Endocrine Traits of Polycystic Ovary Syndrome in Prenatally Androgenized Female Sprague-Dawley Rats

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Abstract. Although hyperandrogenism is an important condition and is considered the possible pathogenesis behind polycystic ovary syndrome (PCOS), data supporting this is still scarce. We sought to determine whether or not prenatal androgen exposure leads to PCOS and the possible cellular mechanisms involved. To induce prenatal androgen exposure, pregnant rats were treated with daily subcutaneous injections of free testosterone (T) or dihydrotestosterone (DHT) from embryonic days 16 to 19, and their female offspring were studied as adults. The mRNA expression of the progesterone receptor (PR) in the preoptic area (POA) hypothalamus was higher in the experimental groups than in the control group after ovariectomy and stimulation with estradiol benzoate. The levels of T, P, luteinizing hormone (LH), and estradiol were higher in the experimental groups than in the control groups. The frequency and magnitude of LH secretion was increased in experimental rats as compared with the control group. The anogenital distance of the experimental groups was prolonged and the nipple number was lower than that of the control group. Almost all experimental rats had prolonged or irregular estrous cycles. The experimental groups had fewer corpus luteum and preovulatory follicles and more preantral follicles and antral follicles than the controls. Our findings are consistent with the hypothesis that excess androgen during the prenatal period may cause PCOS. Additionally, we show that hyperandrogenic interference in the release of preovulatory LH surges is mediated by the suppressive effects of androgens on PR expression in POA-hypothalamic tissue.

Key words: Polycystic ovary syndrome, Luteinizing hormone, Prenatally androgenized, Progesterone receptor, Hypothalamus

POLYCYSTIC OVARY SYNDROME (PCOS) is a common endocrine disorder found in 5-10% of reproductive-age women and 75-80% of anovulatory women. Characteristics of PCOS include amenorrhea/oligomenorrhea, infertility, hyperinsulinemia from insulin resistance, luteinizing hormone (LH) hypersecretion and hyperandrogenism [1]. The etiology and pathophysiology of PCOS are not clear, so the treatments only scratch the surface of the problem and drugs are needed to ease the symptoms. Some scholars have considered that PCOS is a single autosomal dominant genetic disease [2, 3] because of its per-pubertal onset and familial clustering. However, the gene candidates that regulate insulin action, androgen biosynthesis and gonadal function failed to fully explain its heterogeneity [4, 5]. In addition, genotype and phenotype are not related. Therefore, genetic factors as well as environmental factors, such as gestational androgen excess, probably play a role in PCOS [6]. Given the limitations in human studies, establishing a generally accepted PCOS animal model to help in investigating the etiology, pathophysiology and treatment of PCOS is needed.

In the early embryo, because of rapid cell differentiation, the tissues and organs of the body experience a sensitive stage of development. When the body experiences stimulation and injury at this sensitive stage, it has a life-long impact. In a series of epidemiological studies, Barker et al. [7] confirmed the “fetal origins of adult disease.” Both congenital adrenal hyperplasia
caused by 21-hydroxylase deficiency and congenital excess androgen secretion from the adrenal can lead to an increased incidence of PCOS [8]. These studies have shown that androgen excess during the fetal period may be associated with PCOS. Recently, experiments in animals revealed that the etiology of PCOS may be related to excess androgen, which could interfere in the function of the hypothalamus-pituitary-gonad axis and contribute to reproductive dysfunction in adults [9].

Inducing excess androgen in pregnant animals has been used as an animal model of PCOS [10]. The polycystic ovary not only occurs in women but also in many animals. For example, prenatal, perinatal, or postnatal androgen exposure in monkeys, sheep, rats, and mice can prompt the development of many of the symptoms of PCOS [11]. Some scholars believe that rhesus monkeys are the most similar to humans in reproductive physiology. Rhesus monkey models are beneficial for observation and evaluation and are especially suitable to research of reproductive dysfunction. However, the cost and long breeding cycle are not conducive to large-scale research [12]. Sprague-Dawley (SD) rats, with their stable estrous cycle, high sensitivity to sex hormones and economic convenience, can be used to establish a PCOS animal model. This study was performed as a follow-up of other studies, which showed that prenatal androgen excessive can lead to endocrine changes similar to PCOS. Abbott et al [13] found that female rhesus monkeys exposed in utero to levels of testosterone equivalent to those found in fetal males show many clinical and biochemical features of PCOS. To further evaluate the effects of elevated plasma androgens on LH secretion and LH pulse frequency in women with PCOS, Pastor et al. [14] performed a study to determine whether antiandrogen therapy with flutamide could enhance feedback inhibition by estradiol (E2) and progesterone (P) and found decreased sensitivity of the hypothalamic GnRH pulse generator to regulation by E2 and P.

However, data on excess androgen in PCOS is still scarce, and the mechanism by which androgens directly inhibit GnRH-stimulated LH secretion is not well established. We aimed to clarify the endocrine traits of PCOS in prenatally androgenized female SD rats to understand the change in secretion of hormones and the reasons for the change.

Materials and Methods

Hormones and chemicals
Testosterone (T) and dihydrotestosterone (DHT) were from Sigma Chemical Co. (St. Louis, MO). The radioimmunoassay kit for T, E2, P, LH and follicle-stimulating hormone (FSH) was from NIDDK (Baltimore, MD). The PCR amplification system was from Shanghai Biological engineering Company. The primers utilized in RT-PCR, 5’-CCCACAGGAGTTTGCTCAAGCTC-3’ (sense) and 5’-TAACCTGACAATTTCGGC-3’ (antisense), were synthesized by the Shanghai Biological engineering Company.

Animals and hormone treatment
Animals in all experiments were given standard rodent chow and tap, filtered fresh water ad libitum, and were kept in a room with light control (12-h light/12-h dark) and a temperature of 20-22°C with a relative humidity of 40-55%. Day 1 of pregnancy is defined as the day on which sperm are found through a vaginal smear. The estrous cycle was determined by daily vaginal cytology. All animal procedures were approved by the Shantou University Institutional Animal Care and Use Committee.

Forty-five female SD rats were randomly divided into 3 groups (n=15 each): normal control group, T group, and DHT group. The rats of the T group were subcutaneously injected with 3 mg T and 0.2 ml sesame oil each day from day 16 to 19 of gestation. The rats of the DHT group were subcutaneously injected with 3 mg DHT and 0.2 ml sesame oil at the same time. The control group was subcutaneously injected with 0.2 ml sesame oil at the same times. This hormonal paradigm mimics the endogenous T surge observed in male rats, which is elevated between gestational days 16 and 19 as compared with female rats [15]. The doses of T and DHT were chosen on the basis of previous experiments (data unpublished), which showed that administration of a free steroid dose larger than this dose could result in a mortality rate of more than 65% in fetuses, and the female offspring would be entirely masculinized. Moreover, the rats would have a normal estrous cycle with the administration of a dose less than this dose. All the offspring were weaned at 22 days after birth, and the female offspring were selected as experimental animals. The estrous cycle was determined by daily inspection of vaginal smears.
**Measurement of anogenital distance and nipples**

The anogenital distance (AGD) of female offspring in each group was measured on days 2, 22 and 60 after birth, and on day 13, the number of nipples was counted.

**Assessment of estrous cycles**

The estrous cycle patterns were determined by daily observations of vaginal smears of all adult rats (about 60-80 days after birth) for 4 weeks. The collection procedure of vaginal samples involved parting the vulva lips and inserting a cotton-tipped sterile swab into the vagina. The swab was rotated 2-3 times against the vaginal wall and then withdrawn and rolled on a clean glass slide to form two or three parallel tracks of smear material on the glass surface. The smears were air-dried, stained with Giemsa stain and examined under a light microscope. The vaginal smears were classified on cytology [16]. In this study, the appearance of cornified cells was used for the determination of estrous cycle patterns. The recurrence of a cornified cell phase preceded by a nucleated cell phase was considered evidence of estrous cycles. The length of a cycle was determined as the number of consecutive days from the day of one cornified cell phase to the day prior to the next cornified cell phase.

**Hematoxylin & eosin staining of ovarian sections**

At 60-70 days after birth, all rats (the control group rats were in diestrus while the experimental rats were in the corresponding time) were anesthetized by intraperitoneal injection of 2% pentobarbital sodium, ovaries were obtained and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 1 h, then rinsed in PBS, and then dehydrated and embedded in paraffin. Ovarian tissue sections of 4 μm were prepared for hematoxylin & eosin (H&E) staining. Tissue sections of ovaries were deparaffinized in xylene, hydrated through an ethanol series of 100%, 90%, 80%, 70%, and 50%, and stained with H&E. After clearing with fresh xylene, sections were mounted with Canada balsam and observed on light microscopy. The preantral follicles, antral follicles, corpus luteum and preovulatory follicles per ovary were determined by counting 5 representative sections per ovary at least 20 μm apart.

**Semi-quantitative RT-PCR analysis of progesterone receptor mRNA**

At 1100-1130 h at 60-70 days after birth, the rats were ovariectomized (OVX) when they were in diestrus and injected with 30 μg estradiol benzoate (EB) after surgery. At 1200 h on the following day, all animals were killed by decapitation. Preoptic area (POA) hypothalamic tissues were rapidly dissected, put into liquid nitrogen rapidly and processed for RT-PCR analysis of PR mRNA. Total RNA was extracted according to the RNA extraction kit, and 20-30 μl deionized water was added to the RNA and preserved at -80°C. The ratio of OD260:280 was measured by ultraviolet spectrophotometry, and the RNA at 1.8-2.0 ratio was used for reverse transcription. RNA at 10 μg was used in a 20 μl reaction mixture. The reaction mixture was incubated at 42°C for 60 min and at 70°C for 10 min to inactivate reverse transcriptase, and the cDNA was preserved at -80°C. The PR gene was amplified by the PCR amplification system. For semi-quantitative determination of RT-PCR product, 5 μl of the product was put in a 1.2% agarose gel for electrophoresis (60V, 30 min), and the results were analyzed by the gel imaging system and integrated optical density (IOD). The ribosomal protein S16 (RPS16) was used as the internal control.

**Basal hormone secretion**

To determine the hormone level 60-70 days after birth, at 1100 to 1130 h, all rats (the control group rats were in diestrus while the experimental rats were in the corresponding time) were anesthetized by intraperitoneal injection of 2% pentobarbital sodium, ovaries were obtained and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 1 h, then rinsed in PBS, and then dehydrated and embedded in paraffin. Ovarian tissue sections of 4 μm were prepared for hematoxylin & eosin (H&E) staining. Tissue sections of ovaries were deparaffinized in xylene, hydrated through an ethanol series of 100%, 90%, 80%, 70%, and 50%, and stained with H&E. After clearing with fresh xylene, sections were mounted with Canada balsam and observed on light microscopy. The preantral follicles, antral follicles, corpus luteum and preovulatory follicles per ovary were determined by counting 5 representative sections per ovary at least 20 μm apart.

**Frequency and amplitude of LH secretion**

On 60-70 days after birth, all rats (the control group rats were in diestrus while the experimental rats were in the corresponding time) were ovariectomized (OVX) by intraperitoneal injection of 2% pentobarbital sodium, and 7 days later, blood samples were obtained from the orbital vein every 10 min from 1200 to 1400 h. Sera were collected and extracted by centrifugation (3000 rpm, 20 min) and stored at -30°C for subsequent measurement of hormones (E2, P, T, LH, FSH).
The frequency and amplitude of LH secretion were higher in experimental rats than in the control group (Fig. 2).

Anogenital distance and nipples
The agd of T- and dHT-treated fetuses on postnatal days 2, 22 and 60 was significantly higher than in controls (Fig. 3), with no difference between treatments. The number of nipples in the experimental groups was lower than in the control group (Fig. 4), with no difference between experimental groups.

Basal hormone secretion
T, E2, P and LH but not FSH levels were significantly higher in experimental groups than in the control group (Table 1).

Table 1. Hormone characteristics in prenatal androgenized females (mean ± SEM).

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Control group (n=12)</th>
<th>T group (n=10)</th>
<th>DHT group (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T (ng/mL)</td>
<td>0.028±0.002</td>
<td>0.234±0.010 **</td>
<td>0.252±0.020 **</td>
</tr>
<tr>
<td>E2 (ng/mL)</td>
<td>14.54±0.131</td>
<td>20.51±0.582 **</td>
<td>21.28±0.529 **</td>
</tr>
<tr>
<td>P (ng/mL)</td>
<td>14.33±0.205</td>
<td>20.23±0.427 **</td>
<td>21.36±0.523 **</td>
</tr>
<tr>
<td>LH (ng/mL)</td>
<td>0.47±0.019</td>
<td>0.76±0.013 **</td>
<td>0.76±0.011 **</td>
</tr>
<tr>
<td>FSH (ng/mL)</td>
<td>0.27±0.010</td>
<td>0.25±0.003</td>
<td>0.25±0.007</td>
</tr>
</tbody>
</table>

*indicates a significant difference between the experimental groups and the control group (p<0.05) ** p<0.01

Results

Prenatal hyperandrogenism leads to decreased PR mRNA expression in the POA-hypothalamus
After treatments with OVX and injection with EB, the PR mRNA expression in the POA-hypothalamus of control rats was higher than in experimental rats (p<0.05). RT-PCR analysis revealed that androgen exposure in the fetal period significantly reduced the PR expression as compared with control treatment (Fig. 1).

Frequency and amplitude of LH secretion
The frequency and amplitude of the LH secretion were higher in experimental rats than in the control group (Fig. 2).

Analysis involved use of SPSS v13.0 (SPSS Inc., Chicago, IL). Data are presented as the mean ± SEM. A p<0.05 was considered statistically significant.
teum and preovulatory follicles were fewer while the preantral follicles and antral follicles were greater in the experimental groups than in controls (Table 2).

**Discussion**

**PCOS model of prenatal androgenized SD rat**

The animal of this research, SD rats, are altricial animals. At birth, the genitalia and nervous system of the newborn rat are absent and are not functional until they develop after birth. The genitalia and nervous system develop at different rates, with the genitalia developing earlier than the nervous system. The development of the genitalia is important for the development of the reproductive system, and the development of the nervous system is important for the development of behavior.

**Change in estrous cycle**

Although we could not obtain vaginal smears from a small number of prenatal androgenized rats because of vaginal opening atresia, almost all rats had prolonged (6-14 days) or irregular estrous cycles. All control rats had regular estrous cycles (4-6 days).

**Corpus luteum and follicle staining in ovary sections**

H&E staining (Fig. 5) showed that the corpus luteum and preovulatory follicles were fewer while the preantral follicles and antral follicles were greater in the experimental groups than in controls (Table 2).
rats are in the medium-term of differentiation and development and are sensitive to androgen. The female offspring androgenized in utero showed false hermaphroditism as adults, including increased AGD, decreased number of nipples and masculinized genitalia. In addition, a few prenatal androgen-exposed female offspring had no vaginal opening from birth to adulthood, but their ovaries, fallopian tubes, uterus and cervix did not disappear. The situation is similar to false hermaphroditism in the human female, which shows congenital adrenal hyperplasia and 21-hydroxylase deficiency and can lead to excess androgen in the fetal period. Patients with congenital adrenal hyperplasia and 21-hydroxylase deficiency are prone to PCOS [17].

The standards of masculinization include male genitals or a genital combination of male and female. Studies [18] suggest that excess androgen in pregnant animals can induce the masculinized internal and external genitalia of the female fetus, with loss of some genitals of the female. Lacking the gene of testicular decision (male gonad development needs this gene), the female animals have the changes mentioned above, but a testicle cannot develop. In addition, the differentiation of the ovary, fallopian tubes, uterus and upper vagina of the female animals exposed to excess androgen in pregnancy are not suppressed by excessive androgen because of lack of testicular anti-mullerian hormone. In primates and other precocial animals, the sexual differentiation and development of the nervous system has been established at birth, so the impact of androgen continues only to mid-pregnancy. In animals exposed to excess androgen in late pregnancy, the genitilia would not be masculinized. Patients with PCOS and no masculinized genitilia may be protected [6].

Ovulation dysfunction or menstrual disorders are the main features of PCOS patients and are diagnostic criteria for PCOS. In this study, the results showing an irregular estrous cycle and corpus luteum and that preovulatory follicles were lower in experimental rats, combined with the significantly higher levels of LH and P in experimental groups, confirmed that the experimental rats exhibited little ovulation. These were similar to those for patients with PCOS. In other words, prenatal exposure to androgen excess can lead to an alteration mimicking PCOS.

Sullivan et al. [19] found that the estrous cycle of the prenatal androgenized female mice was longer than in controls. Research of the rhesus monkey and sheep also confirm these characteristics. Abbott [6] found that exposure of the fetal rhesus monkey to excessive androgen in early or late pregnancy represents ovulation dysfunction, and the menstrual cycle was decreased by 40%-50% as compared with controls.

Hyperandrogenism is the most important characteristic of PCOS and one of the diagnostic criteria for PCOS. In this research, the serum total testosterone levels and free testosterone index (data not shown) were higher in experimental than control groups. These findings are similar to those for patients with PCOS.

In the present research, the LH level was significantly higher in the experimental groups. Furthermore, the frequency and level of LH was higher in the experimental groups. In the clinic, increased LH secretion can be seen in 70% of women with PCOS. In some of these patients, LH pulse amplitude expansion and frequency increases, which results in a 2-3 times increased ratio of LH and FSH. Women with 21-hydroxylase deficiency (excessive androgen secretion in pregnancy) have high concentrations of LH [20]. The characteristics of LH secretion in adult female rats with androgen exposure during pregnancy resemble the clinical features of PCOS patients.

Hyperinsulinemia and peripheral insulin resistance are common metabolic abnormalities in women with PCOS. Additionally, the opportunity for lipid dysfunction and cardiovascular disease is increased. Studies have shown that more than 40% of obese PCOS women have lower glucose tolerance [17]. These women have almost 3-7 times the risk as normal patients for non-insulin-dependent diabetes mellitus [17]. However, the reasons for these metabolic abnormalities remain unclear. Dysfunction of the post-receptor insulin signaling pathway and insulin secretion abnormalities may play a role. Rhesus monkeys with prenatal androgen exposure also exhibit abnormal insulin secretion or insulin activity dysfunction [20]. Although we did observe any significant effect of excess prenatal androgen on insulin secretion or activity, rats with prenatal androgen exposure showed the trend of abnormal insulin secretion or insulin activity dysfunction (unpublished observations). The experimental animals in this research may not fully exhibit the changes shown by other species, perhaps because of less-than-optimal timing, magnitude, and/or duration of the androgen exposure.

Because of the heterogeneity of the clinical features of PCOS, the diagnostic criteria have been controversial. New diagnostic criteria for PCOS were
interfering with the positive feedback effect of estrogen in the POA-hypothalamus and/or pituitary gland, which results in oligo-ovulation/anovulation.

In the fetal or perinatal phase, there are two kinds of trends for the development of gonadotropin-release in rats. If there is testosterone, a male brain that will not produce the pre-ovulating gonadotropin in adulthood will be formed. If the brain lacks androgen, it will form a female brain, which is conducive to gonadotropin-releasing cyclically in adulthood and maintained by POA and the hypothalamus [28]. In other words, rats receiving T or DHT on pregnancy days 16-19 resulted in decreased PR in the POA-hypothalamus of the female offspring rats in adulthood. This finding suggests that prenatal androgen exposure resulted in decreased PR levels in the POA of rats, so the release of GnRH in the hypothalamus is decreased and the LH surge cannot be formed; that is, the secretion of gonadotropin could be non-cyclical in the adults.

Our findings suggest that excess prenatal androgen decreases the expression of PR in the hypothalamus. In addition, this may explain the gonadotropin abnormalities. At present, the mechanism of PR suppression by androgen is not entirely clear. Sullivan et al. [19] confirmed that prenatal androgen administration can activate the androgen receptor (AR) so that the PR generation by POA induced by E2 is not enough. However, the mechanism of prenatal AR activation and program hyperactivity of the GnRH pulse generator in adulthood is unclear. Prenatal androgenization of female sheep was found to reduce synaptic contacts to GnRH neurons to lower levels than those observed in males [29], which suggests that the effects of androgens on GnRH pulsation may be mediated by alterations in synaptic connectivity. Sullivan and Moenter [19], however, provided evidence that AR activation may produce specific alterations in the drive from gamma-aminobutyric acid-releasing neurons to GnRH neurons, perhaps by altering synaptic connectivity between the two neuronal phenotypes.

Our studies provide evidence that prenatal androgens can de-feminize gonadotropin secretions in the female fetus by rendering POA tissues insensitive to the PR-inducing actions of E2. Excess androgens in pregnancy can induce the alteration in the hypothalamic program of the female fetus animals and eliminate their ability to sustain normal, female-typical, cyclic hormone secretions as adults. These findings, along with previous observations, may have impor-
tant implications for understanding accelerated GnRH pulse generator activity and the oligomenorrhea or amenorrhea commonly observed in PCOS.

Prenatal androgen exposure leads to the negative feedback effect on LH from reduced E2 levels, resulting in excessive secretion of LH. High LH levels, in turn, affect the theca cells for increased secretion of androgen and progesterone. Moreover, excessive androgen transforms into estrogen through cytochrome P450 aromatase; consequently, the level of circulating estrogen is increased.

As mentioned above, reduced sensitivity of the hypothalamus to estrogen would lead to abnormal secretion of GnRH and gonadotropin, which impairs follicular development and increases the ovarian androgen level; therefore, the neuroendocrine abnormalities and oligo-ovulation/anovulation are, at least in part, associated with the negative feedback effect of the sensitive decline of the GnRH pulse generator to ovarian hormones. So, a vicious circle is initiated: excessive androgen causes neuroendocrine dysfunction, and neuroendocrine abnormalities, in turn, maintain the hyperandrogenism.

The results of this study are consistent with other research in sheep and rhesus monkeys. Exposure of sheep and rhesus monkeys to excessive androgen increased LH secretion and conferred resistance to the negative feedback of ovarian hormones [30-32]. Although PCOS is a complex endocrine-disordering disease that may be divided into some heterogeneous items, the obstacles caused by androgen in the fetal or perinatal phase can explain most of the clinical and biological characteristics.

The pathophysiological effects of prenatal hyperandrogenism may be attributed to changes in neural connectivity, cell proliferation, cell death, and/or certain characteristics of various cells in this critical period. In short, the molecular and cellular mechanism is not yet clear, and more research is needed to explore these assumptions to provide the guidance for clinical treatment.

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References


