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2,3,5,4'−Tetrahydroxystilbene−2−O−β−glucoside Alleviates PCOS−like Characteristics by Upregulating The Expression of CYP19A1 in Granular Cells of Sinus Follicles to Inhibit Inflammatory Response *

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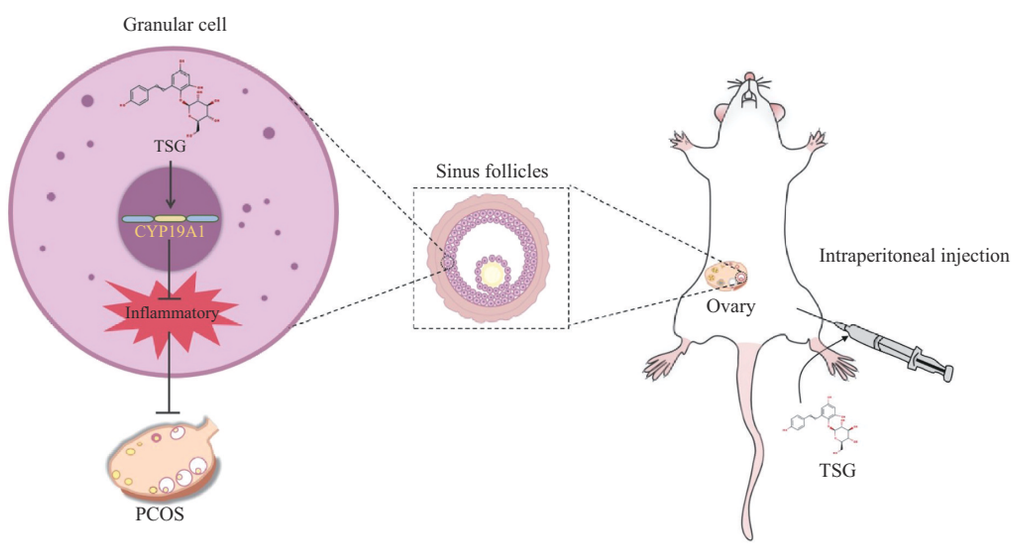
Graphical abstract



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Abstract **Objective** To investigate whether 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside (TSG) ameliorated polycystic ovary syndrome (PCOS) -like characteristics by inhibiting inflammation. **Methods** PCOS models were established by injecting subcutaneously with dehydroepiandrosterone into female Sprague-Dawley rats, followed by receiving intraperitoneal injection of TSG. The granular cells (GCs) KGN were transfected with small interfering RNAs (si-NC and si-CYP19A1). The cells were preincubated with lipopolysaccharide (LPS) and then treated with or without TSG. The estrous cycle was monitored using vaginal exfoliated cells. The morphology of ovarian follicles was analyzed by H&E staining. ELISA was used to analyze estradiol (E2), testosterone (T), follicle stimulating hormone (FSH), luteinizing hormone (LH), IL-6, TNF-α, AGEs, CRP and Omentin-1 levels in serum. Immunohistochemistry was performed to analyze PCNA and CYP19A1 expressions in the GCs of ovaries. Tunel staining was executed to detect the apoptosis of GCs. Quantitative polymerase chain reaction (qPCR) and Western blot were implemented to measure the expression of CYP19A1 in the ovaries and transfected cells. qPCR was used to analyze the expression of IL-6 and TNF-α in the transfected cells treated with LPS and TSG. **Results** The estrous cycles were restored in TSG group. Compared with model group, the sinus follicles were reduced and corpus luteums were increased in TSG group. TSG group showed increased E2, and decreased T and LH, compared with model group. Pro-inflammatory factors (IL-6, TNF-α, CRP and AGEs) were decreased, and anti-inflammatory factor (Omentin-1) was increased in TSG group compared with those in model group. TSG could partially inhibit decrease of PNCA-positive GCs and increase of Tunel-positive GCs caused by PCOS. The CYP19A1 expression of GCs in TSG group was upregulated compared with model group. The expressions of IL-6 and TNF-α in si-CYP19A1 cells were increased compared with si-NC cells. Compared with cells (si-NC and si-CYP19A1) treated without LPS, the expressions of IL-6 and TNF-α cells were increased, and the expression of CYP19A1 was downregulated in LPS-preincubated cells. Compared with cells treated with LPS, the expression of IL-6 and TNF-α were decreased, and the expression of CYP19A1 was increased in cells treated with LPS and TSG. Compared with si-NC cells treated with LPS and TSG, the expressions of IL-6 and TNF-α cells were increased in the si-CYP19A1 cells treated with LPS and TSG. **Conclusion** TSG could alleviate PCOS-like characteristics by increasing the expression of CYP19A1 in GCs to inhibit inflammatory response.

Key words 2,3,5,4'-tetrahydroxystilbene-2-O-β-glucoside, granular cells, CYP19A1, polycystic ovary syndrome, inflammatory response

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Polycystic ovary syndrome (PCOS) is the most common reproductive endocrine disease in women of reproductive age, and is one of the main causes of female infertility. The relevant studies showed that the prevalence of PCOS was as high as 20% in women of reproductive age[1-2]. PCOS was characterized by hyperandrogenemia, polycystic ovary morphology, menstrual disorders, infertility and obesity, often accompanied by metabolic disorders and chronic inflammation[2-3]. This variety of clinical manifestations were related to genetic[2, 4] and unhealthy living habit[5-7], however, the underlying pathogenesis was slurred. The study of the pathological of PCOS is helpful to select a reliable new treatment to promote follicle development and ovulation, which maybe increase the probability of pregnancy and alleviate the trend of fertility decline and population aging.

The reproductive dysfunction of PCOS was mainly manifested by anovulation and hyperandrogenemia[2, 8]. Meanwhile, hyperandrogenemia was proved to be one of the important pathological mechanisms of PCOS[9-10]. Aromatases such as CYP19A1 were known to accelerate the conversion of androgen to estrogen in granular cells (GCs) [11-14]. A study had shown that CYP19A1 transcription was abnormal in PCOS[15-16]. PCOS was accompanied by chronic inflammation[17-20]. At the same time, it was shown that excess androgen could enhance the expression of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), ultimately accelerating the progression of PCOS[21]. Inflammatory markers were increased and the expression of CYP19A1 was downregulated in lipopolysaccharide (LPS) -induced GCs[22-24], which suggested that the expression of CYP19A1 was negatively correlated with the level of inflammatory cytokines in GCs. It was verified that CYP19A1 could improve inflammation by regulating estradiol (E2) synthesis *via* inhibiting the NLRP3 inflammasome signaling pathway in skin of acne mice[25]. However, whether CYP19A1 can also regulate inflammation in GCs still needs further

investigation.

Based on the promotion of inflammatory factors in the PCOS process, the inflammation of PCOS had received much attention by researchers. Increasing evidences showed that traditional Chinese medicine could alleviate ovarian function of PCOS[26-28]. 2,3,5,4'-Tetrahydroxystilbene-2-O-β-glucoside (TSG) was an active monomeric ingredient extracted from *Polygonum multiflorum* and had been proved to be effective in suppressing inflammatory responses of several diseases[29-33]. For example, TSG attenuated a reactive oxygen species (ROS)-dependent inflammatory response in the *P. gingivalis*-infected brain endothelial cells[30]. TSG played a protective role in traumatic brain injury by inhibiting inflammatory response and oxidative stress through PARP1/Ras/JNK pathway[29]. TSG ameliorated periodontitis by decreasing the expression of inflammatory cytokines through enhancing AMPK activation and increasing SirT1 expression[31]. TSG had an effective anti-infrasound drug against central nervous system injury by downregulation of IL-6, IL-8 TNF-α and high-sensitivity C-reactive protein (CRP) [33]. However, whether TSG inhibits inflammatory response to alleviate PCOS-like features needs further investigation. This study aims to explore the impact of TSG on PCOS and whether it inhibits inflammation by up-regulating the expression of CYP19A1, thereby alleviating the polycystic ovary phenotype.

1 Materials and methods

1.1 Animals

Fifteen 3-week-old female Sprague-Dawley (SD) rats were purchased from Hunan SJA Laboratory Animal Co., Ltd (SYXK (Xiang) 2019-0004, Changsha, China) and fed a standard animal diet with adequate food and water for 1 week at a temperature of (22±3)℃, humidity of (55±5)% and 12 h light/dark cycle. All rats were used for subsequent studies. This project was approved by the Ethics Committee of Hunan University of Medicine (No. 2022(A01010)).

1.2 Drug treatment

Dehydroepiandrosterone (DHEA, D4000, Sigma-Aldrich, St Louis, USA) was dissolved in sesame oil to form an 100 g/L solution and TSG (SML0834, Sigma-Aldrich, St Louis, USA) was dissolved in ddH_2O to form a 5 g/L solution. All drugs were stored at −20℃ for subsequent experiments.

The rats were randomly divided into Control, PCOS and TSG groups (n=5 each group). The rats (PCOS and TSG) were injected subcutaneously with DHEA at a dose of 60 mg/kg (based on body mass) every day for 20 d to establish the PCOS model. The rats of control group were subcutaneous injected daily with the same volume of sesame oil for 20 d. For TSG group, the rats were received intraperitoneal injection of TSG at 25 mg/kg (based on body mass) daily from 21 d to 30 d. For PCOS model and control groups, the rats were intraperitoneally injected with the same volume of PBS daily during this period.

1.3 Cell culture and transfection

The human ovarian granular cells (KGN) were obtained from ATCC. KGN cells were cultured in DMEM (11965092, Gibco, USA) supplement with 10% FBS (C0234, Beyotime, Shanghai, China) and 1% penicillin-streptomycin (C0222, Beyotime, Shanghai, China). Cells were cultured in a 5% CO_2 incubator at 37℃ . The CYP19A1 small interfering RNA (siRNA) (si-CYP19A1, sc-41498) and nontargeting siRNA (si-NC, sc-37007) were purchased from Santa Cruz Biotechnology (Texas, USA). KGN cells were transfected with siRNAs (si-CYP19A1 or si-NC) in according to the instructions of Lipofectamine 3000 reagent when cells reached 70% confluence and were cultured for subsequent experiments.

1.4 Estrous cycle assay

The cotton swabs were moistened and disinfected with saline, then inserted into the vagina of rat, gently rotated in the vagina for several times. The cells on the cotton swab were evenly smeared on the slide and stained with meilan (G1185, Solarbio, Beijing, China). The estrous cycle was determined according to the types and characteristics of vaginal smear cells. The rats were in proestrus stage when the number of nucleated epithelial cells was dominant. The rats were in estrus stage when cornified squamous epithelial cells were predominant. The rats were in metestrus stage when the number of cornified squamous epithelial cells decreased, accompanied by a large number of white blood cells and nucleated epithelial cells appeared. The vaginal mucosa was thin and the cells were almost exclusively white blood cells, which indicated that the rats were in the diestrus stage.

1.5 Measurment of body mass, mass and length of ovaries

There was no significant difference in body mass of all rats before the experiment. On the 31 day of treatment, all rats were euthanized by cervical dislocation. The body mass and the ovaries mass of all the rats were measured using an electronic balance. The length of ovaries was measured using a ruler with a scale.

1.6 Hematoxylin and eosin （H&E） staining

The ovaries were fixed with 4% paraformaldehyde and embedded in paraffin. The sections were cut at 2−5 μm thickness on the paraffin microtome and dewaxed. Then, the sections were stained with H&E Kit (G1120, Solarbio, Beijing, China) in according to the protocol of manufacturer. Finally, the images were photographed to assess follicle development by microscope (Carl Zeiss, Germany).

1.7 Enzyme linked immunosorbent assay （ELISA）

All ELISA kits were purchased from Biomatik (Delaware, USA). Blood was collected by eyeball method before the rats were euthanized. Serum was collected after centrifugation at 1 000*g* for 15 min. The serum levels of E2, testosterone (T), follicle stimulating hormone (FSH), luteinizing hormone (LH), IL-6, TNF-α, advanced glycation end-products (AGEs), CRP and Omentin-1 were respectively analyzed by E2 (EKC40197), T (EKC40191), FSH (EKL54320), LH (EKL54776), IL-6 (EKC39275), TNF-α (EKC40834), AGEs (EKF57894), CRP (EKE62152) and Omentin-1 (EKF60603) ELISA kits according to the protocol of manufacturer.

1.8 Immunohistochemistry （IHC）

Sections were dewaxed, treated with 3% H_2O_2 for 10 min to eliminate the activity of endogenous peroxidase and boiled in 0.01 mmol/L sodium citrate buffer for antigen repair. Then the sections were blocked by 10% FBS for 10 min at room temperature (RT) and stained with specific antibodies against PCNA (1∶100, AF1363, Beyotime, Shanghai, China) and CYP19A1 (1∶50, AF6231, Beyotime, Shanghai, China) at 37℃ for 1 h. The sections were subsequently incubated with a secondary horseradish peroxidase (HRP) labeled goat anti-rabbit IgG (H+L) at 37℃ for 10 min and incubated with diaminobenzidine (DAB, DA1010, Solarbio, Beijing, China) at RT for several minutes in according to the protocol of manufacturer. Finally, images were viewed and captured by microscope.

1.9 Tunel staining

The apoptosis of GCs in follicles was analyzed by Tunel Assay Kit (C1091, Beyotime, Shanghai, China) in according to the protocol of manufacturer. Briefly, the sections were dewaxed and treated with 20 mg/L protease K without DNase (ST535, Beyotime, Shanghai, China) at 37℃ for 15 min. The samples were incubated with 3% H_2O_2 at RT for 20 min, and then incubated with Tunel detection solution at 37℃ for 1 h. Subsequently, the sections were incubated with DAB at RT for several minutes. Finally, images were viewed and captured by microscope.

1.10 Quantitative polymerase chain reaction （qPCR）

Total RNA was isolated from ovaries and transfected cells using the TransZol (ET101, TRANSGEN, Beijing, China). The concentration of RNA was determined by the NanoDrop ND-2000 spectrophotometer (Thermo, Massachusetts, USA). The cDNA was synthesized using the EasyScript® First-strand cDNA Synthesis Super Mix (AE301, TRANSGEN, Beijing, China) in according to the protocol of manufacturer. Finally, the expression of CYP19A1 was determined using the PerefectStart® Fast Green qPCR SuperMix (AQ611, TRANSGEN, Beijing, China) in according to the protocol of manufacturer by CFX Connect™ Real-Time System (BIO-RAD, California, USA) with primers of *CYP19A1* (forward: 5'-TTGAACAGAACCTGAGCCTCC-3', reverse: 5'-GTGGCTCCTGTCACTTGGAA-3'), *IL-6* (forward: 5'-AGGATACCACTCCCAACAGACCT-3', reverse: 5'-CAAGTGCATCATCGTTGTTACTAC-3'), *TNF-α* (forward: 5'-CATCTTCTCAAAATTCGAGTGACAA-3', reverse: 5'-CCCAACATGGAACAGATGAGGGT-3'), and *β-actin* (forward: 5'-TGTACCCAGGCATTGCTGAC-3', reverse: 5'-AACGCAGCTCAGTAACAGTCC-3'). The qPCR was performed with following parameters: predenaturation at 95℃ for 1 min; 45 cycles at 95℃ for 5 s, 60℃ for 15 s. *β-Actin* was used as an internal control. The expression of genes were calculated with $2^{-\Delta\Delta Ct}$ method[34].

1.11 Western blot （WB）

The ovaries tissues and transfected cells were lysed in pre-cooled RIPA Lysis Buffer (P0013C, Beyotime, Shanghai, China) on ice for 30 min and centrifuged at 13 000*g* for 30 min at 4℃ . The supernatant of lysates was collected. The protein concentration was measured by a NanoDrop ND-2000 spectrophotometer. 10% SDS-PAGE was performed to separate proteins. Then, the proteins were transferred onto a PVDF membrane (IPVH00010, Millipore, Darmstadt, Germany). Based on the molecular mass of CYP19A1 (53 ku) and β-actin (43 ku), it was difficult to separate both proteins sufficiently to display on the same membrane. In order to eliminate the difference in the proteins on the membranes, the same total protein amount was transferred onto two membranes separately under the same conditions. The membranes were respectively blocked with 5% defatted milk for 1 h at RT, then incubated with specific antibodies against CYP19A1 (1∶1 000, AF6231, Beyotime, Shanghai, China) and β-actin (1∶ 1 000, AF5003, Beyotime, Shanghai, China) for 2 h at RT, following by incubating with a secondary HRP labeled goat anti-rabbit IgG for 1 h at RT. Finally, bands of protein were visualized by chemiluminescence under the gel imaging system (Chemidoc MP, Bio-Rad, California, USA). The images were analyzed by ImageJ software.

1.12 Cell Counting Kit−8（CCK−8）assay

The proliferation of cells was analyzed by CCK-8 assay (C0037, Beyotime, Shanghai, China). Briefly, the cells were implanted in 96-well plates and treated with the corresponding concentration of TSG for 24 h. The absorbance (*A*) values were measured on a microplate reader (SpectraMax® M5, Molecular Devices, San Diego, USA) at the wavelength of 450 nm according to CCK-8 reagent instructions.

1.13 Statistical analysis

Except for WB data of animal and all data of cells from three independent experiments, other data were from 5 independent experiments. All data were presented as mean±standard deviation and analyzed by GraphPad Prism 8.0 software. The difference of two groups was analyzed using student's *t* test. The difference among groups was analyzed using Tukey's multiple comparisons test of one-way ANOVA. It was considered as statistically different when *P* value was less than 0.05.

2 Results

2.1 TSG alleviated PCOS−like characteristics in rats

In order to explore the preventive and therapeutic effects of TSG on the PCOS, SD rats were selected for experiments according to Figure 1a. The vaginal secretions were collected and stained from 25 d to 31 d. The results of vaginal smear showed that the typical estrous cycles were present in the control group, however, the estrous cycle of PCOS rats disappeared and remained in the diestrus. Surprisingly, TSG could redress the disturbance of estrous cycle caused by PCOS (Figure 1b, S1). The body mass of rats was measured. It was found that the mass of PCOS group was observably heavier than that of the control, however there was no significant difference between PCOS and TSG groups (Figure 1c). As shown in Figure 1d, the ovarian mass/body of PCOS group was markedly increased compared to that of control, and ovarian mass/body mass of PCOS group was decreased after TSG treatment. The length of ovary in the PCOS group was longer than that in the control, whereas the length of ovary in the PCOS treated with TSG was less than that in the PCOS group (Figure 1e, f). H&E staining of the ovaries were showed in Figure 2a−c. A small number of sinus follicles and a large number of corpus luteum were observed in the control group. In PCOS group, there were a large number of sinus follicles and a small amount of corpus luteum. In the TSG group, the number of sinus follicles was significantly decreased and that of corpus luteum was increased. The results indicated that TSG alleviated the disturbance of estrous cycle, obesity, enlargement of the ovaries, and promoted follicle development and ovulation in PCOS-like rats.

2.2 TSG could reverse the abnormal of sex hormones in PCOS rats

To investigate the effect of TSG on sex hormones in PCOS rats, serum was collected and the sex hormones of serum were measured. The E2 level of PCOS rats was decreased compared with that of control, whereas E2 level of PCOS rats treated with TSG was markedly increased compared with that of PCOS rats (Figure 3a). T and LH levels in the PCOS rats were higher than that in the control, however T and LH levels in the PCOS rats treated with TSG

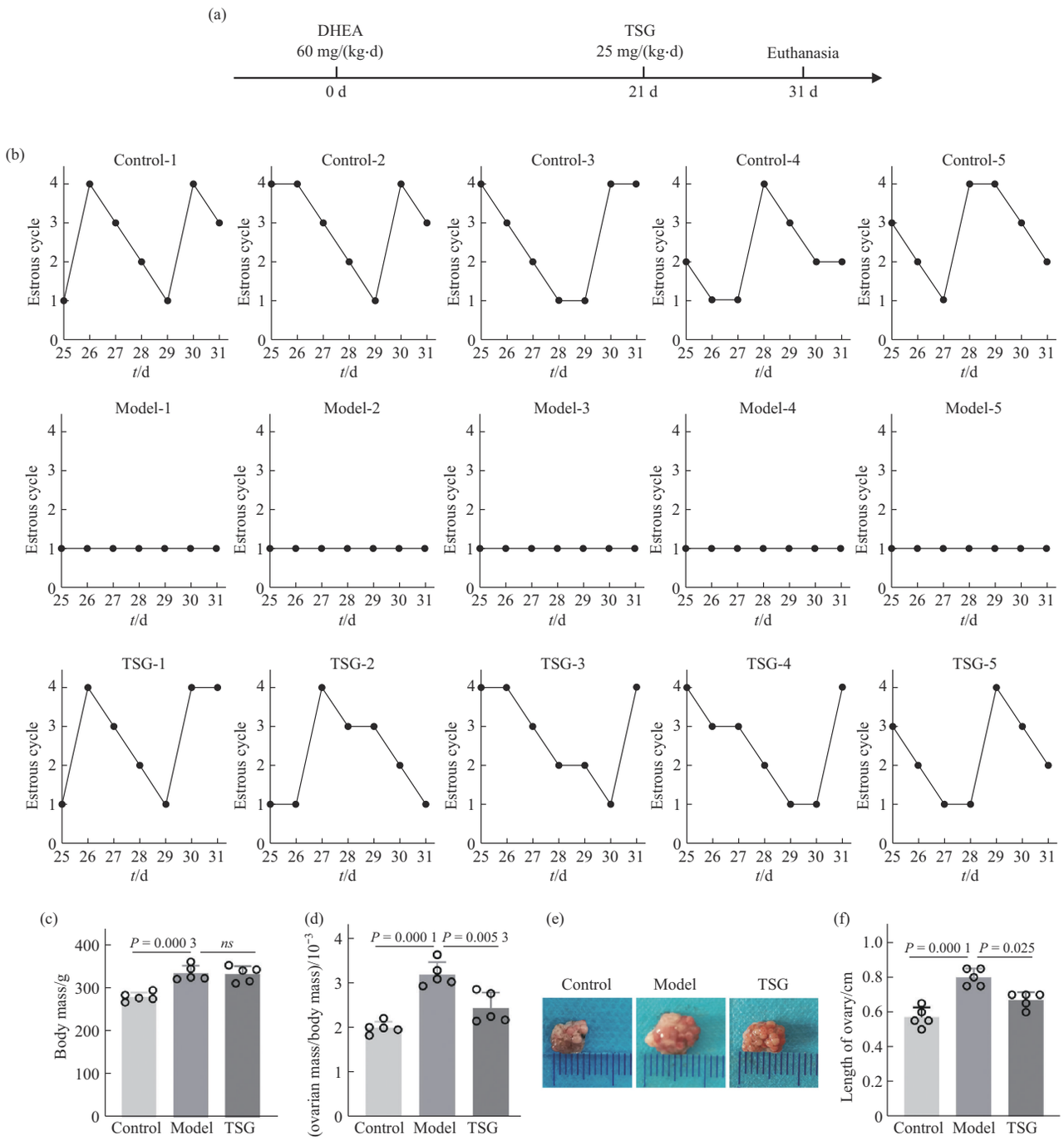

Fig. 1　TSG alleviated the disturbance of estrous cycle and enlargement of ovaries in rats with PCOS

(a) The illustration of PCOS model construction and PCOS treatment with TSG. The estrous cycle (b), body mass (c), ovarian mass/body mass (d) and length of ovary (e, f) were analyzed in PCOS rats treated with or without TSG. The ordinate of estrous cycle is as follows: 1 diestrus; 2 metestrus; 3 estrus; 4 proestrus. *ns*: no significance.

were lower than that in the PCOS rats (Figure 3b, d). There was no significant difference in FSH levels among the groups (Figure 3c). Unsurprisingly, the level of LH/FSH in the PCOS rats was increased compared with that in the control, the LHF/FSH of PCOS rats treated with TSG was decreased (Figure 3e). These results showed that TSG could reverse the decrease of E2 levels and the increase of LH and T levels in the PCOS rats.

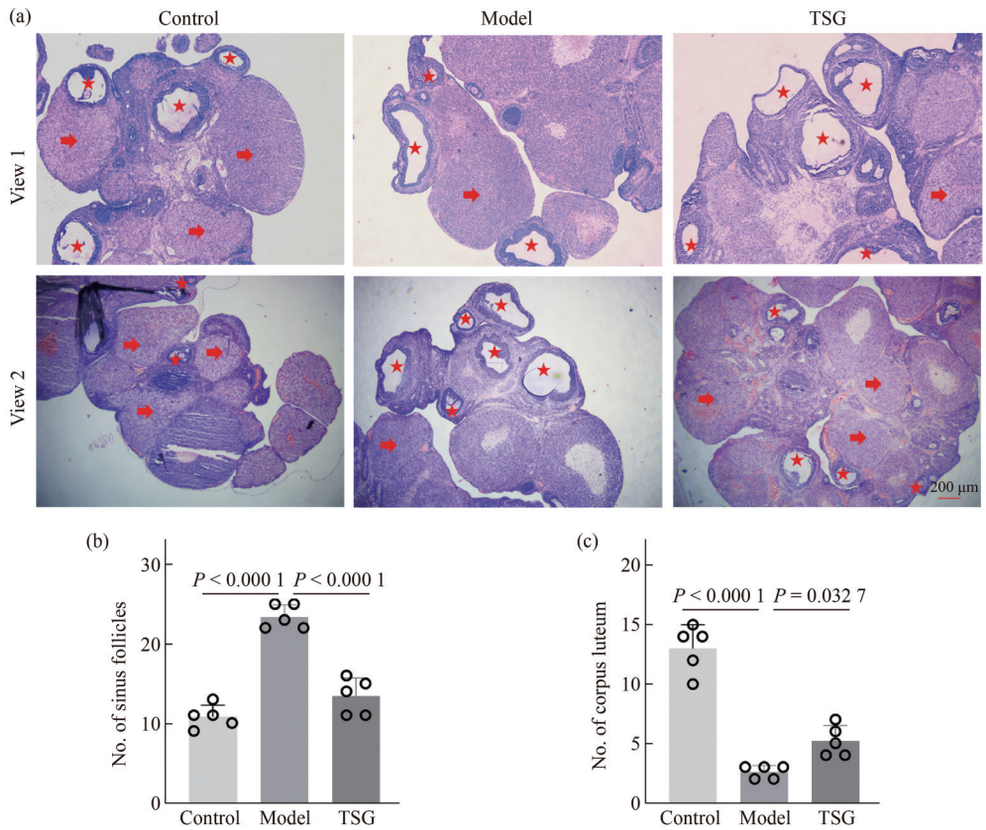

Fig. 2　TSG attenuated the ovarian polycystic characteristics in PCOS rats

(a) The ovaries were stained by H&E staining to show the follicles and corpus luteum in PCOS rats treated with or without TSG. Red stars indicate the sinus follicles. Red arrows indicate the corpus luteum. Differences in number of sinus follicles (b) and corpus luteum (c) among groups were analyzed.

2.3　TSG ameliorated the abnormal inflammatory markers caused by PCOS in rats

To explore the effect of TSG on inflammation, the inflammatory markers, such as IL-6, TNF-α, CRP, Omentin-1 and AGEs, were determined by ELISA assay. As shown in Figure 4a–e, the release of pro-inflammatory factors (IL-6, TNF-α, CRP and AGEs) increased, while the release of anti-inflammatory factor (Omentin-1) decreased in PCOS rats. Compared with PCOS rats, the pro-inflammatory factors were significantly decreased, and Omentin-1 was dramatically increased in the PCOS rats treated with TSG. In conclusion, TSG ameliorated the abnormal inflammatory markers caused by PCOS in rats, in other words, TSG could significantly inhibit the inflammatory response in PCOS rats.

2.4　TSG promoted the proliferation and inhibited the apoptosis of GCs in PCOS rats

To explore the mechanism by which TSG alleviates the PCOS-like features in rats, the proliferation of GCs was analyzed by IHC. As showed in the Figure 5a, b, there were a large number of PNCA-positive GCs in the sinus follicles of control, the positive GCs in the sinus follicles of PCOS rats were significantly reduced. Surprisingly, the positive GCs in the PCOS rats treated with TSG were observably increased compared with those in PCOS rats. The apoptosis of GCs was analyzed by Tunel staining. Compared with control, Tunel-positive GCs were markedly increased in the PCOS rats, while positive GCs of PCOS rats treated with TSG were obviously reduced compared with those in PCOS rats (Figure 5c, d). These results indicated that TSG could accelerate the proliferation and restrain the apoptosis of GCs in PCOS rats.

2.5　TSG could upregulate the expression of CYP19A1 in GCs of PCOS rats

To elucidate why TSG reversed abnormal sex hormones in PCOS, IHC, qPCR and WB were performed to analyze the expression of CYP19A1 in

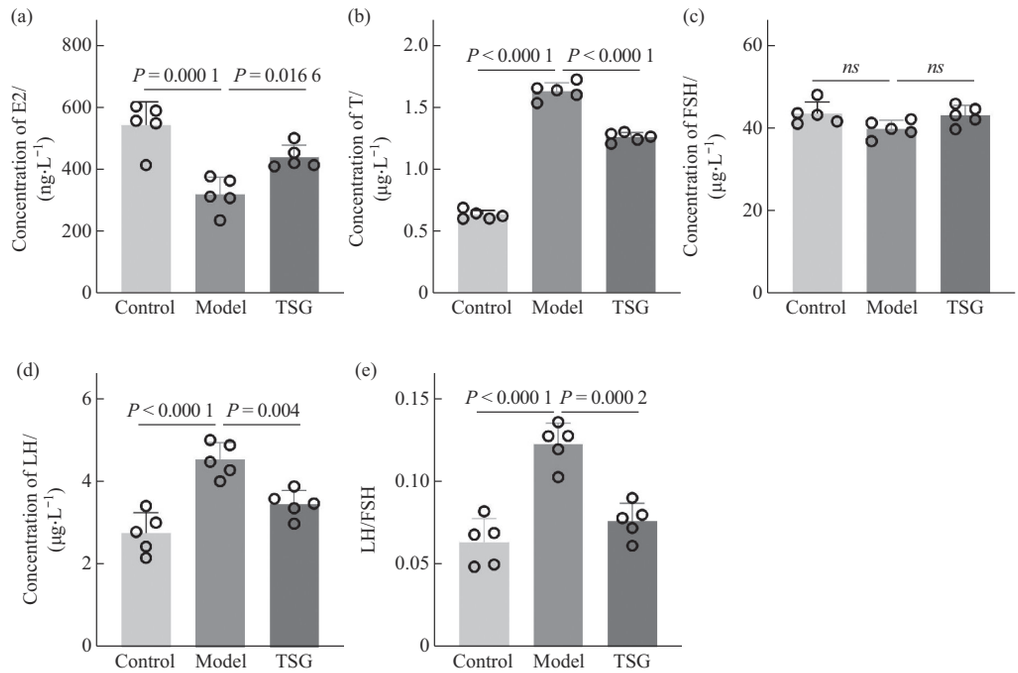

Fig. 3　TSG could rectify the abnormal of sex hormones in PCOS rats

The PCOS rats were treated with TSG. Then, the serum was collected, and the levels of E2 (a), T (b), FSH (c), LH (d) and LH/FSH (e) were analyzed by ELISA. *ns*: no significance.

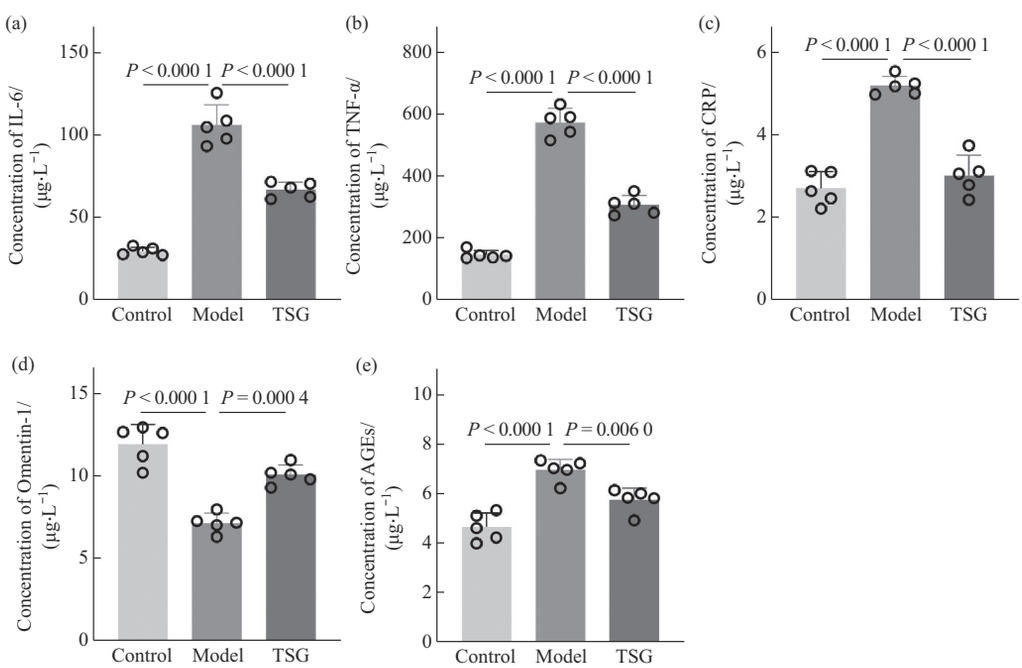

Fig. 4　TSG inhibited inflammatory response in the PCOS rats

The PCOS rats were treated with TSG. Then, the serum was collected, and the levels of IL-6 (a), TNF-α (b), CRP (c), Omentin-1 (d) and AGEs (e) were analyzed by ELISA.

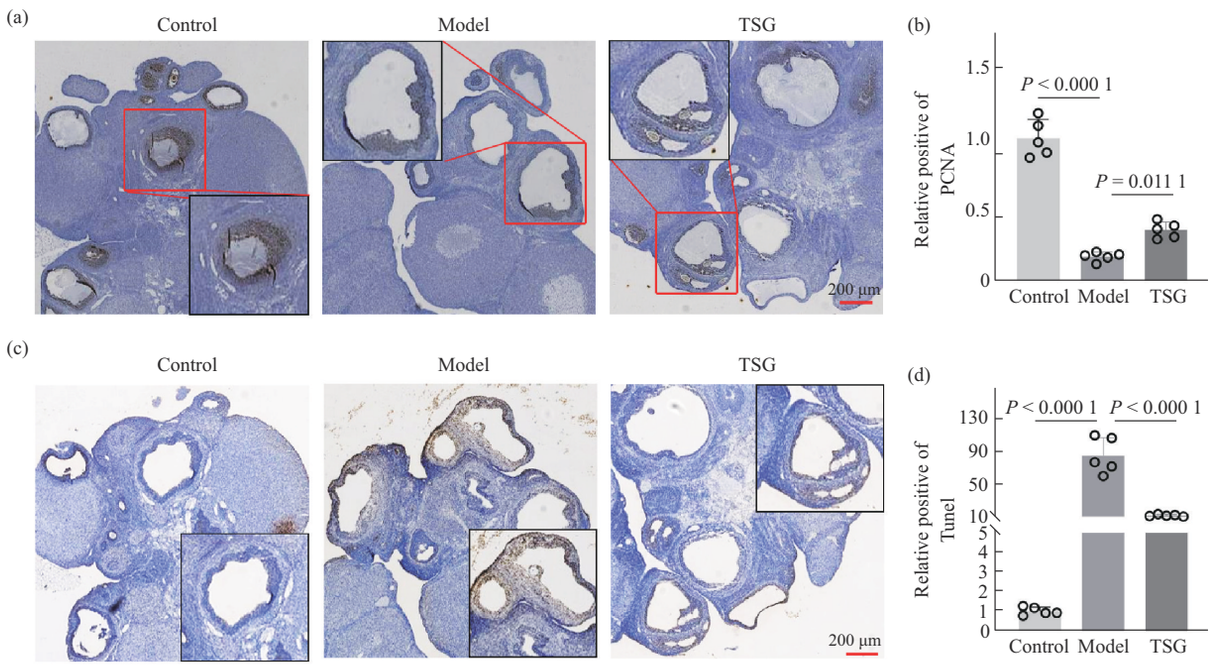

Fig. 5　TSG could promote the proliferation and repress apoptosis of GCs in PCOS rats

(a, b) The proliferation of GCs was analyzed by PCNA IHC. (c, d) The apoptosis of GCs was analyzed by Tunel staining.

GCs of sinus follicles. The results of IHC showed that the expression of CYP19A1 in the PCOS rats was significantly downregulated compared with that in control; however, the expression of CYP19A1 in the PCOS rats treated with TSG was upregulated compared with that in the PCOS rats (Figure 6a, b). The expression of CYP19A1 mRNA (Figure 6c) and protein (Figure 6d, e) were consistent with the results of IHC. These results suggested that the expression of CYP19A1 was downregulated in the GCs of PCOS, and TSG could upregulate the expression of CYP19A1 in GCs of PCOS.

2.6　TSG could inhibit LPS−induced inflammation in KGN cells

To select the appropriate concentration of TSG, KGN cells were treated with different concentrations of TSG (0, 6.25, 12.5, 25, 50, 100 μmol/L) for 24 h, then cell viability was measured by CCK-8 assay. As shown in Figure 7a, TSG could promote the growth of KGN cells. The median effective concentration (EC50) was 17.38 μmol/L and the 95% confidence interval (CI) was 17.95−20.85 μmol/L. So, KGN cells were treated with 20 μmol/L TSG for subsequently experiments.

The KGN cells were pretreated with 10 μmol/L LPS for 24 h[35] and then treated with 20 μmol/L TSG for another 24 h. The expression of IL-6 and TNF-α were detected by qPCR. The expression of IL-6 and TNF-α were significantly upregulated in LPS-treated KGN cells compared with control; excitingly, the expression of IL-6 and TNF-α were inhibited in the TSG treated KGN cells compared with those in LPS treated KGN cells, which suggested that TSG could inhibited increase of IL-6 and TNF-α induced by LPS in KGN cells (Figure 7b, c).

2.7　TSG could attenuate the inflammatory response by upregulating CYP19A1 expression in KGN cells

To explore the underlying mechanism of TSG inhibiting inflammation, KGN cells were transfected with si-CYP19A1 or si-NC. The expression of CYP19A1 was measured by qPCR and WB assays. As shown in Figure 8a, b, compared with si-NC cells, the expression of CYP19A1 mRNA and protein in si-CYP19A1 cells was respectively decreased by 45.48% and 43.83%, which indicated that CYP19A1 had been successfully knocked down in KGN cells. The results of qPCR assay showed that the expression of IL-6 and TNF-α was increased in si-CYP19A1 KGN cells compared with those in si-NC cells, which

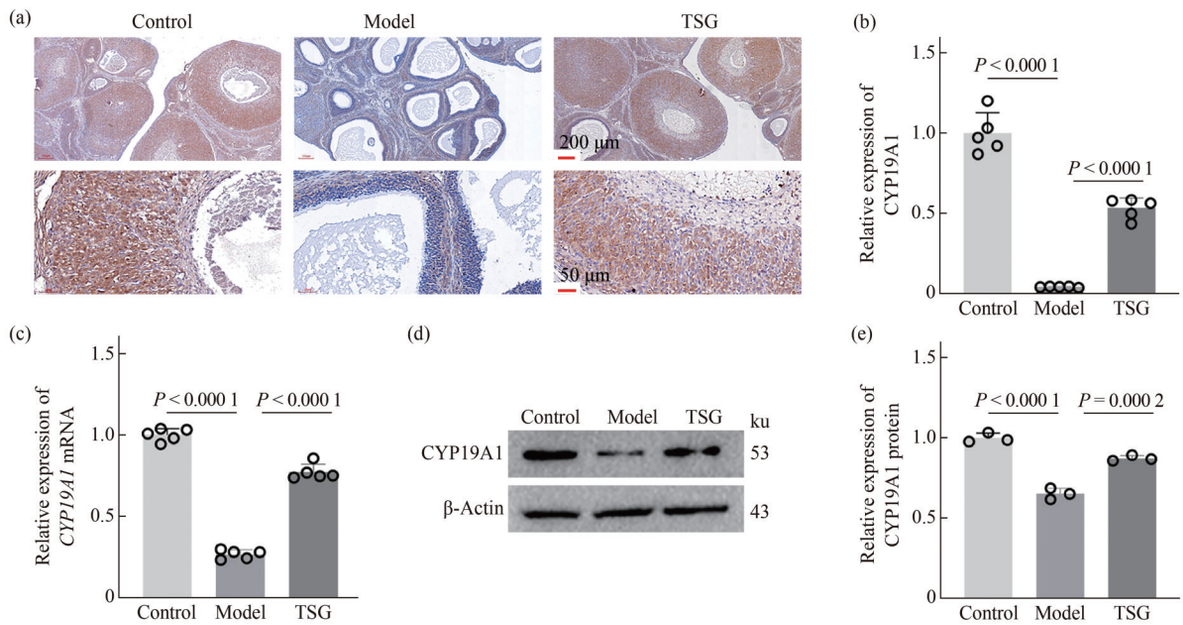

Fig. 6　TSG upregulated CYP19A1 expression in GCs of PCOS rats

The expression of CYP19A1 was analyzed by IHC (a, b), qPCR (c) and Western blot (d, e) assays.

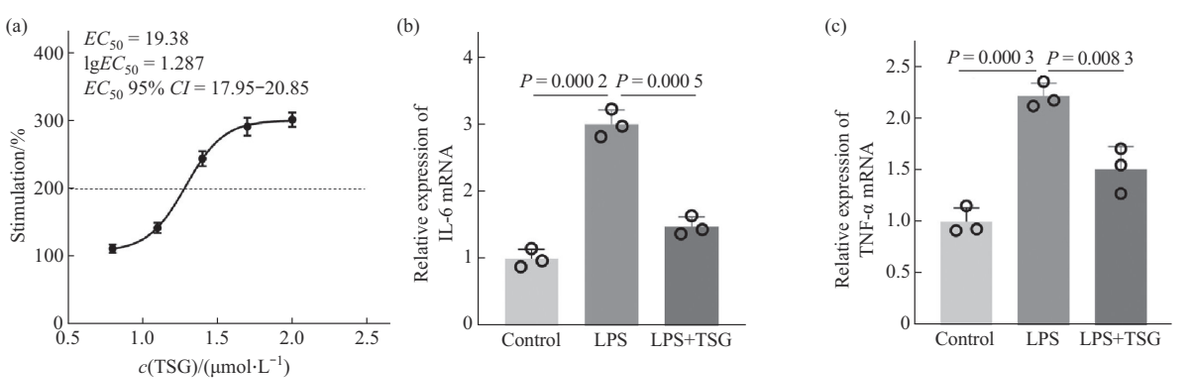

Fig. 7　TSG attenuated LPS−induced the expression of IL−6 and TNF−α in the KGN cells

(a) EC_{50} of TSG was calculated for KGN cells. The expression of IL-6 (b) and TNF-α (c) were measured in KGN cells treated with LPS and TSG.

suggested that knockdown of CYP19A1 could promote the expression of inflammatory cytokines, such as IL-6 and TNF-α, in other words, CYP19A1 could inhibit inflammation (Figure 8c).

Transfected cells were preincubated with LPS and then treated with TSG, CYP19A1, IL-6 and TNF-α were analyzed by qPCR and the results were shown in Figure 8d. The expression of CYP19A1 was downregulated in transfected cells treated with LPS compared with that in cells transfected cells, which indicated that LPS could decrease the expression of CYP19A1 in the KGN cells. Compared with transfected cells treated with LPS, CYP19A1 expression was significantly increased in the transfected cells treated with LPS and TSG, which indicated that TSG could inhibit the decrease of CYP19A1 expression induced by LPS. The expression of IL-6 and TNF-α were significantly upregulated in transfected cells treated with LPS compared with that in transfected cells, which indicated that LPS could induce the inflammatory response in KGN cells. Compared with transfected

cells treated with LPS, the expression of IL-6 and TNF-α were significantly reduced in the transfected cells treated with LPS and TSG, which indicated that TSG could inhibit the inflammation induced by LPS. The expression of IL-6 and TNF-α in the si-CYP19A1 cells treated with LPS were increased compared with those in the si-NC cells treated with LPS, which suggested that CYP19A1 knockdown could enhance the LPS-induced inflammatory response in KGN cells. The expression of IL-6 and TNF-α was decreased in the si-NC cells treated with LPS and TSG compared with those in the si-NC cells treated with LPS, which showed that TSG could inhibit LPS-induced inflammation. However, the expression of IL-6 and TNF-α was increased in the si-CYP19A1 cells treated with LPS and TSG compared with those in the si-NC cells treated with LPS and TSG, which indicated that downregulation of CYP19A1 could weaken TSG inhibiting LPS-induced inflammation, indicating that TSG inhibited LPS-induced inflammation by upregulating the expression of CYP19A1 in KGN cells.

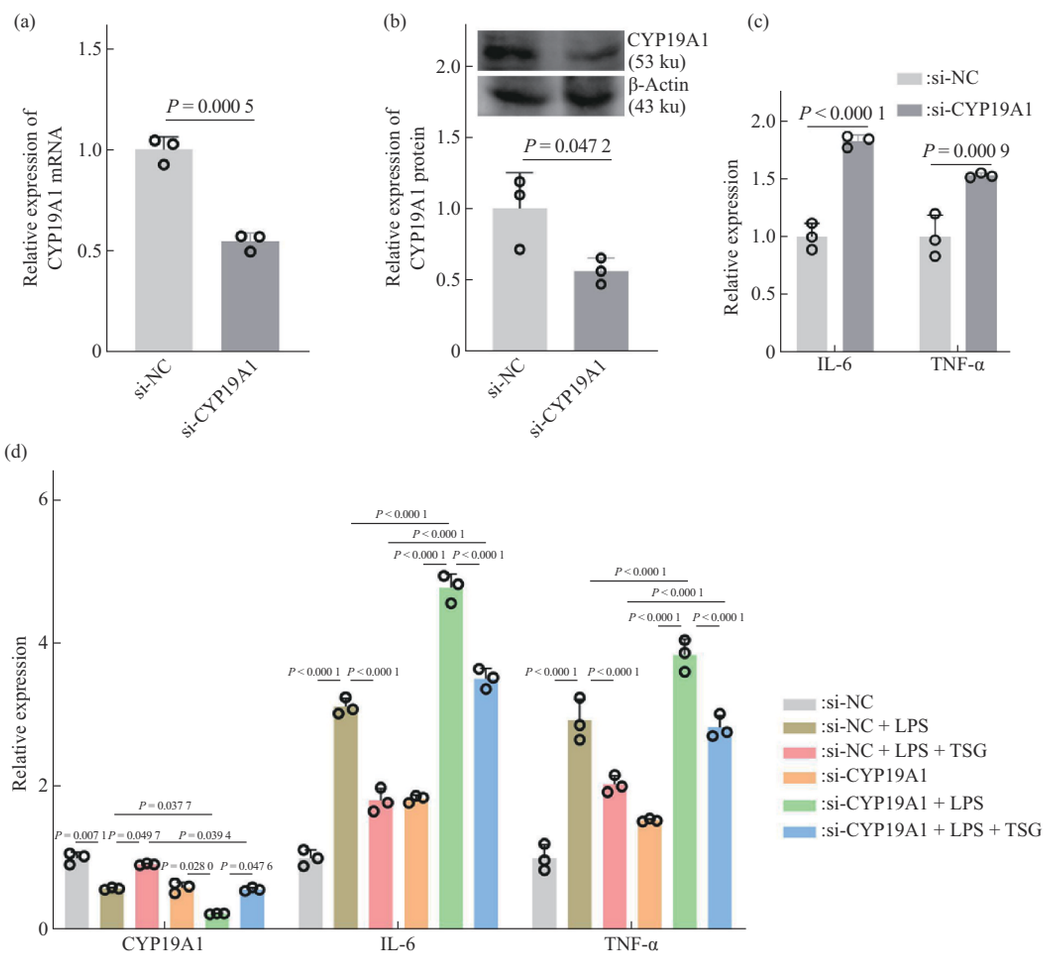

Fig. 8　TSG attenuated LPS−induced inflammation by upregulating CYP19A1 in KGN cells

The KGN cells were transfected with si-NC or si-CYP19A1, the expression of CYP19A1 mRNA (a) and protein (b) were respectively analyzed by qPCR and Western blot. (c) The expression of IL-6 and TNF-α were measured by qPCR. (d) The transfected cells were preincubated with LPS and then treated with TSG. The expression of CYP19A1, IL-6 and TNF-α were detected by qPCR.

3　Discussion

The pathogenesis of PCOS was relatively complex and has a certain relationship with endocrine disorders, it was characterized by disorders of female hormones, polycystic ovary morphology, infertility

and obesity, often accompanied by metabolic disorders and chronic inflammation[3]. Patients with PCOS often presented with ovarian dysfunction, which primarily involves the production of oocytes for successful ovulation and the synthesis of female hormones[36]. Ovarian function has long been considered to be related to hormonal and reproductive disturbances[37]. Folliculogenesis and ovarian activity were disrupted in the women with PCOS, which was emphasized the central role of the ovary in PCOS[38]. The increase of androgen and LH was the main cause of a series of symptoms[39]. The increasing evidences were showed that PCOS was often accompanied by chronic inflammation[18-19]. Therefore, suppressing the inflammatory response and reversing female hormonal abnormalities are the main strategies for the treatment of PCOS.

Cangfudaotan, a common traditional Chinese medicine prescription used for treating female infertility, reduced inflammatory response and inhibited apoptosis of granular cells, thereby improving the ovarian function of PCOS[40]. Saffron petal extract and saffron petal anthocyanins ameliorated symptoms of PCOS by improving dysregulation of ovarian hormone and inflammatory markers in PCOS mice[41]. The crocin treatment had protective effects on increase of IL-6 and TNF-α in the women with PCOS[42]. Curcumin had beneficial effects on markers of inflammation, weight loss and lipid metabolism in PCOS patients, and the incidence of adverse reactions did not increase with the application of curcumin[43]. It is evident that traditional Chinese medicine compound preparations or monomers have been demonstrated to alleviate the symptoms similar to PCOS by suppressing inflammatory responses. In essence, curbing mass loss response is beneficial for ameliorating the PCOS-like manifestations.

TSG extracted from *Polygonum multiflorum* had been proved to be effective in suppressing inflammatory responses of several diseases, except PCOS. In our study, we found that TSG reduced the polycystic characteristics of ovaries and partially restored the ovulation in the PCOS rats. It was confirmed that LH could stimulated the theca cells of ovarian follicle to synthesize androgen[44]. The release of LH depended on E2 level to some extent. Low levels of E2 inhibited LH release, and high levels of E2 strongly stimulated production and release of LH[45]. In our PCOS-like rats, the follicles stagnated in the sinusoidal follicular stage, and E2 levels was decreased. It may be that low concentrations of E2 are not sufficient to promote follicle development, which is consistent with the previous study that E2 maintained different stages of follicular development through binding to a variety of estrogen receptors[44]. TSG could alleviate the disorder of female hormones caused by PCOS, such as the increase of T and LH, and the decrease of E2. A study had showed that abnormal of inflammatory markers, including IL-6, TNF-α, CRP, AGEs and Omentin-1 triggered occurrence of PCOS[20]. TSG significantly reduced the increase of IL-6, TNF-α, CRP and AGEs, and also inhibited the decrease of Omentin-1 in PCOS rats. Based on the fact that TSG could ameliorate polycystic ovarian morphology, disorder of female hormone and inflammatory markers, we speculated that TSG may promote the development of sinus follicles by alleviating the disorder of female hormones and inhibiting inflammation in PCOS rats. The increase of serum T provided important information on the degree of androgen excess[46], which implied the level of T in serum could reflect the level of androgen in body. A study had showed that excessive androgen could accelerate the progression of PCOS by promoting inflammatory response[21]. Thus, we hypothesized that TSG may partially correct sex hormone abnormalities and inhibit the release of inflammatory markers, thereby improving the polycystic ovaries of PCOS rats, but its specific molecular mechanism still needs to be further explored.

CYP19A1 was responsible for the conversion of androgen to estrogen in the GCs[47-48]. A large number of studies had shown that knockdown or loss of CYP19A1 significantly inhibited E2 synthesis in GCs[49], which leaded to increased T levels. E2 synthesis could be promoted by increasing the cells activity or inhibiting cells apoptosis in GCs[50-51]. Our study had confirmed that TSG significantly rectified abnormal of sex hormones, inhibited the apoptosis, promoted the proliferation, and upregulated the CYP19A expression of GCs in PCOS rats. So, we believe that TSG accelerated the transformation of androgen to estrogen by reducing the apoptosis and upregulated the expression of CYP19A1 in GCs of PCOS rats.

In this study, it was confirmed that TSG inhibited

inflammatory response and upregulated the expression of CYP19A1 *in vivo*. Although studies had shown an increase of inflammatory markers and a downregulation of CYP19A1 in GCs treated with LPS, there was no evidence of a regulatory relationship between inflammation and CYP19A1[22-24]. In the cell study, we found that inflammatory markers were increased and CYP19A1 was decreased after GCs were treated with LPS, which was consistent with the results of previous studies. At the same time, we also found that knockdown of CYP19A1 could upregulate the expression of inflammatory markers, and could also exacerbate the increase of inflammatory markers induced by LPS in GCs, which suggested that CYP19A1 knockdown could promote inflammation in GCs. TSG could reverse the downregulation of CYP19A1 and reduce the expression of inflammatory markers induced by LPS. Downregulation of CYP19A1 could alleviate TSG inhibiting inflammatory markers expression. These results indicated that TSG alleviated PCOS-like characteristics by upregulating CYP19A1 in GCs to inhibit LPS-induced inflammatory .

In the existing clinical treatment of PCOS, there are mainly the following strategy. Medical interventions directly treated core symptoms of PCOS patients. Such as, contraceptive medications were used to regulate the menstrual cycle and reduce T levels[52-53]. Ovulatory stimulants like letrozole or clomiphene citrate induced ovulation by stimulating the pituitary gland to produce LH and FSH[54-56]. Laparoscopic ovarian drilling could restore ovulation by destroying a portion of the tissue that produces androgen[56-57]. These treatment strategies improve PCOS-like features only by reversing sex hormone abnormalities in PCOS patients. Our study showed that TSG inhibited the apoptosis and upregulated CYP19A1 expression in GCs of PCOS rats, which triggered the transformation of T to E2, resulting in the decrease of T and the increase of E2. The decreased T inhibited the inflammatory response, and the higher E2 promoted the development of follicles. TSG could also upregulate the expression of CYP19A1, thereby reducing the inflammatory response, which is beneficial to improve PCOS-like characteristics. In other words, our study concluded that TSG can both alleviate sex hormone disorders and suppress inflammatory responses, suggesting that TSG for the treatment of PCOS may be superior to treatment strategies that only improve sex hormone abnormalities.

4 Conclusion

TSG has shown potential in alleviating PCOS-like characteristics by upregulating the expression of CYP19A1 in the GCs of antral follicles, thereby inhibiting the inflammatory response. This suggests that TSG holds promise as a novel therapeutic strategy for PCOS. However, there are limitations in this study. For instance, the mechanisms by which TSG inhibits inflammation through the upregulation of CYP19A1 in vivo, as well as the precise pathways through which TSG upregulates CYP19A1 in granulosa cells, still require further investigation.

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TSG通过上调窦状卵泡中颗粒细胞CYP19A1的表达抑制炎症反应缓解大鼠多囊卵巢综合征样特征*

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摘要　目的　为了探讨2,3,5,4'-四羟基二苯基-2-O-β-葡萄糖苷（TSG）是否通过抑制炎症改善多囊卵巢综合征（PCOS）样特征。**方法**　皮下注射脱氢表雄酮建立雌性SD大鼠PCOS模型，然后腹腔注射TSG。颗粒细胞（GCs）KGN转染小分子干扰RNAs（si-NC和si-CYP19A1）后，先用脂多糖（LPS）预孵育，再用（/不用）TSG处理。阴道脱落细胞监测发情周期；苏木精-伊红（HE）染色分析卵巢卵泡形态；酶联免疫吸附分析（ELISA）法检测血清雌二醇（E2）、睾酮（T）、卵泡刺激素（FSH）、黄体生成素（LH）、IL-6、肿瘤坏死因子α（TNF-α）、晚期糖基化终末产物（AGEs）、C反应蛋白（CRP）、网膜素1（Omentin-1）水平；免疫组化法检测GCs中增殖细胞核抗原（PCNA）和芳香化酶（CYP19A1）的表达；原位末端标记（Tunel）染色检测GCs的凋亡情况；采用实时定量PCR（qPCR）和蛋白质免疫印迹（Western blot）检测卵巢和转染细胞的CYP19A1表达；qPCR分析LPS和TSG处理后转染细胞中IL-6和TNF-α的表达。**结果**　TSG治疗的PCOS鼠恢复了动情周期。与PCOS相比，TSG治疗的PCOS鼠卵巢中窦状卵泡减少，黄体增多。与PCOS相比，TSG处理的PCOS鼠E2升高，T和LH降低；促炎因子（IL-6、TNF-α、CRP、AGEs）降低，抗炎因子（Omentin-1）升高。TSG能部分抑制PCOS引起的PCNA阳性GCs的减少和Tunel阳性GCs的增加。与PCOS相比，经TSG处理后GCs的CYP19A1表达上调。转染si-CYP19A1的细胞中IL-6和TNF-α表达量与si-NC细胞比明显增加。转染细胞经LPS预孵育后，IL-6和TNF-α的表达明显增加，CYP19A1表达下调。与经LPS孵育的细胞相比，经LPS和TSG处理后，细胞IL-6和TNF-α表达量减少，而CYP19A1表达增加。与LPS和TSG处理转染si-NC细胞相比，转染si-CYP19A1的细胞经LPS和TSG处理后，IL-6和TNF-α表达量明显增加。**结论**　TSG通过上调GCs中CYP19A1的表达抑制炎症反应来缓解PCOS样特征。

关键词　2,3,5,4'-四羟基二苯基-2-o-β-葡萄糖苷，CYP19A1，多囊卵巢综合征，炎症反应
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