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Original Article

Activation of NLRP3 inflammasome in the ovaries during the development and treatment of polycystic ovary syndrome

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Abstract: Polycystic ovary syndrome (PCOS) is considered as a highly heterogeneous and complex disease. Dimethyldiguanide (DMBG) is widely used to improve the reproductive dysfunction in women with PCOS. However, the precise mechanism by which DMBG exerts its benefical effect on PCOS remains largely unknown. The present study was designed to explore the effects of DMBG on the changes of oxidative stress and the activation of nucleotide leukin rich polypeptide 3 (NLRP3) inflammasome in the ovaries during the development and treatment of PCOS. A letrozole-induced rat PCOS model was developed. The inflammatory status was examined by analyzing the serum high sensitive C-reactive protein (hsCRP) levels in ras. We found that DMBG treatment rescued PCOS rats, which is associated with the reduced chronic low grade inflammation in these rats. In PCOS rats, the NLRP3 and the adaptor protein apoptosis-associated speck-like protein (ASC) mRNA levels, caspase-1 activation, and IL-1β production were unregulated, which was markedly attenuated by DMBG treatment. Moreover, oxidative stress was enhanced in PCOS rats as shown by increased lipid peroxidation (LPO) and activity of superoxide dismutase (SOD) and catalase. DMBG significantly decreased LPO, while it had no effects on SOD and catalase activities. Together, these results indicate that DMBG treatment may rescue PCOS rats by suppressing oxidative stress and NLRP3 inflammasome activation in PCOS ovaries.

Keywords: NLRP inflammasome, polycystic ovary syndrome, oxidative stress, dimethyldiguanide, ovary

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine and metabolic occurring in women of reproductive age, which increases the occurrence of the risks of other diseases [1-3].

PCOS is characterized by insulin resistance, androgen excess and ovarian dysfunction [1-3]. The complications of PCOS are closely associated with chronic low-grade inflammation, such as obesity, diabetes type 2 and cardiovascular diseases [4]. Kelly et al. demonstrated an elevation of high sensitivity C-reaction protein (hsCRP) in women with PCOS, which suggests that inflammation factors may be involved in the occurrence of PCOS [4].

Inflammasomes are cellular machinery responsible for activation of inflammatory processes [5-7]. There are four types of inflammasomes including nucleotide leukin rich polypeptide 1 (NLRP1), NLRP3, ice protease-activating factor (IPAF), and absent in melanoma 2 (AIM2) inflammasome [8-10]. NLRP3 inflammasome is well characterized in a variety of mammalian cells, and is characteristic of a proteolytic complex which consists of three subunits: NLRP3, the adaptor protein apoptosis-associated speck-like protein (ASC), and caspase-1 [8-13]. Upon activation, NLRP3 binds to the ASC, which in turn recruits pro-caspase-1 to form an integrated inflammsome complex. Activation of NLRP3 inflammasome leads to cleavage of procaspase-1 into activated caspase-1, which subsequently cleaves pro-IL-1β to matured IL-1β

[14], an important inflammatory cytokine with a broad range of biological activities [6-13]. The role of NLRP3 inflammasome in the development of PCOS remains largely unknown.

Oxidative stress is well known to be importantly involved in chronic low-grade inflammation and various metabolic diseases, such as diabetes and obesity. Recent studies also demonstrated a role of oxidative stress in the physiology and pathology of female reproductive system [15-17]. Insulin resistance and hyperandrogenism are the most common features of PCOS. Hyperglycemia by insulin resistance causes cells to release reactive oxygen species leading to oxidative stress [16]. Oxidative stress may enhance the expression of pro-inflammatory cytokines, which in turn promotes insulin resistance and the occurrence of hyperandrogenism [18]. Thus, oxidative stress may contribute to the pathogenesis of PCOS. Recent studies demonstrated that oxidative stress triggers the activation of NLRP3 inflammasome in the renal diseases [6-13]. In a letrozole-induced PCOS rat model, the present study examined whether or not redox-dependent activation of NLRP3 inflammasome contributes to the development of PCOS and dimethyldiguanide (DMBG), an insulin sensitizer, improvs the clinic symptoms of PCOS by targeting the NLRP3 infalmmasome [19-21].

Materials and methods

Animals

Sprague-Dawley rats were purchased from Wushi Experimental Animal Supply Co. Ltd. (Fuzhou, China). The animals were maintained under a 14-h light/10-h dark schedule with continuous supply of chow and water. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee, Fujian Normal University.

Experimental design

Six-week-old female rats with two consecutive 4-day estrous cycles were randomly divided into 3 groups, including control group, PCOS group, and DMBG group. PCOS was induced by i.g. 1 mg/kg/day letrozole dissolved in 1% carboxymethyl cellulose (CMC, 2 ml/kg) for 21 days, while the control was injected with the equal CMC. All rats were detected by vaginal smear to confirm the estrous cycle. Then, DMBG-treated PCOS rats were subsequently received 300 mg/kg DMBG (Shanghai Sangon Biotech Ltd., Shanghai, China) for 4 weeks. Blood was collected for detection of serum hsCRP levels. The left ovary of the experimental animals was fixed and used for histological examination; the right ovary was frozen and used for detecting the expressions of functional proteins and other indexes. The experiment was repeated two times.

Hematoxylin-eosin staining of ovarian histology

The ovaries of these rats were fixed in 4% paraformaldehyde and embedded into paraffin, and then 5-μm sections were cut and mounted on slides. The sections were stained with hematoxylin-eosin staining kit and the images were obtained by microscope (BX51, Olympus Corporation).

Determination of serum hsCRP levels

Serum hsCRP levels were measured by enzyme-linked immunosorbent assay (ELISA) kit (Nanjingjiancheng, Jiangsu, China). The kit was used following the manufacturer's instructions.

RNA extraction and qRT-PCR analysis of NLRP3 and ASC mRNA

Total RNA was extracted using TRIzol solution (Life Technologies, Rockville, MD) and then reverse-transcribed (cDNA Synthesis Kit, Bio-Rad). The products were amplified using a TaqMan Gene Expression Assays kits for NLRP3 and ASC (Applied Biosystems). A kit for detecting the levels of 18S ribosomal RNA was used as an endogenous control. The relative gene expressions were calculated in accordance with the ΔΔCt method. Relative mRNA levels were expressed as $2^{-\Delta\Delta Ct}$ values.

Western blot analysis of caspase-1 proteins

Protein concentrations were determined by a BCA assay kit (Beyotime Biotechnology, Shanghai, China). 20 μg protein samples were subjected to 15% SDS-PAGE gel electrophoresis and then electrophoretically transferred onto a PVDF membrane. The membrane was washed and probed with rabbit anti-caspase-1 antibody (1:500, Abcam) overnight at 4°C. After washing, the membrane was incubated with HRP-

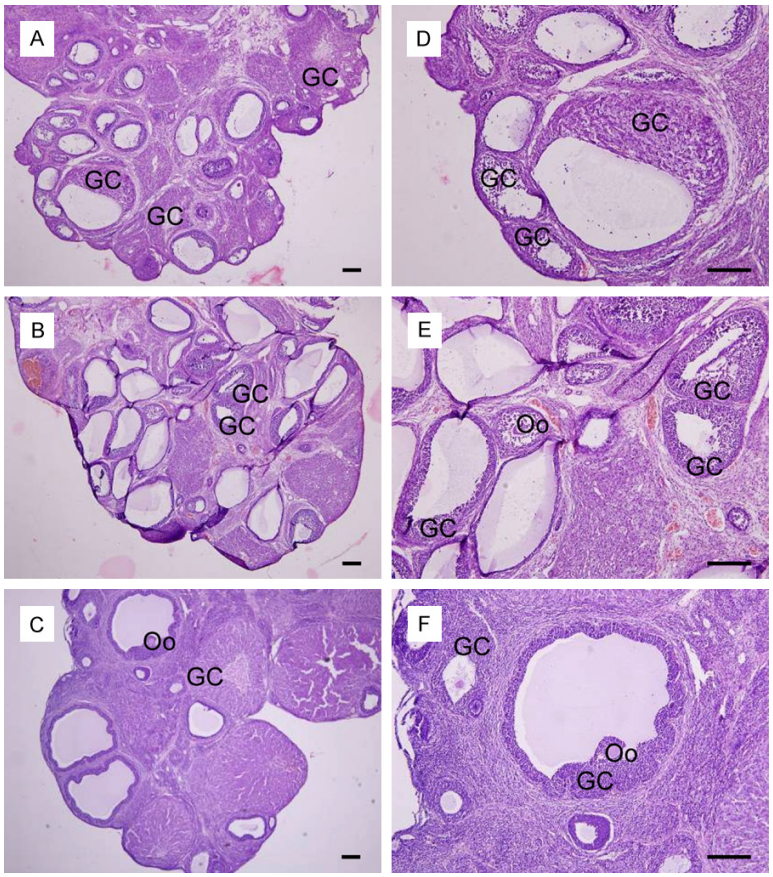

Figure 1. Histological examination of the ovaries during the development and treatment of PCOS. The ovarian sections were stained with hematoxylin-eosin staining and the images were obtained by microscope. The healthy follicles are showed in the control group (A, D), while many follicular cysts existed in PCOS ovaries with a very thin layer of granulosa cells (B, E) and DMBG rescued granulosa cells and follicular development (C, F). Bar = 100 um. GC: granulosa cell, Oo: oocyte.

labeled goat anti-rabbit IgG (1:2000, Beyotime Biotechnology, Shanghai, China) for 60 minutes at room temperature, and then developed the membrane to obtain the images. The membrane was then reprobed for β-actin with anti-β-actin antibody (1:5000, Santa Cruz, Shanghai, China). The immunoblotting signals were detected by enhanced chemiluminescence detection solution. The intensity of the bands was quantified by densitometry with the use of ImageJ software version 1.44p (NIH, Bethesda, MD).

Caspase-1 activity and IL-1β production assay

The ovaries were homogenized to extract proteins for caspase-1 activity assay by using a commercially available kit (Biovision). These data were expressed as the fold change compared with the control. In addition, the supernatant was also collected to measure the IL-1β production by an IL-1β ELISA kit (Bender Medsystems, Burlingame, CA) according to the protocol described by the manufacturer.

Determination of SOD activity, catalase activity and LOP product level in the ovaries

The levels of SOD activity (Nanjingjiancheng, Jiangsu, China), catalase activity (Nanjingjiancheng, Jiangsu, China) and LOP product level (Nanjingjiancheng, Jiangsu, China) were measured by the corresponding kits. All kits were used following the manufacturer's instructions.

Statistics

Data are presented as means ± SE. The significance of differences in mean values within multiple groups was evaluated using a one-way ANOVA, followed by a Tukey's multiple range test. $P<0.05$ was considered statistically significant. Different superscripts denote significant values ($P<0.05$).

Results

Morphological examination of ovarian histology and follicular structure during the development and treatment of PCOS

PCOS rat model was developed by letrozole and confirmed by morphological examination of each ovary. The results show there were healthy follicles with normal structure in the control group (**Figure 1A** and **1D**), whereas many cystic follicles cysts exist in PCOS ovaries with degrading granulosa cells and the very thin layer of granulosa cells (**Figure 1B** and **1E**). In DMBG-treated PCOS rats, some healthy follicles and corpus luteum were found and the number of large cystic follicles decreased compared with the PCOS rats (**Figure 1C** and **1F**). Moreover, the follicular diameter was significantly expanding in PCOS ovaries compared with the control

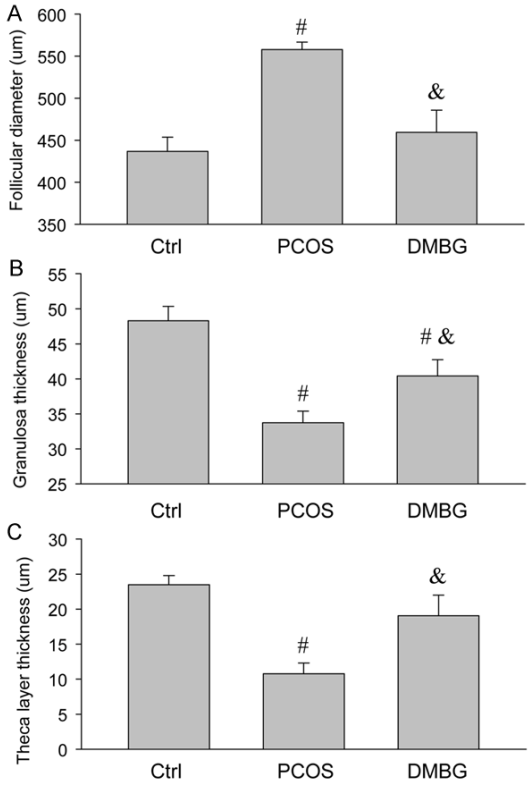

Figure 2. Changes in follicular diameter, granulose thickness and theca layer thickness in the ovaries during the development and treatment of PCOS. All historical sections of the ovaries from each group were stained and counted. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. #: *P<0.05,* vs. the control group, &: *P<0.05,* vs. the PCOS group.

(**Figure 2A**), and rescued by DMBG treatment (**Figure 2A**). The thicknesses of the granulose layer (**Figure 2B**) and theca layer (**Figure 2C**) were significantly decreased in PCOS group compared to control group, which was reversed after DMBG treatment. These results indicated the PCOS rat model was successfully developed and can be rescued by DMBG treatment.

Changes of serum inflammatory status during the development and treatment of PCOS

The serum hsCRP is one of the most important markers of inflammation. The concentrations of serum hsCRP was significantly increased in PCOS rats compared with the control, which was reduced by DMBG treatment (**Figure 3**). Thus, these data implies inflammation was involved in the development and treatment of PCOS.

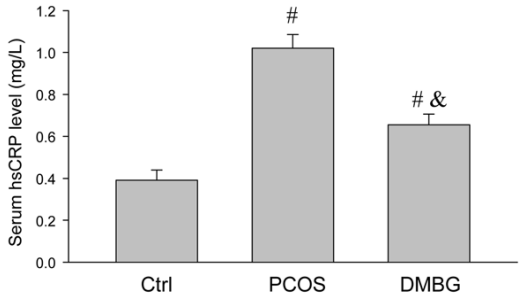

Figure 3. Changes of serum hsCRP level in PCOS rats during the development and treatment of PCOS. The concentrations of serum hsCRP were examined by enzyme-linked immunosorbent assay (ELISA) kits. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. #: *P<0.05,* vs. the control group, &: *P<0.05,* vs. the PCOS group. hsCRP: high sensitive C-reactive protein.

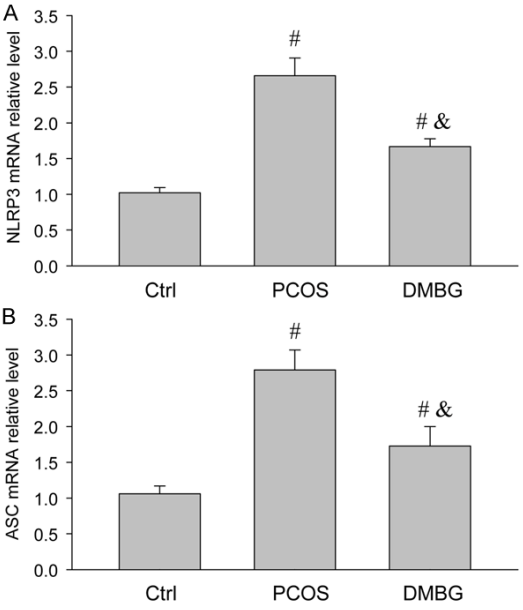

Figure 4. Expression changes of NLRP3 and ASC mRNA in the ovaries during the development and treatment of PCOS. A: The relative mRNA levels of NLRP3 by real-time RT-PCR analysis. B: The relative mRNA levels of ASC by real-time RT-PCR analysis. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. #: *P<0.05,* vs. the control group, &: *P<0.05,* vs. the PCOS group.

Expression changes of NLRP3 and ASC mRNA in the ovaries during the development and treatment of PCOS

NLRP3 and ASC mRNA levels were increased in the ovaries of PCOS rats, which were signifi-

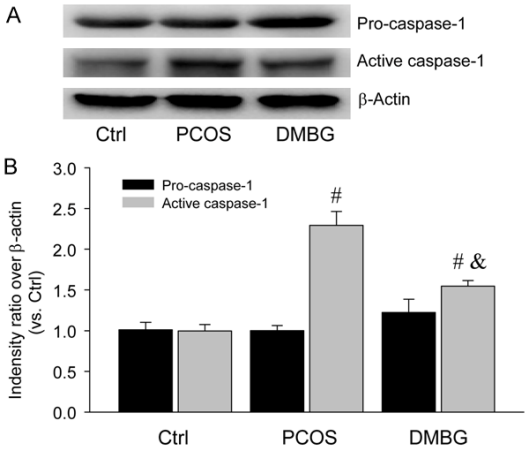

Figure 5. Expression changes of caspase-1 in the ovaries during the development and treatment of PCOS. A: Representative ECL gel documents of Western blot analyses depicting the protein levels of pro-caspase-1 and active caspase-1. B: Summarized intensities of pro-caspase-1 and active caspase-1 blots normalized to the control. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. #: *P<0.05,* vs. the control group, &: *P<0.05,* vs. the PCOS group.

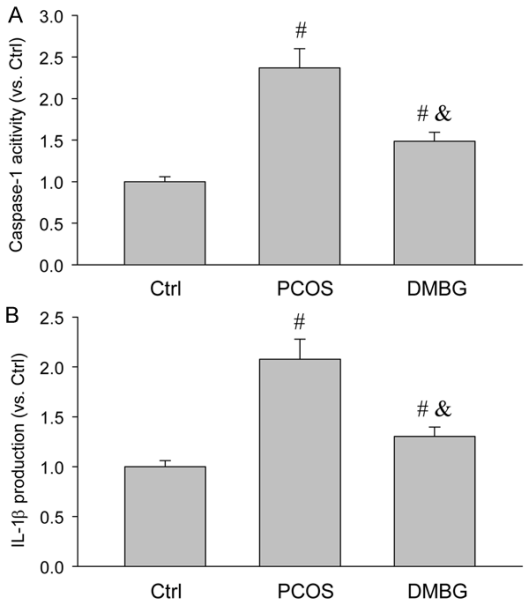

Figure 6. Examination of caspase-1 activity and IL-1β production in the ovaries during the development and treatment of PCOS. A: The data for caspase-1 activity in the ovaries from each group normalized to the control. B: The data for IL-1β production in the ovaries from each group normalized to the control. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. #: *P<0.05,* vs. the control group, &: *P<0.05,* vs. the PCOS group.

cantly decreased after DMBG treatment (**Figure 4**). This result indicates NLRP3 inflammasome is expressed in the ovary and may be involved in PCOS.

Expression changes of caspase-1 in the ovaries during the development and treatment of PCOS

The components of NALP3 inflammasome included NALP3, ASC and caspase-1. The pro-caspase-1 expression was not changed in PCOS ovaries, but the active caspase-1 expressions increased dramatically, which was decreased significantly after DMBG treatment (**Figure 5**). These data imply that the activation of NLRP3 inflammasome in the ovaries of PCOS rats, which was suppressed by DMBG treatment.

Examination of caspase-1 activity and IL-1β production in the ovaries during the development and treatment of PCOS

NLRP3 inflammasome activation is known to cause caspase-1 activation and then cleave pro-IL-1β into the bioactive form IL-1β [6-13]. Caspase-1 activity and IL-1β production were significantly increased in PCOS ovaries, which were reduced after DMBG treatment (**Figure 6A** and **6B**). These data indicate that NLRP3 inflammasome is activated in the ovaries of PCOS rats and subsequently causes caspase-1 activation and IL-1β production.

Examination of SOD activity, catalase activity and LOP concentration in the ovaries during the development and treatment of PCOS

Given the contribution of oxidative stress to the activation of NALP3 inflammasome, the markers of oxidative stress were examined in rat PCOS model. The activities of antioxidant enzymes SOD and catalase and LOP concentration increased in PCOS ovaries of rats (**Figure 7**). Interestingly, DMBG treatment decreased LOP concentration without changing SOD and catalase activities in PCOS rats (**Figure 7**).

Discussion

The present study demonstrated that a rat model of PCOS was developed by letrozole for 12 days with oligo-/anovulation, which meet

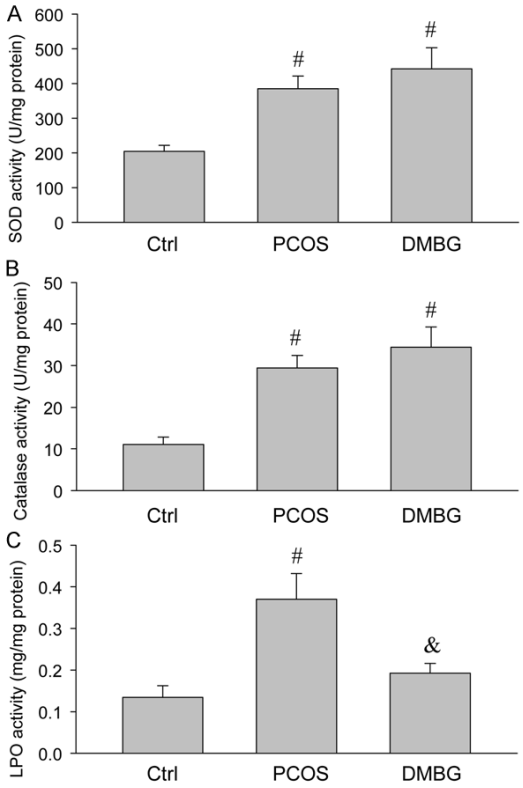

Figure 7. Changes of ovarian superoxide dismutase (SOD), catalase activities and lipid peroxidation (LPO) concentration in PCOS rats without and with DMBG. A: The data for SOD activity examined in the ovaries from each group by total superoxide dismutase assay kit (hydroxylamine method). B: The data for catalase activity examined in the ovaries from each group by catalase assay kits. C: The data for LPO concentration examined in the ovaries from each group by LPO assay kits. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. #: *P<0.05,* vs. the control group, &: *P<0.05,* vs. the PCOS group. SOD: superoxide dismutase, LPO: lipid peroxidation.

the Rotterdam diagnostic criteria. There was chronic low-grade inflammation during the development of PCOS, which is associated with the enhanced oxidative stress and activation of NLRP3 inflammasome. DMBG, an insulin sensitizer, was found to decrease redox-dependent NLRP3 inflammasome activation and rescue PCOS rats.

PCOS is characteristic by insulin resistance, hyperandrogenism, obesity and anovulation. Letrozole-induced rat model was firstly developed by Kafali for the study of polycystic ovaries, which is similar to the human PCOS in several ways. This PCOS rat model is based on the fact that the administration of aromatase inhibitor can cause ovulatory failure and polycystic ovaries [22]. The present study established this letrozole-induced PCOS rat model by confirming the ovarian histology and follicular structure. Chronic low-grade inflammation triggers the inflammatory responses by the excess metabolism of nutrients and leads to several chronic metabolic diseases [23]. Moreover, chronic low-grade inflammation has been increasingly recognized as an interposer in the endocrine, metabolic and reproductive disturbances that characterize PCOS [24-26]. Serum hsCRP level, a most common maker of inflammation, was increased in PCOS animals, and DMBG treatment of PCOS rats resulted in the reduction of hsCRP level [26-28]. The present study reproduced this phenomena in the PCOS rats, which confirmed that the presence of chronic low-grade inflammation in PCOS.

The occurrence and activation of chronic inflammatory process are always initiated and caused by danger signals. Inflammasomes served as an intracellular machinery to initiate inflammatory response to various danger signals. NLRP3 inflammasome have been implicated in the development of obesity and insulin resistance [29, 30]. Here, we demonstrated that the expressions of NLRP3 and ASC were increased obviously in PCOS rats and decreased after DMBG treatment, which is accompanied with the changes of caspase-1 activation and IL-1β production in the ovaries. Our findings suggest that NLRP3 inflammasome may be responsible for the initiation of inflammatory responses during the development and treatment of PCOS.

Oxidative stress influences reproductive functions of a woman, such as folliculogenesis, oocyte maturation, steroidogenesis and luteolysis [31, 32]. Moreover, oxidative stress may contribute to a proinflammatory state that induces insulin resistance and hyperandrogenism in PCOS [33]. Various reactive oxygen species (ROS) and antioxidants are used as biomarkers for PCOS patients [34-37]. In the present study, the ovarian levels of several oxidative stress markers including SOD, catalase, and LPO were significantly higher in the PCOS group when compared to the control group. These findings are consistent with the previous studies [31-37]. The increased antioxidant levels showed that excessive ROS production stimulates the defense systems in PCOS rats.

Moreover, we found that DMBG treatment significantly suppressed the upregulation of LPO levels in PCOS rats without affecting the antioxidant levels. Our data suggest that DMBG may target the production of ROS rather than antioxidants. Previous studies demonstrated that hyperglycemia by insulin resistance or high level of free fatty acids lead to ROS production and oxidative stress, which can enhance the activities of ovarian steroidogenesis enzymes and stimulate androgen production [32-37]. Oxidative stress is also a crucial trigger for the activation of NLRP3 inflammasomes in a variety of cardiovascular, metabolic, and renal diseases [6-13]. Thus, our data support a view that oxidative stress may contribute to the activation of NLRP3 inflammasome, and then leading to the ovarian dysfunction of PCOS, including cystic follicles, ovarian androgen excess and selective ovarian insulin resistance [34-37].

Clinically, DMBG improve IR and hyperinsulinemia, menstrual cycle disorders and hyperandrogenism in PCOS patients [28]. In the present study, DMBG rescued the high levels of inflammation marker and LPO in PCOS rats. Accordingly, DMBG treatment blocked the activation of NLRP3 inflammasomes in PCOS ovaries. Our data indicate DMBG can suppress inflammation in PCOS by inhibiting redox-dependent activation of NLRP3 inflammasomes.

In conclusion, the present study, for the first time, demonstrated the enhanced oxidative stress and activation of NLRP3 inflammasomes in PCOS, which may lead to chronic low-grade inflammation in PCOS. The protective effects of DMBG in the PCOS ovaries are associated with reduction of oxidative stress and NLRP3 inflammasomes. These findings provide novel insights into the understanding of the molecular mechanisms for the occurrence and development of PCOS, and therapeutical basis for the clinical treatment of PCOS.

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Disclosure of conflict of interest

None.

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