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Research Article

Shaoyao-Gancao Decoction alleviated hyperandrogenism in a letrozole-induced rat model of polycystic ovary syndrome by inhibition of NF-κB activation

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Shaoyao-Gancao Decoction (SGD) has been widely used for the treatment of gynopathy. The present study aimed to evaluate the therapeutic effect and potential mechanism of SGD on hyperandrogenism in polycystic ovary syndrome (PCOS) rats. In the present work, SGD was orally administrated to the PCOS rats at the dose of 12.5, 25, and 50 g/kg/d for 14 consecutive days. UPLC–MS/MS was performed to identify the main chemical components of SGD. Body weight, ovarian weight, cystic dilating follicles, and serum levels of steroid hormones were tested to evaluate the therapeutic effect of SGD. In order to further clarify the underlying mechanism, we also measured mRNA and the protein levels of NF-κB, NF-κB p65, P-NF-κB p65, and IκB by RT-qPCR and Western blotting techniques. Our results showed that SGD treatment significantly alleviated hyperandrogenism in PCOS rats as evidenced by reduced serum levels of T and increased E_2 and FSH levels. In addition, SGD effectively reduced the phosphorylation of NF-κB p65 and increased the expression of IκB. Results of the present study demonstrated that SGD could ameliorate hyperandrogenism in PCOS rats, and the potential mechanism may relate to the NF-κB pathway.

Introduction

Polycystic ovary syndrome (PCOS), one of the most frequently reported endocrinopathies in women of reproductive age, is characterized by anovulation, hyperandrogenism, and polycystic ovaries syndrome [1,2]. Epidemiologic studies showed that PCOS affected 5–10% reproductive women and it was the leading cause of nearly 75% cases of anovulatory infertility [3]. In addition to the reproductive abnormalities, affected women are more likely to develop various clinical implications, including hyperandrogenism, cardiovascular disease, and endometrial carcinoma. Despite the high prevalence and marked impact of PCOS in the community, the precise mechanism underlying the pathogenesis of PCOS is still not fully understood [4].

At present, accumulating evidence indicate that PCOS is a chronic low-grade inflammation state, which is associated with an increased risk of ovarian dysfunction, metabolic aberration, and cardiovascular disease [5,6]. Patients with PCOS were reported to have elevated levels of inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-18 [7–9]. IL-18 plays an important role in follicular growth and oocyte maturation [10]. IL-6 is associated with hyperandrogenism and insulin resistance in PCOS patients [11]; local inflammation in PCOS ovaries could impair follicular growth and maturation [11]. Inflammation has now been considered as a potential contributor to the pathogenesis of PCOS. This highlights the need

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to understand the pathological mechanism from the perspective of inflammation and search for early medical intervention.

Shaoyao-Gancao Decoction (SGD), also known as Shakuyaku-Kanzo-to in Japan, is a traditional Chinese herbal prescription, which was first recorded in Treatise on Febrile diseases, a classical work of traditional Chinese medicine (TCM) written by the famous Chinese physician Zhongjing Zhang in Han Dynasty of Chinese history (202 BC-220 AD) [12]. It is a combination of *Radix Paeoniae Alba* (*Paeonia lactiflora Pall, root*) (PA) and *Glycyrrhizae uralensis* (*Glycyrrhiza uralensis Fisch., root and rhizome, honeyed*) (GU) in the ratio of 1:1. It is traditionally used in the treatment of gynecological disorders, such as dysmenorrheal (Treatise on Exogenous Febrile Disease, in 210 CE), PCOS [13], endometriosis, and adenomyosis [14]. Similar herbal prescription has also been used to treat the same diseases in Asia such as Japan. Since 1980s, Japanese researchers have showed that SGD could improve pregnancy rate in oligomenorrheic or amenorrheic women by lowering high serum testosterone levels [15]. Takahashi et al. further conducted a clinical observation study to identify that SGD was effective for achieving pregnancy in patients with PCOS [15,16].

Modern pharmacological studies have showed that SGD possesses multitude of pharmacological activities including analgesia [17] and anti-inflammatory [18] properties. Flavonoids and triterpenoid saponins are reported as the main bioactive compounds of SGD [19,20]. It has been showed that glycyrrhizin and glycyrrhetic acid (triterpenoid saponins) can lower the serum testosterone level *in vitro* and *in vivo* [21–24]. In addition, paeoniflorin, albiflorin, and oxypaeoniflorin are believed to be the main bioactive compounds of PA responsible for anti-inflammation *in vitro* and *in vivo* [25–27]. However, whether the therapeutic effect of SGD on PCOS was achieved by anti-inflammation was still unknown. We all know that rats have regular estrous cycles similar to the women menstrual cycles; thus, rats are suitable for PCOS modeling. Based on the above facts, in the present work we investigated the therapeutic effect of SGD on hyperandrogenism in letrozole-induced PCOS rat model and further elucidated the underlying molecular mechanisms.

Methods

Chemicals and reagents

Letrozole, 2.5 mg/piece, was purchased from HengRui Pharmaceutical Factory (Jiangsu, China). ELISA kits for T, E_2, LH, FSH, and P assays were purchased from Sinobestbio (Shanghai, China). *Radix Paeoniae Alba* (Anhui, China) and *Glycyrrhizae uralensis* (Shanxi, China) were purchased from China National Pharmaceutical Group Co., Ltd (Taiyuan, Shanxi, China). The plants were identified by J.-p.Z. (Department of Pharmacy, Second Hospital of Shanxi Medical University).

Preparation and quantity control of SGD

SGD, which is composed of PA and GU, was prepared according to original Chinese documents. Briefly, PA and GU were mixed together and macerated in deionized water (1:10, w/v) for 1 h. Then SGD was obtained by boiling in water for 1 h each time and repeated for two times. The solution obtained was filtered and concentrated to 4.24 g crude material per ml.

An ABI 5500 QTRAP mass spectrometer was operated in the present work. The chromatography analysis was carried out on a reverse phase XB-C18 (2.6 μm, 100 mm × 2.1 mm, hypersil) column. The mobile phase consisted of 0.1% formic acid in water (A) and methanol (B). The gradient program was as follows: 70% B at 0–5.5 min, 70–15% B at 5.5–6.5 min, 15–10% B at 6.5–7 min, 10% B at 7.5–9.5 min, 10–70% B at 9.5–11 min, 70% B at 11–12 min, flowing at 0.3 ml/min. The injection volume for sample analysis was 5 μl.

Animals and induction of PCOS model

A total of 48 female adult rats were obtained from the Laboratory Animal Center of Chinese Food and Drug Administrator. All rats were allowed to adapt for the environment for 1 week. The breeding room was maintained at constant temperature on a 12–12 h alternating light–dark cycle. All animals had free access to water and food. The Ethics Committee of animal care and use of the Second Hospital of Shanxi Medical University (2015KS001) approved the proposals. The rat model of PCOS was developed by orally administrating with letrozole (Jiangsu HengRui Pharmaceutical Factory, China) at the dose of 1 mg/kg as previously reported. The control group was orally administrated with saline instead of letrozole. To study whether letrozole treatment altered the estrous cycle, vaginal smears were performed every other day by vaginal washing, and then evaluated by microscopy during the treatment period; only the rats with altered estrous cycle were selected for the PCOS rats.

Experimental design

The experiment was aimed to evaluate the effect of SGD on hyperandrogenism in letrozole-induced PCOS rats. A total of 48 rats were randomly assigned 6 groups (n=8 per group): control group, PCOS group, PCOS + SGD (12.5 g/kg) group, PCOS + SGD (25 g/kg), PCOS + SGD (50 g/kg), and PCOS + Diane-35 group. After 2 weeks of drug treatment, the rats were killed for sample collection. The whole blood samples were collected and centrifuged at 3000 rpm for 10 min; both ovaries of all rats were removed; one was collected for histological examination and the other was stored at −80°C until further analysis. At the end of the study, the animals were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.) and killed by cervical dislocation.

Vaginal smears for estrus phase determination

Vaginal smears were carried out every other day on all rats accordingly [28]. Briefly, a sterile cotton swab was inserted into the rat vagina to collect the vaginal secretions. The vaginal secretions were fixed in absolute ethanol and then stained with hematoxylin–eosin (H&E) dye. The estrus phase was evaluated by microscopy with Giemsa staining.

Ovarian histopathology and immunohistochemical (IHC) staining

Ovary tissue from all rats was harvested after killing. After weighing with a precision balance (Sarto-rius, BT 125D, Germany), the ovaries were fixed with 4% formaldehyde buffer, embedded in paraffin, sectioned into 4 μm slides, and stained with H&E, dehydrated in 95%, 90%, and 70% ethanol, cleared in xylene. The sections were observed using a microscope (Carl Zeiss Microscopy GmbH, Goettingen, Germany).

Paraffin-embedded ovary tissues were cut into 5 μm sections. The sections were deparaffinized, rehydrated, and placed in citrate buffer (pH 6.0) for retrieving the antigens. After that, the sections were quenched in 3% H_2O_2 for 15 min and blocked with 5% BSA. Primary antibody for IL-1β, TNF-α, IL-6, and IL-18 (Biosynthesis) was incubated overnight at 4°C in the ratio of 1:2000, respectively. After washing with PBS, the sections were incubated with HRP labeling antibodies for 1 h. DAB solution was applied for the visualization of the reaction product.

Western blot analysis

The ovary tissues were homogenized in the lysis buffer and centrifuged at 10,000 g at 4°C for 10 min. The protein concentration in the supernatants was measured by bicinchoninic acid protein assay reagent. The samples were separated on 10% SDS-PAGE and transferred to polyvinylidenefluoride membrane. Non-specific binding sites were incubated with non-fat milk, and then the membrane was incubated overnight at 4°C with corresponding primary antibodies. The membranes were washed in 1× TBST, and then incubated with the appropriate HRP conjugated secondary antibodies. The immunoreaction was detected using the electrochemiluminescence Western blotting detection kit. The scanned digital images were quantified using NIH Image J software.

Real-time reverse transcriptase quantitative polymerase chain reaction

RT-qPCR was conducted according to a previously described method [29]. Total RNA was extracted from ovary using RNA prep Pure Tissue Kit (Tiangen Biotech, China) according to the manufacturer's instructions and cDNA was transcribed from the total RNA. The primer sequences of TNF-α, IL-1β, IL-6, IL-18, estrogen receptor α (ERα), steroid sulfatase (STS), estrogen sulfotransferase (EST), NF-κB, NF-κB p65, IκB, and β-actin were listed in Table 1. RT-qPCR was carried out as follows: single cycle at 95°C for 15 min; 40 cycles of denaturation at 95°C for 15 s, followed by annealing and elongation at 61°C for 31 s. Results were analyzed using $2^{-(\Delta\Delta C_T)}$ method and mRNA levels were represented as fold changes of β-actin.

ELISA analysis for hormone and inflammatory cytokine levels

Rat serum samples and ovary tissues were collected as previously described. Serum hormone levels (T, E_2, LH, FSH, and P) and inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-18) were assayed using ELISA kits (Expandbio, Beijing, China). ELISA procedures were conducted as manufacturer's instructions.

Statistical analysis

All data were expressed as mean ± SEM. Multiple means were compared with two-way ANOVA. Differences between two groups were analyzed by Student's t-test and a value of $P<0.05$ was considered statistically significant.

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Table 1 List of primers for real-time PCR

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
TNF-α	AGCACGGAAAGCATGATCCG	ACCGATCACCCCGAAGTTCA
IL-1β	CCCTTGTCGAGAATGGGCAG	GACCAGAATGTGCCACGGTT
IL-6	CCCCAACTTCCAATGCTCTCC	AGCACACTAGGTTTGCCGAG
IL-18	CAGCCAACGAATCCCAGACC	AGATAGGGTCACAGCCAGTCC
ERα	TCTGGAGTGTGCCTGGTTGGAG	GCGGAATCGACTTGACGTAGCC
STS	ATGGCTGATGACCTTGGCATTGG	CGCCAGGTGCTGAGTCAACTTC
EST	ACCTGCCAGCTAAGCTCCTTCC	CCAGGAACCATACGGAACTTGCC
NF-κB	TGTGGTGGAGGACTTGCTGAGG	AGTGCTGCCTTGCTGTTCTTGAG
NF-κB p65	GATGGCTTCTATGAGGCTGAACTCTG	CTTGCTCCAGGTCTCGCTTCTTC
IκB	GAAGGACGAGGATTACGAGCAGATG	ATGGTCAGTGTCTTCTCTTCATGGATG
β-Actin	AGATTACTGCCCTGGCTCCT	ACATCTGCTGGAAGGTGGAC

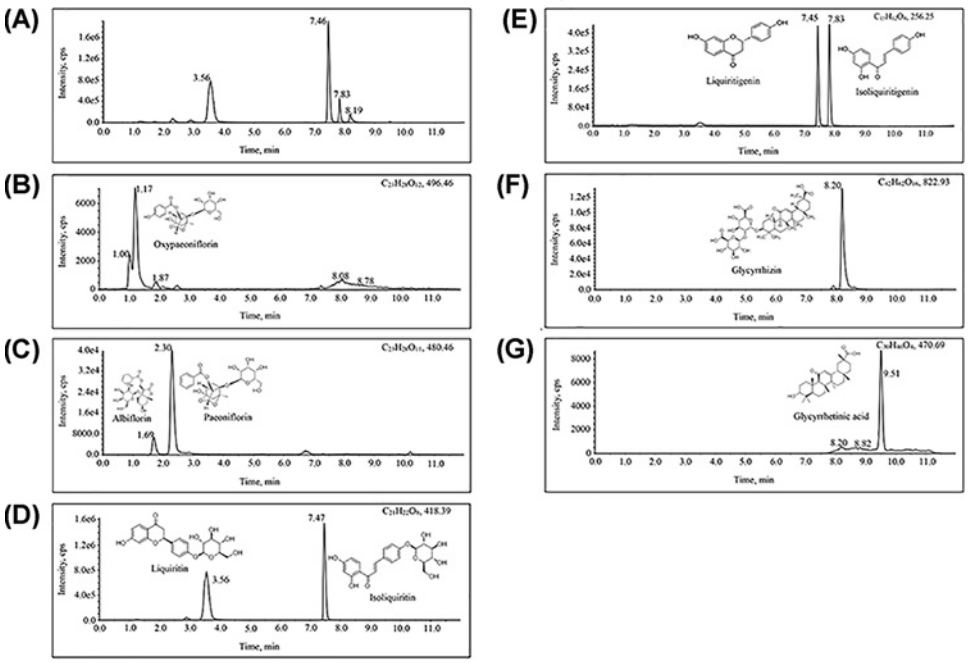

Figure 1. UPLC–MS/MS profiles of the analytes in SGD

(**A**) Total ion chromatograms, (**B**) oxypaeoniflorin, (**C**) albiflorin and paeoniflorin, (**D**) liquiritin and isoliquiritin, (**E**) liquiritigenin and isoliquiritigenin, (**F**) glycyrrhizin, and (**G**) glycyrrhetinic acid.

Results

UPLC–MS/MS analysis of eight components in SGD

For the ionization of compounds in SGD, negative ion polarity modes were used in high and low collision energy test. Oxypaeoniflorin, albiflorin, paeoniflorin, liquiritin, isoliquiritin, glycyrrhizin, and glycyrrhetinic acid were eluted at 1.17, 1.69, 2.28, 3.56, 7.47, 8.19, and 9.51 min, respectively (Figure 1). Liquiritigenin and isoliquiritigenin, a pair of isomers, had identical product ion at MS/MS transitions (m/z) 255. The optimized MS/MS energy parameters declustering potential and collision energy of the analytes were shown in Table 2.

Effect of SGD on the body weight and ovarian weight of PCOS rats

Given that obesity is the most common clinical feature of PCOS, we investigated whether SGD treatment could influence the body weight and ovarian weight in PCOS rats. As shown in Figure 2A, body weight in PCOS rats was significantly elevated when compared with the normal rats ($P<0.05$), indicating that the PCOS rats had gained more weight, indicating the feature of PCOS. However, Figure 2A and B showed that compared with the PCOS rats, body

Table 2 MS/MS transitions and parameters for the detection of the analytes

Analyte	Precursor ion (m/z)	Product ion (m/z)	CE (V)	DP (V)
Paeoniflorin	479.1	121.0	−20.2	−120.0
Albiflorin	479.1	121.1	−80.0	−46.0
Oxypaeoniflorin	495.1	137.1	−150.0	−32.8
Liquiritin/Isoliquiritin	417.3	255.1	−130.0	−26.2
Liquiritigenin/Isoliquiritigenin	255.1	119.0	−80.0	−35.0
Glycyrrhetinic acid	469.3	355.2	−122.0	−63.0
Glycyrrhizin	821.4	350.9	−80.0	−58.8

weight and ovarian weight in SGD-treated rats were significantly and dose-dependently reduced ($P<0.05$), suggesting that SGD could alleviate the symptoms of obesity.

Effect of SGD on estrous cycles of PCOS rats

Since the regular estrous cycle is a main index of the normal ovarian function in humans, to evaluate the effect of SGD on estrous cycle in rats, we verified the stage of estrous cycle by vaginal smear. Among 40 letrozole-induced rats, 85% rats had irregular estrous cycles, namely only 34 rats were selected as PCOS rats. Figure 2 showed that after low-dose SGD treatment, 71.4% PCOS rats had regular estrous cycle. In addition, the population of rats with estrous stages could show recovery up to 85.7% in the middle-dose and high-dose SGD groups. These findings suggested that SGD could regulate the estrous cycles of PCOS rats.

Effect of SGD on ovarian morphological changes of PCOS rats

As the morphological changes of ovary were the major feature of patients with PCOS, we investigated whether SGD changed the appearance and histological structures of ovaries. As shown in Figure 2D, compared with the normal rats, the ovaries in PCOS group showed increased cystic dilating follicles, reduced layers of granular cells (GCs), and disappeared oocytes and corona radiating in the follicles. Compared with the PCOS group, SGD treatment dose-dependently decreased the number of cystic dilating follicles and increased the layers of GCs.

Effect of SGD on steroid hormone levels in PCOS rats

In addition to suppress the increased body weight and ovarian weight of PCOS rats, we further investigated whether SGD could regulate the steroid hormone levels of PCOS rats. As shown in Figure 3, compared with the normal rats, the levels of T and LH in the serum of PCOS rats were all significantly increased ($P<0.05$), whereas the levels of estrogen and FSH were significantly decreased ($P<0.05$), which was in accordance with the characteristic hormone changes in patients with PCOS. Treatment with SGD reversed the increased serum levels of androgen in PCOS rats and the decreased serum levels of estrogen. Moreover, serum levels of progesterone were dose-dependently increased in SGD treatment group, but there was not a significant difference ($P>0.05$).

Effect of SGD on TNF-α, IL-1β, IL-6, and IL-18 levels of PCOS rats

Low-grade chronic inflammation in PCOS could be considered one of the potential links between PCOS and ovarian dysfunction, metabolic aberration, and cardiovascular disease. SGD possesses the pharmacological activities of anti-inflammatory. Thus, we investigated whether administering SGD could regulate the level of pro-inflammatory cytokines. As shown in Figures 4–6, compared with control group, TNF-α, IL-1β, IL-6, and IL-18 levels in serum and ovary tissue were increased in PCOS group. On the contrary, treatment with SGD markedly decreased the levels of TNF-α, IL-1β, IL-6, and IL-18 in serum and ovary tissue ($P<0.05$).

Effect of SGD on NF-κB, NF-κB p65, P-NF-κB p65, and IκB levels in PCOS rats

All our studies so far showed that SGD has therapeutic effect on hyperandrogenism in PCOS rats. In addition, SGD marked decrease in the pro-inflammatory cytokine levels of TNF-α, IL-1β, IL-6, and IL-18. It was well known that NF-κB is a transcription factor that plays a crucial role in controlling the inflammatory process. Therefore, we further investigated whether the anti-inflammation effect of SGD was mediated by inhibiting the phosphorylation of NF-κB. As shown in Figures 6,7, the ovarian mRNA levels of NF-κB and NF-κB p65 were significantly increased ($P<0.01$) in the PCOS group. However, the increased mRNA levels were decreased significantly in the SGD intervention groups.

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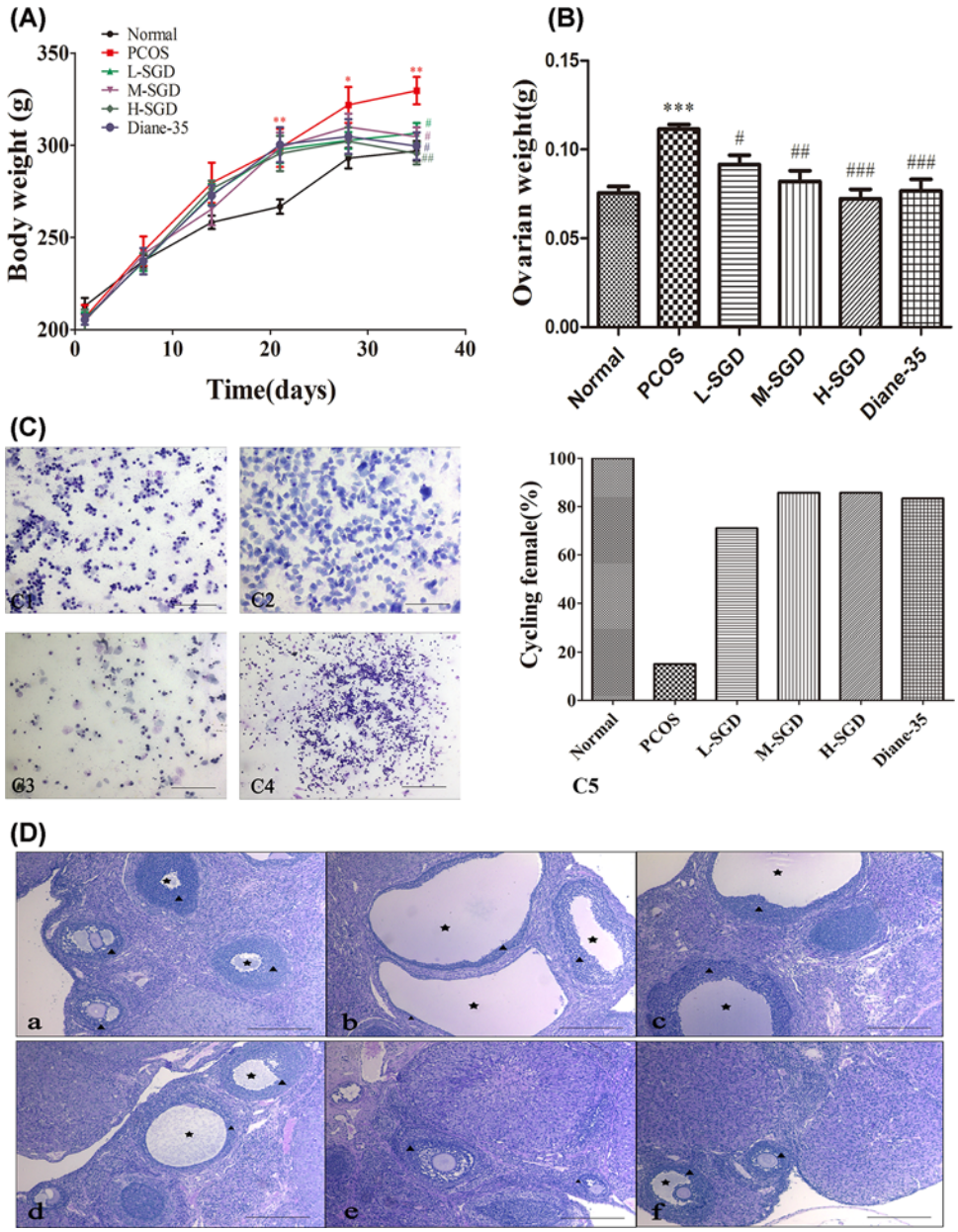

Figure 2. The therapeutic effect of SGD on PCOS rats

(**A and B**) Effects of SGD on body weight and ovarian weight in PCOS rats. (**C**) Effect of SGD in estrous cycle in PCOS rats; (C1–C4) representative photomicrographs of the vaginal smears stained for H&E dye staining proestrus (C1), estrous (C2), metestrus (C3), and diestrus stages (C4); (C5) statistical graphs of estrous cycle in the normal group and PCOS group. (**D**) Effects of SGD on morphological changes in ovarian tissues of PCOS rats, (a) normal group; (b) PCOS group; (c) L-SGD group; (d) M-SGD group; (e) H-SGD group; (f) Diane-35 group. ★ indicated the appearances of cystic follicle, ▲ indicated the appearances of granular cells layer; (magnification, ×100), scale bars, 200 μm. Data are presented as mean ± SEM (n=8). $^{*}P<0.05$ compared with normal group, $^{**}P<0.01$ compared with normal group, $^{***}P<0.001$ compared with normal group, $^{\#}P<0.05$ compared with PCOS rats, $^{\#\#}P<0.01$ compared with PCOS rats, and $^{\#\#\#}P<0.001$ compared with PCOS rats.

In addition, the mRNA and protein levels of IκB were decreased in the ovary tissue of PCOS rats and SGD treatment notably reversed the changes. It is worth noting that the ratio of P-NF-κB p65/NF-κB p65 was increased in the PCOS group and SGD treatment could significantly reduce the ratio, suggesting that the inflammation was triggered in PCOS group and SGD had the ability of inhibiting the phosphorylation of NF-κB p65.

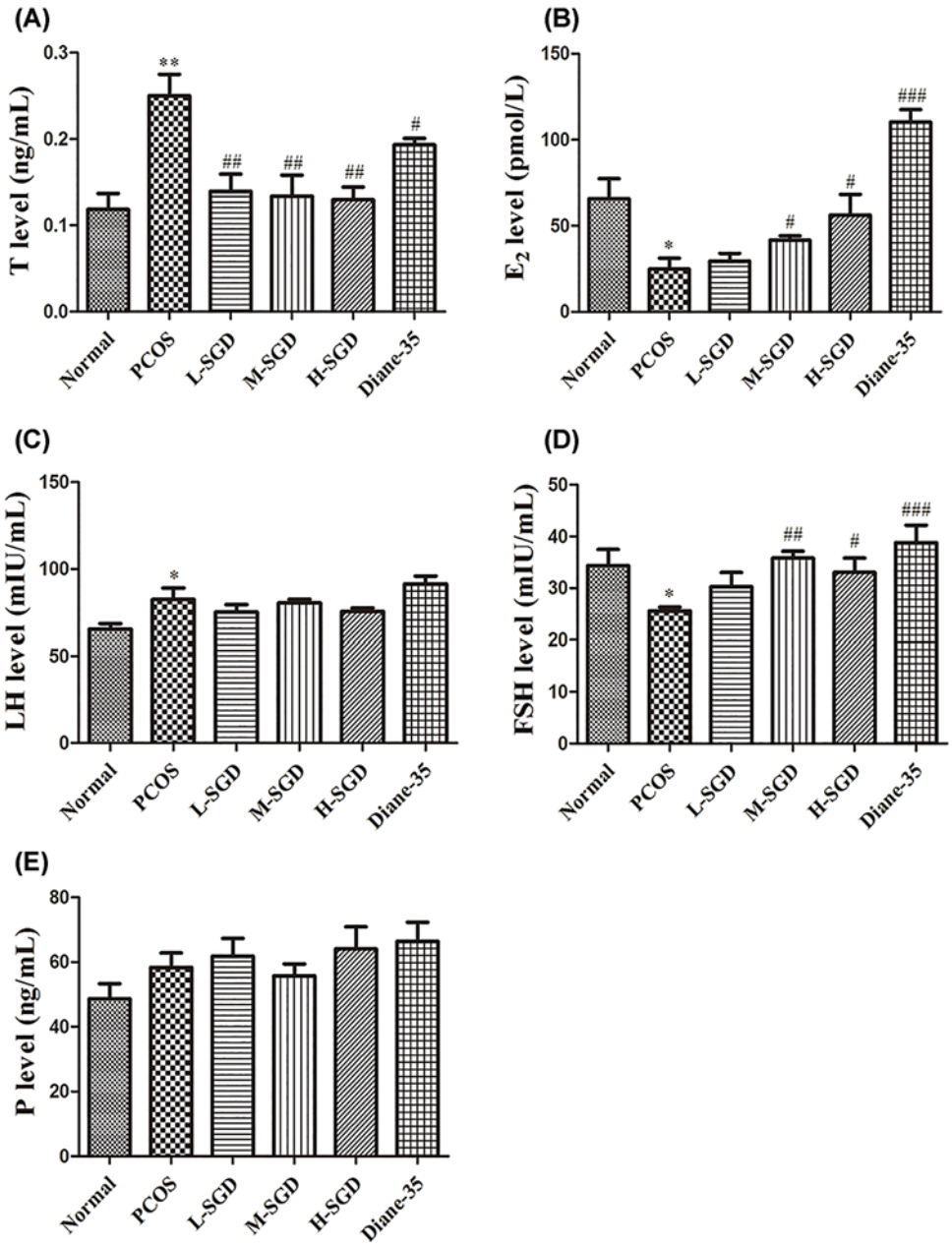

Figure 3. Effects of SGD on the serum levels of hormones in PCOS rats detected by ELISA kit

(**A–E**) represented testosterone (T), 17β-estradiol (E2), luteinizing hormone (LH), follicle stimulating hormone (FSH), and progesterone (P), respectively. Data are presented as mean ± SEM (n=8). $^{*}P<0.05$ versus normal group, $^{**}P<0.01$ versus normal group, $^{\#}P<0.05$ versus PCOS rats, $^{\#\#}P<0.01$ versus PCOS rats, and $^{\#\#\#}P<0.001$ versus PCOS rats.

Discussion

For a long time, the treatment of PCOS relies on application of clomiphene citrate [30]. However, besides the side effect of clomiphene citrate, some patients were non-responsive for clomiphene citrate [31]. Recently, new treatment based on traditional Chinese herbs provided a novel therapeutic way for PCOS. The present study demonstrated that SGD could ameliorate hyperandrogenism in PCOS rats, and the potential mechanism may involve NF-κB pathway.

Steroid hormones such as T, E_2, LH, FSH, and P play key roles in fertility and reproductive function through activating the specific nuclear receptors. For example, abnormal production of LH affects the function of the granulosa and theca interna layers [32]. E_2 influences growth and differentiation of GCs [33]. T plays an important role in

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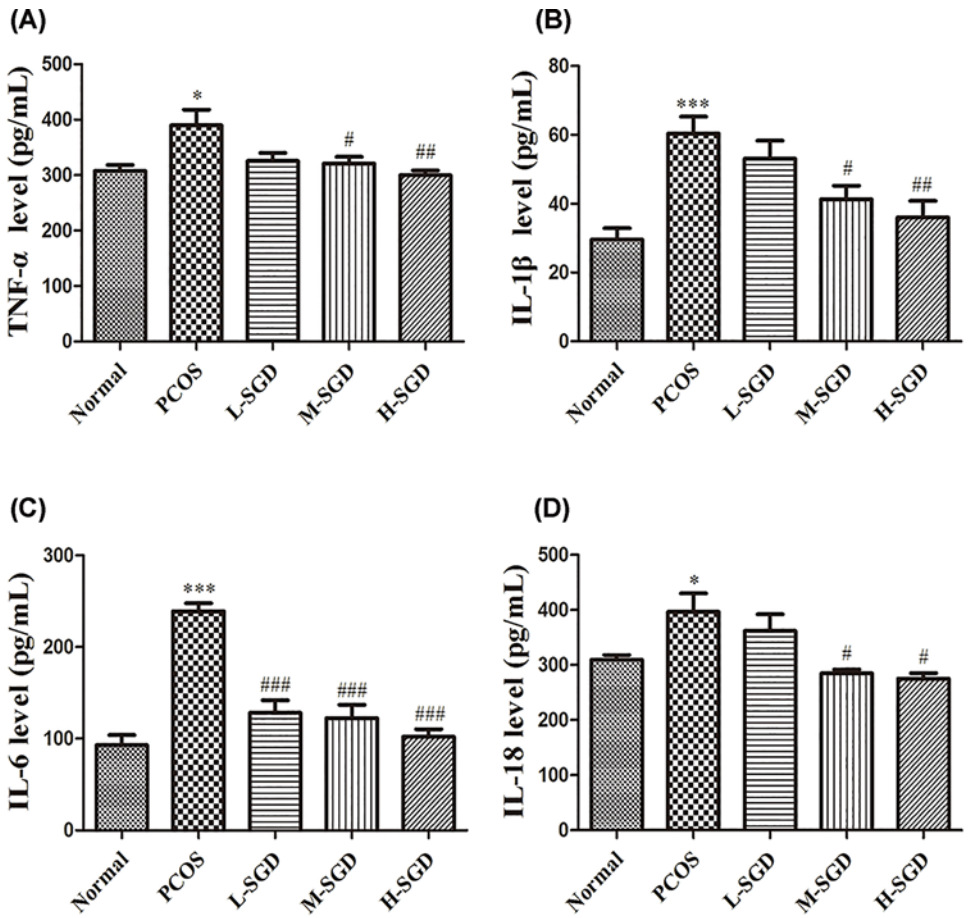

Figure 4. Effects of SGD on the serum levels of inflammatory factors in PCOS rats

The concentrations of (**A**) TNF-α, (**B**) IL-1β, (**C**) IL-6, and (**D**) IL-18 were measured with ELISA kit. Data are presented as mean ± SEM (n=8). $^{*}P<0.05$ compared with normal group, $^{***}P<0.001$ compared with normal group, $^{\#}P<0.05$ versus PCOS rats, $^{\#\#}P<0.01$ compared with PCOS rats, and $^{\#\#\#}P<0.001$ versus PCOS rats.

physiological process of ovarian follicular maturation [34]. Our results showed that the levels of T and LH in the serum of PCOS rats were significantly increased, whereas the levels of E_2 and FSH were significantly decreased. These hormonal profiles are consistent with the hormonal environment described in PCOS patients [35,36]. Moreover, we also showed that SGD treatment dose-dependently and significantly reversed the alteration in serum levels of hormone, i.e., SGD can reduce the serum levels of T and LH and increase the serum levels of E_2 and FSH in PCOS rats. As E_2 level was from the conversion of T, the increased E_2 level was in accordance with the reduced levels of T. These results suggested that, to a certain extent, SGD could rebalance the serum levels of T and E_2. It was reported that the disorder of steroid hormones could be caused by disturbance of the hypothalamus–pituitary–gonadal axis [37]. Alterations in key synthesis or catalysis enzyme activities of hormones also can lead to hormonal environment imbalance in PCOS patients [38]. In addition, abnormal expression level of nuclear receptors, including androgen receptor and ERα, involved in disorder of hormonal environment [39,40]. The results showed that the treatment with SGD could increase the mRNA levels of ERα and EST, and decreased the STS levels (Supplementary Figure S1). As we know, TCM exerts therapeutic effects through multi-target and multi-pathway, the regulation of SGD on hormones disorder may be through hypothalamus–pituitary–gonadal axis or the enzymatic activity of ovarian P450arom and P450c17a or expression level of nuclear receptors. More studies are needed to explore these questions.

The hallmarks of women with PCOS are the presence of polycystic ovaries and hyperandrogenism. It is reported that peroxisome proliferator-activated receptor-γ (PPAR-γ) can affect adipocyte differentiation, glucose and lipid homeostasis, [41] and hyperandrogenism that could down-regulate the expression of PPAR-γ [42]. Thus, it is speculated that obesity in women with PCOS could be partially attributed to hyperandrogenism. Results from the present study showed that body and ovarian weights were markedly increased in letrozole-induced PCOS rats, which are

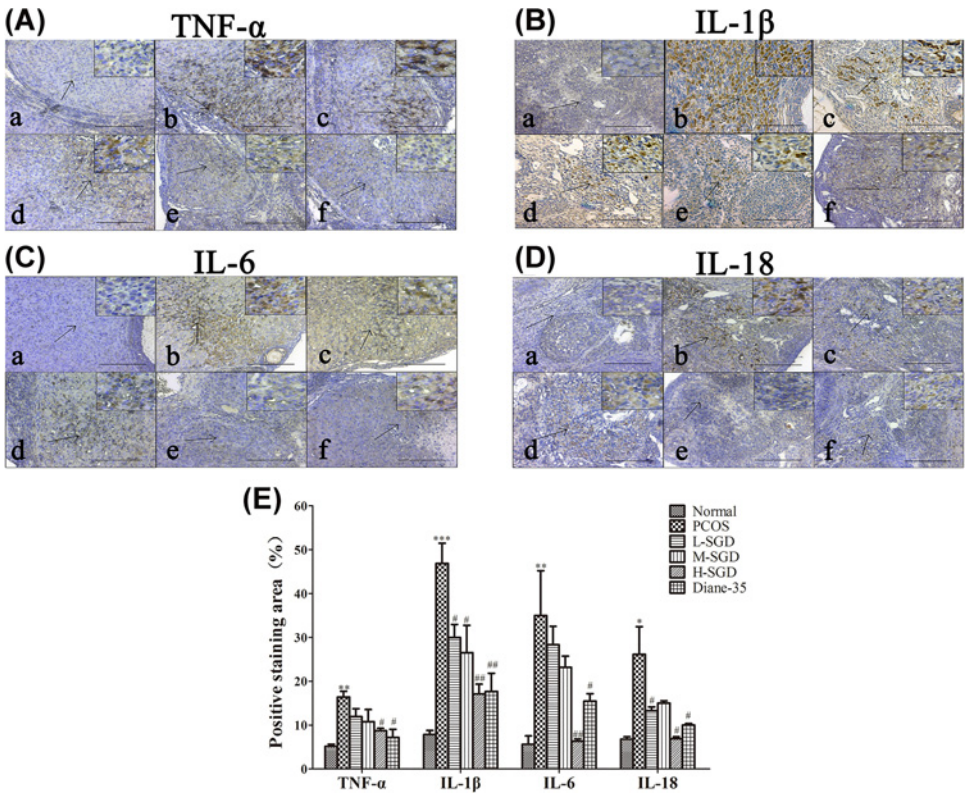

Figure 5. Effects of SGD on the expression of inflammatory factors in the ovarian tissues in PCOS rats determined by IHC staining

(**A–D**) show representative images of immune infiltration areas of TNF-α, IL-1β, IL-6, and IL-18 (marked with arrows in each marker), respectively. Scale bars indicate 100 μm (200×) in large pictures and 50 μm (400×) in small-corned pictures. (**E**) shows quantitative analysis for TNF-α, IL-1β, IL-6, and IL-18 expression. Data are presented as mean ± SEM (n=8). $^{*}P<0.05$ compared with normal group, $^{**}P<0.01$ compared with normal group, $^{***}P<0.001$ compared with normal group, $^{\#}P<0.05$ compared with PCOS rats, and $^{\#\#}P<0.01$ compared with PCOS rats.

consistent with previous findings [43]; we also found that SGD treatment dose-dependently reduced body weight and ovarian weight in PCOS rats, and the reduced body weight and ovarian weight correlate significantly with the alterations of circulation level of T. These results indicated that the active ingredients of this formulation might exert losing weight effects through reducing circulation level of T.

As a typical clinical feature of PCOS, hyperandrogenism could obviously affect the physiological process of ovarian follicular maturation [44]. However, the underlying mechanism has not been clarified. It was found that GCs play a crucial role in promoting preantral follicle growth and prevent follicular atresia, and T could affect GCs differentiation [45]. Evidence also showed that T could cause early luteinization of ovarian granular layer cells, resulting in excessively small follicles [46]. Therefore, hyperandrogenism was believed to play prominent roles in the symptom generation of polycystic ovaries. Our results showed increased number of cystic dilation follicles and fewer layers of GCs in PCOS rats. We also found that SGD treatment dose-dependently decreases the size and number of follicular cysts, and increases the layers of GCs in PCOS rats. Given the vital role of T in the symptom generation of PCOS, it is possible that the decreased T level in SGD-treated rats be responsible for the follicular growth and oocyte maturation of PCOS rats.

To date, the mechanism concerning hyperandrogenism in PCOS is still not clear, but it is reported to be correlated with the increased synthesis of T precursors and excess T secretion from ovary [34]. NF-κB has been regarded as the key mediator of the inflammatory process and regulated a number of genes, including TNF-α, IL-1β, IL-6, and IL-18 [6]. Under normal condition, NF-κB can form an inactive complex with IκB in the cytoplasm. Upon stimulation, NF-κB translocates to the nucleus where it can activate certain genes by binding to promoter regions [47,48]. The activation of NF-κB involves the phosphorylation of the IκB protein on two conserved serine residues, which is carried out by IκB kinase complex. After IκB phosphorylation and subsequent proteolysis, NF-κB transcription factor

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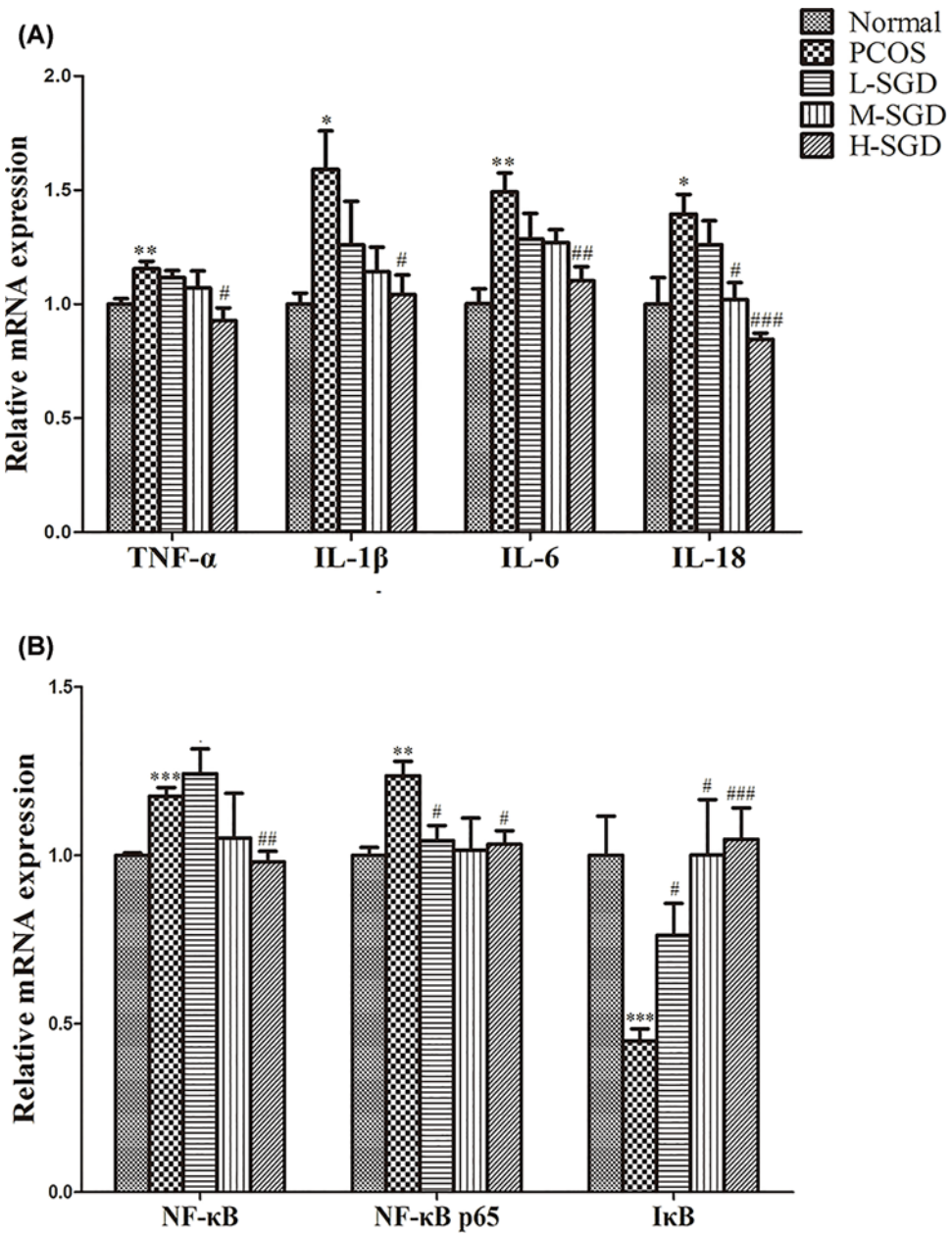

Figure 6. Effects of SGD on NF-κB signaling pathway relative gene mRNA levels in PCOS rats.

The mRNA levels of (**A**) inflammatory factors (TNF-α, IL-1β, IL-6, and IL-18) and (**B**) NF-κB signaling pathway relative genes (NF-κB, NF-κB p65, and IκB) were determined by real-time PCR. The expression levels of target mRNA were standardized by β-actin mRNA level in each sample. Data are presented as mean ± SEM (n=8). $^{*}P<0.05$ compared with normal group, $^{**}P<0.01$ compared with normal group, $^{***}P<0.001$ versus normal group, $^{\#}P<0.05$ compared with PCOS rats, $^{\#\#}P<0.01$ compared with PCOS rats, and $^{\#\#\#}P<0.001$ compared with PCOS rats.

p50/p65 is translocated to the nucleus, activating transcription of its target genes [49,50]. We found that the phosphorylation of NF-κB was significantly increased in PCOS rats. By contrast, SGD treatment obviously suppressed the phosphorylation of NF-κB. Paeoniflorin, albiflorin, and oxypaeoniflorin are the main ingredients of Radix Paeoniae Alba, which are responsible for anti-inflammation in the prescription of SGT. It is reported that these compositions could inhibit the phosphorylation of IκB and block NF-κB transcription factor p50/p65 activation [51]. In addition, paeoniflorin could affect DNA binding ability of nuclear translocation p65 [51,52]. Thus, we speculated that SGD affects NF-κB activation by inhibiting IκB phosphorylation and blocks DNA binding ability of NF-κB transcription

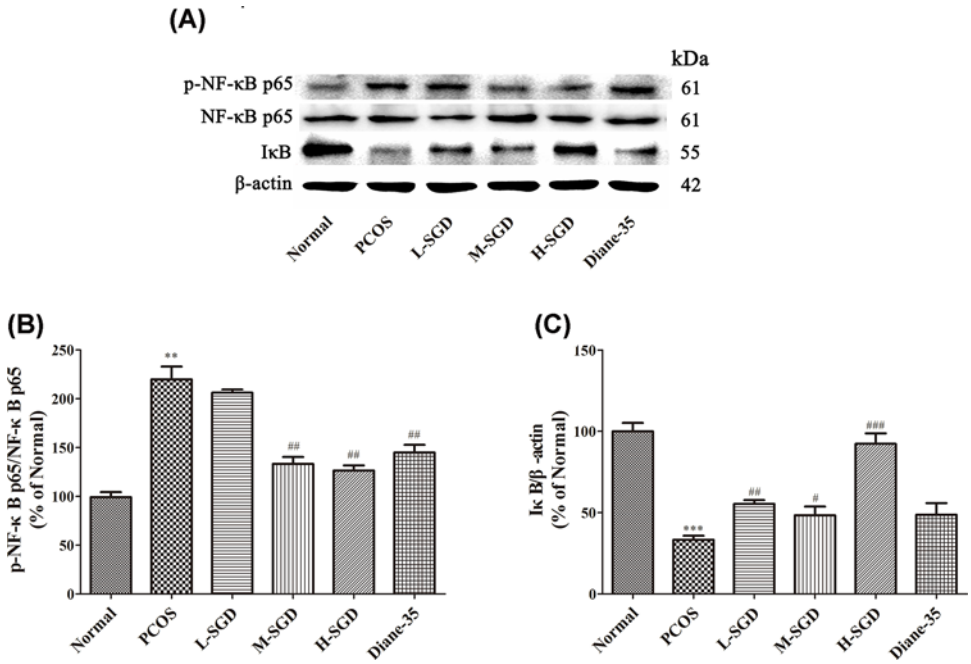

Figure 7. Effects of SGD on NF-κB p65, p-NF-κB p65, and IκB protein expression in PCOS rats

(**A**) Western blot analysis shows the relative expression of NF-κB p65, p-NF-κB p65, and IκB. (**B–C**) Quantitative analysis of NF-κB p65, p-NF-κB p65, and IκB. Data are presented as mean ± SEM of three identical experiments. $^{***}P<0.001$ compared with normal group, $^{\#}P<0.05$ compared with PCOS rats, $^{\#\#}P<0.01$ compared with PCOS rats, $^{\#\#\#}P<0.001$ compared with PCOS rats.

factor p50/p65. The present study also found that SGD treatment decreased the levels of TNF-α, IL-1β, IL-6, and IL-18 in ovary tissue of PCOS rats. These findings are consistent with the previous studies that showed that NF-κB can regulate the inflammatory factor genes including TNF-α, IL-1β, IL-6, and IL-18 [53,54]. We all knew that local inflammation in PCOS ovaries could impair follicular growth and maturation. It is possible that the increased inflammatory cytokine levels affected follicular development. These findings indicated that SGD may reduce the size and number of follicular cysts in PCOS rats by reducing the T level and the inhibited NF-κB activation may contribute to the regulatory effect. As we all know that each TCM comprises more than one herb and it exerts therapeutic effect by using individual herbs in combination, i.e., SGD consists of two herbs: *Radix Paeoniae Alba* and *Glycyrrhizae uralensis*. In this prescription, paeoniflorin and glycyrrhizin act as the main components and they were reported to have the capacity of lowering the serum levels of androgen. We believe that the main reason for the efficacy of SGD on PCOS relates to the active ingredients of each herb and these ingredients exert therapeutic effects through multi-target and multi-pathway. To confirm the key active ingredients that are responsible for the therapeutic effect, further study is needed.

Conclusion

Our study demonstrated that SGD could ameliorate hyperandrogenism in PCOS rats, and the potential mechanism may involve in the inhibited NF-κB activation. Results from this present study provide experimental evidence about the therapeutic potential of SGD in PCOS.

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Author Contribution

Rui-gang Hou and Yun-yun Shao conceived and designed the study; Zhuang-peng Chang, Yao Cheng, Xin-chun Wang, Jing-ping Zhang, Xiao-juan Feng and Yi-ting Guo performed the experiments; Jun-jin Liu and Zhuang-peng Chang wrote the paper; Rui-gang Hou reviewed and edited the manuscript. All authors discussed the results and approved the final manuscript.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

ERα, estrogen receptor α; EST, estrogen sulfotransferase; GC, granular cell; H&E, hematoxylin–eosin; IHC, immunohistochemical; PCOS, polycystic ovary syndrome; PPAR-γ, peroxisome proliferator-activated receptor-γ; SGD, Shaoyao-Gancao Decoction; STS, steroid sulfatase; TCM, traditional Chinese medicine.

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