

Follicular hyperandrogenism downregulates aromatase in luteinized granulosa cells in polycystic ovary syndrome women

Fang Yang*, Ye-Chun Ruan^{1,2,*}, Yun-jie Yang, Kai Wang, Shan-shan Liang, Yi-bin Han, Xiao-Ming Teng and Jian-Zhi Yang

Shanghai First Maternity and Infant Hospital, School of Medicine, Tongji University, Shanghai, China and

¹Epithelial Cell Biology Research Centre, School of Biomedical Sciences, Faculty of Medicine and

²Shenzhen Research Institute, The Chinese University of Hong Kong, Hong Kong, China

Correspondence should be addressed to J-Z Yang; Email: yangjianzhi@hotmail.com

*(F Yang and Y-C Ruan contributed equally to this work)

Abstract

Women with polycystic ovary syndrome (PCOS) undergoing IVF–embryo transfer based-assisted reproductive technology (ART) treatment show variable ovarian responses to exogenous FSH administration. For better understanding and control of PCOS ovarian responses in ART, the present study was carried out to compare the follicular hormones and the expression of granulosa cell genes between PCOS and non-PCOS women during ART treatment as well as their IVF outcomes. Overall, 138 PCOS and 78 non-PCOS women were recruited for the present study. Follicular fluid collected from PCOS women showed high levels of testosterone. The expression of aromatase was found significantly reduced in luteinized granulosa cells from PCOS women. In cultured luteinized granulosa cells isolated from non-PCOS women, their exposure to testosterone at a level that was observed in PCOS follicles could decrease both mRNA and protein levels of aromatase *in vitro*. The inhibitory effect of testosterone was abolished by androgen receptor antagonist, flutamide. These results suggest that the hyperandrogenic follicular environment may be a key hazardous factor leading to the down-regulation of aromatase in PCOS.

Reproduction (2015) 150 289–296

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder found in 6–10% women of reproductive age and represents a leading cause of female infertility (Goodarzi *et al.* 2011, Jayasena & Franks 2014). Ovarian follicular development is altered in PCOS, where most ovarian follicles, after leaving the primordial follicle resting pool, are arrested at the small antral stage and fail to complete their maturation for ovulation. This results in the accumulation of a high number of small antrum follicles in the ovary, a morphology of polycystic ovary (Dumesic *et al.* 2008, Dumesic & Richards 2013).

The ovarian morphological abnormality is accompanied with hormonal disruption in PCOS, including significant increases in androgens and luteinizing hormone (LH), and reduction in follicle-stimulating hormone (FSH) (McCartney *et al.* 2002). LH is one of the pituitary-released gonadotropins, which, by activating its receptors in theca cells of small secondary follicles, induces the production of theca cell-derived

androgens (Richards *et al.* 1987). In PCOS, LH is hypersecreted contributing to the excessive androgen level (Blank *et al.* 2006). FSH is another gonadotropin essential to both follicle maturation and ovarian steroidogenesis (Payne & Hales 2004). It activates FSH receptors (FSHRs) in granulosa cells of antral follicles leading to the transcription of aromatase (CYP19), a key enzyme that catalyzes the conversion of androgens to estrogens (Payne & Hales 2004, Stocco 2008), which is required for follicle maturation (Chen *et al.* 2012). In PCOS, the expression of FSHR in granulosa cells appears to be up-regulated (Cateau-Jonard *et al.* 2008), which is believed to be responsible for the observed hyperresponsiveness of PCOS granulosa cells to FSH both *in vitro* and *in vivo* (Erickson *et al.* 1992, Mason *et al.* 1994, Coffler *et al.* 2003). Although FSH hypersensitivity accelerates estrogen production, PCOS women were found unable to sustain their estrogen level (Coffler *et al.* 2003).

PCOS women often seek fertility treatment by assisted reproductive technology (ART), where pituitary

suppression followed by exogenous FSH administration is a standard procedure to stimulate the ovary and maximize follicle maturation and ovulation for IVF. We have observed in the IVF clinic that PCOS patients undergoing ART treatment have various ovarian responses to exogenous FSH, from insufficient to overstimulated, probably because of the complicated FSH-regulatory system in PCOS (Erickson *et al.* 1992, Mason *et al.* 1994, Coffler *et al.* 2003). The following procedures for ART often had to be cancelled either due to the lack of oocyte numbers or to avoid possible development of ovarian hyperstimulation syndrome, a life-threatening complication.

For better understanding and control of PCOS ovarian responses in ART, the present study was carried out to compare the follicular hormones and granulosa cell gene expression between PCOS and non-PCOS women during their ART treatment as well as their IVF–embryo transfer (ET) outcomes, including the number of oocytes retrieved, fertilization rate, percentage of high quality grade embryo, implantation, and clinical pregnancy rates. Follicular fluid collected from PCOS women, particularly small follicles, showed high levels of testosterone. Whereas, the expression of aromatase was found significantly reduced in luteinized granulosa cells from PCOS women. In isolated non-PCOS human luteinized granulosa cells, we also demonstrated that exposure of the cells to testosterone at a level that was observed in PCOS follicles down-regulated aromatase *in vitro*.

Materials and methods

Patients

A total of 216 female patients at the IVF center of Shanghai First Maternity and Infant Hospital from June 2012 to June 2013 were recruited for the present study. Among the patients, 138 were diagnosed with PCOS based on the presence of at least two of the following criteria (Ng *et al.* 2005): i) ovulatory disturbance (oligomenorrhea or amenorrhea), ii) hyperandrogenism as defined by hirsutism, seborrhea, and/or testosterone >0.7 ng/ml and/or androstenedione >2.2 ng/ml as measured on day 3 of the menstrual cycle, and iii) the presence of more than 12 follicles of 2–9 mm in diameter in each ovary under B-ultrasound and/or ovarian volume higher than 10 ml. The other 78 women met the following inclusion criteria and were grouped as non-PCOS: i) both ovaries were present, ii) menstrual cycle length was between 25 and 35 days, iii) there were no current or past diseases affecting the ovaries, gonadotropin or sex steroid secretion, iv) there were no clinical signs of hyperandrogenism, and v) FSH levels were ≤10 mIU/ml on day 3 of the cycle. Causes of infertility in the non-PCOS group include tubal blockage ($n=46$), pelvic adhesions ($n=11$), or male factor ($n=21$). All the procedures were approved by the Ethics Committee of Tongji University and all patients have given informed consents.

Clinical data collection

A blood test was performed on the 2nd–5th day of the menstrual cycle before the treatment to determine basal levels of estradiol (E_2), testosterone, progesterone, LH, and FSH in the patients. During treatment, patient data were documented, including age and BMI. IVF results include follicle number, numbers of collected oocytes, fertilization rate, cleavage rate, number of available embryos and transferred embryos, good embryo quality rate (grades I and II), cryopreserved embryo number, and implantation rate.

Ovarian hyperstimulation

Diphereline (Ipsen Pharma Biotech, Cambridge, UK; 1.25 or 1.88 mg), a gonadotropin-releasing hormone agonist, was intramuscularly injected at the mid-luteal phase of the menstrual cycle in each patient, to suppress pituitary secretion of gonadotropin hormones and prevent premature ovulation. After pituitary suppression was achieved as evidenced by plasma E_2 levels of ≤50 pg/ml, the absence of ovarian follicles and endometrial thickness ≤6 mm by transvaginal ultrasound examination (Barash *et al.* 1998), patients were daily injected with Gonal-F (Merck–Serono), a recombinant human FSH (rhFSH), starting from the 5th day of the menstrual cycle. The initial rhFSH dose was determined by a variety of factors, including age, number of antral follicles, basal FSH level, and history of ovarian response. Trans-vaginal ultrasound using a 5-MHz vaginal transducer attached to a sector scanner (Model SSD-620, Aloka, Tokyo, Japan) was performed and serum sex hormones were assessed to monitor follicular development and adjust the dose of rhFSH. When the lead follicle achieved 18 mm in diameter, the lead two were 17 mm or the lead three were 16 mm, patients were subcutaneously injected with recombinant human chorionic gonadotropin (hCG, Ovidrel, Merck–Serono, 250 µg) to trigger oocyte maturation. Blood was collected right before the injection of first rhFSH and hCG respectively.

Oocyte retrieval, follicle fluid, and luteinized granulosa cell collection

Thirty-four to 36 h after the administration of hCG, ovarian follicles were aspirated using a single-lumen, 17-gauge needle (Cook Medical, Bloomington, IN, USA) guided by trans-vaginal ultrasonography. Follicles were classified into two groups, the large follicle group with the diameter >18 mm and the small follicle group with diameter <10 mm as measured by ultrasonography. All the samples were collected by the same operator to ensure the size of the follicles. Large follicles were used to isolate luteinized granulosa cells. The oocytes were dissected from the collected follicles under a dissecting microscope. After collecting the oocytes, the remaining granulosa cells with fluid were transferred into sterile tubes (Axygen Scientific, Union City, CA, USA) and centrifuged at 200 g for 10 min. Afterwards, the supernatant was aspirated and collected as follicular fluid and cell pellets were washed with PBS (Beyotime, Shanghai, China) and subsequently a red blood cell lysis buffer (Beyotime) to eliminate red blood cells. The cells were stored at –80 °C or re-suspended for culture.

Table 1 Sequences of primers.

Gene	Forward sequence	Reverse sequence	Product size (bp)
<i>FSHR</i>	TCTGTCAGTCTCTAACAGGG	TGCACCTTTTGGATGACTCG	131
<i>AR</i>	CCTGGCTTCGCAACTTACAC	GGACTTGTGCATGCGGTACTCA	168
<i>LHCGR</i>	ATTTGTCAATCTCCTGGAGGC	CACTCAGTTCCTCTCAGCA	191
<i>FLK</i>	GGCCAATAATCAGAGTGGCA	TGTCAATTTCCGATCACTTTTGGGA	105
<i>IGF1R</i>	TGGTGGAGAACGACCATATCC	CGATTAAGTGAAGAGGAGTTTCCGA	123
<i>CYP19</i>	TGGAAATGCTGAACCCGATAC	AATTCATGCGAGTAGCCAGG	161
<i>CYP17</i>	GCTGCTTACCCTAGCTTATTGT	ACCGAATAGATGGGGCCATATT	174
<i>PPARG</i>	GGGATCAGCTCCGTGGATCT	TGCACCTTTGGTACTCTTGAAGTT	186
<i>β-actin</i>	TCATGAAGTGTGACGTGGACATC	CAGGAGGAGCAATGATCTTGATCT	156

IVF and ET

The oocytes isolated were used for IVF or ICSI based on the condition of semen. No more than three embryos were transferred into the uterine cavity 2–3 days after IVF or ICSI. Patients were intramuscularly injected with progesterone (60 mg/day, Tongyong Pharmaceutical Co., Shanghai, China) from the day oocyte retrieval was performed till 14 days after ET. Serum β -hCG was measured 14 days after ET and clinical pregnancy was defined by the presence of an intrauterine gestational sac with fetal heartbeats 3–4 weeks after ET.

Luteinized granulosa cell culture

The isolated luteinized granulosa cells from non-PCOS group were seeded at 0.75×10^6 cells/ml into six-well plates and cultured in DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin and streptomycin sulfate (Life Technologies, Inc.) at 37 °C in a 5% CO₂ incubator. On the next day after seeding the cells, testosterone (5, 15, or 60 ng/ml, Sigma) or the solvent was added into the culture medium. Forty-eight hours after the treatment with testosterone, the cells were collected for subsequent analysis.

RNA isolation and real-time PCR analysis

RNAs were extracted from cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instruction. The extracted RNAs that show distinct 18s and 28s bands in RNA electrophoresis were used in subsequent experiments. The quantity of RNA was measured by the NanoDrop 2000c U.v.–Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNAs were reversely transcribed into cDNA with random primers (Takara, Shiga, Japan) in a 20 μ l reaction buffer at 37 °C for 15 min, and terminated by heating at 85 °C for 5 s followed by cooling at 4 °C. The sequences of primer pairs used in the present study are shown in Table 1. The primers were verified with their efficiency close to 100%. Quantitative real-time PCR was performed on the PCR system (Roche Diagnostics Ltd) with the SYBR reagent (Takara Bio, Inc., Otsu, Japan). The reaction was performed at 95 °C for 10 s and followed by 40 cycles of heating at 95 °C for 5 s and subsequently 60 °C for 34 s. The dissociation stage was initiated at 95 °C for 15 s, followed by one cycle of 60 °C for 1 min and 95 °C for 15 s. PCRs were carried out in triplicate for each sample. Gene expression was analyzed using housekeeping gene β -actin and cycle threshold (Ct) method. Fold-induction values (x) were calculated using the following formula, $x = 2^{-\Delta\Delta Ct}$, where Ct is the mean value of all replicates of a given gene, ΔCt is the

difference between the Ct value of the gene in target and that of β -actin, and $\Delta\Delta Ct$ is the difference between ΔCt values of the samples for each target and the ΔCt of a control sample.

Western blot

Cells were lysed in RIPA buffer (Beyotime) with proteases inhibitor and phenylmethylsulphonyl fluoride for 30 min on ice before centrifuged at 15 000 *g* for 20 min at 4 °C. Afterwards, the supernatant was collected and the protein concentration was determined by a BCA assay (Pierce Biotechnology, Rockford, IL, USA) and using a microplate reader (Multiskan MK3, Thermo Scientific). Proteins were mixed with the SDS sample buffer and boiled for 10 min before being separated by SDS–PAGE with 5% stacking gel and 10% separating gel (Beyotime) at 100 V for around 2 h and subsequently transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 5% non-fat milk in TBST (0.01 M Tris–HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.4, Beyotime) for 1 h at room temperature and subsequently the antibody against aromatase (Cell Signaling Technology, Danvers, MA, USA, 1:1000) at 4 °C overnight. The membranes were then washed with TBST three times before being incubated with the HRP-conjugated secondary antibody (Proteintech, Chicago, IL, USA, 1:2000) for 1 h at room temperature. The blots were visualized by ECL (Millipore, Watford, UK) and the images were taken by FluorChem E (Protein Simple, San Francisco, CA, USA). Quantification of the blots was performed by the freely available ImageJ Software.

Steroid hormone assay

E₂, testosterone, progesterone, LH, and FSH were measured by an automated chemiluminescence immunoassay analyzer

Table 2 Basic characteristics of PCOS and non-PCOS women.

Variables	Non-PCOS (n=78)	PCOS (n=138)	P value
Age (years)	31.93 \pm 0.46	29.57 \pm 0.54	<0.05
BMI (kg/m ²)	21.33 \pm 0.28	22.62 \pm 0.64	>0.05
Years of infertility	5.08 \pm 0.29	4.29 \pm 0.29	>0.05
Initial dose of rhFSH (IU)	181.7 \pm 5.46	149.1 \pm 4.64	<0.001
Total dose of rhFSH (IU)	2177.83 \pm 88.04	2058.9 \pm 140.40	>0.05
Duration of rhFSH (day)	11.82 \pm 0.30	13.2 \pm 0.65	<0.05

rhFSH, recombinant human FSH. Data other than P values are mean \pm s.e.m., n = number of patients.

Table 3 Hormonal levels in PCOS and non-PCOS women.

Variables	Non-PCOS (n=78)	PCOS (n=138)	P value
Basal			
FSH (IU/l)	7.53±0.18	6.39±0.25	<0.01
LH (IU/l)	4.53±0.25	8.67±1.57	<0.01
LH/FSH	0.57±0.05	1.3±0.18	<0.001
E ₂ (ng/ml)	61.53±6.10	63.09±11.50	>0.05
Initial day			
FSH (IU/l)	3.21±0.21	3.05±0.22	>0.05
LH (IU/l)	1.99±0.12	1.44±0.19	>0.05
E ₂ (ng/ml)	22.15±1.07	22.53±2.22	>0.05
hCG day			
Progesterone (ng/ml)	1.17±0.29	0.81±0.16	>0.05
LH (IU/l)	1.81±0.18	1.18±0.19	<0.05
E ₂ (ng/ml)	2125.23±113.9	2744.96±311.05	>0.05

Data other than *P* values are mean±s.e.m., *n*=number of patients.

(Siemens, Tarrytown, NY, USA). Intra- and inter-assay coefficients of variations were 6.1–8.7, 5–6.7, 1.4–2.9, 1.6–3.3, and 2.2–5.7% respectively. All samples were operated at least three times and the mean values were used for statistical analysis. The plates were read by an ELISA reader (Toyo-Sokuki Inc., Tokyo, Japan).

Statistical analysis

Data are mean±s.e.m. Student's *t*-test was used to compare PCOS and non-PCOS groups. One-way ANOVA followed by Tukey's *post hoc* tests was used to compare more than two groups. *P*<0.05 was considered statistically significant.

Results

Clinical results

Basic clinical characteristics of the patients for the present study are shown in Table 2. The PCOS patients were about 3 years younger than the non-PCOS patients on average. No significant difference in years of infertility was observed between the two groups. Although obesity is often seen in PCOS cases, the PCOS patients recruited in the present study showed normal BMI with no significant difference with the non-PCOS group. The PCOS patients were treated with the rhFSH at a lower initial dose for a longer administration duration in comparison to the non-PCOS patients, although similar total amounts of rhFSH was administered to patients of both groups.

Serum levels of the hormones measured are shown in Table 3. The PCOS group showed significantly higher basal LH and lower basal FSH levels as compared to the non-PCOS group (*P*<0.01), although basal E₂ levels were found to be similar between the two groups. After pituitary suppression (initial day), all the hormones measured including LH, FSH, and E₂ dropped to a low level in both PCOS and non-PCOS groups and no significant difference in the residual hormones was found between them. On the day when hCG was injected (hCG day), no difference in progesterone and E₂ between the two groups was found. The LH level

remained low on the hCG day in both groups with the non-PCOS group showing relatively higher LH levels.

ART related parameters are shown in Table 4. A larger number of follicles were collected from the PCOS than non-PCOS group, although similar numbers of oocytes were obtained from the collected follicles of the two groups. Thus, the oocyte obtaining rate (oocyte number per follicle number) was lower in the PCOS than non-PCOS group. The oocytes obtained from the PCOS group showed no difference in fertilization rate and cleavage rate as compared with non-PCOS ones. The embryos derived from PCOS oocytes showed similar good quality rate, implantation rate, and pregnancy rate to those in non-PCOS ones.

Analysis of follicular fluid

We were able to collect a sufficient amount of follicular fluid from 25 patients in each group, PCOS or non-PCOS, of the cohort for the analysis of follicular hormones. Both PCOS and non-PCOS groups showed low follicular LH levels (below 0.5 IU/l, Fig. 1A) with no difference between the two groups probably due to pituitary suppression. The FSH levels in both small and large follicles were found significantly reduced in PCOS compared to that in non-PCOS patients (Fig. 1B). The testosterone levels in both large and small follicles from PCOS patients were found to be respectively higher than those in non-PCOS patients, although only in small follicles, the difference between PCOS and non-PCOS is statistically significant (Fig. 1C). The E₂ level in follicles showed no significant difference between PCOS and non-PCOS (Fig. 1D). In both small and large follicles, the progesterone levels were found significantly lower in PCOS than those in non-PCOS (Fig. 1E).

Gene expression in luteinized granulosa cells and down-regulation of aromatase in PCOS

Genes that are associated with granulosa cell functions including FSHR, androgen receptor (AR), insulin-like

Table 4 IVF outcomes of PCOS and non-PCOS women.

Variables	Non-PCOS (n=78)	PCOS (n=138)	P value
Follicle number	11.95±0.62	18.0±1.23	<0.001
Oocyte number	9.00±0.85	10.26±0.96	>0.05
Oocyte obtaining rate (oocyte number per follicle)	0.89±0.06	0.60±0.05	<0.01
Fertilization rate	0.70±0.02	0.77±0.04	>0.05
Cleavage rate	0.84±0.03	0.92±0.02	>0.05
Available embryo number	3.99±3.03	5.6±4.16	<0.05
Good quality embryo rate	0.69±0.06	0.61±0.05	>0.05
Cryopreserved embryo number	2.39±3.06	4.64±4.31	<0.05
Embryo transfer number	1.6±1.08	0.95±1.01	<0.01

Data other than *P* values are mean±s.e.m., *n*=number of patients.

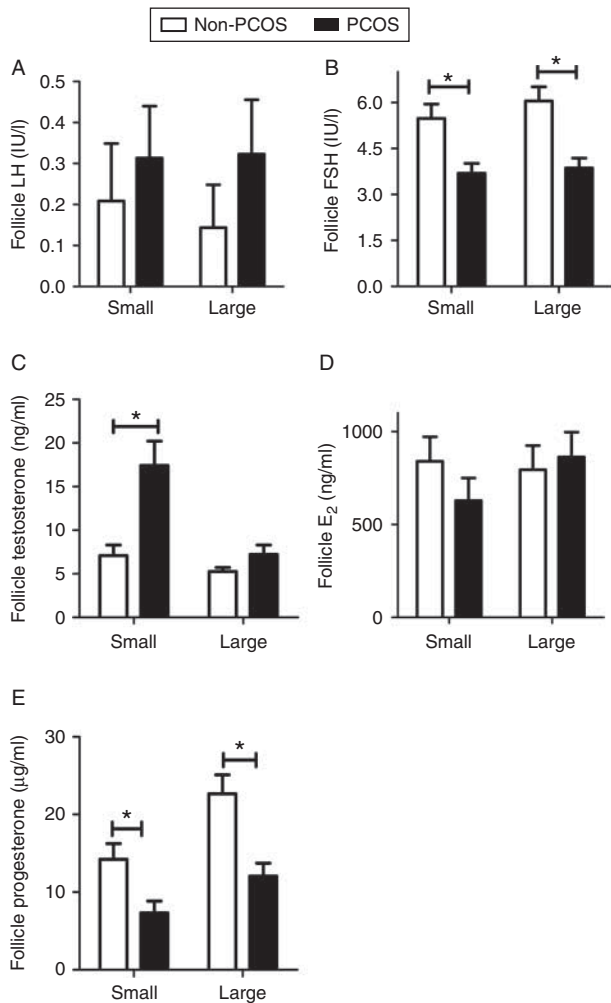


Figure 1 Hormones in follicles from PCOS and non-PCOS patients with ART treatment. The concentration of LH (A), FSH (B), testosterone (C), E₂ (D), and progesterone (E) was measured in small and large follicles collected from PCOS and non-PCOS patients during their ART treatment. Data are means \pm s.e.m., $n=25$. * $P<0.05$, t -test.

growth factor 1 receptor (IGF1R), vascular endothelial growth factor receptor (FLK), LH receptor (LHCGR), CYP17, CYP19, and peroxisome proliferator-activated receptor gamma (PPARG) were compared between PCOS and non-PCOS. We were able to collect a sufficient number of luteinized large (>18 mm) follicle granulosa cells from 20 to 35 patients in each group, PCOS or non-PCOS, of the whole cohort for real-time PCR analysis of each gene. Among these genes, PCOS luteinized granulosa cells showed significantly increased transcripts of FSHR, and decreased LHCGR and CYP19 as compared to the non-PCOS group (Fig. 2). To confirm that aromatase (CYP19) expression is affected in PCOS, proteins were extracted from luteinized large follicle granulosa cells for western blot analysis, which showed that the protein level of aromatase was also down-regulated in PCOS group as compared to the non-PCOS group (Fig. 3).

Testosterone-induced downregulation of aromatase in human granulosa cells in vitro

The transcription of CYP19 is known to be promoted by FSH. Since that similar total amount of exogenous FSH (Table 2) was used in PCOS and non-PCOS patients, and that PCOS granulosa cells showed higher level of FSHR expression (Fig. 2), the downregulation of CYP19 in PCOS luteinized granulosa cells might be the result from factors other than FSH. Given the increased testosterone levels observed in PCOS follicles, we hypothesized that CYP19 expression in granulosa cells might be affected by testosterone. In order to test this, we isolated and cultured human luteinized granulosa cells from the non-PCOS patients. Since the testosterone level in small follicles of PCOS patients was determined to be around 15 ng/ml (Fig. 1C), we treated the cells with testosterone at a similar dose range. Testosterone (5, 15, and 60 ng/ml) was added to the culture medium to treat these cells. After 48 h, both mRNA (Fig. 4A) and protein (Fig. 4B) levels of aromatase in the cells were found to be significantly decreased by testosterone in a dose-dependent manner. Moreover, pretreatment of the cells with flutamide (2 μ M, 24 h), an antagonist of AR, abolished the testosterone-induced reduction in CYP19 transcription (Fig. 4C), suggesting the role of AR in mediating the inhibitory effect of testosterone on CYP19. In addition, we also tested whether testosterone would influence the effect of FSH on aromatase in these cells. As shown in Fig. 4D, incubation with FSH (0.04–0.2 IU) for 48 h increased the mRNA level of CYP19 in these cells in a dose-dependent manner. Testosterone (15 ng/ml) was added in together with FSH (0.04 IU) to incubate these cells for 48 h. As shown in Fig. 4E, in the presence of testosterone (15 ng/ml), the FSH-induced

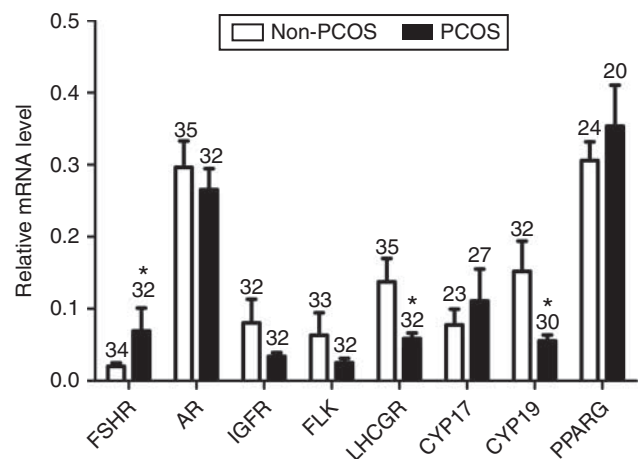


Figure 2 Real-time PCR analysis of genes expression in granulosa cells freshly isolated from PCOS and non-PCOS patients with ART treatment. Data are means \pm s.e.m., n is indicated above each bar. β -actin was used as internal control for relative mRNA level measurement by real-time PCR. * $P<0.05$, t -test.

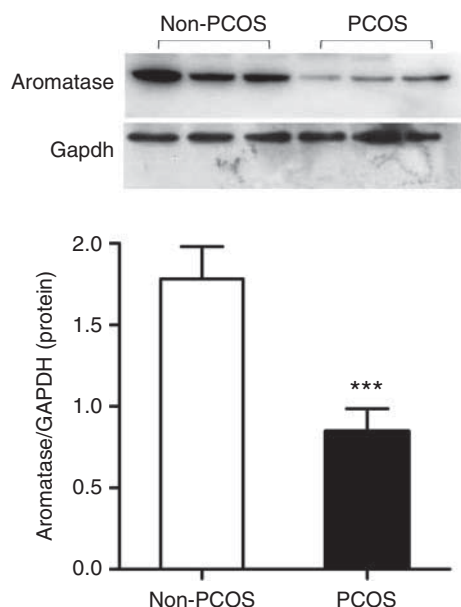


Figure 3 Western blot analysis of aromatase expression in granulosa cells freshly isolated from PCOS and non-PCOS patients with ART treatment. Gapdh was used as a loading control. Data are means \pm S.E.M., $n=12$. *** $P<0.001$, t -test.

CYP19 transcription was found to be slightly reduced ($P=0.0528$).

Discussion

The present study compared patients under ART treatment with and without PCOS, and the results show that, despite the controlled LH and FSH levels in the circulation system during ART treatment, PCOS patients have significantly higher follicular testosterone and reduced expression of aromatase in luteinized granulosa cells. Treating luteinized granulosa cells isolated from non-PCOS women with a high level of testosterone similar to that observed in PCOS small follicles decreased the aromatase expression in luteinized granulosa cells *in vitro*. These results suggest for the first time that the down-regulation of aromatase in PCOS may be a result of the hyperandrogenic follicular environment.

Consistent with previous studies (Dickerson *et al.* 2010, de Resende *et al.* 2010, Nejad *et al.* 2011, Zhong *et al.* 2012), the present study has shown that PCOS women have similar fertilization rates, good embryo quality rates and clinical pregnancy rates in ART as compared to non-PCOS women (Table 4). A possible reason for this might be that the pituitary suppression treatment necessary for ART have overcome the high LH level in PCOS (Table 3). In addition, IVF and *in vitro* pre-implantation embryo culture for ART have released oocytes/embryos from pathological *in vivo* environment in PCOS, such as the high level testosterone. Given these

reasons, ART is suggested to be a suitable treatment strategy for PCOS-associated infertility.

Although not all the samples from the present cohort contained a sufficient amount of materials for the analysis of follicular fluid, the available samples showed that hormonal levels in PCOS follicles remained remarkably different from that in non-PCOS even after pituitary suppression and exogenous FSH treatment in ART. For instance, progesterone is lower in PCOS follicles (Fig. 1E) suggesting impaired granulosa cells function, which may account for the lower oocyte obtaining rate in PCOS (Table 4). In addition, the FSH level in PCOS follicles was found to be lower than that in non-PCOS ones (Fig. 1B), even though the two groups had been treated with a similar total amount of

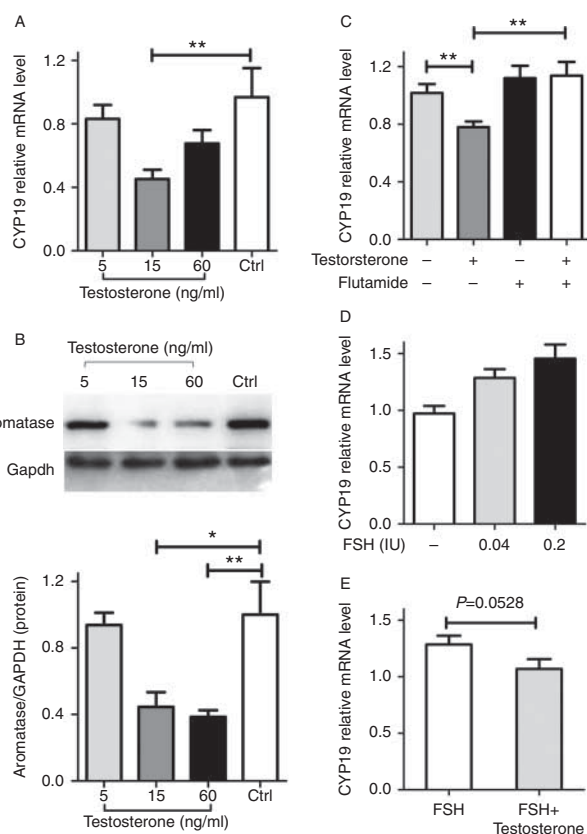


Figure 4 Effect of testosterone on the expression of aromatase in cultured non-PCOS human granulosa cells. (A and B) Real-time PCR of CYP19 (A) and western blotting for aromatase (B) in non-PCOS granulosa cells treated with testosterone (5, 15, and 60 ng/ml). Gapdh was used as a loading control for western blot. (C) Real-time PCR of CYP19 in non-PCOS granulosa cells treated with or without flutamide (2 μ m, 24 h pretreatment) or testosterone (15 ng/ml, 48 h). (D) Cells were treated with FSH (0.04–0.2 IU) for 48 h before CYP19 mRNA level was measured by real-time PCR. (E) Cells were treated with FSH (0.04 IU) in the presence or absence of testosterone (15 ng/ml) for 48 h before the CYP19 mRNA level was measured. β -actin was used as internal control for relative mRNA level measurement by real-time PCR. Data are means \pm S.E.M., $n=7-9$ (A), 4 (B), 9 (C), and 12 (D and E). * $P<0.05$ and ** $P<0.01$, one-way ANOVA (A, B, C and D), t -test (E).

exogenous FSH, which may suggest that not only the production of FSH but also other regulatory factors for FSH, such as its bio-degeneration, might have been altered resulting in a reduced FSH level in PCOS. Further investigation is required to clarify the mechanisms underlying FSH disruption in PCOS. Moreover, the testosterone level in PCOS follicles is high (Fig. 1C) even with a reduced low level of circulating LH (Table 3), which may suggest that theca cells, where most androgens are derived from, may have been intrinsically changed in PCOS. Alternatively, the processing of testosterone, such as its conversion to estrogens by granulosa cells, may have been inhibited in PCOS. This seems to be the likely case given the down-regulation of aromatase in PCOS granulosa cells as observed in the present study (Figs 2 and 3).

In a previous study, primary cultured luteinized granulosa cells from PCOS patients showed increased aromatase activity *in vitro* (Andreani *et al.* 1997). The present study using freshly isolated granulosa cells from luteinized follicles in PCOS, however, showed significantly reduced mRNA and protein levels of aromatase. Another previous study has also demonstrated that freshly isolated follicles from PCOS women have low mRNA expression of aromatase (Jakimiuk *et al.* 1998). Since freshly isolated cells/follicles may be more representative of *in vivo*, it is thus suggested that aromatase may be suppressed by *in vivo* environmental factor(s) in PCOS. For instance, it could be a result of the reduced follicular FSH in PCOS. However, FSHR was found up-regulated in PCOS luteinized granulosa cells (Fig. 2) which might compensate for the effect of low FSH in PCOS suggesting down-regulation of aromatase may be caused by factor(s) other than a low FSH level. The present results support that testosterone is a key factor responsible for down-regulation of aromatase in PCOS. First, at the averaged level in small follicles in PCOS patients, testosterone down-regulated both mRNA and protein levels of aromatase in cultured non-PCOS luteinized granulosa cells. Second, AR antagonist, flutamide, could abolish the inhibitory effect of testosterone. Moreover, in the presence of FSH, testosterone also showed some inhibitory effect on aromatase transcription in these cells *in vitro* (Fig. 4E). The present study is the first to show the effect of a high level of testosterone on the expression level of aromatase in human luteinized granulosa cells, which may provide explanation to the previous observation that the activity of aromatase in human granulosa cells was inhibited by androgens at high doses (Kirilovas *et al.* 2003). Since these PCOS patients had been controlled to have a reduced LH level during ART treatment, the follicular testosterone level in untreated PCOS patients may have gone higher than 15 ng/ml resulting in further inhibition of aromatase expression. The key role of aromatase is to convert androgens to estrogens. With lower aromatase expression, androgens would be accumulated.

Therefore, the inhibition of aromatase by high testosterone as observed in the present study may also suggest a vicious cycle leading to hyperandrogenism, a key feature in PCOS (McCartney *et al.* 2002).

Of note, aromatase inhibitors have been proposed to treat PCOS patients, since they may stimulate pituitary secretion of FSH by reducing circulating estrogen levels (Polyzos *et al.* 2008, Misso *et al.* 2012, Pavone & Bulun 2013, Legro *et al.* 2014). However, clinical trials of aromatase inhibitors for PCOS yield controversial results regarding pregnancy rates (Badawy *et al.* 2009, Kar 2012, Roy *et al.* 2012). The present study has shown that aromatase expression in luteinized granulosa cells is down-regulated by high testosterone in PCOS patients. Whether further inhibition of residual aromatases in PCOS by these aromatase inhibitors would be effective as proposed requires further investigation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported in part by the National Major Basic Research Program of China (2012CB944903) and the Natural Science Foundation of China (no. 81471460).

References

- Andreani CL, Diotallevi L, Lazzarin N, Pietro E, Giannini P, Lanzone A, Capitanio P & Mancuso S 1997 The cellular activity of different sized follicles in cycles treated with gonadotrophin-releasing hormone analogue. *Human Reproduction* **12** 89–94. (doi:10.1093/humrep/12.1.89)
- Badawy A, Shokeir T, Allam AF & Abdelhady H 2009 Pregnancy outcome after ovulation induction with aromatase inhibitors or clomiphene citrate in unexplained infertility. *Acta Obstetrica et Gynecologica Scandinavica* **88** 187–191. (doi:10.1080/00016340802638199)
- Barash A, Weissman A, Manor M, Milman D, Ben-Arie A & Shoham Z 1998 Prospective evaluation of endometrial thickness as a predictor of pituitary down-regulation after gonadotropin-releasing hormone analogue administration in an *in vitro* fertilization program. *Fertility and Sterility* **69** 496–499. (doi:10.1016/S0015-0282(97)00542-6)
- Blank SK, McCartney CR & Marshall JC 2006 The origins and sequelae of abnormal neuroendocrine function in polycystic ovary syndrome. *Human Reproduction Update* **12** 351–361. (doi:10.1093/humupd/dml017)
- Catteau-Jonard S, Jamin SP, Leclerc A, Gonzales J, Dewailly D & di Clemente N 2008 Anti-Müllerian hormone, its receptor, FSH receptor, and androgen receptor genes are overexpressed by granulosa cells from stimulated follicles in women with polycystic ovary syndrome. *Journal of Clinical Endocrinology and Metabolism* **93** 4456–4461. (doi:10.1210/jc.2008-1231)
- Chen H, Guo JH, Lu YC, Ding GL, Yu MK, Tsang LL, Fok KL, Liu XM, Zhang XH, Chung YW *et al.* 2012 Impaired CFTR-dependent amplification of FSH-stimulated estrogen production in cystic fibrosis and PCOS. *Journal of Clinical Endocrinology and Metabolism* **97** 923–932. (doi:10.1210/jc.2011-1363)
- Coffler MS, Patel K, Dahan MH, Malcom PJ, Kawashima T, Deutsch R & Chang RJ 2003 Evidence for abnormal granulosa cell responsiveness to

- follicle-stimulating hormone in women with polycystic ovary syndrome. *Journal of Clinical Endocrinology and Metabolism* **88** 1742–1747. (doi:10.1210/jc.2002-021280)
- Dickerson EH, Cho LW, Maguiness SD, Killick SL, Robinson J & Atkin SL** 2010 Insulin resistance and free androgen index correlate with the outcome of controlled ovarian hyperstimulation in non-PCOS women undergoing IVF. *Human Reproduction* **25** 504–509. (doi:10.1093/humrep/dep393)
- Dumesic DA & Richards JS** 2013 Ontogeny of the ovary in polycystic ovary syndrome. *Fertility and Sterility* **100** 23–38. (doi:10.1016/j.fertnstert.2013.02.011)
- Dumesic DA, Padmanabhan V & Abbott DH** 2008 Polycystic ovary syndrome and oocyte developmental competence. *Obstetrical & Gynecological Survey* **63** 39–48. (doi:10.1097/OGX.0b013e31815e85fc)
- Erickson GF, Magoffin DA, Garzo VG, Cheung AP & Chang RJ** 1992 Granulosa cells of polycystic ovaries: are they normal or abnormal? *Human Reproduction* **7** 293–299.
- Goodarzi MO, Dumesic DA, Chazenbalk G & Azziz R** 2011 Polycystic ovary syndrome: etiology, pathogenesis and diagnosis. *Nature Reviews. Endocrinology* **7** 219–231. (doi:10.1038/nrendo.2010.217)
- Jakimiuk AJ, Weitsman SR, Brzechffa PR & Magoffin DA** 1998 Aromatase mRNA expression in individual follicles from polycystic ovaries. *Molecular Human Reproduction* **4** 1–8. (doi:10.1093/molehr/4.1.1)
- Jayasena CN & Franks S** 2014 The management of patients with polycystic ovary syndrome. *Nature Reviews. Endocrinology* **10** 624–636. (doi:10.1038/nrendo.2014.102)
- Kar S** 2012 Clomiphene citrate or letrozole as first-line ovulation induction drug in infertile PCOS women: a prospective randomized trial. *Journal of Human Reproductive Sciences* **5** 262–265. (doi:10.4103/0974-1208.106338)
- Kirilovas D, Naessen T, Bergstrom M, Bonasera TA, Bergstrom-Pettermann E, Holte J, Carlstrom K, Simberg N & Langstrom B** 2003 Effects of androgens on aromatase activity and 11C-vorzole binding in granulosa cells *in vitro*. *Acta Obstetrica et Gynecologica Scandinavica* **82** 209–215. (doi:10.1034/j.1600-0412.2003.00144.x)
- Legro RS, Brzyski RG, Diamond MP, Coutifaris C, Schlaff WD, Casson P, Christman GM, Huang H, Yan Q, Alvero R et al.** 2014 Letrozole versus clomiphene for infertility in the polycystic ovary syndrome. *New England Journal of Medicine* **371** 119–129. (doi:10.1056/NEJMoa1313517)
- Mason HD, Willis DS, Beard RW, Winston RM, Margara R & Franks S** 1994 Estradiol production by granulosa cells of normal and polycystic ovaries: relationship to menstrual cycle history and concentrations of gonadotropins and sex steroids in follicular fluid. *Journal of Clinical Endocrinology and Metabolism* **79** 1355–1360. (doi:10.1210/jcem.79.5.7962330)
- McCartney CR, Eagleson CA & Marshall JC** 2002 Regulation of gonadotropin secretion: implications for polycystic ovary syndrome. *Seminars in Reproductive Medicine* **20** 317–326. (doi:10.1055/s-2002-36706)
- Misso ML, Wong JL, Teede HJ, Hart R, Rombauts L, Melder AM, Norman RJ & Costello MF** 2012 Aromatase inhibitors for PCOS: a systematic review and meta-analysis. *Human Reproduction Update* **18** 301–312. (doi:10.1093/humupd/dms003)
- Nejad ES, Saedi T, Saedi S, Rashidi BH, Nekoo ZA & Jahangiri N** 2011 Comparison of *in vitro* fertilisation success in patients with polycystic ovary syndrome and tubal factor. *Gynecological Endocrinology* **27** 117–120. (doi:10.3109/09513590.2010.501872)
- Ng EH, Chan CC, Yeung WS & Ho PC** 2005 Comparison of ovarian stromal blood flow between fertile women with normal ovaries and infertile women with polycystic ovary syndrome. *Human Reproduction* **20** 1881–1886. (doi:10.1093/humrep/deh853)
- Pavone ME & Bulun SE** 2013 Clinical review: The use of aromatase inhibitors for ovulation induction and superovulation. *Journal of Clinical Endocrinology and Metabolism* **98** 1838–1844. (doi:10.1210/jc.2013-1328)
- Payne AH & Hales DB** 2004 Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocrine Reviews* **25** 947–970. (doi:10.1210/er.2003-0030)
- Polyzos NP, Tsappi M, Mauri D, Atay V, Cortinovis I & Casazza G** 2008 Aromatase inhibitors for infertility in polycystic ovary syndrome. The beginning or the end of a new era? *Fertility and Sterility* **89** 278–280. (doi:10.1016/j.fertnstert.2007.10.016)
- de Resende LO, dos Reis RM, Ferriani RA, Vireque AA, Santana LF, de Sa Rosa e Silva AC & Martins Wde P** 2010 Concentration of steroid hormones in the follicular fluid of mature and immature ovarian follicles of patients with polycystic ovary syndrome submitted to *in vitro* fertilization. *Revista Brasileira de Ginecologia e Obstetrícia* **32** 447–453. (doi:10.1590/S0100-72032010000900006)
- Richards JS, Jahnsen T, Hedin L, Lifka J, Ratoosh S, Durica JM & Goldring NB** 1987 Ovarian follicular development: from physiology to molecular biology. *Recent Progress in Hormone Research* **43** 231–276. (doi:10.1016/B978-0-12-571143-2.50012-5)
- Roy KK, Baruah J, Singla S, Sharma JB, Singh N, Jain SK & Goyal M** 2012 A prospective randomized trial comparing the efficacy of letrozole and clomiphene citrate in induction of ovulation in polycystic ovarian syndrome. *Journal of Human Reproductive Sciences* **5** 20–25. (doi:10.4103/0974-1208.97789)
- Stocco C** 2008 Aromatase expression in the ovary: hormonal and molecular regulation. *Steroids* **73** 473–487. (doi:10.1016/j.steroids.2008.01.017)
- Zhong YP, Ying Y, Wu HT, Zhou CQ, Xu YW, Wang Q, Li J, Shen XT & Li J** 2012 Comparison of endocrine profile and *in vitro* fertilization outcome in patients with PCOS, ovulatory PCO, or normal ovaries. *International Journal of Endocrinology* **2012** 492803. (doi:10.1155/2012/492803)

Received 2 February 2015

First decision 23 February 2015

Revised manuscript received 17 July 2015

Accepted 21 July 2015