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High Levels of Testosterone Inhibit Ovarian Follicle Development by Repressing the FSH Signaling Pathway*

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Summary: The effect of high concentrations of testosterone on ovarian follicle development was investigated. Primary follicles and granulosa cells were cultured *in vitro* in media supplemented with a testosterone concentration gradient. The combined effects of testosterone and follicle-stimulating hormone (FSH) on follicular growth and granulosa cell gonadotropin receptor mRNA expression were also investigated. Follicle growth in the presence of high testosterone concentrations was promoted at early stages (days 1–7), but inhibited at later stage (days 7–14) of *in vitro* culture. Interestingly, testosterone-induced follicle development arrest was rescued by treatment with high concentrations of FSH (400 mIU/mL). In addition, in cultured granulosa cells, high testosterone concentrations induced cell proliferation, and increased the mRNA expression level of FSH receptor (FSHR), and luteinized hormone/choriogonadotropin receptor. It was concluded that high concentrations of testosterone inhibited follicle development, most likely through regulation of the FSH signaling pathway, although independently from FSHR downregulation. These findings are an important step in further understanding the pathogenesis of polycystic ovary syndrome.
Key words: testosterone; follicle; follicle-stimulating hormone; polycystic ovary syndrome

Ovarian follicle development plays a central role in mammalian fertility. Follicles go through multiple stages of maturation before formation of an antral cavity, a process that is regulated by the interplay of multiple hormones. In a normal menstrual cycle, follicle-stimulating hormone (FSH) promotes follicle growth during the early stages of antral follicle recruitment and development. Meanwhile, luteinizing hormone (LH) regulates the development and selection of the dominant follicle and ovulation, as well as the regression of smaller follicles during later stages of pre-ovulatory follicular development[1–5]. In this regard, the theca cells of antral follicles express LH receptors and produce androgens in response to LH stimulation. Conversely, granulosa cells express FSH receptors (FSHRs), the stimulation of which facilitates the conversion of androgens into estrogens via increased aromatase activity in these cells[6]. Interestingly, there appears to be a synergistic effect between androgens and gonadotropins in the ovary, with the former exerting a control over the initial stages of follicle maturation. In this regard, androgens enhance FSH-driven granulosa cell differentiation during the antral stage of follicle differentiation[7, 8].

The important role of androgens on normal ovarian function has been demonstrated in several *in vitro* and *in vivo* models. In all species studied, androgens promote their effects upon early follicular growth acting via the nuclear androgen receptor (AR) in granulosa cells[9]. However, in large antral follicles, androgens can also exert effects via their aromatization into estrogens, mainly estrone and 17β-estradiol[10]. In primates and rodents, androgens seem to have a stimulatory effect on the growth of small follicles[11], with subsequent AR downregulation as the follicles mature[12–14]. Similarly, in the bovine species, androgens support the transition from primary to secondary follicles[15]. In fact, *in vitro* folliculogenesis experiments in mice have shown that anti-androgenic compounds reduced follicle growth during

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the preantral phase with a consequent arrest in oocyte meiotic maturation[7]. Moreover, female mice in which the AR expression was specifically knocked out in granulosa cells and oocytes presented signs of subfertility such as premature ovarian failure, longer estrous cycles and fewer ovulated oocytes, more preantral and atretic follicles, as well as fewer antral follicles and corpora lutea[16]. Conversely, high androgen concentrations either from endogenous production in pathological processes or from pharmacological administration can also exert detrimental effects upon folliculogenesis. For instance, the high levels of testosterone present in a situation of hyperandrogenemia are typically converted into estrone via aromatase activity in peripheral adipose tissue[17]. The excessive estrone produced as a result continues to act upon the hypothalamic-pituitary axis, leading to a positive feedback on LH secretion and a negative feedback on FSH secretion. This leads to a situation of constitutively high LH levels and low FSH levels, eventually leading to follicular dysplasia[18].

In this context, a model representing endogenous hyperandrogenemia is that of polycystic ovary syndrome (PCOS), which is the most common endocrinopathy among women of reproductive age with a 5%–10% reported incidence worldwide. This syndrome is associated with an increased risk of type 2 diabetes and cardiovascular disease, and is an important contributor to anovulatory infertility and menstrual cycle abnormalities[19, 20]. Reportedly, PCOS patients frequently have increased concentrations of circulating testosterone[21, 22]. While it has been suggested that high androgen levels are associated to abnormal follicular development and decreased oocyte quality in PCOS patients[23, 24], the mechanism of action remains unclear. In support of these assumptions, testosterone induced follicle development arrest in a mouse PCOS model[25]. In addition, hyperandrogenemia increased follicular atresia and inhibited the proliferation of FSH-induced granulosa cells in rats; notably, of the two classical androgens, testosterone was more effective than dihydrotestosterone (DHEA) in promoting these effects[26]. Moreover, FSH treatment may have a positive effect on follicle recruitment and ovulation in patients with PCOS, consistent with an interaction between FSH and androgens during folliculogenesis[27, 28].

In this study, we investigated the role of testosterone in follicle development using an *in vitro* culture system.

1 MATERIALS AND METHODS

1.1 Animals

This study was approved by the Institutional Ethical Committee for the Use and Care of Laboratory Animals at the Chinese Academy of Science, Beijing, China. Fourteen-day-old CD1 female mice were purchased from the Animal Facility of the Institute of Genetics and Development Biology and housed in a temperature- and light-controlled facility with free access to water and food.

1.2 Follicle Isolation and *In Vitro* Culture

Ovary processing medium was prepared with α-minimum essential medium (α-MEM; Hyclone, USA) by the addition of 10% heat-inactivated fetal bovine serum (FBS; Hyclone, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. Culture medium Ⅰ contained α-MEM supplemented with 10% FBS, ITS-mix (insulin 5 mg/mL, transferrin 5 mg/mL, and selenium 5 ng/mL; Sigma-Aldrich, USA), and 100 mIU/mL recombinant FSH (Livzon Pharmaceutical Group Co., Ltd., China) or LH (Merck Serono, Switzerland). Culture medium Ⅱ was similar to culture medium Ⅰ except for containing 5% FBS instead.

Follicle culture was carried out as previously reported[5]. Ovaries were removed aseptically from 14-day old mice. The tissue adhering to ovaries was removed using the beveled edges of two 25 Ga syringe needles. The ovaries were transferred to a dish with prewarmed (37°C) ovary processing medium and mechanically dissected using the beveled edges of two 25 Ga syringe needles. Follicles with one layer of granulosa cells, a centrally placed oocyte, an intact basal membrane, and some attached theca cells were collected and cultured individually in 10-μL droplets of culture medium Ⅰ overlaid with paraffin oil (Sigma-Aldrich, USA). Culture dishes were kept in the incubator at 37°C and with 5% CO_2 in humidified air. After 4 days of culture, 10 μL of culture medium Ⅰ was added to each droplet. From day 6 onward, culture medium Ⅰ was replaced with culture medium Ⅱ; subsequently, medium in each droplet was completely replaced every other day. Follicles were measured on an inverted microscope using a SPOT camera and image analysis software (Diagnostic Instruments, Sterling Heights, USA). Diameters were calculated by taking the average of two measurements, aligned at right angles to each other, passing through the center of the follicle. Viable follicles were defined as those that retained an oocyte completely embedded within the granulosa cell mass, and the survival rate was expressed as a percentage of all plated follicles.

To study the effects of testosterone on follicular growth, a concentration gradient of 1×10^{-6}, 1×10^{-5}, and 1×10^{-4} mol/L of testosterone was tested. These concentrations were chosen based upon the study by Duda *et al*[29], where hyperandrogenism was defined by a concentration of 1×10^{-5} mol/L of testosterone. Follicles were incubated for 14 days in medium containing no testosterone (negative control) or one of the three test concentrations of testosterone.

To investigate the potential antagonistic effects of FSH on testosterone-induced abnormal follicular growth, FSH was added to the culture medium at concentrations of 100 (baseline), 200, 400, 800, 1000, or 10 000 mIU/mL. A concentration of testosterone of 1×10^{-5} mol/L was chosen based upon the results of the previous experiments. Controls contained no testosterone and only baseline FSH concentration in the follicle culture medium. Follicular growth was checked every day during 14 days.

1.3 Granulosa Cells Culture

Granulosa cells were collected from primary follicles as previously reported[30]. Briefly, after mechanical dissection, follicles were digested in DMEM/F12 (Hyclone, USA) medium containing 1 mg/mL collagenase Ⅰ, 0.025% trypsin, and 0.02 mg/mL deoxyribonuclease Ⅰ (Sigma-Aldrich, USA) for 30–45 min at 37°C. After

two washes, granulosa cells were seeded with DMEM/F12 culture medium, supplemented with 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate, and cultured overnight for adhesion. Then, cells were cultured in fresh medium with or without the addition of the various reagents for the indicated time period.

1.4 MTT Test

Granulosa cells were seeded in 96-well plates and incubated in fresh medium in the presence of testosterone (1×10^{-7}, 1×10^{-6}, 1×10^{-5}, 1×10^{-4}, or 1×10^{-3} mol/L) for 24 and 48 h to monitor cell proliferation using the MTT assay. At each reading time, the MTT reagent (20 μL) was added into each well and incubated for 4 h. The supernatant was removed and the cells were treated with 150 μL/well DMSO for 10 min. Absorbance (*A*) was recorded at 570 nm using an ELISA plate reader (BioTek ELx808, USA).

1.5 RNA Extraction and Real-time PCR

Follicles and granulosa cells were lysed with TRIzol reagent (Invitrogen, USA). Total RNA was extracted according to the manufacturer's instructions. Two micrograms of total RNA were reverse-transcribed in a final volume of 25 μL containing random primers, Moloney murine leukemia virus reverse transcriptase, reaction buffer, deoxynucleotide triphosphate, and ribonuclease inhibitor. Primer pairs used for real-time PCR were as follows: FSHR [forward primer sequence: (5'–3') TGCTACACCCACATCTACCT, reverse primer sequence: (5'–3') GCACCTCATAACAGCCAAAC]; luteinizing hormone/choriogonadotropin receptor (LHCGR) [forward primer sequence: (5'–3') AATCTCTCCTTTGCAGACTTTTG, reverse primer sequence: (5'–3') AGCATAGGTGATGGTGTGCCA]. Real-time PCR was carried out using LightCycler®480 sequence detection system (Roche Applied Science, Germany). The mRNA expression levels of the test transcripts were normalized against those of the housekeeping control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and analyzed using the comparative cycle threshold method.

To test the effects of FSH or FSH and testosterone on FSHR and LHCGR expression in granulosa cells, the following experimental groups were set up: control group (CTR, 100 mIU/mL FSH); testosterone group (T, 100 mIU/mL FSH+T); 200, 400, 800, and 1000 mIU/mL FSH+testosterone groups (200, 400, 800, and 1000 mIU/mL FSH+T).

1.6 Statistical Analysis

The data were evaluated for statistical differences using SPSS 13.0 computer software (SPSS Inc., USA). One-way ANOVA followed by a least-significant-difference test was used for statistical comparisons among multiple groups. $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 Effect of Testosterone On the Development of *In Vitro* Cultured Follicles

To examine the effect of high concentrations of testosterone on ovarian follicle development, primary follicles isolated from the ovaries of 2-week-old female mice were cultured and treated with serial concentrations of testosterone. During the early growth stages, follicle development was not markedly different between the testosterone treated and control groups (fig. 1). Interestingly, while testosterone treatment increased follicle size at 7 days, treatment for a longer period (i.e. 14 days) had a negative effect on follicle size in comparison to the control group (table 1). Moreover, both the positive and negative effects of testosterone were directly proportional to the concentration used. In addition, the number of abnormal follicles was greatest ($P<0.05$) with the 1×10^{-4} mol/L testosterone treatment (table 2). Based upon our findings, we selected 1×10^{-5} mol/L of testosterone for further experiments.

Table 1 The effect of different concentrations of testosterone on the diameter of *in vitro* cultured follicles

Groups	Total follicle number	Follicle mean diameter (μm)			
		Day 7	*P*	Day 14	*P*
Control	30	100.0±2.3		521.3±3.3	
T (mol/L)					
10^{-6}	30	130.2±5.7*	0.043	512.3±2.9*	0.049
10^{-5}	30	138.5±2.2*	0.035	433.4±5.3*	0.033
10^{-4}	30	146.7±6.2*	0.019	332.8±7.1*	0.008

Results are shown as $\bar{x}\pm s$. *$P<0.05$ *vs.* control group
T: testosterone

Table 2 The effect of different concentrations of testosterone on the follicle development

Groups	Total follicle number	Day 7		Day 14	
		Live follicle number (%)	Dead follicle number (%)	Live follicle number (%)	Dead follicle number (%)
Control	30	29 (96.67)	1 (3.33)	29 (96.67)	1 (3.33)
T (mol/L)					
10^{-6}	30	27 (90.00)	3 (10.00)	26 (86.67)	4 (13.33
10^{-5}	30	26 (86.67)	4 (13.33)	25 (83.33)	5 (16.67)
10^{-4}	30	24 (80.00)*	6 (20.00)*	22 (73.33)*	8 (26.67)*

Results are shown as $\bar{x}\pm s$. *$P<0.05$ *vs.* control group
T: testosterone

2.2 Effect of FSH Addition in Testosterone-supplemented Follicle Culture Medium

A concentration gradient FSH was tested in testosterone-supplemented culture medium to assess the interaction of the two hormones upon follicle development. Interestingly, we found that testosterone-induced follicle development arrest was rescued by 400 mIU/mL FSH, with an increase (P<0.05) in the number of normal follicles at the 14-day time point (fig. 2; table 3). Conversely, treatment with 800 mIU/mL FSH significantly inhibited follicle development, so that approximately 50% of follicles had stopped developing or were dead by 14 days. These results were consistent with the notion that high concentrations of circulating testosterone may inhibit follicle development by exerting a negative feedback on FSH secretion.

Table 3 Effect of FSH addition to testosterone-supplemented culture medium on *in vitro* follicle development

Groups	Total follicle number	Live follicle number (%)	Dead follicle number (%)
Control	11	10 (90.9)	1 (9.1)
T	11	8 (72.7)	3 (27.3)
200 mIU/mL FSH+T	11	8 (72.7)	3 (27.3)
400 mIU/mL FSH+T	12	10 (83.3)*	2 (16.7)*
800 mIU/mL FSH+T	12	4 (33.3)#	8 (66.7)#

Results are shown as $\bar{x}\pm s$. *P<0.05 *vs.* T group, #P<0.05 *vs.* control group
T: 10^{-5} mol/L testosterone

2.3 Effect of Testosterone on Granulosa Cell Proliferation

The experiments above showed that exposure to high testosterone concentrations for longer than 7 days had a detrimental effect on follicular growth, which was consistent with previous studies showing that high androgen concentrations could induce mouse granulosa cell apoptosis *in vitro*, leading to follicular atresia[31]. In this study, we assessed the effect of a concentration gradient of testosterone on granulosa cell proliferation at both 24 and 48 h in the culture. The lowest concentration tested (1×10^{-7} mol/L) yielded a significant increase in granulosa cell proliferation at both time periods as compared to control untreated cells (fig. 3). The response was gradually declined with higher concentrations of testosterone. Notably, the highest testosterone concentration used (1×10^{-3} mol/L) inhibited cell proliferation.

2.4 Effect of Testosterone and FSH Treatment on the Expression of FSHR and LHCGR in Granulosa Cells

In an attempt to understand the mechanism of action of testosterone upon granulosa cell proliferation and potential interaction with gonadotropins, we used RT-qPCR to assess FSHR and luteinizing hormone/choriogonadotropin receptor (LHCGR) expression in treated cells. We found that FSHR expression was increased after testosterone treatment, although increasing the dose of FSH above baseline did not have any further effects. Conversely, while testosterone also increased LHCGR expression, high doses of FSH did block testosterone-induced LHCGR expression.

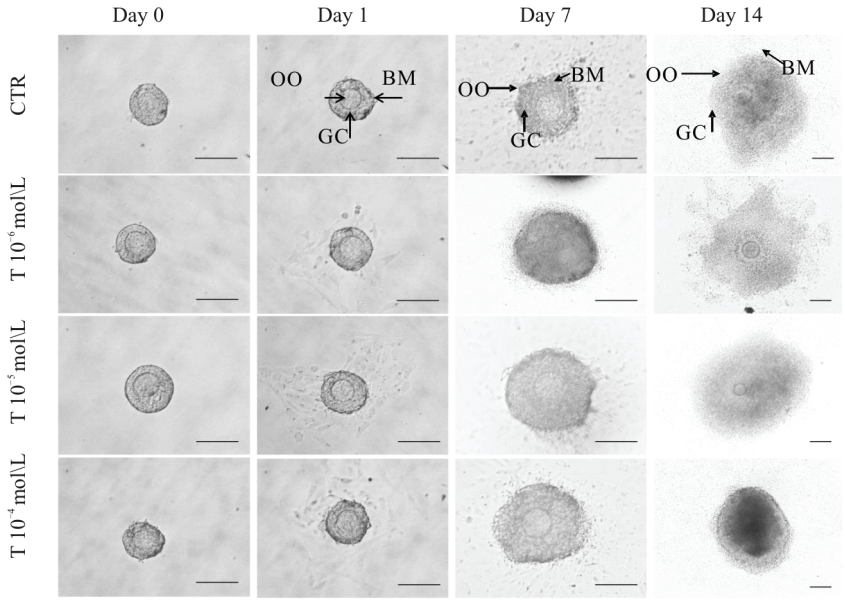

Fig. 1 Representative images of primary follicles cultured *in vitro* in the absence or presence of different concentrations of testosterone
Follicles were cultured with medium containing 0 (CTR) or 1×10^{-6}, 1×10^{-5} or 1×10^{-4} mol/mL of testosterone (T). Follicle development was recorded by daily measurements of diameter using phase contrast microscopy at day 1–14.
OO: oocytes; BM: basement membrane; GC: granulosa cells. Scale bar: 100 μm

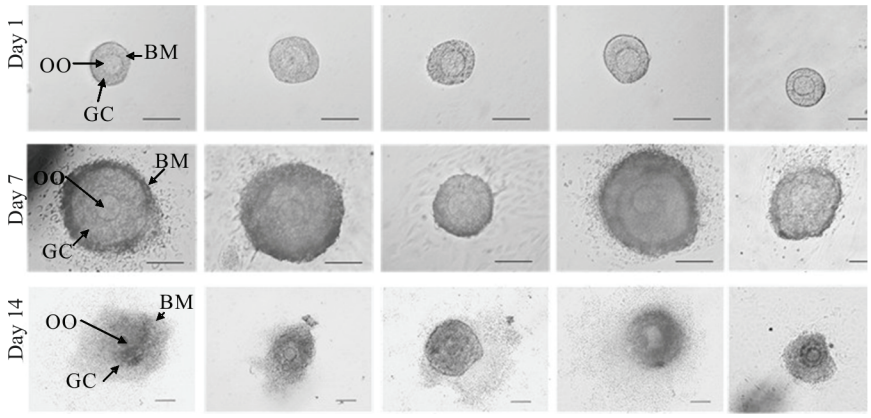

Fig. 2 Representative images of primary follicles cultured in the presence of testosterone and different FSH concentrations
The culture medium contained no testosterone and baseline FSH (100 mIU/mL; CTR), or 1×10^{-5} mmol/L testosterone (T) with 100, 200, 400 or 800 mIU/mL FSH respectively. Follicle development was recorded by daily measurements of diameter using phase contrast microscopy at days 1–14. The arrest of follicle development induced by high concentrations of testosterone after 14 days of culture was partially rescued by treatment with 400 mIU/mL FSH. Conversely, 14-day culture in the presence of 800 mIU/mL of FSH inhibited follicle growth. OO: oocytes; BM: basement membrane; GC: granulosa cells. Scale bar: 100 μm

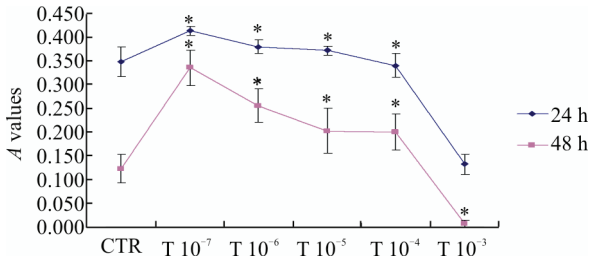

Fig. 3 Effect of different testosterone concentrations on proliferation of granulosa cells cultured *in vitro* for 24 or 48 h
Cell proliferation was promoted by 1×10^{-7}–1×10^{-4} mol/L of testosterone (T), but inhibited by 1×10^{-3} mol/L of testosterone. $^{*}P<0.05$ *vs.* control (CTR) samples without testosterone

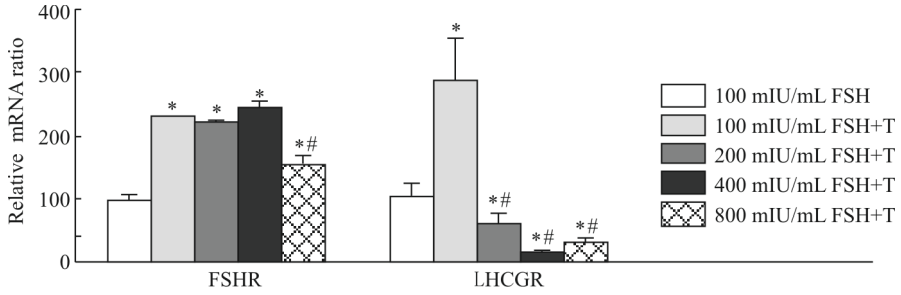

Fig. 4 FSHR and LHCGR expression in granulosa cells cultured *in vitro* in medium containing testosterone and different concentrations of FSH-treated granulosa cells
Control (CTR) samples contained 100 mIU/L FSH and no testosterone. Treated samples contained 1×10^{-5} testosterone (T) and 100, 200, 400, or 800 mIU/mL FSH. Testosterone treatment increased FSHR expression, with no difference observed when increasing the concentration of FSH. Conversely, testosterone increased LHCGR expression, which was blocked by high FSH doses. $^{*}P<0.05$ *vs.* CTR group, $^{\#}P<0.05$ *vs.* 100 mIU/mL FSH+T

3 DISCUSSION

Corroborating the statements above, in this study we sought to analyze the potential effects of high testosterone upon follicle growth and granulosa cell proliferation in an attempt to understand the potential pathogenic effects of high androgen concentrations on ovarian function. This is particularly relevant in women with PCOS, a common endocrine disorder characterized by a status of hyperandrogenemia[32]. The scenario of high androgens is also worth considering in the context of dehydroepiandrosterone (DHEA) administration during stimulation protocols for assisted reproduction in women with diminished ovarian reserve. In fact, long term DHEA administration may result in the development of a PCOS-like syndrome both in mice and women[33–36]. While different animal models have been used to assess the effects of high concentration androgen treatment, none of them successfully mimicked all features of human PCOS[37]. Moreover, there is no consensus regarding the effect of high androgen concentrations on follicle growth. For instance, administration of high testosterone concentrations, at least in a short term basis, induced granulosa cell proliferation and prolonged follicle life

span in prepuberal primates[38]. Moreover, hyperandrogenemia inhibited apoptosis of granulosa and theca-interstitial cells, and promoted follicle development in several mammalian species[39–41]. However, in another study, high androgen concentrations increased follicular atresia and inhibited the proliferation of FSH-induced granulosa cells in rats[26]. Herein we used an *in vitro* model of primary follicle culture to test the effects of high testosterone concentrations on follicle growth in mice. Our experiments clearly revealed that a high concentration of testosterone initially promoted the early stages of follicle development, but then inhibited and even arrested follicle growth at later stages of follicle development. This is consistent with the findings that administration of DHEA to mice not only increased the growth rate of primary follicles, but also had adverse effects upon follicular growth, and increased oxidative stress and apoptotic rate in ovarian cells[42, 43]. Altogether, these results support the assumption that high concentrations of testosterone may play a role in the follicle development disorders observed in PCOS patients.

Notably, in clinical practice, high doses of FSH are required for controlled ovarian hyperstimulation in PCOS patients, suggesting a lower sensitivity of the follicle to FSH in PCOS as compared to unaffected patients. We therefore speculated that high androgen levels may block the FSH signaling pathway and consequentially inhibit follicle development. To test this hypothesis, granulosa cells were cultured in the presence of testosterone and an increasing FSH concentration gradient. We found that high doses of FSH partially rescued the defect in follicle development induced by testosterone. To further understand the mechanism by which FSH could exert this effect, we tested FSHR expression in treated granulosa cells. We concomitantly checked the expression of the LHCGR because of the close link between pituitary gonadotropin signaling. Interestingly, we found that the FSHR expression was increased in granulosa cells after testosterone treatment, and this did not change with increasing concentrations of FSH. Testosterone treatment also increased expression of LHCGR in follicles, while this effect was counteracted by high FSH concentrations. Androgens may affect folliculogenesis directly via ARs or indirectly through aromatization to estrogen. Moreover, ARs are highly expressed in the granulosa and theca cells of early stage follicles, with lower expression in mature follicles. Thus, short-term androgen exposure augmented FSH receptor expression in the granulosa cells of developing follicles and enhanced the FSH-induced cAMP formation necessary for the transcription of genes involved in the control of follicular cell proliferation and differentiation[44], corroborating our findings. In this study, we also found that testosterone induced granulosa cell proliferation. In this regard, high levels of androgen promoted recruitment of large numbers of immature follicles through the inhibition of granulosa cell apoptosis, and the suppression of the FSH-induced aromatase activity[45–49]. Altogether, these results are consistent with the findings of high testosterone concentrations inhibiting later stages of follicle development through repression of FSH signaling independent from receptor downregulation. Although the exact mechanism requires further investigation, previous results in our laboratory also elulidated that FSHR and LHCGR gene were closely related to the etiology of PCOS[50].

In summary, we show that high levels of testosterone inhibit follicle development presumably via FSH signaling inhibition, although independently from LHCGR downregulation. High levels of FSHR and/or LHCGR expression are likely involved in the pathogenesis of PCOS[50]. Therefore, our study provides further insight into the mechanisms underlying PCOS in women, and hence this information may be useful for developing treatment strategies for this disorder.

Conflict of Interest Statement

The authors declare that they have no competing interests.

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