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Original Research

Evidence for Increased Expression of *SIRT3* Associated with Hyperandrogenism in Granulosa Cells of Non-Obese PCOS Patients

Shen Zhang[1,2,*], Wenli Deng[3], Qiongyou Liu[4], Wei Yang[5]

[1]Reproductive Medicine Center, Department of Obstetrics and Gynecology, The Second Affiliated Hospital, Chongqing Medical University, 400010 Chongqing, China
[2]Reproductive Medicine Center, The First Affiliated Hospital, Wenzhou Medical University, 325000 Wenzhou, Zhejiang, China
[3]The Fist Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology and Chongqing Eye Institute, 400016 Chongqing, China
[4]School of Basic Medical Sciences, Zunyi Medical University, 563003 Zunyi, Guizhou, China
[5]Institute of Biomedical Engineering, Shenzhen People's Hospital (The Second Clinical Medical College, Jinan University; The First Affiliated Hospital, Southern University of Science and Technology), 518020 Shenzhen, Guangdong, China
*Correspondence: shenzhang@cqmu.edu.cn (Shen Zhang)
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Abstract

Background: *SIRT3* regulates the generation of reactive oxygen species (ROS) in human granulosa cells (GCs). Increased levels of oxidative stress may cause follicular dysplasia in GCs of polycystic ovary syndrome (PCOS) patients. However, expression and regulation of *SIRT3* in GCs of PCOS patients have not yet been investigated. The present study is conducted to determine the correlation between *SIRT3* and hyperandrogenism in luteinized GCs of PCOS patients. **Methods**: The mRNA and protein expression of *SIRT3* were analysed in the luteinized GCs from the controls and non-obese PCOS patients. Dihydrotestosterone (DHT) was added to the primary cultured GCs to test the effects of androgen excess on intracellular ROS and *SIRT3* expression. A DHT-induced PCOS murine model was used to confirm the effects *in vivo*. **Results**: In the matched case-control study including 32 pairs of the controls and non-obese PCOS patients, we showed that the expression of *SIRT3* was increased in luteinized GCs of non-obese PCOS patients compared with normovulatory controls. Moreover, DHT induced oxidative stress and *SIRT3* expression in human GCs, which was further confirmed in a murine PCOS model. **Conclusions**: These results indicated that the increased expression of *SIRT3* was induced by hyperandrogenism in GCs of non-obese PCOS patients.

Keywords: *SIRT3*; polycystic ovary syndrome; luteinized granulosa cells; hyperandrogenism; oxidative stress

1. Introduction

Polycystic ovary syndrome is one of the most prevalent endocrine disorders in women of reproductive age. Women with PCOS are characterized by hyperandrognism, abnormal folliculogenesis and ovulatory dysfunction. Granulosa cells (GCs) play an important role in folliculogenesis. Altered gene expression was reported in GCs of PCOS patients [1]. Although the pathogenesis of PCOS remains unclear, dysregulation of GCs may affect follicular environment and follicular growth.

Hyperandrogenism is one of the main features of PCOS. Hyperandrogenism induces oxidative stress which has detrimental effects on GCs and oocytes [2,3]. Theca cells have an intrinsic defect accounting for excess androgen production in PCOS patients [4]. Excess androgen induces FSH receptor expression in GCs, which in turn induce LH receptor expression in theca cells [5]. Therefore, GC dysfunction also contributes to hyperandrogenism in PCOS patients.

Oxidative stress is caused by excess production of reactive oxygen species (ROS) relative to antioxidants. A 20-fold increase of ROS levels was detected and reported to induce cell apoptosis in GCs of PCOS patients [6,7]. Oxidative stress initiates GC apoptosis during follicular atresia [2]. Thus, increased apoptosis of GCs may contribute to follicular atresia in PCOS. These findings suggest that oxidative stress-induced apoptosis may play an important role in follicular dysplasia in PCOS patients.

Sirtuins belong to a family of conserved nicotinamide adenine dinucleotide (NAD)-dependent protein deacytylases. Among the sirtuins, *SIRT3* mainly located in mitochondria plays a critical role in maintenance of the homeostasis of ROS in oocytes and embryos [8,9]. *SIRT3* deacytylate and activate mitochondrial metabolic enzymes such as isocitrate dehydrogenase 2 and glutamate dehydrogenase [10]. It is reported that *SIRT3* is involved in the folliculogenesis and luteinization processes in GCs [11]. However, expression and regulation of *SIRT3* in GCs of PCOS patients is still unknown.

We hypothesized that high levels of androgen induced *SIRT3* expression in GCs of PCOS patients. To exclude the effect of obesity, we recruited non-obese women with PCOS and the matched controls. We analyzed the expression of *SIRT3* in luteinized GCs of PCOS patients and

the controls. The dihydrotestosterone (DHT) was used to mimic the hyperandrogenic state of PCOS patients. We showed that DHT induced oxidative stress and *SIRT3* expression in GCs of PCOS patients and the controls. Additionally, *SIRT3* expression was increased in GCs of DHT-treated mice. These results indicated that the elevated expression of *SIRT3* was induced by androgen excess in GCs of non-obese PCOS patients.

2. Materials and Methods

2.1 Patient Recruitment

Women with male infertility or tubal factor infertility, regular menstrual cycles, serum testosterone (T) level below 2 nmol/L, and body mass index (BMI) below 27 served as controls. PCOS was diagnosed refer to the Rotterdam revised criteria [12]. PCOS patients with high LH received oral contraceptive pretreatment (OCP) to reduce the basal LH before controlled ovarian stimulation (COS). The non-obese PCOS patients enrolled had oligo- or anovulation, serum T level above 2 nmol/L, polycystic ovary morphology and BMI below 27. The exclusion criteria were abnormal serum level of prolactin, endometriosis, dysfunctional thyroid or systemic diseases. Plasma levels of luteinizing hormone (LH), anti-Müllerian hormone (AMH), T, estradiol (E2) and follicle-stimulating hormone (FSH) were collected and measured between day 3 and day 5 of the menstrual cycle before controlled ovarian stimulation.

2.2 Measurement of Hormones

Serum levels of AMH, LH, FSH, T, E2 and prolactin (PRL) were determined by chemiluminescence immunoassay (CLIA). The inter-assay coefficients were 5.9% for AMH, 6.8% for LH, 5.6% for FSH, 12.1% for T and 9.2% for E2. The intra-assay coefficients were 3.6% for AMH, 5.1% for LH, 4.4% for FSH, 7.9% for T, 7.0% for E2 and 11.2% for PRL.

2.3 Ovarian Stimulation

All patients underwent ovarian stimulation using the long gonadotrophin-releasing hormone (GnRH) agonist (Ferring, Kiel, Germany) protocol according to our previous work [13]. Briefly, patients were given daily subcutaneous injections of 0.1 mg GnRH agonist from day 20 of a menstrual cycle until the day of human chorionic gonadotrophin (hCG) trigger. Ovarian stimulation was initiated with 150 international unit (IU) recombinant human FSH (rhFSH, Merck Serono, Geneva, Switzerland) per day. Recombinant hCG (Ovidrel, Merck Serono) was injected, when at least three follicles reached $\geq$17 mm diameter. Oocyte retrieval was performed 34–36 h after hCG injection.

2.4 GCs Isolation and Primary Cell Culture

Follicular fluids from follicles reached $\geq$17 mm diameter were collected. After centrifugation at 340 g for 8 min to pellet the GCs, the cell pellets were resuspended with 1$\times$PBS solution (Gibco, Carlsbad, CA, USA) and subsequently layered on 40%/80% gradient solution (PureCeption, SAGE) followed by centrifugation at 320 g for 20 min. The GC layers in the interface were collected and washed with 1$\times$PBS solution at 340 g for 5 min. Then, the purified GCs were cultured as previously described [13].

2.5 RNA Extraction and Real-Time Quantitative RT-PCR Analysis (qRT-PCR)

For the GC pellet harvested from follicular fluids and ovaries from the murine model, total RNA was extracted by the Total RNA Kit II Kit (Omega Bio-Tek). For the primary cultured GCs, total RNA was extracted by MicroElute Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA). The methods were performed from a protocol in our previous study [13]. The efficiencies of the PCR reaction were above 95%. The primers were hSIRT3-F and hSIRT3-R for human *SIRT3*, hGAPDH-F and hGAPDH-R for human *GAPDH*, mAMH-F and mAMH-R for mouse *AMH*, mFOXO1-F and mFOXO1-R for mouse *FOXO1*, mFOXO3A-F and mFOXO3A-R for mouse *FOXO3A*, mSIRT3-F and mSIRT3-R for mouse *SIRT3*, mGAPDH-F and mGAPDH-R for mouse *GAPDH*. The sequences of the primers are listed in Table 1.

Table 1. The oligonucleotide sequences of primers used for RT-PCR.

Primer name	Sequence (5′→3′)
hSIRT3-F	CGGCTCTACACGCAGAACATC
hSIRT3-R	CAGCGGCTCCCCAAAGAACAC
hGAPDH-F	GAAGGTGAAGGTCGGAGTC
hGAPDH-R	GAAGATGGTGATGGGATTTC
mAMH-F1	GGGGCACACAGAACCTCT
mAMH-R1	GCACCTTCTCTGCTTGGTTG
mFOXO1-F1	CGTGCTTACAGCCTTCTA
mFOXO1-R1	ACCTCCATCGTGACAAAA
mFOXO3A-F1	GATAAGGGCGACAGCAACA
mFOXO3A-R1	CCGTGCCTTCATTCTGAAC
mSIRT3-F	TATGGGCTGATGTGATGGCG
mSIRT3-R	AGTCGGGGCACTGATTTCTG
mGAPDH-F1	TGGCAAAGTGGAGATTGTTGCC
mGAPDH-R1	AAGATGGTGATGGGCTTCCCG

2.6 Western Blot Analysis

The method was performed as described previously [13]. The murine monoclonal antibody against *SIRT3* (Santa Cruz Biotechnology, sc-365175, Santa Cruz Biotechnology, Dallas, TX, USA) and beta-actin (Proteintech, 60008-1-Ig, Proteintech, Wuhan, Hubei, China) were

used. The signal intensities of the bands were measured by ImageJ software (Bethesda, Maryland, NIH). The protein expression of *SIRT3* was normalized to β-Actin (internal control, v1.8.0, NIH, Bethesda, MD, USA).

2.7 Measurement of Intracellular ROS

The intracellular ROS of GCs were examined by the general oxidative stress indicator (CM-H2DCFDA, Invitrogen). The CM-H2DCFDA was prepared in DMSO immediately before use. The cultured GCs were incubated with 10 μM CM-H2DCFDA for 30 minutes at 37 °C and then observed under a confocal microscope (TCS SP8, Leica, Wetzlar, Germany) with an excitation wavelength of 490 nm and an emission wavelength of 525 nm. The fluorescence intensity was analyzed and quantified by ImageJ software according to a previous study [11].

2.8 Animals and Establishment of a Mouse Model for PCOS

All procedures were reviewed and approved by the Animal Ethical Committee of the Laboratory Animals Center of Chongqing Medical University. Female C57BL/6J mice were purchased from Shanghai slake experimental animal Co., Ltd (Shanghai, China). Mice were bred and housed in a temperature-control room (20–22 °C, on a 12/12 h light/dark cycle. The protocol of generating a PCOS mouse model was modified from the previous study [14]. After acclimating to the laboratory conditions with a standard diet and water for 3 days, mice (19 days of age) arranging from 8.6 to 10.5 g weight were randomly divided into two groups (n = 8 per group). The dihydrotestosterone (DHT) group was implanted with a DHT release pellet (Innovative Research of America, Sarasota, FL, USA). The pellets contained 2.5 mg of DHT with a 90-day continuous release. The control group was treated with a placebo pellet. After 90 days of treatment, mice were killed by cervical dislocation, and the ovaries were collected.

2.9 Determination of Estrous Cycle

Vaginal smear analysis was performed to assess the estrous cycle for 10 consecutive days before the mice were killed. Briefly, vaginal cells were collected by saline lavage and placed on a glass slide. After air-drying, the slides were stained with 0.1% methylene blue solution (Sigma-aldrich, St. Louis, MO, USA). The stages of the estrous cycle were identified by the relative ratio of cell types observed in the slides as described previously [15].

2.10 Measurements of Serum T Levels in PCOS Mice

Total serum T was assayed using RIA kit with 125I-labelled ligands (Beijing North institute of Biological Technology) according to the manufacturer's instruction. The radioactivity was measured by a Wallac Wizard 1470 automatic gamma counter (PerkinElmer). The detection efficiency was 75%. The extraction efficiency was 92%. The detection limit was 0.02 ng/mL.

Table 2. Clinical parameters in normovulatory women and PCOS patients.

Variable	Controls	PCOS patients
N	32	32
Age (yr)	30.33 $\pm$ 4.27	29.17 $\pm$ 2.04
BMI (kg/m^2)	22.73 $\pm$ 2.89	25.43 $\pm$ 2.21
AMH (ng/mL)	4.52 $\pm$ 1.89	10.35 $\pm$ 1.70^a
AFC	19.60 $\pm$ 7.30	33.80 $\pm$ 7.26^a
LH (IU/L)	4.57 $\pm$ 1.39	4.48 $\pm$ 1.53
FSH (IU/L)	7.03 $\pm$ 1.36	6.40 $\pm$ 1.62
T (nmol/L)	1.45 $\pm$ 0.70	2.72 $\pm$ 0.52^a
E2 (pmol/L)	159.33 $\pm$ 44.92	169.83 $\pm$ 66.78
PRL (mIU/L)	298.12 $\pm$ 184.68	352.97 $\pm$ 96.93
Insulin 0 (mU/L)	7.35 $\pm$ 3.09	9.56 $\pm$ 3.98^a
Insulin 120 (mU/L)	33.32 $\pm$ 25.68	63.47 $\pm$ 42.51^a
Glucose 0 (mmol/L)	4.94 $\pm$ 0.61	5.38 $\pm$ 0.69
Glucose 120 (mmol/L)	5.88 $\pm$ 1.57	6.73 $\pm$ 1.46^a
HOMA-IR	1.69 $\pm$ 1.43	2.49 $\pm$ 1.80^a

BMI, Body mass index; AFC, Antral follicle count; PRL, Prolactin. $^a p < 0.05$ vs the controls.

2.11 Statistical Analysis

Data are presented as the means $\pm$ SD. The normality and homogeneity of variance of the data were assessed. Differences between groups were determined by Student's *t*-test (for only two groups) or one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test (for comparing all groups) using SPSS 16.0 software package (IBM Corp., Chicago, IL, USA). Significance was set at $p < 0.05$.

3. Results

3.1 High Expression of SIRT3 is Associated with PCOS in the Luteinized Granulosa Cells

The clinical parameters of patients were summarized in Table 2. Compared with the normovulatory controls, basal levels of AMH and T as well as antral follicle count (AFC) were significantly increased in non-obese PCOS patients ($p < 0.05$). The insulin-glucose parameters showed the PCOS patients had insulin resistance. Moreover, there was a positive correlation between T and HOMA-IR levels. *SIRT3* expression in controls and PCOS patients was examined using qRT-PCR and western blot. *SIRT3* expression was significantly elevated at both mRNA and protein levels in PCOS patients compared with the controls (Fig. 1A,B). Western blot quantification revealed that *SIRT3* protein expression was approximately 4-fold higher in GC of PCOS patients than in the controls (Fig. 1C). Additionally, the Pearson's correlation was performed to evaluate the relationship between the mRNA levels of *SIRT3* and total antral follicle counts, testosterone levels. We found mRNA expression of *SIRT3* was correlated with testosterone levels, but not total antral follicle counts.

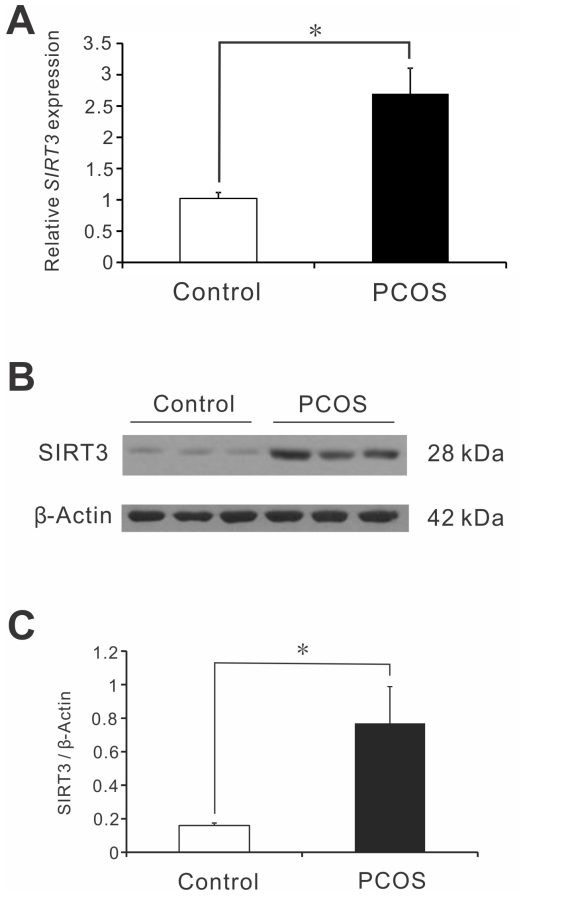

Fig. 1. Expression of *SIRT3* in human GCs between normovulatory women and non-obese PCOS patients. (A) Differential expression of *SIRT3* mRNA in GCs between the two groups. Data were expressed as fold changes relative to the control. Bars represent means ± SD, n = 32. *$p < 0.05$ vs the control. (B) *SIRT3* protein in GCs of the two groups determined by Western blot analysis. The β-Actin was served as the internal control in both groups. (C) The signal intensities of the western blot bands were assessed by the ImageJ software (NIH). The protein expression of *SIRT3* was normalized to β-Actin. Bars represent means ± SD, n = 3. *$p <$ 0.05 vs the control.

3.2 Androgenic Induction of ROS and SIRT3 Expression in Granulosa Cells

To assess the effects of hyperandrogenism, GCs from the controls and PCOS patients were treated with vehicle or DHT, a nonaromatizable androgen, and the intracellular ROS levels were measured by CM-H2DCFDA (Fig. 2A–D). Then, the fluorescence intensity was quantified. GCs of the controls showed scant ROS generation, while ROS was significantly increased in GCs from PCOS patients (Fig. 2E). And DHT treatment significantly stimulates ROS generation in GCs from both the controls and PCOS patients. Next, we performed quantitative RT-PCR analysis to assess the effects of DHT on the expression of *SIRT3* (Fig. 2F). The result revealed that DHT up-regulated the expression of *SIRT3*.

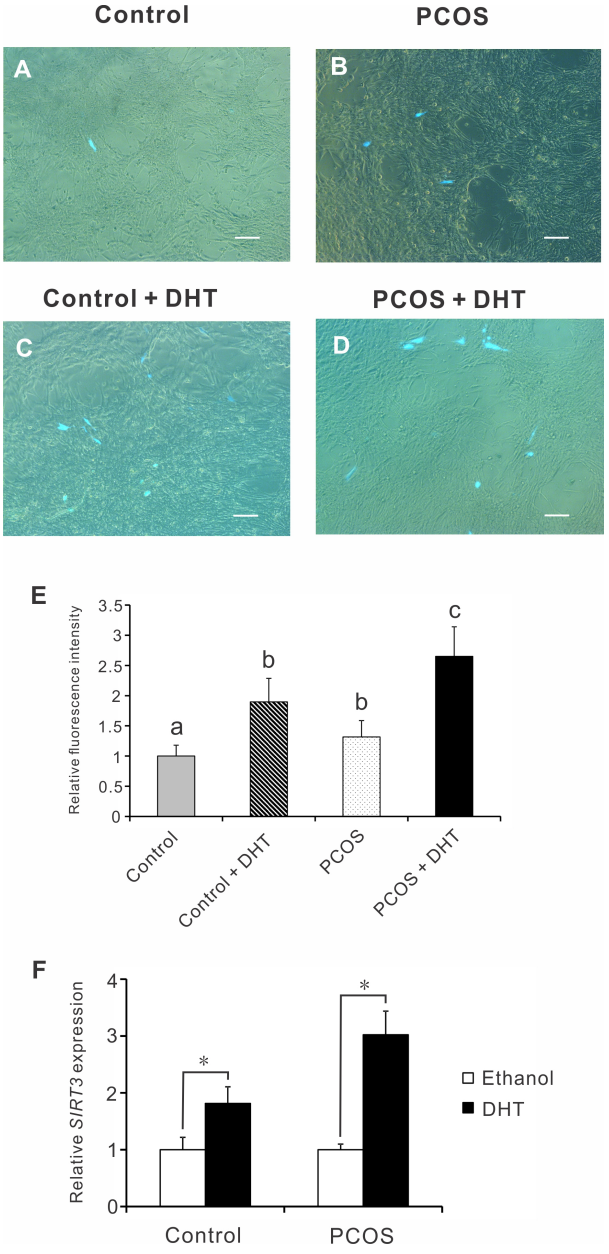

Fig. 2. Effects of DHT on intracellular ROS and *SIRT3* expression in the luteinized GCs of the controls and PCOS patients. The primary cultured GCs were treated with vehicle or DHT (10 nM) for 24 h before further analysis. Intracellular ROS levels were measured by CM-H2DCFDA. Representative images are shown as following: (A) GCs of the controls treated with vehicle. (B) GCs of PCOS patients treated with vehicle. (C) GCs of the controls treated with DHT. (D) GCs of PCOS patients treated with DHT. The fluorescence intensities were assessed by the ImageJ software (E). Bars represent means ± SD, n = 5. Alphabets a–c indicate significant differences between the groups ($p < 0.05$). The mRNA levels of *SIRT3* were quantified with qRT-PCR analysis (F). Data were expressed as fold changes relative to the ethanol vehicle control. Bars represent means ± SD, n = 5. *$p < 0.05$ vs the control.

3.3 SIRT3 Expression is Increased in DHT-Induced PCOS Mice

For *in vivo* experiments, a DHT-induced PCOS murine model was established. Menstrual irregularities are a key feature of PCOS patients. We performed vaginal smears experiments to determine the estrous cycles of the mice. The control mice showed regular estrous cycles, while the DHT-treated mice exhibited disrupted estrous cycles (Fig. 3A,B). No cystic follicles were detected in the control mice. In contrast, such follicles were observed in DHT-treated mice (Fig. 3C,D). The number of antral follicles was obviously increased in DHT-treated mice compared with the controls (Fig. 3D). To confirm that the DHT treatment caused hyperandrognism, the serum T level was measured. As expected, the serum T level was dramatically raised in DHT-treated mice (Fig. 3E). The expression of specific genes such as AMH, FOXO1 and FOXO3A were associated with PCOS in ovaries [16,17]. Our results showed that the expression of these genes was significantly enhanced in the ovaries of the DHT-treated mice compared with the controls (Fig. 3F). Consistent with the results in primary culture cells, the expression of *SIRT3* was also significantly elevated in DHT-treated mice (Fig. 3F).

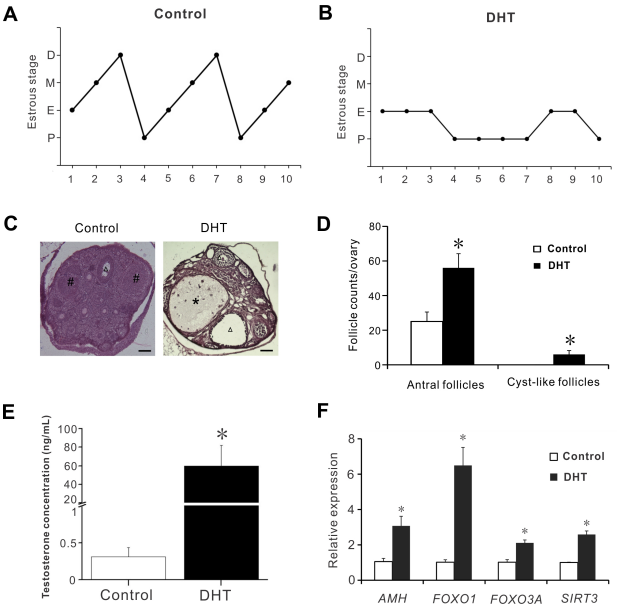

Fig. 3. Ovarian morphology, serum testosterone levels and ovarian gene expression in a murine model of PCOS. (A) Representative estrous cycle of the control mice. (B) Reprentative estrous cycle of the DHT-treated mice. (C) Representative HE staining of ovarian sections from the controls and DHT-treated mice. Asterisk indicates cyst-like follicles. Triangle indicates antral follicles. # indicates corpora lutea. Scale bar = 50 μm. (D) Effects of DHT treatment on the number of antral follicles and cyst-like follicles. (E) Serum testosterone levels in the two groups. (F) Expression of *AMH*, *FOXO1*, *FOXO3A* and *SIRT3* mRNA in ovaries determined by qRT-PCR. Bars represent means $\pm$ SD, n = 8. *$p < 0.05$ vs the control.

4. Discussion

Oxidative stress induced by ROS plays a pivotal role in apoptosis, and has detrimental effects on oocytes [3,18]. PCOS patients had higher levels of oxidative stress and apoptosis in the follicles compared to non-PCOS patients [19,20]. A previous report showed the presence of *SIRT3* expression in human GCs [11]. A deletion of *SIRT3* results in oxidative stress eliciting in GCs. *SIRT3* scavenges ROS by activating SOD2 [21]. A meta-analysis showed that SOD activity was higher in PCOS patients than in the controls [22]. Consistent with the previous study, we showed elevated oxidative stress in GCs of PCOS patients [19]. We further found that the expression of *SIRT3* was enhanced in GCs of PCOS patients. Although high levels of oxidative stress are detected in women with PCOS, it remains inconclusive whether PCOS patients have poor outcomes of assisted reproductive techniques (ART) treatment [23–27]. Non-obese PCOS patients have significantly higher total oxidant status and total antioxidant capacity levels compared with the matched controls [28,29]. Therefore, in our study, higher levels of *SIRT3* expression in non-obese PCOS patients may account for the elevated total antioxidant status.

Improvement of insulin resistance decreases the levels of androgen in PCOS patients [30]. Our study showed a positive correlation between T and HOMA-IR levels. Thus, hyperandrogenism may be related to insulin resistance in PCOS patients. Androgens exert their effects by binding to their receptor. Androgen receptor is mainly expressed in GCs throughout follicular development. Therefore, excess androgen may cause GC dysfunction in PCOS patients. In our study, excess androgen induced *SIRT3* expression, implying alterations in GC function of PCOS patients.

Communication between the oocyte and GCs is essential for oocyte development [31]. Downregulation of *SIRT3* expression altered mitochondrial metabolism and affected the oocyte quality in GCs from patients with reduced ovarian reserve or advanced maternal age [32]. These results suggest high expression of *SIRT3* may cause alterations of mitochondrial metabolism in GCs of PCOS patients.

5. Conclusions

In summary, we showed that *SIRT3* expression was increased in luteinized GCs of PCOS patients. Hyperandrogenism promoted oxidative stress and *SIRT3* expression in GCs of human and mice. These results suggest that *SIRT3* may be involved in the regulation of oxidative stress and GC function in luteinized GCs of non-obese PCOS patients and may be a novel therapeutic candidate for PCOS.

Author Contributions

SZ and WD designed the research study. SZ, WD and QL performed the research. SZ, WD and WY analyzed the

data. SZ wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

For human samples, ethics approval was obtained from the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (Approved number: YS2016-063 and YS2019-046). For animal experiments, all procedures were reviewed and approved by the Animal Ethical Committee of the Laboratory Animals Center of Chongqing Medical University (Approved number: 2021-184).

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Conflict of Interest

The authors declare no conflict of interest.

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