.919, -0.779$, $P = 0.0020, 0.0001, 0.0020$, respectively). There was no significant correlation between the expression levels of AQP-9 mRNA and the TT, SHBG and FAI levels in FF of the controls (Pearson $r = 0.195, 0.307, -0.118$, $P = 0.2930, 0.0930, 0.5270$, respectively).

The effects of DHT on AQP-9 expression in GCs *in vitro*

As shown in Fig. 4, in GCs from control women, DHT at concentrations of $10^{-12}, 10^{-11}, 10^{-10}$ and 10^{-9} mol/l significantly decreased AQP-9 mRNA levels compared with the untreated control cells ($P = 0.0355, 0.0112, 0.0016, 0.0005$, respectively). DHT only at 10^{-9} mol/l significantly decreased AQP-9 protein expression compared with the untreated control cells ($P = 0.0247$).

As shown in Fig. 5, AQP-9 mRNA expression was significantly decreased after the treatment of GCs with DHT at 10^{-9} mol/l for 2, 6, 12 and 24 h ($P = 0.0308, 0.0306, 0.0130, 0.0023$, respectively) and the lowest expression level was observed at 12 h (Fig. 5A). After the cells were treated with DHT at 10^{-9} mol/l and LY294002, AQP-9 mRNA expression was significantly increased compared with those treated with DHT alone ($P = 0.0013$). There was no significant alteration in the AQP-9 mRNA level after the GCs were treated with H89 or forskolin in the presence of DHT at 10^{-9} mol/l compared with those treated with DHT alone ($P = 0.5709, 0.5370$, respectively). However, the GCs treated with H89 or forskolin in the presence of DHT at 10^{-9} mol/l still decreased AQP-9 mRNA expression, compared with the controls, not treated with DHT ($P = 0.0411, 0.0156$, respectively) (Fig 5B).

As shown in Fig. 6, in the swelling assay, both 50 μ M PMB and 10^{-9} mol/l DHT significantly blunted the GCs swelling in the hypotonic medium ($P = 0.0350, 0.0027$, respectively). There were no significant differences among the three groups in the volumes of GCs

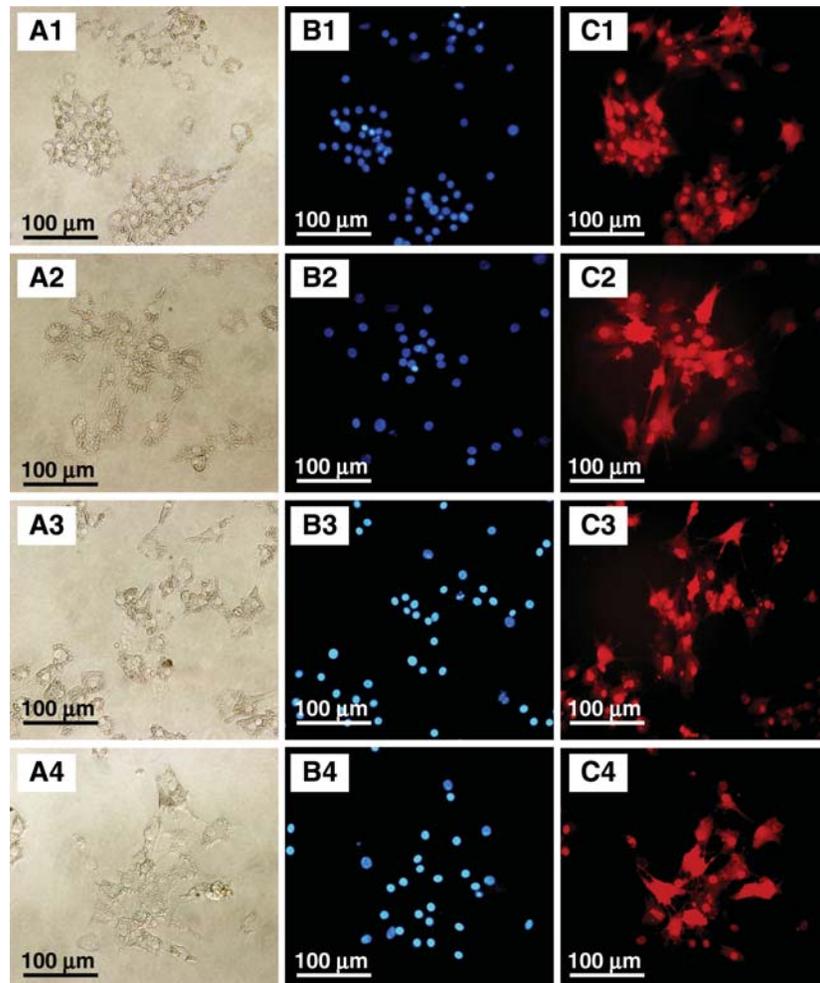


Figure 1 Representative images of indirect immunofluorescence staining for aquaporin-9 (AQP-9) in the human granulosa cells (GCs) from infertile women with tubal blockage (controls). (A1–A4) Under optical microscope; (B1–B4) 4',6-Diamidino-2-phenylindole-labeled nucleus (blue); (C1–C4) expression and location of AQP-9 protein (red). All the images of each group were observed in the same field ($\times 400$). No immunofluorescence staining was detected in the negative control (data not shown).

before treatment. After exposure to the hypotonic medium, the volume of the control cells ($-PMB$) reached $127 \pm 4\%$ of the initial volume, and volume of the GCs treated with DHT and PMB reached 116 ± 3 and $110 \pm 10\%$, respectively, of their initial volumes.

Discussion

In the present study, significantly higher follicular TT and LH levels and FAI, and lower SHBG level were observed in women with PCOS compared with controls, and AQP-9 mRNA levels were significantly lower in GCs of the women with PCOS than controls. The AQP-9 mRNA expression in GCs of the patients with PCOS was significantly correlated with the TT and SHBG levels and FAI in FF. *In vitro*, using GCs from control women, DHT markedly lowered AQP-9 mRNA levels at all the concentrations tested and this effect peaked at 10^{-9} mol/l. Consistent with the mRNA data, DHT at 10^{-9} mol/l significantly decreased the AQP-9 protein. The AQP-9 mRNA expression was rapidly down-regulated after treatment of GCs with DHT at

10^{-9} mol/l for 2–24 h, and the peak value was at 12 h. Moreover, in the swelling assay, we found DHT at 10^{-9} mol/l significantly blunted the GC swelling, which was similar to PMB, a specific AQP inhibitor.

Some studies have revealed higher levels of TT and E_2 in the FF of patients with PCOS (Volpe *et al.*, 1991; Ledee-Bataille *et al.*, 2001). Additionally, another study showed that the LH levels in FF of the anovulatory patients with PCOS were significantly higher than the controls, yet the FSH and E_2 contents in their FF did not differ from controls (Mason *et al.*, 1994), although the FFs were not collected in stimulated cycles. In agreement with above findings, the present study showed that the TT and LH levels were significantly higher in FF of the women with PCOS than the controls, whereas the levels of FSH and E_2 in FF were similar between two groups. It may be the high FF level of LH, instead of FSH, that leads to the hyperandrogenism in FF of the women with PCOS.

In the present study, TT, SHBG, FAI, LH and FSH in FF were chosen as the main parameters to reflect the hyperandrogenism in FF.

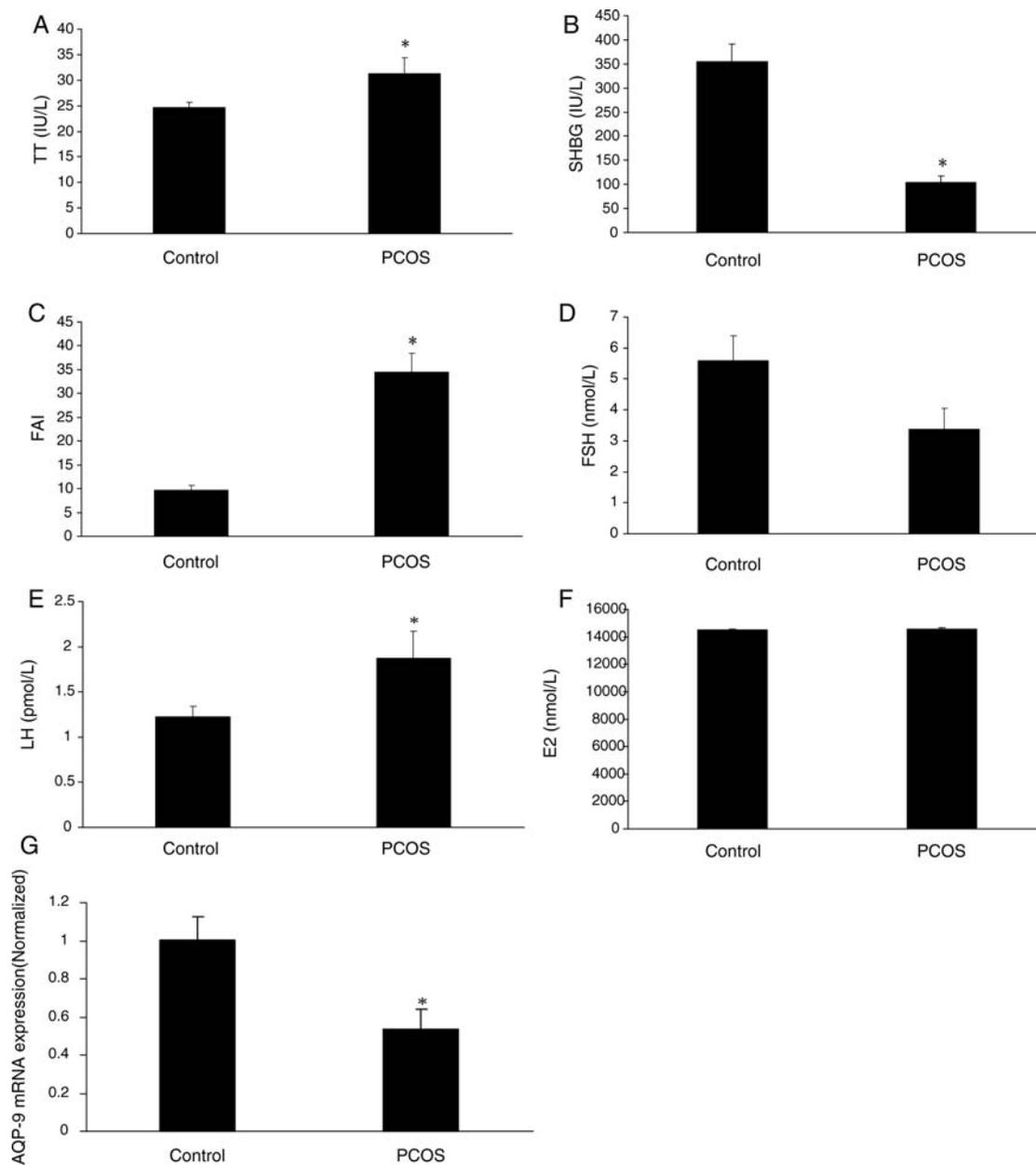


Figure 2 Total testosterone (TT), sex hormone-binding globulin (SHBG), free androgen index (FAI), FSH, LH and E₂ levels in follicular fluids (FF) and AQP-9 mRNA levels in human GCs, as assessed by PCR. (A–F) The TT, FAI and LH levels were all higher and the SHBG level was lower in FF of the women with polycystic ovary syndrome. (PCOS, $n = 14$) than those of the controls ($n = 31$) ($*P = 0.0145, 0.0001, 0.0191, 0.0001$, respectively, versus control). There were no differences in the FF levels of FSH and E₂ between two groups ($P = 0.0859, 0.6673$). (G) AQP-9 mRNA expression was lower in GCs of the women with PCOS than controls ($*P = 0.0257$). Infertile women with tubal blockage who were referred to our department for IVF served as controls. Data are mean \pm SEM.

The 2003 criteria for PCOS did not define which biochemical parameters of hyperandrogenism most accurately represent androgen excess and which cutoff levels should be used for identifying women with PCOS. Koskinen et al. (1996) found that a combination of LH, FSH and androstendione values had the highest clinical utility. Other hormonal parameters, such as SHBG and FAI, have also been used for evaluating PCOS-associated hyperandrogenism (Escobar-Morreale et al., 2001). The follicular concentration of SHBG in human antral

follicles was similar to its serum level, suggesting that it might derive from blood (Martin et al., 1981). SHBG expression has been found in human granulosa-lutein cells and the follicular SHBG is probably involved in the broader ovarian physiological functions other than modulating the follicular level of unbound steroid (Forges et al., 2004). To our knowledge, ours is the first report describing follicular SHBG and FAI levels in the patients with PCOS. We herein demonstrated that the FAI was significantly higher, and SHBG levels were

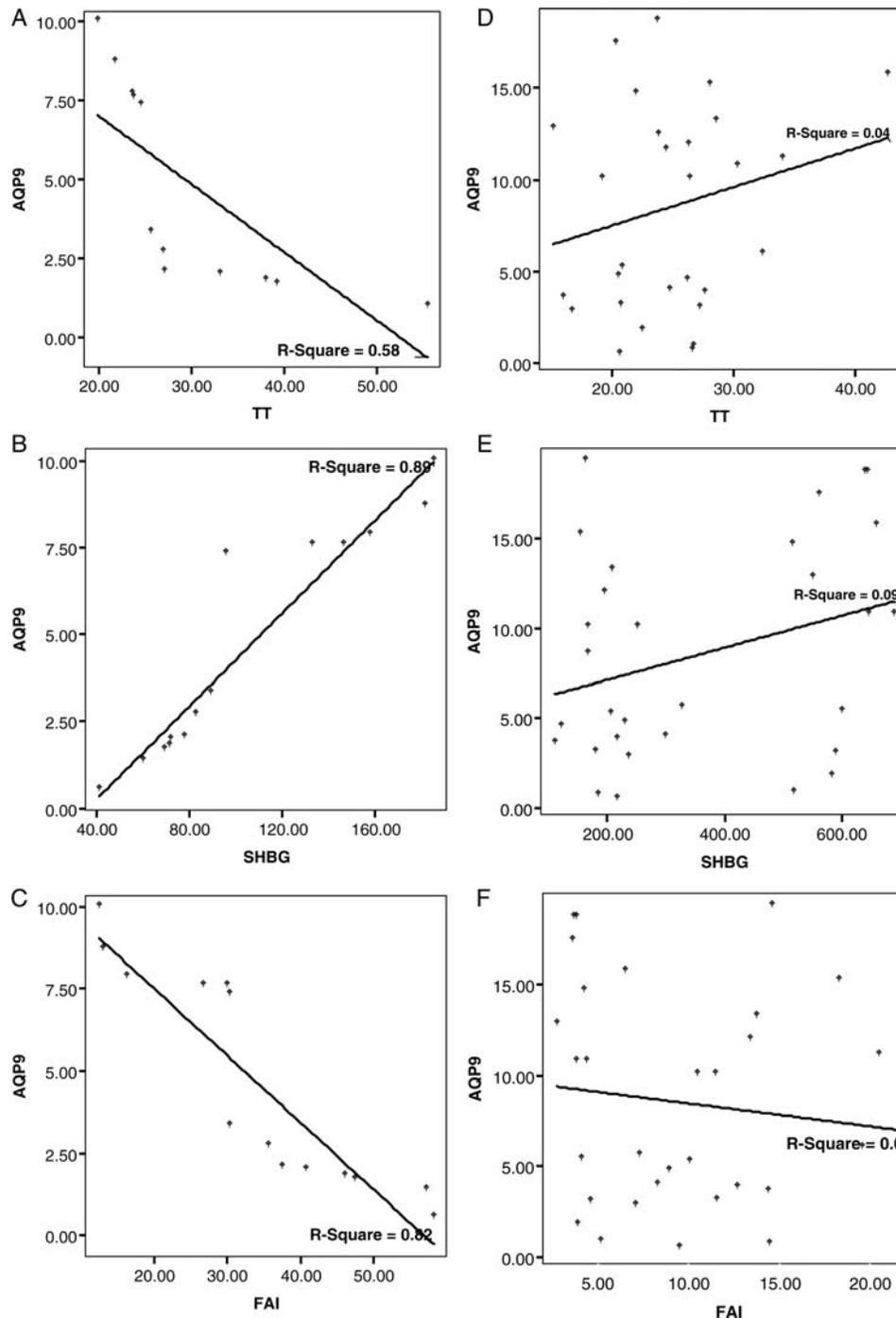


Figure 3 Correlation between AQP-9 mRNA in GCs and the TT, SHBG and FAI levels in human FF. (A–C) AQP-9 mRNA levels in GCs of the patients with PCOS was significantly correlated with the TT and SHBG levels, and FAI in FF (Pearson $r = -0.725, 0.919, -0.779, P = 0.0020, 0.0001, 0.0020$, respectively). (D–F) There was no correlation between AQP-9 mRNA and TT, SHBG and FAI in FF of the controls (Pearson $r = 0.195, 0.307, -0.118, P = 0.2930, 0.0930, 0.5270$).

significantly lower, in FF of the women with PCOS than controls. Our findings suggest that the hyperandrogenism in FF of the patients with PCOS may lead to the low expression of AQP-9 in GCs, possibly impairing its function in GCs and hampering follicular development. It is possible that the women with PCOS had abnormal antral follicles

with lower fluid contents. However, for the control ovaries, as there was no hyperandrogenism in FF, no such correlation existed.

In the present study, AQP-9 protein was present and localized in the nucleus, cytoplasm and cell membrane of the human GCs. The expression of AQP-9 in the membrane of GCs is likely to be

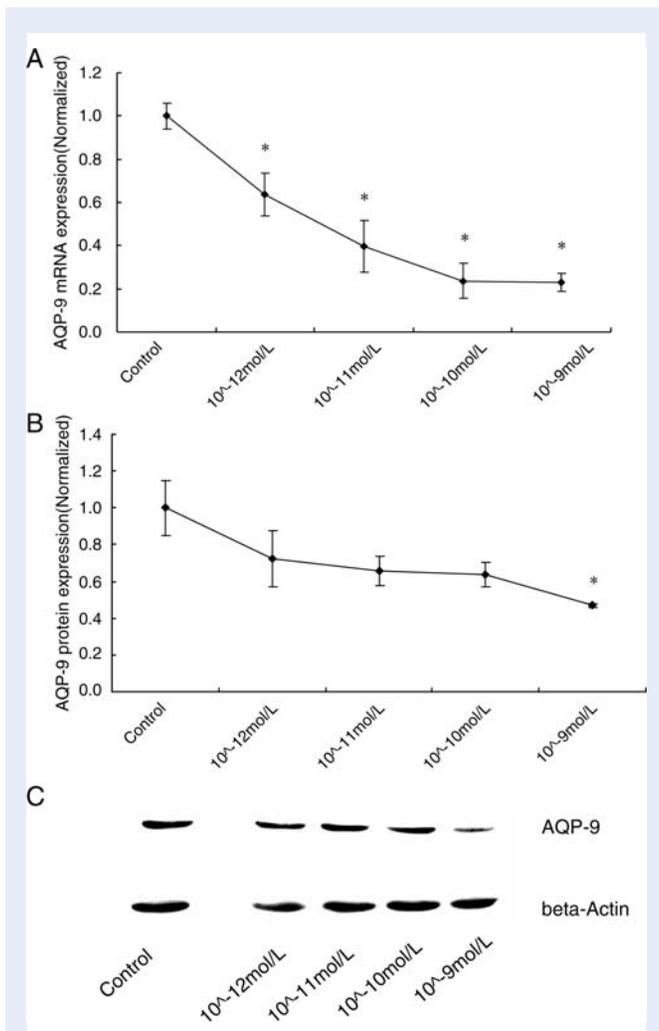


Figure 4 The effects of dihydrotestosterone (DHT) on AQP-9 mRNA and protein levels in human GCs from an additional 37 infertile women with tubal blockage. **(A)** DHT at concentrations of 10^{-12} , 10^{-11} , 10^{-10} , and 10^{-9} mol/l all decreased AQP-9 mRNA compared with the untreated control cells (* $P = 0.0355$, 0.0112 , 0.0016 , 0.0005 , respectively). **(B)** DHT at 10^{-9} mol/l decreased AQP-9 protein compared with the untreated control cells (* $P = 0.0247$). **(C)** AQP-9 and β -actin (internal control) in GCs were assessed by western blotting. Data are mean \pm SEM.

related to the water metabolism of GCs, whereas in the cytoplasm it may be related to the synthesis of steroid hormones. To explore which signaling pathway is involved in the process of DHT decreasing AQP-9 mRNA expression in GCs, three kinds of inhibitors were chosen in the present study. Although AQP-9 mRNA in GCs was decreased by treatment with DHT, this inhibitory effect was significantly attenuated by LY294002, a specific PI3K inhibitor. Our results therefore indicate that the PI3K pathway may be involved in the decrease in AQP-9 mRNA caused by DHT in GCs. However, in the presence of DHT at 10^{-9} mol/l, there was no significant alteration in AQP-9 mRNA expression after the GCs were treated either by H89, a PKA inhibitor or by forskolin, an activator of adenylate cyclase. Forskolin is known to elevate intracellular levels of cAMP

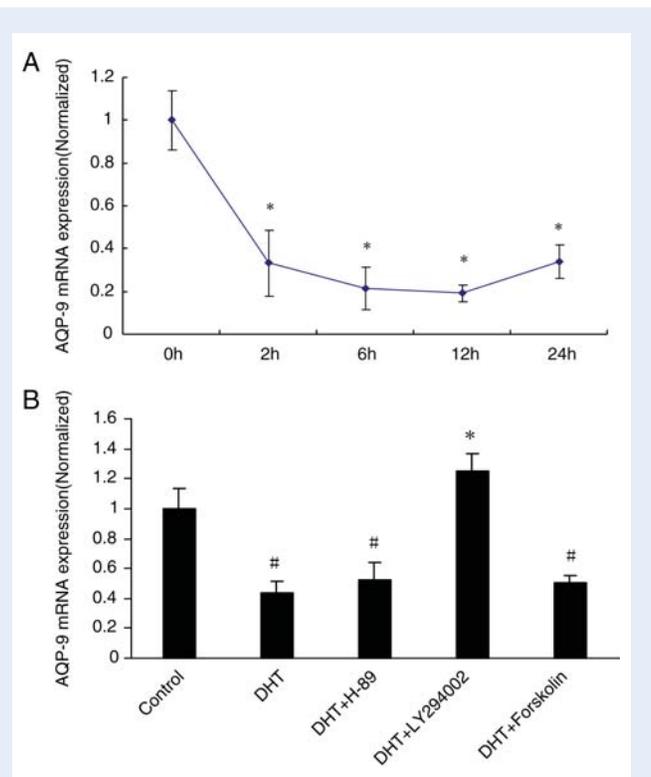


Figure 5 The effects of DHT treatment for various times on AQP-9 mRNA levels in human GCs. **(A)** AQP-9 mRNA decreased after treatment with DHT at 10^{-9} mol/l for 2, 6, 12 and 24 h (* $P = 0.0308$, 0.0306 , 0.0130 , 0.0023 , respectively) and was the lowest at 12 h. **(B)** The effects of DHT plus H-89, LY294002 or forskolin on AQP-9 mRNA in GCs. DHT at 10^{-9} mol/l plus LY294002, increased AQP-9 mRNA compared with DHT alone (* $P = 0.0013$). H89 or forskolin plus DHT at 10^{-9} mol/l had no effect, compared with DHT alone (* $P = 0.5709$, 0.5370 , respectively) but decreased mRNA levels versus controls, without DHT treatment (# $P = 0.0411$, 0.0156 , respectively). Data are mean \pm SEM.

and activate PKA. Our findings indicate that adenylate cyclase and the PKA pathway might not be involved in the inhibitory effect of DHT on AQP-9 mRNA expression in GCs.

The swelling assay is usually used to evaluate the function of AQP (McConnell et al., 2002), and is based upon the fact that fluid transport occurs across an intact cell membrane under hypoosmotic conditions until equilibrium is reached. Owing to the influx of fluid, the cell will expand and bulge, and this change can be readily observed with a phase contrast microscope (Bachtell et al., 1999). In this context, the functions of mercury-sensitive AQPs are evaluated by mercury, which has widely been used as an effective inhibitor of most AQP isoforms (McConnell et al., 2002). The PMB used in the present study contains more than 95% mercury. Dramatic swelling of GCs induced by hypotonic medium could be inhibited by the pretreatment of GCs with PMB, indicating that the contribution of AQP-9 to water transport in GCs is physiologically substantial.

As the rapid and massive transport of water is needed to increase the size of the antral cavity in order to develop into an antral follicle under gonadotrophin stimulation, AQP-9 may play a role in this

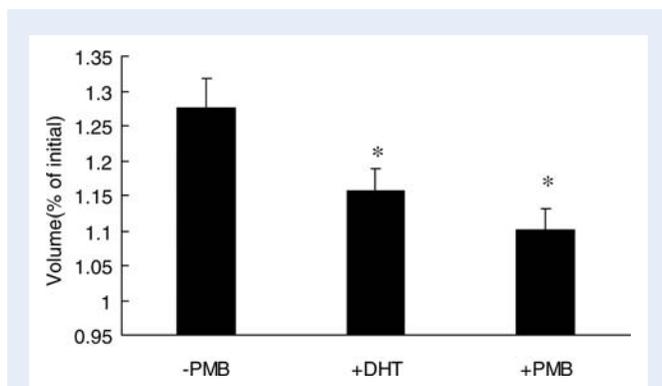


Figure 6 The effects of DHT on the swelling of GCs in hypotonic medium. In the assay, the GCs were randomized into three groups. DHT and 4-(hydroxymercuri) benzoic acid sodium salt (PMB) were used to treat the cells. The first group was treated with DHT at 10^{-9} mol/l for 24 h beforehand. At the beginning of the assay, all the GCs were resuspended and the initial photographs were taken. The second and the third groups (both without the treatment with DHT) were, respectively, incubated for 20 min in the presence (+PMB) and absence (-PMB) of 50 μ M PMB. Then all the GCs were washed three times with PBS and exposed to hypotonic medium (H_2O : rPMI 1640 = 3:4, osmotic pressure: 161 mosm) for 30 s. Photographs ($\times 40$) were taken again from 0 to 30 s at an interval of 3 s. Image-Pro Plus version 6.0 (Media Cybernetics, Inc. Silver Spring, MD, USA) was used to measure the volume of the cells. Data are mean \pm SEM of 12 separate measurements from three separate experiments performed on different days. After exposure to the hypotonic medium, the volumes of the control cells (-PMB) increased to $127 \pm 4\%$ of the initial volumes, and the volumes of the GCs treated with DHT and PMB increased to 116 ± 3 and $110 \pm 10\%$ of their initial volumes, respectively. Volumes of GCs treated with DHT and PMB both differed from the control cells (* $P = 0.0350, 0.0027$, respectively).

process (McConnell *et al.*, 2002; Huang *et al.*, 2006). The present study showed that AQP-9 mRNA levels were significantly lower in GCs of the women with PCOS than controls, and were significantly correlated with the TT and SHBG levels, and FAI in FF. Furthermore, *in vitro*, DHT markedly inhibited the expression of AQP-9 in control GCs. Taken together, these findings demonstrate that the hyperandrogenism in FF of patients with PCOS leads to the reduced expression of AQP-9, as well as impaired function in GCs, thus hampering follicular development. Our study provides a potential new aspect for exploring the pathogenesis of PCOS.

As the number of GCs obtained from the patients with PCOS is limited, in the present study we could not compare the protein levels of AQP-9 between the patients with PCOS and controls. However, *in vitro*, DHT at 10^{-9} mol/l significantly decreased both the AQP mRNA and protein in control GCs. Further research is needed to explore whether there are significant differences in AQP-9 protein levels between the patients with PCOS and controls.

Authors' roles

F.Q.: contributed to the conception and design of the present research, acquisition of data, analysis and interpretation of data;

drafting the article and revising it critically for important intellectual content; and final approval of the version to be published. F.-F.W.: contributed to the acquisition of data, analysis and interpretation of data; drafting the article and final approval of the version to be published. X.-E.L.: contributed to the conception of the present research, acquisition of data and final approval of the version to be published. M.-Y.D.: contributed to the analysis and interpretation of data; and revision of the paper critically for important intellectual content; and final approval of the version to be published. J.-Z.S.: contributed to the conception and design of the present research, analysis and interpretation of data; and final approval of the version to be published. P.-P.L.: contributed to the acquisition of data, analysis of data and final approval of the version to be published. G.-L.D.: contributed to the acquisition of data, analysis of data and final approval of the version to be published. B.-W.S.: contributed to the acquisition of data, analysis of data and final approval of the version to be published. D.Z.: contributed to the analysis of data and final approval of the version to be published. H.-F.H.: contributed to the conception and design of the research, and revision of the paper critically for important intellectual content and final approval of the version to be published.

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