Mitochondrial abnormality in ovarian granulosa cells of patients with polycystic ovary syndrome

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Abstract. The quality of oocytes in patients with polycystic ovary syndrome (PCOS) decreases, which is closely related to the function of oocytes' mitochondria. If mitochondrial dysfunction is involved in PCOS, it is likely to affect the function of cumulus cells. However, the mechanism of mitochondrial dysfunction remains unclear. In the present study, granulosa cells were collected from women attending the Hebei Reproductive Health Hospital and were divided into the normal ovarian reserve group (CON group) and the PCOS group. The mitochondrial ultrastructure was observed by transmission electron microscope, and the mitochondrial function was determined by detecting the ATP content, reactive oxygen species levels, the number of mitochondria and the mitochondrial membrane potential. Additionally, western blotting was used to compare the expression levels of mitochondrial kinetic protein, the related channel protein, between the two groups. In the present study, it was found that patients with PCOS had abnormal granulosa cell morphology, increased mitochondrial abnormalities, decreased mitochondrial function and disturbed mitochondrial dynamics. In addition, the silent information regulator 1/phosphorylated-AMP-activated protein kinase/peroxisome proliferator-activated receptor-γ coactivator 1α pathway expression was decreased, and it was hypothesized that the decreased mitochondrial mass in the PCOS group was associated with it.

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Introduction

Polycystic ovary syndrome (PCOS) is the most common and complex endocrine disorder, affecting up to 10% of women, and may present with an irregular menstrual cycle (oligo-ovulation), hyperandrogenism, hirsutism and polycystic ovaries (1,2). Long-term risks conferred by PCOS include metabolic disorders such as type 2 diabetes and cardiovascular disease (3). Additionally, PCOS has been confirmed to be related to the quality of oocytes (4).

Cells with the granulosa support oocyte development. There are two types of granulosa cells: Granulosa cells distributed in and around the follicle wall (mural granulosa cells, mGCs) and granulosa cells in the follicle wall (cumulus granulosa cells, cGCs). Oocytes are enclosed within cGCs, providing necessary growth factors, hormones and other nutrients for oocytes (5).

Mitochondria not only supply the energy needed for the proliferation and metabolism of cGCs, but also regulate important physiological processes including antioxidation, apoptosis and autophagy. Mitochondria are the main source of reactive oxygen species (ROS). Through the production of ROS, mitochondria are the major determinant of numerous factors involved in reproduction such as oocyte quality, follicular growth and development, and granulosa cell proliferation (6).

Silent information regulator 1 (SIRT1), a deacetylase enzyme that requires NAD^+, regulates mitochondrial biogenesis, oxidative stress defense and energy homeostasis during folliculogenesis (7,8). Moreover, SIRT1 deacetylates peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α) and is involved in mitochondrial biogenesis and activity. Among the proteins that mediate the SIRT1-dependent action on mitochondria, a key role is played by peroxisome proliferator-activated receptor PGC1α (9,10), along with mitochondrial transcriptional factor A (TFAM), which is a downstream target gene of SIRT1/PGC1α (11,12). SIRT1 can deacetylate PGC1-α through the AMP-activated protein kinase (AMPK) pathway to affect the function and quality of the mitochondria of ovarian GCs (13). However, there have been no studies on the impact of mitochondrial biogenesis in granulosa cells of PCOS patients, and it is unknown whether mitochondrial dysfunction in cGCs leads to a decrease in oocyte quality in PCOS patients. As a

result of the present study, it was investigated whether regulation of a SIRT1/AMPK axis in ovaries may be associated with mitochondrial biogenesis in patients with PCOS.

Materials and methods

Patients. The participants were recruited from women who attended the Reproductive Medicine Center of Hebei Reproductive Health Hospital (Shijiazhuang, China) between June 2019 and June 2022. The diagnosis of PCOS was based on the Rotterdam criteria. Patients who were included in the study presented at least two of the following conditions: i) oligo-and/or anovulation; ii) androgen excess; iii) polycystic ovaries. Patients with an ovarian cyst, diminished ovarian reserve, hydrosalpinx, endometriosis, chromosome abnormality, or other systemic diseases which could affect granulosa cell gene expression were excluded. Infertile patients who have common ovulation with tubal issues were recruited as controls. The final study group included 66 PCOS group and 63 control (CON) group. The patient information statistics are shown in Table I.

Ethical approval. The present study was approved by the Ethics Committee of the Hebei Reproductive Health Hospital (approval no. KYY-2021-LW-005; Shijiazhuang, China). Written informed consent was obtained from each patient. The research followed the Helsinki Declaration standards (1964).

Isolation of GCs. The oocyte-cumulus complexes (OCCs) were retrieved 36 h after human chorionic gonadotropin injection and washed with phosphate-buffered saline (PBS) at 4˚C to remove any remaining mGCs, blood cells, or debris. The cumulus complex was selected under a light microscope, and the cGCs around the oocyte were carefully removed mechanically.

Transmission electron microscopy (TEM). The cGCs were collected and centrifuged at 400 x g for 5 min at room temperature. Cells were obtained in 2.5% glutaraldehyde solution at 4˚C for 30 min and were then dehydrated with a gradient of ethyl alcohol series, embedded in Araldite, then sliced into 60-nm sections. Uranyl acetate and lead citrate were used to stain the sections at room temperature for 15 min, after which, the sections were examined under a transmission electron microscope (Hitachi, Ltd.) and images were captured. Overall, the TEM experiment involved 3 patients with CON and 3 patients with PCOS.

Detection of intracellular ROS levels. The 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe (Cayman Chemical Company) was employed to detect the intracellular ROS levels. Briefly, cGCs were resuspended using 10 μmol/l DCFH-DA, incubated for 30 min at 37˚C in the dark, and then washed thrice with PBS buffer (cat. no. 02-024-1ACS; Biological Industries; Sartorius AG). cGCs were resuspended using 10 μg/ml Hoechst 3342 (Cayman Chemical Company) and incubated for 10 min in the dark at 37˚C, and then washed thrice with PBS buffer. The cell fluorescence intensity was detected with an ImageXpress confocal microscope (Molecular Devices, LLC), and the ROS levels were expressed as the mean green fluorescence intensity. This experiment involved 7 patients with CON and 5 patients with PCOS.

Mito-Tracker Red CMXRos staining. cGCs were cultured with 10 μm/l Mito-Tracker Red CMXRos staining solution for 30 min at 37˚C in 5% CO_2. After washing thrice with PBS buffer. Then, cGCs were stained using 10 μg/ml Hoechst 3342, incubated for 10 min at 37˚C in 5% CO_2, and then washed thrice with PBS buffer (Seville) before placing in fresh medium. The fluorescence intensity of mitochondrial Mito-Tracker Red CMXRos was detected immediately with an ImageXpress confocal microscope (Molecular Devices, LLC). The captured images were assessed, using ImageJ Imaging System software (version 1.51; National Institutes of Health). Mito-Tracker Red levels were assessed by calculating the mean red fluorescence intensity. This experiment involved 3 patients with CON and 4 patients with PCOS.

Measurement of cellular ATP levels. With the ATP Assay kit (cat. no. A095-1-1; Nanjing Jiancheng Bioengineering Institute), it was possible to measure cellular ATP levels. The ATP value of cGCs was assessed by measuring the absorbance at a wavelength of 636 nm using a microplate reader (Spectra Max iD3; Molecular Devices, LLC). To avoid errors caused by differences in protein content, the bicinchoninic acid (BCA) protein Assay kit (Beyotime Institute of Biotechnology) was used to measure sample protein concentrations. This experiment involved 11 patients with CON and 10 patients with PCOS.

Mitochondrial membrane potential (MMP) assay-JC-1. The MMP of cGCs was measured using the fluorescent dye 5,6-dichloro-2-[3[(5,6-dichloro-1,3-diethyl-1,3-dihydro-2H-benzimidazole-2-ylidene)-1-propen-1-yl]-1,3-diethyl-1H benzim-idazolium, monoiodide (JC-1; Cayman Chemical Company). cGCs were collected and labeled with 10 μmol/l JC-1 fluorescent probe in 5% CO_2 at 37˚C for 30 min. Then were washed thrice with PBS buffer. The aforementioned images were captured using ImageXpress confocal microscope (Molecular Devices, LLC) to demonstrate green fluorescence and red fluorescence. Then, captured images were further analyzed using ImageJ Imaging System software (version 1.51). The fluorescence was expressed as a ratio of red to green. This experiment involved 4 patients with CON and 3 patients with PCOS.

Reverse-transcription-quantitative PCR (RT-qPCR). Total RNA was purified with an RNA extract kit (cat. no. TR254-2; Genstone Biotech) according to the manufacturer's instructions, and cDNA was synthesized using the Supersmart™ 6 min 1st Strand cDNA Synthesis kit (cat. no. ZS-M14003M-50T; ZHONGSHI TONTRU) according to the manufacturer's instructions. For qPCR, each 20 μl reaction system contained 2 μl cDNA and 10 μl 2X SYBR® Green PCR Master Mix (cat. no. RK21203; ABclonal Biotech Co., Ltd.), forward primer 0.4 μl, reverse primer 0.4 μl and ddH_2O 7.2 μl. The qPCR was carried out with the following conditions: Initial denaturation at 95˚C for 3 min followed by 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec for annealing, and extension at 72˚C for 1 min. Each sample was analyzed in triplicate, and

Table I. Comparison of general condition between two groups (mean ± SD).

General condition	CON group (n=66)	PCOS group (n=63)	P-value
Age, years	30.90±3.81	31.14±3.23	0.728
BMI, kg/m^2	23.44±2.60	24.79±3.95	0.042
Basal FSH, IU/l	7.27±2.13	7.38±3.19	0.826
Basal LH, IU/l,	4.61±3.29	5.64±3.50	0.109
FSH/LH	2.02±1.14	1.70±0.96	0.114
Basal E2, pg/ml	38.07±17.38	38.42±11.27	0.904
Basal P, ng/ml	0.70±0.60	0.62±0.50	0.413
Basal PRL, ng/ml	15.46±11.41	12.53±5.85	0.109
Basal T, ng/ml	0.36±0.15	0.46±0.21	0.005

FSH, follicular stimulating hormone; LH, luteinizing hormone; E2, estradiol; P, progesterone; PRL, prolactin level; T, testosterone.

the relative fold expression of each gene was calculated using the comparative critical threshold (Cq) method based on the $2^{-\Delta\Delta Cq}$ calculation (14), relative to the housekeeper gene β-actin. Primer sequences of the related genes analyzed are described in Table SI. This experiment involved 11 patients with CON and 11 patients with PCOS.

Western blotting. cGCs were homogenized in 50-100 μl RIPA buffer (50 μM Tris/HCl, pH 7.4; 150 μM NaCl; 1% NP-40; 0.5% deoxycholate; 0.1% sodium dodecyl sulfate; 1% protease inhibitor cocktail; cat. no. RW0001; Report Biotech). After centrifugation at 12,000 x g at 4˚C for 10 min, the supernatant was collected and the protein concentration was determined using a BCA kit. Protein samples (5-8 μg) were separated by 12% SDS-PAGE at 120 V for 60 min. Subsequently, the separated proteins were transferred onto a polyvinylidene difluoride membrane (Sigma-Adrich; Merck KGaA) at 400 mA for 2 h (Bio-Rad Laboratories, Inc.). Afterward, a blocking buffer containing 5% non-fat dry milk (Inner Mongolia Yili Industrial Group Company Ltd.) was provided for 45 min to block the PVDF membrane. The Membrane was then incubated with primary antibodies for immunoblotting against SIRT1 (1:1,000; cat. no. ab110304; Abcam), AMPK (1:1,000; cat. no. AF6423; Affinity Biosciences, Ltd.), phosphorylated (P-)AMPK (1:1,000; cat. no. JJ08-19; Huabio), PGC-1α (1:500; cat. no. sc-518038; Santa Cruz Biotechnology, Inc.), optic nerve atrophy 1 (OPA1) (1:500; cat. no. ET1705-9; Huabio), dynamin-related protein 1 (DRP1) (1:1,000; cat. no. ab199722; Abcam), mitofusin 2 (MFN2) (1:1,000; cat. no. 9482S; Cell Signaling Technology, Inc.), mitochondrial fission 1 (FIS1) (1:500; cat. no. ET7109-17; Huabio) and β-actin (1:5,000; cat. no. AC026; ABclonal Biotech Co., Ltd.) overnight at 4˚C. Following the primary incubation, the membrane was incubated with the HRP-goat anti-rabbit IgG (H + L) (1:3,000; cat. no. RS0002; Immunoway) or the HRP-goat anti-mouse IgG (H + L) (1:3,000; cat. no. S1001; Report Biotech) for 1.5 h at room temperature. The protein bands were detected using a Minichemi 320 chemiluminescence imaging system (Beijing sage creation) and quantified with ImageJ 1.8.0 software. This experiment involved 27 patients with CON and 27 patients with PCOS.

Statistical analysis. Descriptive analyses were performed for the variables studied. The results are presented as the mean ± SD for continuous variables and absolute numbers and relative frequencies for categorical variables. Comparisons of unpaired continuous data were conducted using the unpaired Student's t-test or Mann-Whitney U test, depending on how the data were distributed. Comparisons of paired continuous data were conducted using the paired t-test or Mann-Whitney U test, depending on the data distribution. the chi-square test was used to analyze qualitative variables. Analysis was conducted using SPSS 20.0 software (SPSS, Inc.; IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of the general conditions between two groups. In the present study, a total of 66 patients with PCOS and 63 control patients were included. A comparative study of clinical characteristics between PCOS patients and controls is presented in Table I. Age (30.90±3.81 vs. 31.14±3.23 years; P=0.728), basal follicular stimulating hormone (FSH) level (7.27±2.13 vs. 7.38±3.19; P=0.826), basal luteinizing hormone (LH) level (4.61±3.29 vs. 5.64±3.50; P=0.109), FSH/LH ratio (2.02±1.14 vs. 1.70±0.96; P=0.114), basal estradiol (E2) level (38.07±17.38 vs. 38.42±11.27; P=0.904), basal progesterone (P) level (0.70±0.60 vs. 0.62±0.50; P=0.413) and basal prolactin level (15.46±11.41 vs. 12.53±5.85, P=0.109) had no significant difference between these two groups, while BMI (23.44±2.60 vs. 24.79±3.95; P=0.042) and basal testosterone (T) (0.36±0.15 vs. 0.46±0.21; P=0.005) were increased in the PCOS group.

Injured mitochondria are observed in the GCs of the patients with PCOS. To investigate the changes in mitochondrial ultrastructure, TEM was used. In the control group, all the cGCs had a normal nucleus with intact and continuous nuclear membranes, and most round or rod-shaped mitochondria with a clear crista around the nucleus. However, the cGCs of patients with PCOS presented mitochondrial exhibited edema, reduced crista and fracture, and mitochondrial vacuoles. It was suggested that the mitochondrial structure of GCs in the ovary of PCOS patients was abnormal and seriously damaged (Fig. 1).

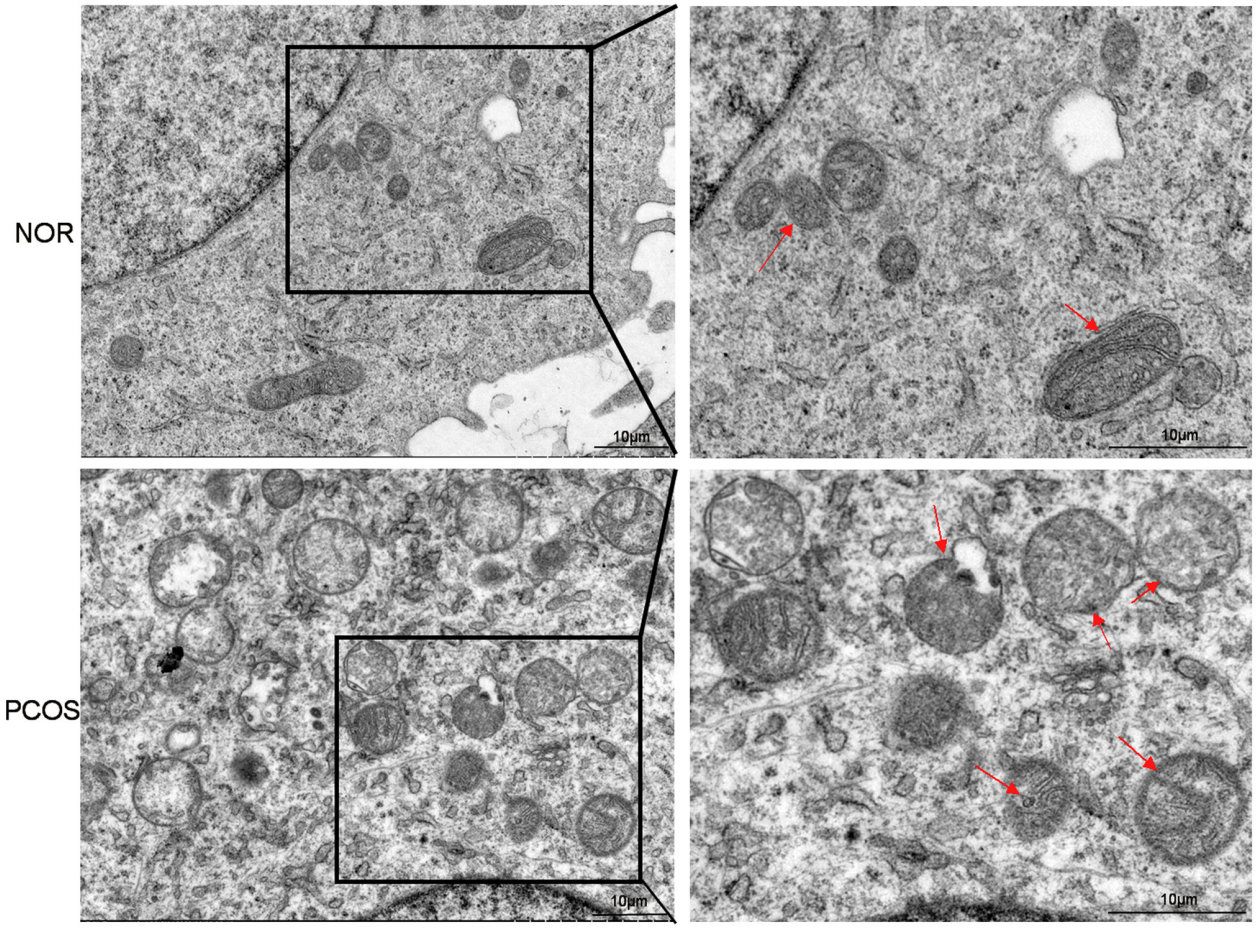

Figure 1. Observation of the granular cell mitochondria ultrastructure in the CON and PCOS groups by transmission electron microscopy (n=3 vs. n=3). As shown by the red arrow in the images, cGCs had a normal nucleus with a clear nucleoli and double nuclear membranes, and the mitochondria had a clear mitochondrial crista around the nucleus in control group. However, the cGCs presented mitochondrial aggregated distribution, crista dissolution and fracture and mitochondria with vacuoles in PCOS group. CON, normal ovarian reserve; PCOS, polycystic ovary syndrome; cGCs, cumulus granulosa cells.

Detection of intracellular ROS levels. When mitochondria produce excessive ROS, GCs become dysfunctional and apoptotic. After incubating the probe, the cGCs in the CON group showed weak fluorescence, while those in the PCOS group demonstrated clearer fluorescence (Fig. 2A). As revealed in the bar graph, mean green fluorescence intensity for cGCs from the patients of the CON group was 2.51±0.46, which was significantly lower than that in patients of the PCOS group (3.35±0.36) (P=0.001) (Fig. 2B).

The ATP content of cGCs in patients with PCOS is reduced. According to the analyses of the ATP bioluminescence detection kit, the ATP content (0.70±0.35 mm) of cGCs in the PCOS group was significantly reduced compared with the control group (1.55±0.78 mm; P=0.006) (Fig. 3A). To investigate the expression of ATP synthesizing genes, a RT-qPCR was conducted. The mRNA transcription levels of ATP5A1 and ATP5I were not significantly different between the two groups (P>0.05) (Fig. 3B and C).

The numbers of mitochondria of cGCs in patients with PCOS are reduced. Mito-Tracker Red CMXRos staining revealed the presence of bioactive mitochondria with a stronger red-light intensity, and compared with the CON group, the cells in the PCOS group revealed weaker fluorescence (Fig. 4A). As shown in the bar graph, the mean red fluorescence intensity of the cGCs from the patients of the PCOS group was decreased, but no statistical significance was observed (16.05±3.63 vs. 13.62±1.29; P=0.259) (Fig. 4B).

Increased mitochondrial membrane damage in patients with PCOS. MMP was also monitored; the JC-1 staining reveals a higher MMP when red fluorescence is present, and a lower MMP when green fluorescence is present. A lower MMP was observed in GCs of women with PCOS as compared with controls (Fig. 4C and D). The results showed that the mean red/green fluorescence intensity ratio for cGCs from the patients of the CON group was 1.47±0.12, which was significantly higher than that (0.37±0.04) of patients of the PCOS group (P=0.002) (Fig. 4D). These observations indicated that mitochondrial dysfunction in GCs appears to be accompanied by PCOS.

The mitochondrial dynamics are unbalanced in cGCs of the patient with PCOS. Due to mitochondrial dysfunction, ROS are produced, which in turn exacerbate mitochondrial damage. Therefore, mitochondrial function was further investigated. It was found that the protein levels of mitochondrial fusion-related genes MFN2 (1.49±0.81 vs. 2.69±1.69;

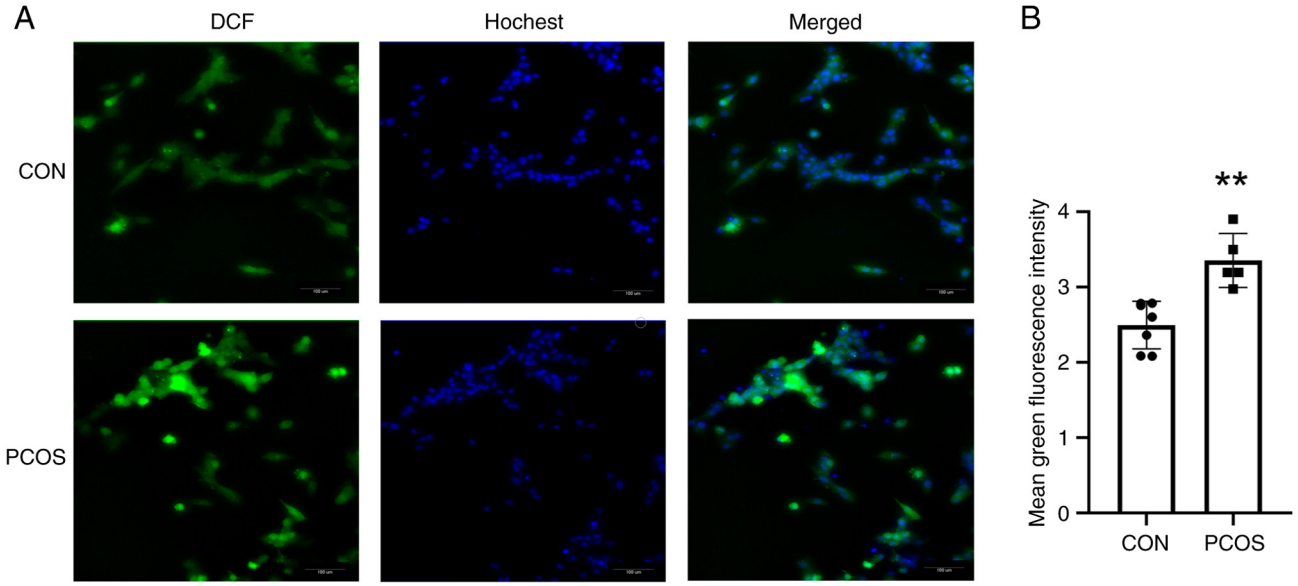

Figure 2. ROS levels in cGCs from the CON and PCOS groups. (A) DCFH-DA staining was used to measure the concentrations of ROS in the CON and PCOS groups, and the staining was visualized using ImageXpressMicro confocal microscope. Scale bar, 100 μm. (B) Data are expressed as the mean ± SEM (n=7 vs. n=5). The cGCs in the CON group demonstrated weak green fluorescence, while those in the PCOS group showed clearer green fluorescence. **P<0.01. ROS, reactive oxygen species; cGCs, cumulus granulosa cells; CON, normal ovarian reserve; PCOS, Polycystic ovary syndrome; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate.

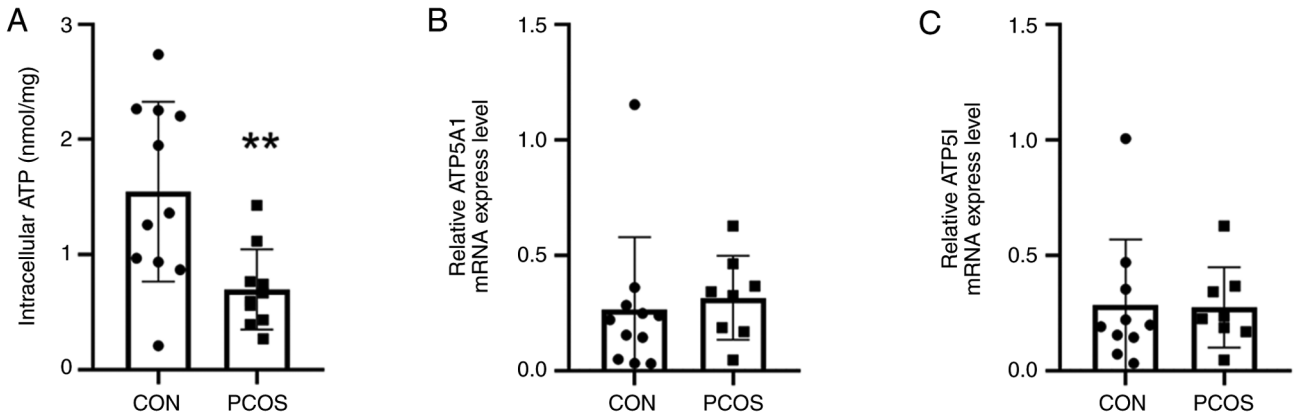

Figure 3. (A) Intracellular ATP concentration in cGCs of the CON and PCOS groups (n=11 vs. n=10). The ATP content of cGCs in the PCOS group was significantly reduced compared with the control group. (B) The mRNA transcription level of ATP5A1 in the CON and PCOS groups was detected by RT-qPCR (n=11 vs. n=8). (C) The mRNA transcription of ATP5I in the CON and PCOS groups was detected by RT-qPCR (n=10 vs. n=8). The mRNA transcription levels of ATP5A1 and ATP5I were not different between the two groups. **P<0.01. cGCs, cumulus granulosa cells; CON, normal ovarian reserve; PCOS, polycystic ovary syndrome; RT-qPCR, reverse transcription-quantitative PCR.

P=0.002; Fig. 5A and E) and genes associated with mitochondrial fission FIS1 (1.93±0.87 vs. 3.47±2.20; P=0.009; Fig. 5A and C) were increased in the PCOS group compared with the CON group. No statistical significance were observed in the expression level of OPA1 and DRP1 between the PCOS and the CON groups (Fig. 5A, B and D). Repeated experiments were conducted for numerous patients and the results revealed the same trend. Results of repeated western blot experiments regarding the proteins DRP1, FIS1, OPA1, MFN2 are included in Figs. S1-S4, respectively. The mRNA transcription levels of genes including Opa1 (0.00081±0.00053 vs. 0.00064±0.00054; P=0.560; Fig. 5F), DRP1 (0.014±0.016 vs. 0.021±0.020; P=0.503; Fig. 5G), FIS1 (0.042±0.0080 vs. 0.070±0.049; P=0.002; Fig. 5H) and MFN2 (0.00031±0.00035 vs. 0.0030±0.0042; P=0.188; Fig. 5I), did not demonstrate a statistically significant difference between the two groups.

The expression of proteins regulating mitochondrial biogenesis in patients with PCOS. Given the initial results of the present study, the molecular regulatory mechanisms of mitochondrial function were then explored. The mRNA transcription level of the mitochondrial transcription factor A (TFAM) was detected, which is crucial for the replication and transcription of mitochondrial DNA-encoded genes. PGC-1α acts as a transcription coactivator by binding directly to the transcription factor TFAM in the DNA promoter region. SIRT1 and P-AMPK affect mitochondrial function by activating PGC-1α.

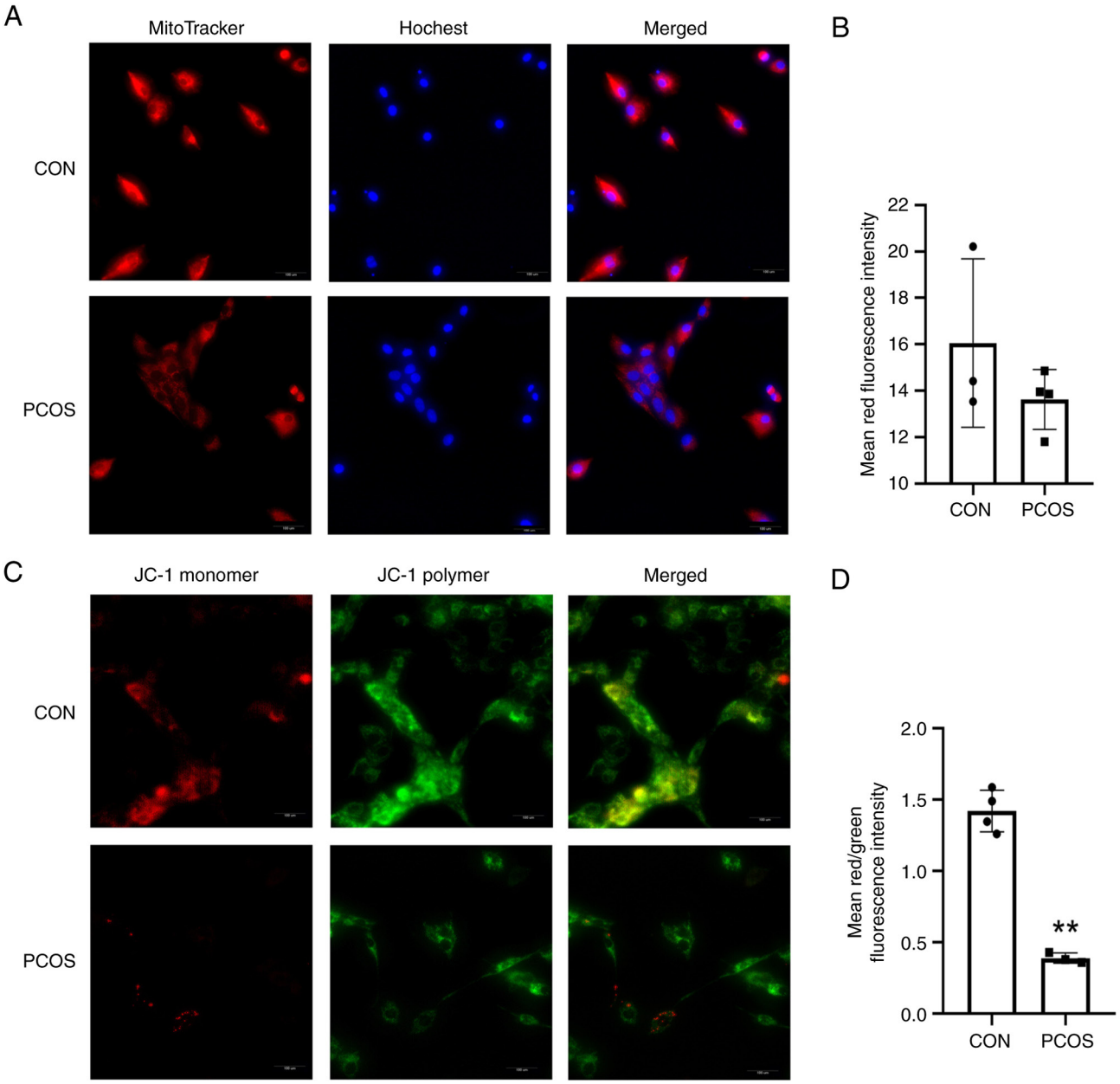

Figure 4. Changes of mitochondrial number and MMP in the CON and PCOS groups. (A) Mito-Tracker Red CMXRos was used to measure the number of mitochondria detected with ImageXpress confocal in cGCs from the CON and PCOS groups. Cells Scale bar, 100 μm. (B) Statistical data are presented as the mean ± SEM (n=3 vs. n=4). The red fluorescence of cGCs in the control group was stronger than that in PCOS group, but there was no statistical difference between the two groups. (C) MMP was detected using ImageXpress confocal microscope and analysis of MMP by JC-1 staining in cGCs from the CON and PCOS groups. Both red and green fluorescence were observed in the cGCs from the NOR group after staining with JC-1. The intensity of red fluorescence excited in the cGCs of the PCOS group was weaker when compared with the NOR group while an increase of green fluorescence was observed in the PCOS group. (D) Statistical data are presented as the mean ± SEM (n=4 vs. n=3). **P<0.01. cGCs, cumulus granulosa cells; CON, normal ovarian reserve; PCOS, Polycystic ovary syndrome; MMP, mitochondrial membrane potential.

The results of the statistical analysis revealed that the mRNA transcription level of TFAM in the cells of the PCOS group was significantly lower than that in the cells of the CON group (0.0090±0.0046 vs. 0.0063±0.0073; P=0.045; Fig. 6H). Moreover, the levels of energy depletion signaling-related factors proteins in the CON group were higher than those in the PCOS group, including SIRT1 (2.29±0.43 vs. 0.86±0.20; P<0.0001; Fig. 6A and B), AMPK (5.78±2.72 vs. 3.69±0.98; P=0.020), P-AMPKα (10.18±3.97 vs. 2.60±1.53, P=0.006) and PGC-1α (0.77±0.29 vs. 0.34±0.11; P=0.015; Fig. 6A and D). The protein ratio of P-AMPKα to AMPK in PCOS group was significantly lower than in the CON group (2.42±1.21 vs. 0.71±0.47; P=0.015; Fig. 6C). Repeated experiments were performed in numerous patients, and the results revealed the same trend. Results of repeated western blot experiments regarding the proteins SIRT1, AMPK, PGC-1 and P-AMPKα are included in Figs. S5-S8, respectively. There was no statistically significant difference in SIRT1 (0.0035±0.0022 vs. 0.0057±0.0030; P=0.159; Fig. 6E), AMPK (0.0038±0.0027 vs. 0.0045±0.0025; P=0.657; Fig. 6F) and PGC-1α (0.015±0.012 vs. 0.0065±0.0070; P=0.162; Fig. 6G) mRNA transcription levels.

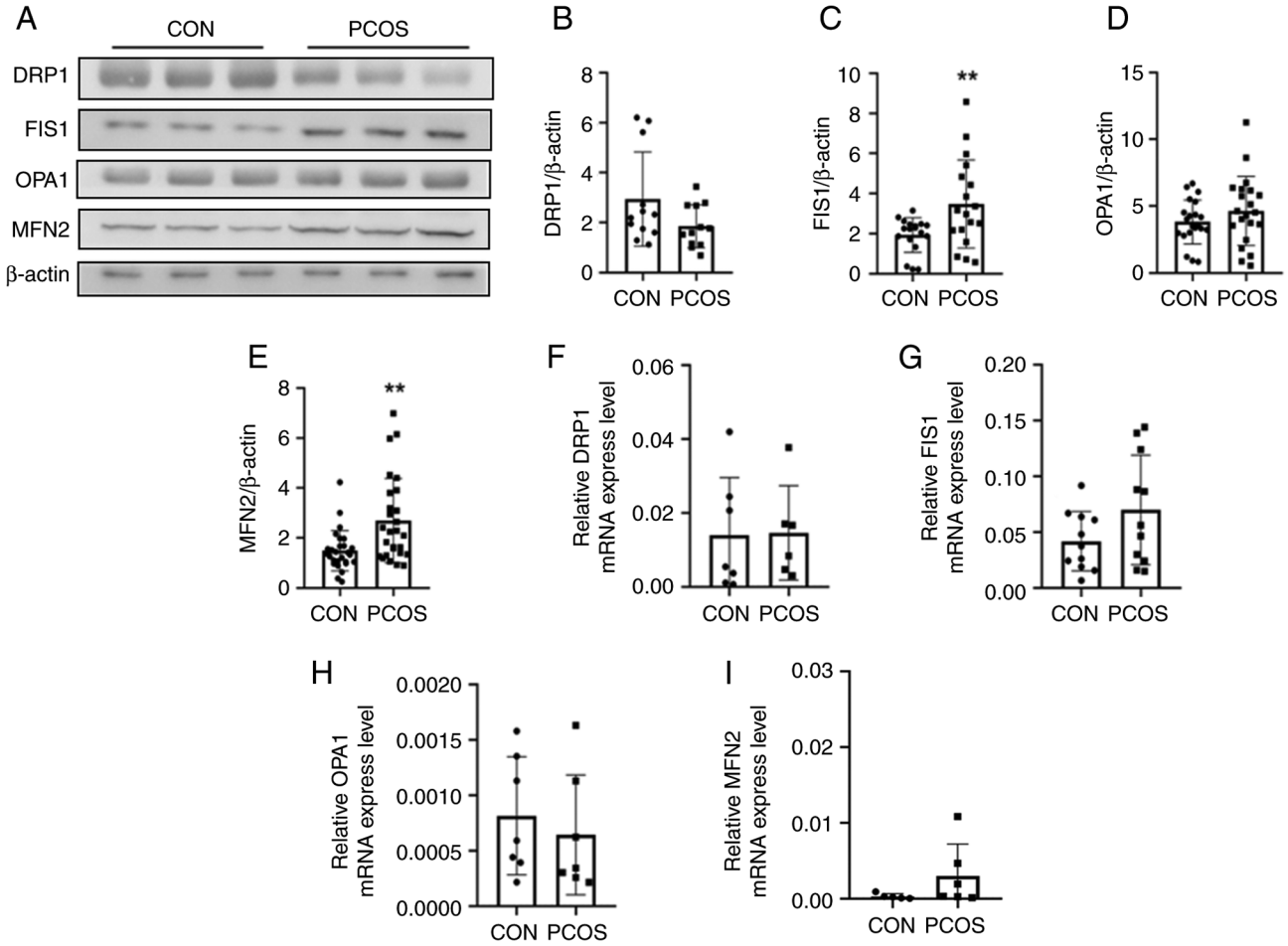

Figure 5. Western blot analysis and RT-qPCR comparison of DRP1, FIS1, OPA1 and MFN2 between the CON and PCOS groups. (A) Mitochondrial protein expression, including DRP1, FIS1, OPA1 and MFN2 was detected in the two groups by western blotting. (B) Gray intensity of DRP1 expression relative to β-actin (n=3 vs. n=3). No statistical significance was observed in the expression levels of DRP1 between the PCOS and the CON groups. (C) The gray intensity of FIS1 expression relative to β-actin (n=3 vs. n=3). The protein expression of FIS1 in the cGCs of the PCOS group was significantly increased compared with the CON group. (D) The gray intensity of OPA1 expression relative to β-actin (n=3 vs. n=3). (E) The gray intensity of MFN2 expression relative to β-actin (n=3 vs. n=3). The protein expression of MFN2 in the cGCs of the PCOS group was significantly increased compared with the CON group. (F) The bar chart shows the mRNA transcription of DRP1 in the CON and PCOS groups by Q-PCR (n=7 vs. n=6). (G) The bar chart shows the mRNA transcription of FIS1 in the CON and PCOS groups by RT-qPCR (n=11 vs. n=11). (H) The bar graph shows the mRNA transcription of OPA1 in the CON and PCOS groups by RT-qPCR (n=7 vs. n=7). (I) The bar chart shows the mRNA transcription of MFN2 in the CON and PCOS groups by RT-qPCR (n=5 vs. n=6). The mRNA transcription levels of the genes DRP1, FIS1, OPA1, MFN2 did not demonstrate statistically significant difference between the two groups. **P<0.01. RT-qPCR, reverse transcription-quantitative PCR; DRP1, dynamin-related protein 1; FIS1, mitochondrial fission 1; OPA1, optic nerve atrophy 1; MFN2, mitofusin 2; CON, normal ovarian reserve; PCOS, polycystic ovary syndrome; cGCs, cumulus granulosa cells.

Discussion

PCOS is one of the most common hormonal disorders of women during the reproductive years. In patients with PCOS, the mitochondrial state of GCs is altered, the spindle assembly disordered, and the quality of oocytes decreases (15-17). Any problems of GCs in growth differentiation, metabolism and apoptosis will directly or indirectly affect the quality of follicular (18-20).

In the present study, the mitochondrial structure and function of cGCs were investigated. As the TEM revealed increased abnormal mitochondrial in cGCs of the patients with PCOS, which was in accordance with the study by Zhang *et al* (21), MMP decreased, and mitochondrial swelling was found in the patients with PCOS.

Mitochondria play a major role in intracellular redox metabolism and are producers of ROS, which mediate oxidative stress, affect cell development and maturation and aggravate cell apoptosis (6,22,23). The present results revealed that the level of ROS in the PCOS group was significantly higher than in the CON group, suggesting that the cells had oxidative damage in the PCOS group.

Intracellular ATP levels are often used to assess mitochondrial function. Studies have shown that ATP deficiency increases aneuploidy during oocyte development and embryonic development, which is a common cause of chromosome segregation (24,25). The decrease in mitochondrial ATP levels in GCs ultimately limits oocyte development and maturation. The ATP level in cGCs cells of the CON group was 2.21 times higher than that of the PCOS group. ATP production is a complex process involving four protein complexes and ATP synthase; the ATP5A1 gene encodes the α-subunit of ATP synthase F1 complex, and the ATP5I gene encodes the e-subunit of ATP synthase F0 complex (26); notably, the present results revealed that the mRNA transcription levels of

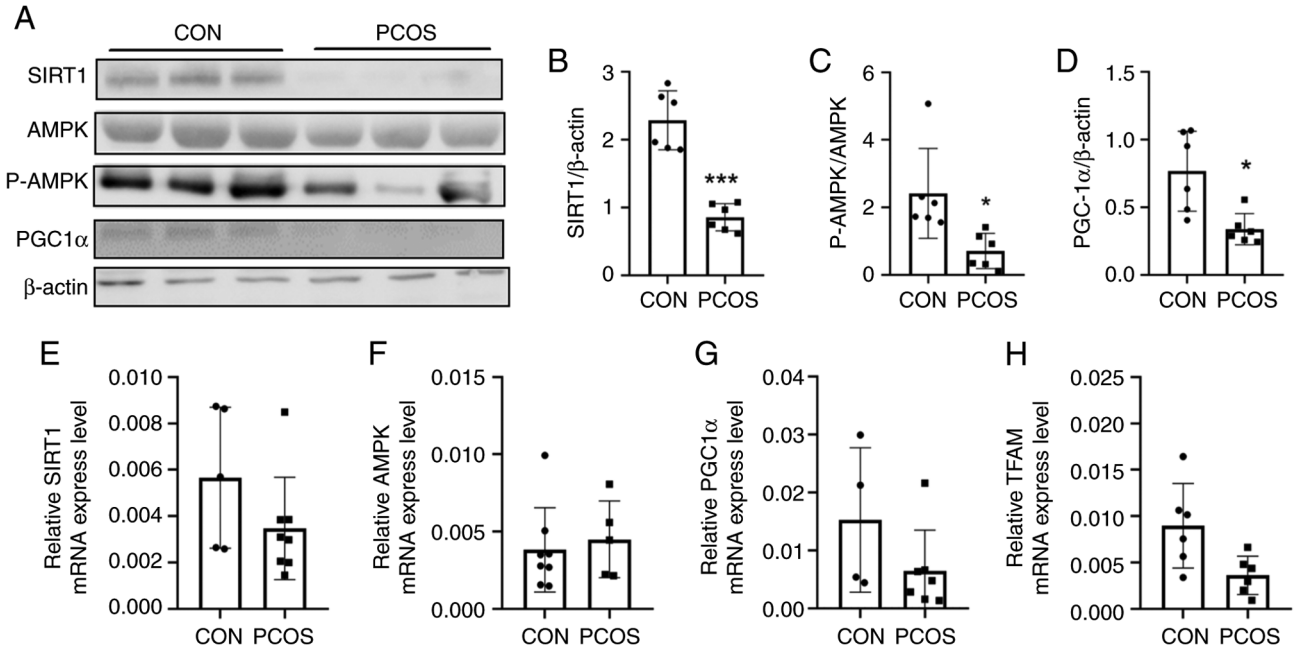

Figure 6. Western blot analysis and RT-qPCR comparison of pathway proteins between the CON and PCOS groups. (A) Mitochondrial protein expression of the pathway protein was detected by western blotting. (B) The gray intensity of SIRT1 expression relative to β-actin (n=3 vs. n=3). (C) The ratio of P-AMPKα to AMPK expression (n=3 vs. n=3). The protein ratio of P-AMPK to AMPK in the cGCs of the PCOS group was significantly lower than in NOR group. (D) The gray intensity of PGC1α expression relative to β-actin (n=3 vs. n=3). The expression protein levels of the energy depletion signaling-related factors SIRT1, AMPK, p-AMPK and PGC-1α were decreased in the cGCs of the PCOS group. (E) mRNA transcription level of SIRT1 by RT-qPCR (n=5 vs. n=8). (F) mRNA transcription level of AMPK by RT-qPCR (n=8 vs. n=5). (G) mRNA transcription level of PGC-1α by RT-qPCR (n=4 vs. n=7). (H) mRNA transcription level of TFAM detected by RT-qPCR (n=6 vs. n=6). There was no statistically significant difference observed in SIRT1, AMPK and PGC-1α mRNA transcription level. *P<0.05 and ***P<0.001. RT-qPCR, reverse transcription-quantitative PCR; CON, normal ovarian reserve; PCOS, polycystic ovary syndrome; SIRT1, silent information regulator 1; AMPK, AMP-activated protein kinase; P-AMPKα, phosphorylated AMPKα; cGCs, cumulus granulosa cells; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; TFAM, mitochondrial transcription factor A.

ATP5A1 and ATP5I exhibited no difference between the two groups, thus indicating that some other enzyme may have a role in ATP synthesis.

Recent studies suggested that there is a crucial role for mitochondrial dynamics by fission and fusion in the maintenance of healthy follicular development. Mitochondrial fission is mediated by DRP1 and FIS1, while mitochondrial fusion is promoted by MFN2 and OPA1. Inner membrane fusion requires OPA1 (27), whereas outer membrane fusion requires MFN2 (28). However, MFN2 and OPA1 showed an obvious imbalance in the expression and interaction according to the results of the present study. In the present study, the expression of MFN2 and OPA1 in the control group was lower. The balance of inner and outer membrane fusion maintains the normal function of mitochondria (29). DRP1 is recruited from the cytosol to the outer membrane by mitochondrial dynamics proteins 49 and 51 (Mid49 and 51), FIS1 and MFF (30). Drp1 downregulation was also associated with mitochondrial elongation. FIS1 protein expression value of the PCOS group was significantly higher than that of the CON group in the aforementioned experiment.

However, the molecular mechanisms controlling mitochondrial function remain unclear. A mitochondrial protein encoded by nuclear genetic material, PGC-1α belongs to the family of nuclear-derived proteins. PGC1α tunes the activity of a range of nuclear transcription factors, including nuclear respiratory factor 1 (NRF1) and NRF2, to control expression of nuclear-encoded OXPHOS subunits and genes associated with mitochondrial replication, transcription and translation, including mitoribosomal proteins. PGC-1α regulates mitochondrial biogenesis and function through mitochondrial transcription factor A (TFAM), downstream transcription factors (31). In the present study, it was revealed that the expression of PGC1-α decreased in the PCOS ovaries when compared with normal ovaries. One of these main control factors is TFAM which binds mitochondrial DNA and regulates mitochondrial transcription and mitochondrial DNA maintenance (32). It was revealed that the expression of TFAM decreased in the granulosa cells of PCOS ovaries when compared with healthy normal ovaries.

Recent studies showed that alterations in the level of mitochondrial biogenesis can give rise to hyperandrogenism, and an increase in mitochondrial biogenesis can ameliorate PCOS by reducing ROS in granulosa cells, thus highlighting the potential role of mitochondria in PCOS (33,34). A large number of mitochondrial proteins are acylated, allowing sirtuins to act as NAD^{+}-dependent deacetylase enzymes, thus regulating, including deacetylation, demalonylation and desuccinylation. Recent studies demonstrated that activation of SIRT1 alleviated mitochondrial damage and enhanced mitochondrial recovery, to attenuate ischemic liver injury and diabetic neuropathy (35,36), suggesting that SIRT1 plays an important role in mitochondrial quality control. In the present study, it was found that the excessively increased mitochondrial damage may be caused by decreased expression of SIRT1 in the GCs of PCOS.

Chronic activation of AMPK is associated with increased mitochondrial biogenesis, probably mediated by increased expression of PGC-1a (37). AMPK phosphorylates DNA methyltransferase 1 (DNMT1) and RbAp46 in human umbilical vein endothelial cells (HUVECs). This triggers the inhibition of DNMT1 and leads to important nucleosomal remodeling. This results in the upregulation of the nuclear genes that encode the proteins involved in mitochondrial biogenesis and function, including TFAM and PGC-1a (38). PGC-1α has the only target of Sirt1 (39). In the present study, the following explanations were provided for the inconsistent expression levels of proteins AMPK, DRP1, OPA1 and mRNA: First, the expression of genes is divided into two levels: Transcription and translation, namely mRNA level and protein level. There is a spatiotemporal interval between the time and location of transcription and translation of eukaryotic gene expression; Second, after transcription, there are several levels of post-transcriptional processing, degradation of transcriptional products, translation, post-translation processing and modification. Translation occurs by a universally conserved set of stages that can be divided into i) initiation, whereby the mRNA is loaded onto the ribosome and the start codon is selected; ii) elongation of the polypeptide by selective addition of amino acids; and iii) termination of translation, followed by recycling of the ribosome. Each step is mediated by nuclear-encoded translation factors that operate in conjunction with the mitoribosome. Therefore, the level of transcription and the level of translation are not exactly consistent; Third, the half-life of mRNA is different from that of protein. mRNA is highly degradable and exists for a short time in tissues, while its properties are stable after being translated into protein; and fourth, the timing of the detection may vary, as the mRNA has already degraded when the protein reached its peak value, or the protein amount is still increasing when the mRNA reached its peak value.

In the present study, it was identified that the low expression of SIRT1/P-AMPK-PGC-1α pathway protein may lead to decreased expression of mitochondrial transcription factors in GCs, which in turn affects mitochondrial function and ultimately affects the quality of oocytes (Fig. S9). It is possible to explore ways to improve the function of patients with PCOS by studying the mechanism of this pathway.

However, the present study has some limitations. Firstly, the phosphorylation of the DRP1 protein on S616 was not detected; this may be due to the small number of cumulus granulosa cells, low protein content or the relatively low proportion of phosphorylation. Secondly, no in-depth study was performed on the structure and function of mitochondria in oocytes from patients with PCOS. Thirdly, further research should be carried out on how to improve the quality of cGCs in patients with PCOS and thus improve the quality of oocytes. In conclusion, the present study revealed that the mitochondria of ovarian GCs in patients with PCOS exhibit disordered structure and dysfunction; it was deduced that the decrease in quality of GCs in patients with PCOS may be caused by the SIRT1-P-AMPK-PGC-1α pathway. Research has suggested that improving GC mitochondrial function will improve the quality of oocytes in patients with PCOS, thereby improving clinical outcomes.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CX, HL, XZ, ZA, TC, WY, SW, DS and XW contributed to the study conception and design. CX performed experiments. XZ, WY, TC and ZA are responsible for the collection of cumulus granulosa cells. HL and XW performed data collection. CX, SW, DS and XW analyzed data. CX wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. CX and XW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Hebei Reproductive Health Hospital (approval no. KYY-2021-LW-005; Shijiazhuang, China). Written consent was obtained from each patient or subject after full explanation of the purpose and nature of all procedures used. The present study has followed procedures that were in accordance with the ethical standards as formulated in the Helsinki Declaration (1964).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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