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Effects of *Stachys sylvatica* hydroalcoholic extract on the ovary and hypophysis-gonadal axis in a rat with polycystic ovary syndrome

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Abstract

Background: Polycystic ovary syndrome (PCOS) is the most common endocrinopathy affecting women. The purpose of this study is to evaluate the effects of *Stachys sylvatica* hydroalcoholic extract on biochemical and histological parameters in a rat model of polycystic ovary syndrome. Thirty adult female Wistar rats with an average weight of 180 g were divided into five groups of six rats. Animals were divided into groups of control, PCOS (intramuscular injection of 2 mg estradiol valerate, Aburaihan Co., Iran/rat, once), and treated with hydroalcoholic extract of *Stachys sylvatica*. After 60 days of PCOS induction, 100, 250, and 500 mg/kg BW *Stachys sylvatica* hydroalcoholic extract were injected intraperitoneally. The control group was just injected with olive oil as a solvent. After 10 days of treatment, animals were weighed and then sacrificed and blood samples were taken from their heart for hormonal studies. Ovaries of all groups were cut for histological studies and fixed in formaldehyde, and 7-μm sections were prepared by microtome and stained using hematoxylin–eosin. The data were analyzed by one-way ANOVA and Tukey-Kramer post hoc test with the SPSS software. The significant level was $p < 0.05$.

Results: The *Stachys sylvatica* extract can improve obesity in the PCOS group. The histological and hormonal results showed that PCOS induction can decrease the number of preantral, antral, and Graafian follicles as well as corpus luteum in comparison with the control ($p < 0.001$), whereas the number of cystic follicles increased significantly ($p < 0.001$). After treatment with *Stachys sylvatica* hydroalcoholic extract (500 mg/kg), a significant increase was observed in all these parameters. In addition, the number of cystic follicles decreased significantly ($p < 0.001$). The level of LH and FSH hormones decreased significantly ($p < 0.01$) in the PCOS group compared with the control. In contrast, the estrogen level increased significantly ($p < 0.01$). In the 250 and 500 mg/kg group, treatment with the extract could increase FSH and decrease estrogen concentration significantly relative to the PCOS group ($p < 0.01$). Results of antral follicle morphometry indicated an increase in follicle diameter and theca thickness, but the thickness of the granulosa layer decreased significantly. An improvement in these measurements was observed in the treated PCOS groups with all doses.

Conclusions: It can be concluded that *Stachys sylvatica* hydroalcoholic extract can improve some symptoms of polycystic ovary syndrome because of components such as iridoids, flavonoids, and sesquiterpenes with antioxidant and anti-inflammatory effects. In this experiment, 500 mg/kg dose of extract was considered as the most effective dose.

Keywords: Estradiol, Gonadotropins, Polycystic ovary syndrome (PCOS), *Stachys sylvatica*, Rats

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Background

Polycystic ovary syndrome (PCOS) is a hormonal disorder that affects one in ten women of reproductive age (18–44). Women with PCOS have symptoms such as menstrual abnormalities, high levels of androgen, and polycystic ovary. PCOS is the most common cause of infertility. It also raises the chances for some health problems, including diabetes mellitus, high blood pressure, cardiovascular disease, and endometrial cancer. This disorder has some physical signs in appearance such as hirsutism (excess facial and body hair), severe acne, and obesity [1, 2].

For the first time, PCOS was described by Stein and Leventhal in the mid-1930s, but the exact cause of PCOS is unclear yet [3]. Both genetic and environmental factors (obesity and lack of physical exercise) can be significantly involved, and most treatments of PCOS are palliative which focuses on the symptoms of this disease and cannot completely cure PCOS [4].

In this regard, some studies indicate that certain nutrition and dietary factors can decrease symptoms of the PCOS. Based on Yang et al.'s study in 2018, omega-3 fatty acid may be recommended for the treatment of PCOS in women with high insulin resistance as well as high cholesterol and triglyceride [5]. Other studies have indicated that herbal medicines especially herbs with anti-inflammatory effects can also be effective [6, 7].

In some related literature, different biological properties such as antiseptic, antitoxic, antianoxia, and anti-inflammatory have been attributed to the *Stachys* genus [8]. Besides, in Iranian traditional medicine, the aerial parts of *Stachys sylvatica* (one of the species of this genus) are recommended for the treatment of women with PCOS by herbalists.

Stachys sylvatica (*Lamiaceae* family) is an erect perennial herb, native to Central and Western Asia and Europe. It grows in the banks of rivers and streams, flowering from July to August, and can grow up to a meter in height. The stem has glandular hairs on the upper part of the plant, and stalked leaves are in opposite parts. This plant has a slightly unpleasant fragrance. The common name is Hedge Woundwort as it is useful for wound healing [9]. In folk medicine and phytotherapy, diuretic, digestive, emmenagogue, antispasmodic, anti-inflammatory, sedative, and tonic properties have been attributed to this plant [10]. *Stachys silvatica* contains polyphenols, phenolic acids, and flavonoids. Essential oil compositions of leaves are germacrene D, *n*-tetracosane, mint sulfide, α-pinene, and β-pinene. It was reported that these compounds are responsible for the biological activity of the plant [8, 11].

Regarding the benefits of this herb in Iranian traditional medicine in improving PCOS, the aim of the present research is the scientific evaluation of the treatment effect by *Stachys sylvatica* hydroalcoholic extract to improve PCOS syndrome in an animal model of this disorder.

Methods

Preparation of *Stachys sylvatica* extract

The herb was collected from the Alialbolagh region (Ardebil, Iran) in the spring and recognized by a systematic expert in Central Tehran Branch, Islamic Azad University. Then, the plant was dried in shade. Afterward, 40 g of plant is powdered and extracted by 70% ethanol using a soxhlet extractor. Every 40 g of the powdered plant had yield 5 g of hydroalcoholic extract. The extract was kept in 4 °C for the next experiments.

Animals

Female Wistar rats weighing 160–200 g were obtained from the Pasteur Institute, Tehran, Iran. Every six animals were housed in a cage and kept in standard condition including light 06:00–20:00 h, temperature 22 ± 2 °C, and 50 ± 5% humidity. The animals had free access to food and water 1 week before and during experiments.

Experimental design

Animals were checked 15 days for regular cycles by smear test [12]. Thirty adult female rats were randomly divided into five groups: control, PCOS, and experimental groups. The control group received just olive oil (i.p, 0.2 ml) as a solvent. PCOS and experimental groups were induced by one intramuscular injection of 2 mg/kg estradiol valerate solved in olive oil at the estrous stage based on Brawer et al. with some modifications [13]. The most important sign of PCOS was irregular estrous cycles and persistent vaginal cornification (PVC) which was confirmed by vaginal smear. After 60 days of PCOS induction, experimental groups received 200, 250, and 500 mg/kg doses of *Stachys sylvatica* hydroalcoholic extract intraperitoneally. After 10 days of daily injections, animals were weighed and sacrificed by cervical dislocation. Then, blood samples were collected from their heart, and the hormone levels (FSH, LH, and estrogen) in the plasma were quantified by ELISA method. The kit used for the hormone experiments was DiaSorin, LIAISON, Italy. Rat ovaries were removed, washed in normal saline, and fixed in formaldehyde (10%) fixative for 48 h. Seven-micrometer sections of ovaries were prepared using microtome and stained by hematoxylin and eosin method. Histological assessment was performed under light microscopy. Follicles were recognized and counted in different stages including: primary (possess mitotic, cuboidal granulosa cells), preantral (presence of theca and multiple layers of granulosa cells), antral

(formed an antrum), Graafian (characterized by a large follicular antrum), cystic (full of liquid) follicles, and corpus luteum. All microscopic measurements for ovary morphometry were done using an eyepiece reticle. The experimental protocol was approved by the ethical committee of Tehran Islamic Azad University of Medical Sciences (IR.IAU.TMU.REC.1398.091) and performed in conformity with the National Institute of Health Guide for Care and Use of Laboratory Animals.

Statistical analysis

Statistical analysis was done by the SPSS software version 26. Data were analyzed using one-way ANOVA and followed by the Tukey-Kramer post hoc test. The significant level was considered $p < 0.05$.

Results

Effects of *Stachys sylvatica* hydroalcoholic extract on animal weight and serum hormone concentrations in the PCOS-induced rats

Measurement of animal weight before and after treatment showed a significant increase in the PCOS and 100 mg/kg of extract groups in comparison with other groups (Fig. 1).

As shown in Table 1, FSH, LH, and estrogen hormone levels were measured in the serum of female PCOS-induced rats. Treatment with *Stachys sylvatica* hydroalcoholic extract at all doses caused a significant increase in FSH levels compared with the PCOS while a decrease was observed in the PCOS group compared with the control group. In contrast, the estrogen hormone level had a significant decrease compared with the PCOS group. However, the estrogen level significantly increased in PCOS compared with the control group.

Table 1 Mean hormone concentrations in different groups (mean ± SEM)

Groups	FSH (mIU/ml)	LH (mIU/ml)	Estrogen (ng/ml)
Control	4.63 ± 0.31	6.64 ± 0.03	0.78 ± 0.08
PCOS	1.69 ± 0.08^{+++}	6.29 ± 0.04^{++}	1.42 ± 0.05^{+++}
Extract 100 mg/kg	2.7 ± 0.31*	6.35 ± 0.08	0.85 ± 0.03***
Extract 250 mg/kg	3.67 ± 0.12***	6.5 ± 0.05	0.99 ± 0.04***
Extract 500 mg/kg	5.95 ± 0.02***	6.48 ± 0.09	0.9 ± 0.07***

$^{++}$ $P < 0.01$, $^{+++}$ $P < 0.01$ PCO versus control, * $P < 0.05$, *** $P < 0.001$ extract versus PCO

Effects of *Stachys sylvatica* hydroalcoholic extract on folliculogenesis in the PCOS-induced rats

The number of cystic follicles significantly increased in the PCOS group in comparison with the control and 500 mg/kg groups (Table 2 and Figs. 2 and 3).

In 500 mg/kg dose of extract, the number of preantral, antral, Graafian follicles, and corpus luteum significantly increased compared with the PCOS group (Fig. 4). In the PCOS group, these factors significantly decreased in comparison with control.

As it is shown in Table 3, the diameter of antral follicles as well as the thickness of the theca layer in the PCOS group had a significant increase ($p < 0.001$), compared with the control. In contrast, the thickness of the granulosa layer significantly decreased ($p < 0.001$). After treatment with *Stachys* extract, the diameter of the antral follicle and theca layer decreased and the diameter of the granulosa increased significantly versus the PCOS group. Therefore, the data became closer to the results of the control group.

Discussion

PCOS is a common metabolic disorder in women of reproductive age that affects all physiological, social, and

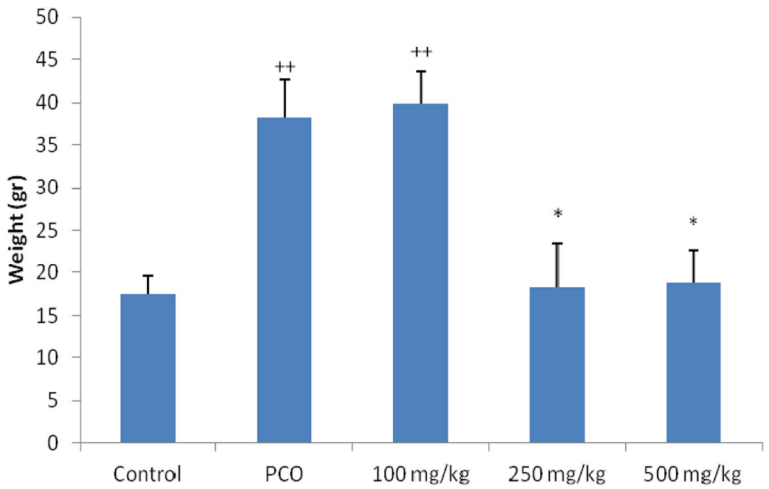

Fig. 1 Weight difference of animals before and after treatment in the groups (mean ± SEM). $^{++}$ $P < 0.01$ PCO and 100 mg/kg of extract versus control, * $P < 0.05$, extract versus PCO

Table 2 Mean follicle numbers in different groups (mean ± SEM)

Groups	Primary follicle	Preantral follicle	Antral follicle	Graafian follicle	Cystic follicle	Corpus luteum
Control	17.25 ± 0.81	53.26 ± 1.81	11.42 ± 0.92	1.65 ± 0.73	0	10.41 ± 0.71
PCOS	17 ± 0.87	39.61 ± 1.75^{+++}	3 ± 0.04^{+++}	0	5.66 ± 0.49^{+++}	3 ± 0.18^{+++}
Extract 100 mg/kg	16.05 ± 0.63	38.56 ± 0.36	5.65 ± 0.47*	1 ± 0.35	4.65 ± 0.73	3.15 ± 0.85
Extract 250 mg/kg	16.98 ± 0.38	31.65 ± 0.96**	4.65 ± 0.28	1.5 ± 0.46	3.65 ± 0.52	4.85 ± 0.59
Extract 500 mg/kg	17.48 ± 0.85	48 ± 1. 96**	6 ± 0.48**	1.83 ± 0.3*	1.28 ± 0.42***	6.42 ± 0.64**

$^{+++}$ $P < 0.001$ PCO versus control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ extract versus PCO

economic aspects of a person's life. Because of the heterogeneous condition, there are no definite diagnostic criteria for this disorder. But these two signs are required to confirm (1) hyperandrogenism and (2) polycystic ovary [2, 4]. Therefore, biochemical markers and ovarian biopsy are usual evidence for PCOS diagnosis. For this reason, we take into consideration both histological and biochemical evaluations in the present study. We focused on two main features: blood sexual hormone (estrogen), gonadotropins (FSH and LH), and ovarian histology. Obesity is one of the symptoms of PCOS which is observed in rats in this study. Two hundred fifty and 500 mg/kg doses of extracts could prevent weight gain in rats.

There are different methods for the induction of PCOS in the experimental animals. One of the common ways is treatment with estrogen and drugs with estrogenic effects. These compositions induce continuous estrous and cyclic follicles with morphologic effects like what was observed in PCOS women [14]. In this experiment, we tried to induce a PCOS-like phenotype by a single-dose injection of estradiol valerate (2 mg/kg) in Wistar rats.

In normal cases, the ratio of LH/FSH is almost 1:1, but in PCOS women, this ratio is 2 times or 3 times higher (2:1 or 3:1), [7]. The results of our experiment also demonstrated that the ratio of LH/FSH in PCOS rats was 2.5 times more than the control. On the other hand, the estrogen level significantly increased in the PCOS group. On the basis of the previous reports, the dominance of estrogen is the most common cause of ovulatory disorder and even anovulatory. This high level of estrogen finally results in insufficient progesterone, elevated LH, and infertility. Most of the follicles make cystic follicles and arrested to form adult follicles. Also, the reduction of corpora lutea is associated with these changes [15].

The study of ovaries sections showed a significant increase in the number of cystic follicles in the PCOS group in comparison with the non-injected control group. In contrast, the number of other (preantral, antral, and Graafian) follicles and corpus luteum significantly decreased.

Data of our study indicated a significant decrease in the thickness of the granulosa layer and a significant increase in the thickness of the theca layer in the PCOS group which is consistent with previous studies [16, 17]. Researchers indicated an abnormality in the granulosa cells of cystic follicles. Also, increased staining in the thecal layer may be described by the carbohydrate which is associated with an increased amount of collagen [16, 18].

The *Stachys* extract caused significant FSH increases and estrogen decrease in comparison with the PCOS group, in the way that the ratio of LH/FSH became close to 1:1 (6.48/5.59) at high doses of extract (500 mg/kg).

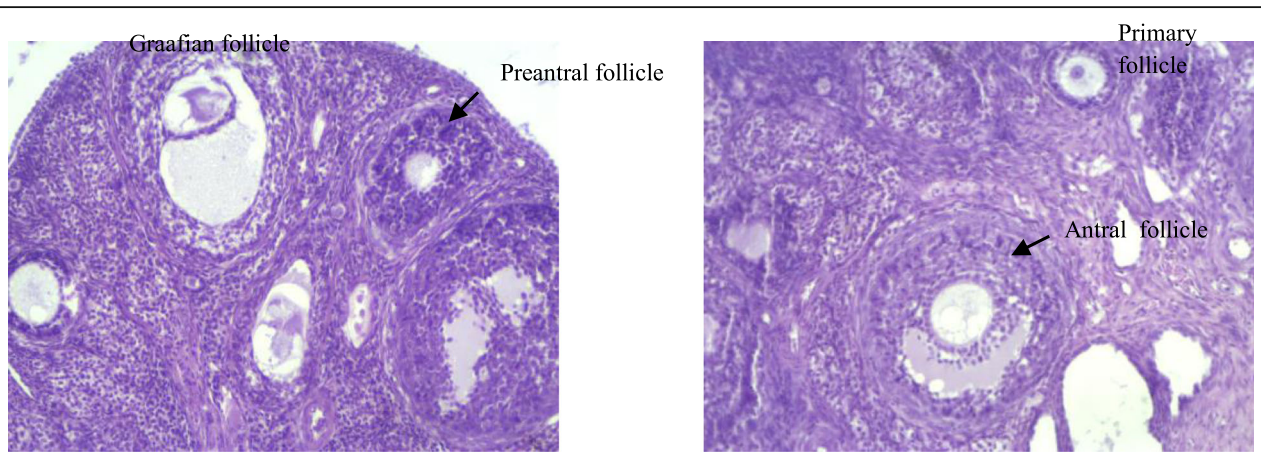

Fig. 2 Cross-section of rat ovary in the control group (× 100, H&E). Primary, preantral, antral, and Graafian follicle

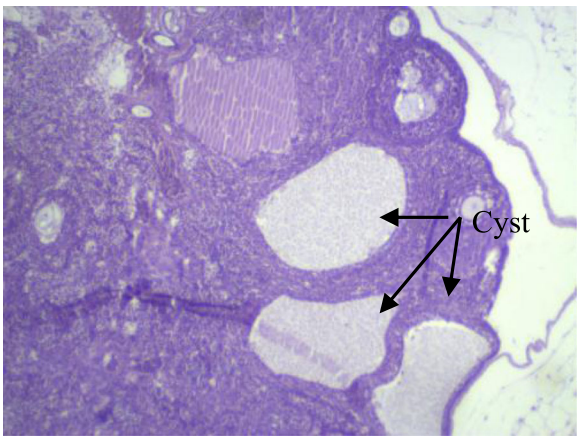

Fig. 3 Cross-section of rat ovary in the PCOS group (× 100, H&E). Cystic follicles are clearly visible in the section of ovary

Therefore, the number of follicles in the different stages and corpora lutea increased significantly. These findings are in agreement with the results of some other useful herbal extract for PCOS treatment [14, 16].

Stachys sylvatica contains iridoids, flavonoids, phenolic acids, sesquiterpenes, and diterpenoids as secondary metabolites [8]. It was reported that flavonoids have an aromatase inhibitory effect [14]. The action of the aromatase enzyme is the biosynthesis of estrogen from the androgen. As it is known, one of the distinct symptoms of PCOS is high levels of androgen which affects follicle development and change of the hypothalamic-pituitary ovarian axis. Chemical aromatase inhibitor drugs like letrazole (prescribed for PCOS patients, off-label usage) can decrease estrogen by preventing androgen to estrogen alteration pathway [19]. It seems that the reduction of estrogen in our experiments after treatment by *Stachys* extract is due to the flavonoid constituents.

On the other hand, some flavonoids can bind to the benzodiazepine site of GABA (gamma amino butyric acid) receptor and act as GABA receptor agonists. Many documents suggest that GABAergic regulations of GnRH (gonadotropin-releasing hormone) neurons are involved in LH surge alteration. It was reported that the inhibitory effect of GABA on GnRH neurons leads to LH surge [20, 21]. This can justify a decrease in LH/FSH ratio in the PCOS groups treated with high doses of *Stachys* extract.

Considering the importance of *Stachys sylvatica* in the Iranian folk medicine for the treatment of some illness, their essential oil compositions were analyzed accurately. β-Caryophellene was the major component, also 9 sesquiterpenes, 13 monoterpenes, δ-cadinene, and bicyclogermacrene were detected [11]. It was demonstrated that sesquiterpenes such as ß-caryophellene and δ-cadinene are responsible for *Stachys* extract anti-inflammatory, antirheumatic, and diuretic activity [22].

Studies on PCOS women have shown the ability of some dietary factors like anti-inflammatory materials to improve the metabolic disorder of patients [5, 23]. Women with PCOS have demonstrated a higher level of inflammatory mediators such as tumor necrosis factor, C-reactive protein, and tissue plasminogen activator in their blood which is associated with insulin resistance [17]. Some in vitro experiments have revealed that pro-inflammatory stimuli can upregulate the steroidogenic enzyme response in the ovarian theca cells in order to produce androgen [19]. Anti-inflammatory factors like flavonoids operate through reducing the synthesis of prostaglandins that have a role in the gonadotropin synthesis. In consequence, *Stachys* extract could be effective in the PCOS treatment through reducing gonadotropin levels.

Háznagy-Radnai et al. in 2011 investigated the iridoid constituents of *Stachys* species for the anti-inflammatory

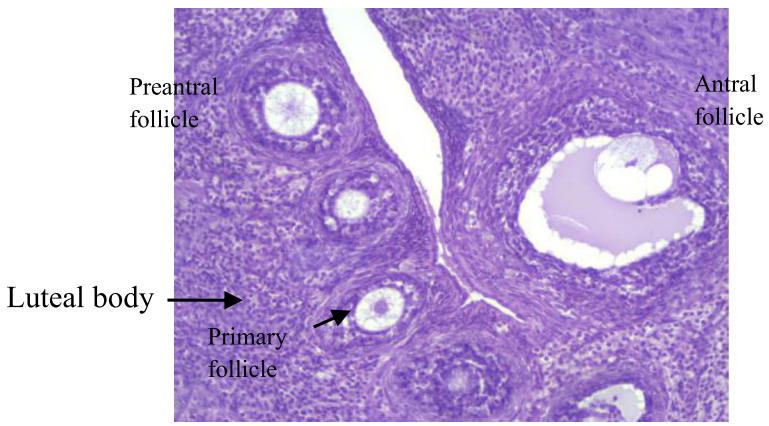

Fig. 4 Cross-section of rat ovary in the extract group, dose 500 mg/kg (× 100, H&E). The cysts disappeared and the number of luteal bodies and follicles in different stages increased

Table 3 Morphometry of antral follicles in different groups (mean ± SEM)

Groups	Antral follicle diameter (μm)	Thickness of the granulosa layer (μm)	Thickness of the theca layer (μm)
Control	385 ± 0.63	58.65 ± 0.04	20.85 ± 0.35
PCOS	600.61 ± 0.74+++	32 ± 0.87^{+++}	48.96 ± 0.19^{+++}
Extract 100 mg/kg	565.56 ± 0.36***	48.05 ± 0.9***	41.65 ± 0.47***
Extract 250 mg/kg	500.65 ± 0.69***	42.98 ± 0.38***	35.65 ± 0.96***
Extract 500 mg/kg	495.29 ± 1.28***	38.48 ± 0.85***	28.55 ± 0.75***

$^{+++}$ $P < 0.001$ PCO versus control, *** $P < 0.001$ extract versus PCO

properties [24]. The results showed that iridoids are responsible for herb anti-inflammatory effects but other active constituents or their synergism must also be involved.

Hajdari et al., exhibited potent antioxidant activity of *Stachys sylvatica* [25]. As it is known, oxidative stress can cause hyperinsulinemia and disorder of folliculogenesis [26]. The extract could have some beneficial effects by high degree of materials for extracting free radical scavengers. Moreover, products from arachidonic metabolism (lipoxigenase pathway), including inflammatory prostaglandins seem to play a crucial role in the inflammation [27]. In fact, the *Stachys* extract by providing potent antioxidant properties has an inhibitory role in this metabolic pathway [28].

Conclusion

The present study for the first time confirms that *Stachys sylvatica* hydroalcoholic extract can be useful as a therapy for the PCOS patients by the improvement of folliculogenesis and regulation of gonadotropins synthesis. It seems that the anti-inflammatory and antioxidant properties are responsible for the effectiveness of the *Stachys* extract. Different constituents of the extract, especially flavonoids, iridois, and sesquiterpenes, and their synergism may have a role in this activity.

Abbreviations

ANOVA: Analysis of variance; ELISA: Enzyme-linked immunosorbent assay; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; PCOS: Polycystic ovary syndrome; PVC: Persistent vaginal cornification

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Authors' contributions

This study was designed and executed by MR and ZP. FA did the experiments and analyzed the data. All authors have read and approved the manuscript.

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Availability of data and materials

The supporting data or findings can be found if it is needed.

Ethics approval and consent to participate

The experimental protocol was approved by the ethical committee of Tehran Islamic Azad University of Medical Sciences (IR.IAU.TMU.REC.1398.091). Consent to participate is not applicable.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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