RESEARCH ARTICLE

Functional Investigation on Aromatase in Endometrial Hyperplasia in Polycystic Ovary Syndrome Cases

Pan-Lin Zhao^{1, 2}, Qiu-Fang Zhang¹, Li-Ying Yan¹, Shuo Huang¹, Yuan Chen¹, Jie Qiao^{1*}

Abstract

<u>Objective</u>: To explore the possible significance of aromatase P450 in endometrial hyperplasia with a background of polycystic ovary syndrome (PCOS). <u>Methods</u>: Immunohistochemistry was used to determine the expression of aromatase P450 in endometrium of PCOS patients. Semiquantitative analysis of aromatase P450 expression of mRNA and protein level wasalso carried out by real-time quantitative RT-PCR method. After endometrial cells were stimulated by testosterone and letrozole *in vitro*, the estradiol (E_2) level was determined, and the expression of cell aromatase P450 mRNA was assessed. <u>Results</u>: The aromatase P450 mRNA level was increased in endometria of PCOS patients. When endometrial cells were cultured with 10⁻⁶ M testosterone, the E_2 level in the culture medium increased. An inhibitory effect on E_2 generation and expression of aromatase P450 mRNA was observed when the endometrial cells were treated with 10⁻⁵ M letrozole. <u>Conclusions</u>: There is an increased expression of aromatase P450 in PCOS patient endometrium. Androgen stimulation could enhance the synthesis of aromatase P450 mRNA and the production of E_2 in endometrial cells *in vitro* while letrozole could do the reverse.

Keywords: Polycystic ovary syndrome - endometrial hyperplasia - aromatase P450 - letrozole -testosterone

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most common diseases in gynecology and endocrinology clinic (Lin et al., 2014; Tokmak et al., 2014). Its main manifestations are menstrual disorder, continuous anovulation, hyperandrogenism and polycystic ovary etc. As PCOS patients suffer from long-term anovulation and the endometrium lacks the effect of progestin due to long-term exposure to estrogen, the morbidity of endometrial hyperplasia and endometrial cancer increases significantly, up to 23% (Park et al., 2011). The abnormality of steroid hormone is one of the manifestations of PCOS. Therefore, aromatase, the key rate-limiting enzyme in the synthesis of steroid hormone, becomes a focus of numerous research.

Aromatase cytochrome P450 (P450arom, CYP) encoding by CYP19 gene is a rate-limiting enzyme in the final step of the estrogen biosynthesis related to estrogendependent diseases, such as breast cancer, hysteromyoma, endometriosis and endometrial cancer (Sen et al., 2012). The promoters of CYP19 gene are tissue-specific (Bulun et al., 2004). The promoter II and promoter I.3 are used in human malignant endometrial tumors (Bulun et al., 1994). In 1982, Tseng firstly reported the existance of the aromatase in endometrial tissues (Tseng et al., 1982). In 1995, Watanabe reported that there was no expression of P450arom in normal endometrium and proliferative endometrium, but overexpression in stromal cells of cancer infiltration sites with enhanced activity (Watanabe et al., 1995). In recent years, Maia had found that aromatase expression in the endometrium was associated with the presence of dysmenorrheal and endometriosis and there was a tendency for aromatase expression to be positively correlated with dysmenorrheal severity (Maia et al., 2012). Some scholars investigated aromatase expression of patients with endometrial cancer and concluded that endometrioid endometrial cancer showed relatively higher expression of aromatase compared with healthy mucosa (Knapp et al., 2013) and aromatase inhibitors showed promise in treatment (Gao et al., 2014).

Recent researches also indicate that hyperandrogenism, hyperinsulinemia and abnormality of the insulin-like growth factors system can have a certain effect upon the nosogenesis of PCOS in addition to the absence of antiestrogen environment, but it still needs further exploration whether these factors are related to the expression of aromatase and how aromatase works in abnormal endometrial hyperplasia of PCOS.

This research aims to study the expression and possible effect of aromatase in the abnormal endometrial hyperplasia of PCOS patients by immunohistochemical and quantitative RT-PCR method.

¹Reproductive Center, Peking University Third Hospital, ²Reproductive Center, Airforce General Hospital, PLA, Beijing, China *For correspondence: jie.qiao@263.net

Materials and Methods

Objects

Patients with PCOS in Reproductive Center of Peking University Third Hospital from Mar., 2012 to Mar., 2013 were selected as research group. All the patients were diagnosed according to diagnostic criteria for PCOS resulted from ESHRE/ASRM consensus conference in May 2003, that is, the diagnosis was finalized if any two of the following three were met and other causes were ruled out: 1.The presence of polycystic ovary according to ultrasound indication, 2. clinical or biochemical hyperandrogenism, 3. anovulatory dysmenorrhea. The included cases in this research all suffered from hyperandrogenism. Another 20 female patients with infertility in the same period due to male factors or/and oviduct factors were selected as control group in which female patients with adenomyosis and submucous uterine myoma were ruled out. All patients in control group had regular menstruation with the menstrual period of 3~7 days and the menstruation cycle of 28~32 days.

Methods

Specimen collection: 1. Endometria in proliferative period: A total of 10 patients in research group without adenomyosis or submucous uterine myoma and 10 patients in control group were performed hysteroscopy and endometrial biopsy during the period of Day 7~12 in one menstrual cycle and collected a small amount of endometria. Afterwards, pathologic diagnosis confirmed that they were proliferative endometria without endometrial lesions. 2. Endometria in secretory period: The ovulation of 13 cases in research group and 10 cases in control group who had no sexual life for 2 weeks were observed through vaginal ultrasound. A small amount of endometria were collected 6~8 days after ovulation. Afterwards, pathologic diagnosis confirmed that they were secretory endometria without endometrial lesions. After the acquisition of the above materials, 4% paraformaldehyde was used immediately to fix some tissues for immunohistochemical analysis; other tissues were washed rapidly with physiological saline and placed into the liquid nitrogen for mRNA extraction.

Preparation of endometrium for in vitro culture: Females in control group who received hysteroscopy and endometrial biopsy due to suspected diagnosis of uterine cavity structural abnormalities during the period of 7~12 days in one menstrual cycle were selected and collected a small amount of endometria. Afterwards, pathologic diagnosis confirmed that they were proliferative endometria without endometrial lesions. Each 2~4 specimens were mixed and placed in precooled PBS at the temperature of 4°C, and then fractional cultivation of endometrial epithelial cells and stromal cells was performed.

Immunohistochemistry: Endometrial tissues fixed by 4% paraformaldehyde were embedded by paraffin, cut into slices, and then dewaxed. Antigens were repaired, incubated by primary antibody (aromatase polyclonal antibody stemming from rabbits, 1:75, 4°C incubation overnight), washed by PBS, incubated by second antibody, washed by PBS, incubated by horseradish peroxidase,

washed by PBS, stained by DAB, counterstained with Hematoxylin, mounted and analyzed.

Primary culture of endometrium cells: Endometrial tissues were digested in vitro with 0.1% collagenase IV and filtered by microporous membrane to separate the stromal cells from the gland clumps of epithelial cells. Both cells were incubated evenly in the culture dish for 24 hours, and then cultured in serum free DMEM/F 12 culture solution for another 24 hours.

<u>Testosterone intervention</u>: In research group, the culture solution was changed into serum free DMEM/F12 culture solution with testosterone at a final concentration of 10⁻⁶, 10⁻⁷, ⁻⁸ and 10⁻⁹ M respectively. In control group, the equal amount of anhydrous ethanol was added instead of testosterone. The cultivation was kept for 48 hours. The culture solution was collected for the determination of the concentration of estradiol (E₂) and cells for the extraction of total RNA.

Letrozole intervention: In research group, the culture solution was changed into serum free DMEM/F12 culture solution with a final concentration of 10^{-7} M testosterone, 10^{-7} M testosterone + 10^{-5} M letrozole, 10^{-7} M testosterone+ 10^{-6} M letrozole, 10^{-7} M testosterone+ 10^{-7} M letrozole respectively. In control group, the equal amount of anhydrous ethanol was added. The cultivation was kept for 48 hours. The culture solution was collected for the determination of the concentration of E₂ and cells for the extraction of total RNA.

Detection of mRNA level of aromatase in tissues or cells: Total RNA of tissues or cells was extracted and its concentration was measured. Internal reference gene GAPDH was used in reverse transcription of 2.5 ug RNA for the synthesis of cDNA and real-time quantitative PCR as a reference to calculate the relative CT value of each target gene. $\Delta\Delta$ Ct=Ct value of target gene in research group - Ct value of corresponding reference value - Ct value of target gene in control group. Use $2^{-\Delta\Delta Ct}$ to express the ratio of relative amount of gene template. CYP19I.3 primer sequence: Forward CAC TCT ACC CAC TCA AGG GCA, Reverse TTG GCT TGA ATT GCA GCA TTT; CYP19IIa primer sequence: Forward CAG GAG CTA TAG ATG AAC CTT TTA GGG, Reverse CTT GTG TTC CTT GAC CTC AGA GG; GAPDH primer sequence: Forward AAC AGC GAC ACC CAC TCC TC, Reverse CAT ACC AGG AAA TGA GCT TGA CAA.

<u>Determination of E_2 in culture solution</u>: Culture solution was absorbed and centrifugated at 300rcf for 5 min. Then E_2 was determined by chemoluminescence method.

Statistical data analysis

SPSS 19.0 statistical analysis software was applied. Measurement data were expressed by Mean \pm SD and analyzed by t test. *P*<0.05 was considered to be statistically different.

Results

Expression of aromatase in endometria of research group and control group

Comparison of aromatase mRNA expression: The ratio



Figure 1. Expression of Aromatase mRNA in Endometria of Research Group and Control Group



Figure 2. Expression of Aromatase in Endometria ($\times 20$). (A) Proliferative endometrium of control group; (B) Secretory endometrium of control group; (C) Proliferative endometrium of research group; (D) Secretory endometrium of research group; (F) Positive control of placental tissue; (G) Negative control of placental tissue; (H) Negative control of endometrial tissue

of CYP19I.3 mRNA in proliferative period in research group and control group of was 2.76 ± 0.51 and the value of CYP19 II.3 mRNA was 5.46 ± 0.78 . The relative amount of CYP19I.3 mRNA and CYP19 II.3 mRNA in research group was significantly higher than that in control group (*P*<0.05) (Figure 1).

Expression of aromatase protein: Aromatase was expressed both in endometria of control group and research group. The expression level of aromatase in proliferative endometrium was higher than that in secretory endometrium; the expression level of aromatase in glandular epithelia and stromal cells in proliferative endometrium of research group was considerably higher than that in control group; in terms of secretory endometrium, there was no significant difference between research group and control group. The specifics were shown in Figure 2.

Changes of aromatase mRNA after endometrial cells were treated with testosterone and letrozole

Changes of aromatase level after dealing with



Figure 3. Changes of Expression of CYP19I.3 mRNA and CYP19IIa mRNA in Endometrial Epithelial Cells and Stromal Cells After Treating with Different Concentration of Testosterone. Compared with control group, *P<0.05



Figure 4. Changes of Expression of CYP19I.3 mRNA of Endometrial Epithelial Cells and Stromal Cells After 24, 48, 72 Hours' Being Dealt with 10⁻⁷ M Testosterone



Figure 5. Changes of Expression of CYP19I.3 and O CYP19II2a mRNA After the Endometrial Epithelial Cells and Stromal Cells were Dealt with 10⁻⁷ M Testosterone and Different Concentrations of Letrozole

testosterone: Results of real-time quantitative RT-PCR indicated that the expression level of CYP19I.3 mRNA and CYP19IIa mRNA in research group increased compared with control group, and the increase was the most obvious when the concentration of testosterone was 10⁻⁷ M (Figure 3). When endometrial epithelial cells were treated with 10⁻⁷ M testosterone for 24, 48 and 72 hours, the expression level of CYP19I.3 mRNA increased by 1.12 folds, 2.01 folds and 1.53 folds, respectively. And the values of endometrial stromal cells increased by 2.21 folds, 5.58 folds and 7.69 folds, respectively (Figure 4).

<u>Changes of aromatase levels after being dealt with</u> <u>testosterone and letrozole</u>: After 48 hours' treatment with 6

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mixture of 10⁻⁷ M testosterone and 10⁻⁵ M, 10⁻⁶ M, 10⁻⁷ M letrozole respectively, the expression of CYP19I.3 mRNA of endometrial stromal cells dropped progressively, only those dealt with 10⁻⁵ M letrozole had statistical significance, CYP19IIa mRNA expression showing no significant difference (Figure 5).

Changes of E_2 in endometrial cells after being dealt with testosterone and letrozole

After being treated with 10^{-6} M testosterone, the concentration of E_2 in endometrial epithelial cells and stromal cells increased significantly while after being dealt with 10^{-7} M, 10^{-8} M and 10^{-9} M testosterone, the E_2 content had no significant change (Table 1). When endometrial epithelial cells were dealt with 10^{-5} M letrozole + 10^{-6} M testosterone, the drop of E_2 content was the most significant. When stromal cells were dealt with 10^{-5} M letrozole + 10^{-6} M testosterone and 10^{-6} M letrozole + 10^{-6} M testosterone, the E_2 content dropped, the difference was statistically significant and the difference generated by 10^{-5} M letrozole + 10^{-6} M testosterone was more significant (Table 2).

Discussion

Aromatase is the rate-limiting enzyme in the last step of transformation from androgen to estrogen and it is widely present at various tissues, including placenta, sex gland, epithelial and stromal cells of mammary gland etc. (Ghosh et al., 2010; Kim et al., 2012; et al., 2013; et al., 2013; Yoshimoto et al., 2014). When it comes to the expression of aromatase in normal endometrium and whether it has activity, it is commonly considered that endometrial cancer is an estrogen-dependent disease and aromatase is widely expressed in endometrial cancer tissues (Hosono et al., 2011; Liu et al., 2013; Thangavelu et al., 2013). This research confirms that aromatase is expressed in both endometrial epithelial cells and endometrial stromal cells. The expression in epithelial cells mainly occurs in cell membrane and cell nucleus, while the expression in stromal cells mainly occurs in cytoplasm and cell membrane, which may be associated with the function. Aromatase is secreted beside stromal cells and acts on epithelial cells, and the expression of aromatase in epithelial cells mainly takes place in cell membrane and the nucleus, which is closely associated with the fact that local androgen is converted into estrogen and the epithelial cells proliferate.

It is universally known that, as for PCOS patients, the possibility of endometrial hyperplasia and endometrial cancer increases, which is considered to be associated with the long-term anovulation and long-term exposure of endometrium to estrogen (Peigné et al., 2014). This research selects endometria of PCOS patients with hyperandrogenism as subjects. The experimental results showed that the expression of local aromatase in endometria of PCOS patients was greater than that in endometria of normal women, indicating that the possibly increased androgen was converted to estrogen catalyzed by local aromatase in endometria. The excessive simulation of local estrogen for a long time is also a mechanism for the occurrence of endometrial hyperplasia and endometrial cancer.

Aromatase is coded by CYP19 gene located in human chromosome 15q21.2, with a full length of 123kb, consisting of a 30kb coding region and a 93kb regulatory region containing at least 10 promoters (Anthoni et al., 2012). The promoter in the regulatory region has the tissue specificity. In terms of female cancers such as endometrial cancer and breast cancer, the expression of aromatase mainly relies on the effect of Promoter *P* II and *P* I.3 (Bulun et al., 2008; Khan et al., 2011). The results of this research indicated that as for endometria in control group and research group, *P* II a and *P* I.3 might jointly played a role, while as for endometria in research group, the higher expression of *P* II a might be associated with the PCOS endometrial hyperplasia and a high incidence of canceration.

It is still unclear that the state of hyperandrogenism of PCOS patients is directly associated with endometrial hyperplasia. Endometrium has aromatase activity locally, and whether the enhanced androgen will be aromatized into estrogen and locally stimulate the cell growth of endometrium becomes a common concern for researchers. Some scholars believe that estrogen synthesis in local endometrium plays an important role in the occurrence of endometrial cancer. In the presence of androgen substrate, the aromatase activity plays a certain role in cell proliferation. It is considered that the transcription of genes related to cell cycle will be activated hereby so that the endometrial hyperplasia will occur.

Letrozole is a third generation of non-steroid aromatase inhibitor, and it can effectively combine with the active center of aromatase with an inhibiting effect upon over 99% enzymes (Li et al., 2014). There have been clinical researches claiming that letrozole is effective in treating endometrial hyperplasia and endometrial carcinoma (Barker et al., 2009; Tabatabaie et al., 2013). This research indicates that as for the endometrial epithelial cells and stromal cells, adding 10⁻⁵ M letrozole can considerably reduce the E_2 content due to the addition of testosterone. The mechanism of letrozole regulating the expression of aromatase mRNA is not clear currently. It has been known that the expression regulation of aromatase is connected with COX-2-PGE, system, .but whether letrozole interferes with a certain segment in the regulatory pathway for expression needs to be further explored.

To sum up, this research indicates that the aromatase expresses in the endometrium, the state of hyperandrogenism for PCOS can induce the overexpression of aromatase mRNA, and the generation amount of local E_2 will increase. It also demonstrates that letrozole plays a down-regulatory role in the expression of aromatase mRNA in endometrial cells, and can also reduce the generation amount of local PGE₂. However, there is no profound study on the mechanism of this action.

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