Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Growth Hormone Activates PI3K/Akt Signaling and Inhibits ROS Accumulation and Apoptosis in Granulosa Cells of Patients With Polycystic Ovary Syndrome

Yan Gong
Sichuan University
Shan Luo
Sichuan University
Ping Fan
Sichuan University
Huili Zhu
Sichuan University
Yujing Li
Sichuan University
Wei Huang (weihuang64@163.com)
1. Department of Obstetrics and Gynecology, West China Second University Hospital of Sichuan University; 2. Key Laboratory of Birth Defects and Related Disease of Women and Children(Sichuan University), Ministry of Education. https://orcid.org/0000-0001-9306-588X

Research

Keywords: polycystic ovary syndrome, growth hormone, reactive oxygen species, apoptosis, PI3K/Akt signaling

Posted Date: September 16th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-76999/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Version of Record: A version of this preprint was published on December 7th, 2020. See the published version at https://doi.org/10.1186/s12958-020-00677-x.

Abstract

Background: Growth hormone (GH) can reduce oxidative stress (OS) induced apoptosis in some types of cells by activating the PI3K/Akt signaling pathway. This study investigated the role and underlying mechanism of GH in OS and apoptosis in GCs of patients with polycystic ovary syndrome (PCOS).

Methods: Primary GCs were collected from patients with and without PCOS (controls, n = 32) during oocyte retrieval. The patients with PCOS were randomly assigned to receive treatment with GH (PCOS-GH, n = 30) or without GH (PCOS-C, n = 31). Reactive oxygen species (ROS) level was determined by spectrophotometry and fluorescence microscopy. GC apoptosis and mitochondrial membrane potential (MMP) were detected by Annexin V-FITC/PI double-staining and JC-1 staining, respectively (flow cytometry). The expression of apoptosis-related genes and proteins involved in PI3K/Akt signaling was determined by quantitative reverse-transcription polymerase chain reaction and western blotting, while active caspase-9 and caspase-3 levels were determined by enzyme-linked immunosorbent assay.

Result(s): The present study found that compared with those in the non-PCOS and PCOS-GH groups, the ROS levels and apoptotic rates were significantly increased, whereas MMP was significantly decreased in the PCOS-C group GCs ($P < 0.05$). Compared with those in non-PCOS and PCOS-GH groups, mRNA levels of FOXO1, Bax, caspase-9, and caspase-3 were significantly increased, whereas Bcl-2 was decreased in the GCs of the PCOS-C group ($P < 0.05$). The protein levels of FOXO1, Bax, cleaved caspase-9/caspase-9 and cleaved caspase-3/caspase-3 were increased, whereas p-PI3K/PI3K, p-Akt/Akt, p-FOXO1 and Bcl-2 were decreased in the GCs of the PCOS-C group, compared with those in the non-PCOS and PCOS-GH groups ($P < 0.05$).

Conclusion: OS induced apoptosis and inactivated the PI3K/Akt signaling pathway in patients with PCOS. GH could improve apoptosis and activate the PI3K/Akt signaling pathway.

Clinical Trial Registration Number: Chinese Clinical Trial Registry (www.chictr.org.cn/index.aspx). ChiCTR1800019437. Prospectively registered on October 20, 2018, http://www.chictr.org.cn/edit.aspx?pid=28663&htm= 4

Introduction

Polycystic ovarian syndrome (PCOS) is the most common endocrinopathy that affects 5–10% women of reproductive age and is characterized by hyperandrogenemia, polycystic ovaries, and/or ovulation dysfunction [1]. Chronic anovulation results in infertility, and some of these patients need *in vitro* fertilization (IVF) and embryo transfer (ET). Reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) are produced in many physiological processes. The antioxidant mechanism existing in the body can maintain ROS and RNS at low concentrations, which is beneficial for normal cell function [2]. Oxidative stress (OS) occurs when the balance of maintaining low concentrations of these reactive species is destroyed. Excessive ROS/RNS levels can induce mitochondrial dysfunction and cell apoptosis [3]. Some studies have reported OS state, mitochondrial dysfunction, and OS-induced apoptosis are

present in the granulosa cells (GCs) of patients with PCOS [4-6]. Through bidirectional communication, GCs play important role in oocyte maturation, fertilization, and subsequent implantation [7,8]. Therefore, apoptosis in GCs is associated with poor oocyte quality and IVF outcomes in patients with PCOS [6,8].

Growth hormone (GH) can reduce OS-induced apoptosis in some types of cells including vascular endothelium, cardiomyocytes, and neural and skeletal muscle cells by activating the PI3K/Akt signaling pathway [9-12]. Hence, GH has been widely applied to treat pathologies associated with OS [12]. GH receptors are expressed in human GCs and oocytes. Exogenous GH administration alleviates mitochondrial dysfunction and improves oocyte quality and IVF outcomes among older women and/or patients with poor ovarian response [13].

PCOS is a disease involving multiple genes and environmental factors [14]. Microarray data of GCs from patients with PCOS indicated that the markedly changed genes are mainly related to diabetes, inflammation, and OS [15]. The PI3K/Akt signaling pathway is dysregulated in both patients with PCOS and animal models of PCOS [16,17]. Activation of the PI3K/Akt signaling pathway can reduce apoptosis induced by downstream signaling molecules [18-20], and consequently, not only protect GCs from OS injury but can also improve oocyte quality and IVF outcomes [18]. However, the antioxidant effects of GH in GCs of patients with PCOS and related signaling pathways have not been investigated yet. Therefore, this study investigated the effects of GH on ROS levels, apoptosis of GCs, and the PI3K/Akt signaling pathway.

Materials And Methods

Clinical samples

From November 2018 to November 2019, patients with PCOS (aged 22–36 years) diagnosed using the Rotterdam criteria [21] were randomly assigned (using computer-generated random numbers) to undergo treatment with GH (PCOS-GH) or without GH (PCOS-C). Written informed consent was obtained from each participant. The study also conforms to the Declaration of Helsinki for Medical Research involving Human Subjects (2013 revision). Age-matched women (aged 25–37 years) who underwent *in vitro* fertilization and embryo transfer (IVF-ET) because of tubal infertility were recruited as non-PCOS controls. Primary GCs were collected from PCOS-C (n = 31), PCOS-GH (n = 30), and non-PCOS groups (n = 32). Exclusion criteria included hydrosalpinx, systemic lupus erythematosus, or sicca syndrome; uncontrolled endocrinopathy such as diabetes, hyperthyroidism, hypothyroidism, and hyperprolactinemia; or currently taking anti-OS medicine such as vitamin E, vitamin C, and Coenzyme Q10. Medical history-related information such as menstrual cycle regularity, duration of infertility, and treatment was collected from all the participants. Physical examinations included measurements of height, body weight, waist circumference, and hip circumference. Body weight index (BMI) was calculated as weight divided by height squared (kg/m^2). The waist-to-hip ratio (WHR) was calculated as the waist circumference divided by the hip circumference. Androgen-related symptoms of hirsutism and acne were evaluated as

previously reported [22]. Antral follicle count (AFC) were assessed by performing transvaginal ultrasound examination on days 2–3 of menstruation or progestin-induced withdrawal bleeding.

Plasma glucose, estradiol (E_2), progesterone, total testosterone (TT), luteinizing hormone (LH), follicle-stimulation hormone (FSH), sex hormone binding globulin (SHBG), and fasting insulin (FINS) levels were measured as reported previously [22]. The free androgen index (FAI) was calculated as TT (nmol/L)/SHBG (nmol/L) × 100. The homeostasis model assessment (HOMA-IR) index was calculated as fasting glucose (mmol/L) × fasting insulin (mIU/L)/22.5 [22]. The intra- and inter-assay coefficients of variation for these values were <5% and <10%, respectively.

All the patients underwent IVF-ET according to the gonadotropin-releasing hormone antagonist protocol. Recombined follicular stimulation hormone (rFSH) (Gonal-F; Merck-Serono KGaA., Darmstadt, Germany) was administered starting from day 2 of the menstrual cycle. The dose of rFSH was adjusted according to follicular growth. Other than rFSH, patients in the PCOS-GH group were subcutaneously administered with 4 IU/d of recombinant human GH (Jintropin, Changchun GeneScience Pharmaceutical Co., Ltd., Changchun, Jilin, China) until the trigger day. Cetrorelix (Cetrotide; Merck-Serono KGaA.) was administered when one of the criteria was met: serum E_2 > 300 pg/mL, leading follicle diameter reached 13–14 mm, LH > 10 IU/L. Recombinant human chorionic gonadotropin (Ovitrelle®; Merck-Serono KGaA., Darmstadt, Germany) was administered as the trigger when the diameters of at least two follicles reached ≥ 18 mm. After 36 hours, oocytes were retrieved under transvaginal ultrasound guidance.

Primary GC isolation

After oocyte retrieval, follicular fluid (FF) was collected from follicles with a diameter ≥ 16 mm measured on the retrieval day and immediately separated by centrifugation at 700 ×*g* for 5 min at room temperature. The precipitates were suspended in left 2 ml of FF and gently layered into 3 mL of 50% lymphocyte separation medium (Solarbio Science and Technology Corporation, Beijing, China). After centrifugation at 700 ×*g* for 10 min at room temperature to remove red blood cells and debris, GCs layered at the interface of the gradient were collected and washed twice with 5 mL of phosphate-buffered saline (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, Jiangsu, China). Furthermore, residual red blood cells were removed using red blood cell lysis buffer (Solarbio Science and Technology Corporation). GCs from each patient were collected separately and considered as one sample. Intracellular ROS levels, mitochondrial membrane potential (MMP) and apoptosis of a portion of GCs were examined immediately. The remaining GCs were stored at −80°C immediately for mRNA and protein detection.

Detection of intracellular ROS levels

ROS generation in GCs was estimated using 2',7'-diclorodihydrofluorescein di-acetate (H2-DCFDA) method by ROS assay kit (Beyotime Biotechnology Co., Ltd., Shanghai, China). Briefly, GCs were resuspended in PBS and incubated with 10 μM H2-DCFDA in the dark for 25 min at 37°C and then incubated together with 10 μg/mL 4',6-diamidino-2-phenylindole (DAPI) (NeoFroxx, Frankfurt, Germany) for 5 min. After the cells were washed three times with PBS, GC suspensions were added to glass slides,

and examined by fluorescence microscopy (Olympus Corporation, Tokyo, Japan). The examination wavelength was 488 nm, and the emission wavelength was 525 nm.

Similar with the above protocol but without DAPI, NanoDrop UV-Vis spectrophotometry (Thermo Scientific, MA, USA) was used to measure the intracellular ROS level in another part of GCs. The fluorescence intensities are calculated as the intensity of the PCOS group relative to that of the control group (non-PCOS group).

Apoptosis assay

Apoptosis of GCs were detected using the Annexin V-FITC apoptosis detection kits (KeyGEN Bio TECH Co., Ltd.). Briefly, 1×10^5/ Test of GCs were resuspended in 500 μL binding buffer, then labeled with Annexin Ⅴ-FITC (5 μL) and propidium iodide (PI) (5 μL) for 15 min in the dark at room temperature. After 1 hour, the green (Annexin V-FITC) and red (PI) fluorescence were examined by flow cytometry (MilliporeSigma Co., Ltd., Burlington, MA, USA). The examination wavelength was 488 nm, and the emission wavelength was 530 nm.

Detection of MMP

The MMP of GCs was examined using JC-1 Apoptosis Detection Kits (KeyGEN Bio TECH Co., Ltd.). In brief, 1×10^5/ Test of GCs were resuspended and incubated with 500 μL JC-1 reagent solution at 37˚C in the dark for 15 min. JC-1 accumulates in functional mitochondria with high ΔΨm and forms aggregates that emit red fluorescence. When mitochondrial transmembrane potential is depolarized with low ΔΨm, JC-1 releases from the mitochondria and forms monomers that emit green fluorescence. After washed two times with incubation buffer, the green and red fluorescence were examined by flow cytometry (MilliporeSigma Co., Ltd., Burlington, MA, USA). The examination wavelength was 488 nm, and the emission wavelength was 530 nm.

Reverse-transcription and quantitative real-time polymerase chain reactions (RT-qPCR)

GCs were rapidly thawed and total RNA was isolated using the RNAprep Pure Micro Kit (Tiangen Biotech Co., Ltd., Beijing, China). The quality of RNA was checked at an absorbance of 260 nm/280 nm by Nanodrop-2000 (ThermoFisher Scientific, Waltham, MA, USA). Total RNA was reverse transcribed to cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Tokyo, Japan). Polymerase chain reaction (PCR) was performed using TB Green™ Premix Ex Taq™ II (TaKaRa) on a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 65°C for 5 s. The PCR system (20 μL) comprised RNase free dH_2O (6.4 μL), cDNA (2 μL), forward primer (0.8 μL), reverse primer (0.8 μL) and 2 ×TB Green Premix Ex Taq II (10 μL). All PCR reactions were conducted in triplicate. Each experiment was repeated at least three times. Glyceraldehyde-phosphate dehydrogenase (GAPDH) was used as the internal control as indicated and fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method. Primers were designed and synthesized at Beijing Tsingke Biologocal technology (Beijing, China). Primers used in the RT-qPCR are shown in Table 1.

Table 1 Sequences of primers used in the qRT-PCR

Gene	Primer (5'→3')	Product size (bp)	Annealing temperature (°C)
FOXO1	F: TTTGCCCCAGATGCCTATAC	114	57.5
	R: GGAGAGTCAGAAGTCAGCAAC		
Bax	F: TTTCCGAGTGGCAGCTG	74	55.8
	R: CAAAGTAGAAAAGGGCGACAAC		
Bcl-2	F: GGATGCCTTTGTGGAACTGT	135	57.4
	R: CACTTGTGGCTCAGATAGGC		
caspase-9	F: TAACAGGCAAGCAGCAAAGT	139	53.4
	R: ACCAAATCCTCCAGAACCAA		
caspase-3	F: AGAACTGGACTGTGGCATTG	111	55.4
	R: TAACCAGGTGCTGTGGAGTA		
GAPDH	F: ACGGATTTGGTCGTATTGGG	214	57.4
	R: CGCTCCTGGAAGATGGTGAT		

Western blotting

Total protein was isolated from the GC samples using the RIPA lysis buffer (KeyGen Biotech. Co., Ltd.) containing Halt™ Protease Inhibitor Cocktail (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Total protein concentration was determined using a quantitative BCA protein kit (Thermo Scientific). The total proteins (60 μg/lane) were subsequently subject to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked with Tris-buffered saline with Tween-20® that contained 5% bovine serum albumin (Bio-Rad) for 1 h at room temperature and subsequently incubated with a primary antibody according to the manufacturer's instructions at 4°C overnight. Specific primary antibodies included PI3K (1:2000; ab140307, Abcam, Cambridge, MA, USA), p-PI3K (Tyr607, 1:1000; ab182651, Abcam), Akt (1:10000; ab179463, Abcam), p-Akt (Ser473, 1:2000; ab81283, Abcam), FOXO1 (1:1000; 2880, Cell Signaling, Beverly, MA, USA), p-FOXO1 (Ser 256, 1:1000; 9461, Cell Signaling), Bax (1:1000; 5023, Cell Signaling), Bcl-2 (1:500; 01556, Wanleibio, Shenyang, China), caspase 9 (1:1000; 9502, Cell Signaling), cleaved caspase-9 (Asp330, 1:1000; 7237, Cell Signaling), caspase 3 (1:1000; ab32351, Abcam), cleaved caspase-3 (Asp175, 1:1000; 9661, Cell Signaling), and GAPDH (1:2000; 2188R, Bioss, Beijing, China). On the day after washing, the membranes were incubated with secondary antibodies for 2 hours at room temperature. SuperSignal® West Pico Trial Kit (ThermoFisher Scientific) was used for signal detection and the protein bands were visualized using a

GelDoc XR densitometer (Bio-Rad). The relative intensities of each protein band were determined using the GAPDH band as an internal reference.

Concentrations of active caspase-9 and caspase-3 in GCs were measured by enzyme-linked immunosorbent assay (ELISA)

The cleaved caspase-9 and caspase-3 have bioactivity to induce apoptosis. The concentrations of active caspase-9 and caspase-3 in GC lysates were determined using human caspase-9 ELISA kit and caspase-3 ELISA kit (Elabscience Biotechnology Co., Ltd., Hubei, China), respectively, and a 450-nm Perlong DNM-9602G microplate spectrophotometer (Beijing Perlong New Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. The amount of protein loaded in each well was the same (100 μg/well) and each sample was detected in duplicate. The intra- and inter-assay coefficients of variation for these values were < 5% and < 10%, respectively. The sensitivities of the caspase-9 and caspase-3 assays were 0.99 ng/mL and 0.19 ng/mL, respectively.

Statistical analysis

All data were statistically analyzed using SPSS 17.0 software (SPSS Inc., Chicago IL, USA). Continuous variables are expressed as means ± standard deviation. The normality of data distribution was assessed using Kolmogorov–Smirnov tests. Between-group comparisons were assessed using one-way ANOVA with *post-hoc* Bonferroni tests. Categorical data were compared using Chi-squared tests. Two-tailed *P* values < 0.05 were considered statistically significant.

Results

Clinical, endocrine, and metabolic characteristics of the patients

Among the patients with PCOS, 48, 13, and 32 patients had abnormal menstrual cycles, hirsutism, and acne, respectively. Clinical, endocrine, and metabolic characteristics were not significantly different between the PCOS-GH and PCOS-C groups ($P > 0.05$). The rates of abnormal menstrual cycles, hirsutism, and acne, and WHR, AFC, LH/FSH ratio, TT, FAI, FINS, and HOMA-IR values were significantly higher, whereas SHBG was significantly lower in patients with PCOS than in the non-PCOS controls ($P < 0.05$) (Table 2). The baseline characteristics of these patients are described in Table 2.

Table 2 Clinical, endocrine, and metabolic characteristics of the study population

	Non-PCOS (n = 32)	PCOS-C (n = 31)	PCOS-GH (n = 30)
Age (yrs)	29.59 ± 3.02	28.90 ± 2.86	28.17 ± 3.52
Irregular menstrual cycle (n) [a,b]	0	25	23
Hirsutism (n) [a,b]	0	7	6
Acne (n) [a,b]	0	16	16
BMI (kg/m^2)	21.76 ± 2.50	22.12 ± 3.13	23.10 ± 2.27
WHR [a,b]	0.82 ± 0.05	0.85 ± 0.05	0.87 ± 0.06
AFC [a,b]	15.75 ± 4.52	24.03 ± 7.95	25.40 ± 6.24
LH/FSH ratio [a,b]	1.08 ± 0.56	1.55 ± 0.90	1.64 ± 1.00
E_2（pg/ml）	46.52 ± 12.25	49.00 ± 13.33	45.14 ± 14.84
P（ng/ml）	0.52 ± 0.27	0.57 ± 0.23	0.45 ± 0.21
TT（ng/ml）[a,b]	0.49 ± 0.16	0.65 ± 0.32	0.66 ± 0.25
SHBG (nmol/l) [a,b]	83.43 ± 25.24	42.64 ± 25.02	48.09 ± 22.11
FAI [a,b]	2.13 ± 0.67	7.22 ± 4.23	7.13 ± 6.64
FPG (mmol/l)	4.80 ± 0.41	5.04 ± 0.52	5.11 ± 0.63
FINS (mIU/l) [a,b]	8.11 ± 2.57	11.51 ± 6.52	11.51 ± 6.40
HOMA-IR [a,b]	1.76 ± 0.70	2.54 ± 1.37	2.65 ± 1.55

Data are presented as mean ± SD or number (percentage). Abbreviations: BMI, body mass index; WHR, waist-to-hip ratio; AFC, antral follicle count; FSH, follicle stimulating hormone; LH, luteinizing hormone; E_2, estradiol; P, progesterone; TT, total testosterone; SHBG, sex hormone binding globulin; FAI, free androgen index; FPG, Fasting plasma glucose; FINS, Fasting insulin; HOMA-IR, homeostatic model assessment of insulin resistance.

[a] $P < 0.05$, non-PCOS group versus PCOS-C group.

[b] $P < 0.05$, non-PCOS group versus PCOS-GH group.

GH inhibited ROS accumulation in GCs of patients with PCOS

The green fluorescence intensity of ROS in GCs was significantly stronger in the PCOS-C group than in the PCOS-GH and non-PCOS groups as visualized by fluorescent microscopy. Quantitative detection by

using a spectrophotometer indicated that the ROS intensity was significantly higher in the PCOS-C group than in the non-PCOS group (2.78 ± 0.35 vs. 1.00 ± 0.21) ($P < 0.05$). The ROS intensity was significantly reduced in the PCOS-GH group compared with the PCOS-C group (1.10 ± 0.21 vs. 2.78 ± 0.35) ($P < 0.05$). The fluorescence intensity of ROS is expressed as the fold change relative to the control (Figure 1).

GH improved MMP and inhibited GC apoptosis in patients with PCOS

The MMP reflected the mitochondrial function of GC. OS could induce mitochondrial dysfunction and apoptosis in GC. Figure 2 (A and B) shows that the MMP was significantly lower (0.22 ± 0.18 vs. 0.79 ± 0.21) in the PCOS-C than in the non-PCOS group ($P < 0.05$). Compared with PCOS-C group, MMP was significantly higher in the PCOS-GH group (0.22 ± 0.18 vs. 0.94 ± 0.26) ($P < 0.05$).

Figure 2 (C and D) showed that the rates of early and late apoptosis were significantly higher (28.18% vs. 11.07%, and 19.01% vs. 11.48%, respectively) in the PCOS-C group than in the non-PCOS group ($P < 0.05$). Compared with PCOS-C group, early and late apoptotic rates were significantly decreased in the PCOS-GH group (28.18% vs. 7.20% and 19.01% vs. 9.37%, respectively) ($P < 0.05$). The results suggest that GH could improve OS-induced mitochondrial dysfunction and apoptosis in GCs.

GH enhanced PI3K/Akt signaling

To study the mechanisms of GH alleviates OS and improve mitochondrial dysfunction in GCs, the important genes and proteins involved in PI3K/Akt signaling were determined by RT-qPCR and western blotting, respectively. The PI3K/Akt pathway plays a crucial role in the regulation of GC growth and apoptosis during follicular development [18-20]. When PI3K is phosphorylated and activated by a variety of growth factors, it can phosphorylate Akt protein. p-Akt phosphorylates FOXO1, and p-FOXO1 is inhibited by unclear localization and transcription activity [23]. FOXO1 is a member of the forkhead box O (FOXO) family of transcription factors. OS may upregulate FOXO1, active FOXO1 can upregulate several proapoptotic genes, such as Bax and Bcl-2, then induce apoptosis in GC [24].

Figure 3 showed that the mRNA and protein levels of FOXO1 were increased, whereas the protein levels of p-PI3K/PI3K, p-Akt/Akt and p-FOXO1 were decreased in the GCs of the PCOS-C group compared with those in the non-PCOS and PCOS-GH groups ($P < 0.05$). The results suggest that PI3K/Akt signaling was downregulated in GCs of patients with PCOS, and GH might enhance the signaling.

GH regulated apoptosis-related genes and proteins in GCs of patients with PCOS

The Bax/Bcl-2 heterodimer increases the membrane's permeability, releases cytochrome c, and initiates apoptosis[25]. Figure 4 shows the significantly increased both mRNA and protein levels of Bax, and decreased Bcl-2 in the GCs of the PCOS-C group compared with those in the non-PCOS and PCOS-GH groups ($P < 0.05$). Furthermore, GH significantly decreased both mRNA and protein levels of Bax and increased Bcl-2 in the GCs of the PCOS-GH group ($P < 0.05$).

Upon apoptotic stimulation, caspase-9 and its down signal caspase-3 is activated and cleaved, resulting in apoptosis. Figure 4 showed significantly increased caspase-9 and caspase-3 mRNA levels, and cleaved caspase-9/caspase-9 and cleaved caspase-3/caspase-3 protein levels in the GCs of the PCOS-C group compared with those in the non-PCOS and PCOS-GH groups ($P < 0.05$).

Figure 4 B showed that the protein bands of cleaved caspase-9 and cleaved caspase-3 were decreased to almost undetectable levels in the PCOS-GH and non-PCOS groups. For quantitative analysis, we measured the concentration of active caspase-9 and active caspase-3 in the cell lysate by ELISA. In Figure 4 D, the concentrations of active caspase-9 (22.39 ± 2.79 ng/mL vs. 6.99 ± 1.08 ng/mL) and active caspase-3 (15.88 ± 2.11 ng/mL vs. 5.35 ± 1.06 ng/mL) can be seen to be significantly higher in the GCs of the PCOS-C group compared with those in the non-PCOS group ($P < 0.05$). Compared with those in the PCOS-C group, the concentration of active caspase-9 (22.39 ± 2.79 ng/mL vs. 7.11 ± 1.31 ng/mL) and caspase-3 (15.88 ± 2.11 ng/mL vs. 5.90 ± 1.42 ng/mL) were significantly decreased in the PCOS-GH group ($P < 0.05$).

These results suggest that apoptosis-related genes and proteins were dysregulated in GCs of patients with PCOS. GH could upregulate the expression of anti-apoptosis genes and downregulate the expression of pro-apoptosis genes.

Discussion

In this study, we found increased ROS levels and apoptotic rates, decreased MMP, inactivated PI3K/Akt signaling pathway, and abnormal apoptosis-associated gene and protein levels in the GCs of patients with PCOS who underwent IVF. To the best of our knowledge, this is the first study to report that GH improved mitochondrial dysfunction, OS-associated apoptosis and activated the PI3K/Akt signaling pathway in GCs. We suggest that GH might alleviate OS in patients with PCOS, and that the PI3K/Akt signaling pathway may be involved in this mechanism.

During hyper-ovarian stimulation, ROS accumulates with accelerated metabolic rates for more energy and nutrients [26]. Ovarian antioxidants are numerous, and OS occurs when the natural antioxidant system cannot balance excessive ROS. In this study, the intracellular ROS level was increased by almost threefold in the GCs of patients with PCOS compared with that of non-PCOS controls. This finding is consistent with the notion that PCOS is associated with OS [4-6]. Excessive ROS leads to mitochondrial dysfunction and apoptosis [8,27]. In this study, early and late apoptotic rates increased by about two times, MMP decreased by 72% in the GCs of patients with PCOS, which is in accordance with previous reports [8,14]. GCs are steroidogenic cells surrounding the oocyte. GCs play important role in oocyte maturation, fertilization, and subsequent implantation [8]. Thus, apoptotic GCs may impair oocyte quality, and induce low rates of fertilization and pregnancy in patients undergoing IVF-ET [14,28].

The PI3K/Akt pathway plays a crucial role in the regulation of GC growth and apoptosis during follicular development [18-20]. When PI3K is phosphorylated and activated by growth factors, it can phosphorylate Akt protein[29]. p-Akt phosphorylates FOXO1 and inhibits its nuclear localization and transcription activity

[23]. FOXO1 is a member of the forkhead box O (FOXO) family of transcription factors. OS may upregulate FOXO1, active FOXO1 can upregulate several proapoptotic genes, such as Bax [24]. Homodimers of Bcl-2 associate with the mitochondrial membrane and stabilize MMP. Upon apoptotic stimulation, Bax/Bcl-2 heterodimer decrease MMP, increase membrane's permeability and release cytochrome c, then activate caspase family [25,30]. Caspase-9 is cleaved and initiates caspase cascade to induce apoptosis [31]. Pro-caspase-3 is cleaved only when apoptosis occurs.

We found that the expression of FOXO1, Bax, caspase-9 and caspase-3 were increased, whereas PI3K, Akt, Bcl-2 were decreased in GC from patients with PCOS. The results suggested that PI3K/Akt signaling and anti-apoptosis genes were downregulated, and pro-apoptosis genes were upregulated in PCOS. Which is in agreement with some reports in patients with PCOS or animal models of PCOS [16,17]. However, other studies reported that the PI3K/Akt signaling is overactivated in PCOS [32,33]. PCOS is a complex disease, and the PI3K/Akt signaling pathway is complicated and dependent on different cells and conditions, which may be the reason of the discrepancy results. In brief, under the condition of IVF, OS-related apoptosis was accompanied with inactivated PI3K/Akt signaling and dysregulated apoptosis related genes in GC from patients with PCOS. Activating PI3K/Akt signaling may alleviate GC apoptosis.

GH functions as an antioxidant in some types of cells, including oocytes, vascular endothelium, cardiomyocytes, and neural and skeletal muscle cells [9-12,34]. In this study, we found that in patients with PCOS, GH apparently decreased ROS production by > 50%, and significantly increased MMP and lowered the early and late apoptotic rates. Women with PCOS have impaired GH secretion[35,36], which is another basis for administering GH treatment. The mechanisms by which GH alleviates OS may be involved in the PI3K/Akt pathway.

GH both directly connects with the cell-surface GH receptor and indirectly improves the expression of insulin-like growth factor (IGF-1); IGF-1 then binds its cell-surface receptor [29]. Consequently, the insulin receptor substrate is activated and PI3K produces PI-3,4,5-trisphosphate (PIP3) [29]. PIP3 phosphorylates Akt and p-Akt downregulates FOXO1, inhibits Bax expression and increases Bcl-2 expression [37]. Increased levels of Bcl-2 homodimers stabilize the permeability of mitochondrial membrane and prevent the release of cytochrome c [25]. Furthermore, the caspase cascade is blocked and apoptotic rate is decreased. GH alleviates OS-induced apoptosis in some types of cells by activating the PI3K/Akt signal pathway [9-11]. We also found the same effects in the GCs of patients with PCOS. GH apparently improved the expression of PI3K, Akt and Bcl-2, whereas decreased FOXO1, Bax, caspase-9 and caspase-3. By performing ELISA, we found that GH reduced active caspase-9 level by 15.28 ng/Ml and caspase-3 level by 9.98 ng/mL. Therefore, activated PI3K/Akt signaling may be one of the mechanisms by which of GH alleviates OS-associated apoptosis in GCs.

The limitation of this study lies in its relatively small sample size. The precise mechanism through which GH alleviates OS remains obscure, and further basic investigations at the cellular level *in vitro* are needed.

Conclusion

In conclusion, this study demonstrated the presence of OS state, mitochondrial dysfunction, apoptosis and inactivated PI3K/Akt signaling in the GCs of patients with PCOS undergoing IVF. GH administered *in vivo* markedly alleviated OS related apoptosis and activated PI3K/Akt signaling.

Declarations

Ethics approval and consent to participate: All patients signed written informed consent forms for participation, and this study was approved by the Chinese Ethics Committee of Registering Clinical Trials (ChiECRCT-20180176). The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Competing interests: The authors declare that they have no conflict of interest.

Funding: This study was funded by the Key Research and Development project of Science and Technology Bureau of Sichuan (2019YF S0406), the Scientific Research Project of Sichuan Provincial Health Commission (20PJ123), the Technology Innovation Project of Science and Technology Bureau of Chengdu (2018-YF05-00247-SN) and the Scientific Research Project of Sichuan Medical Association (S17060).

Authors' contributions: Yan Gong designed the study and wrote the manuscript. Shan Luo and Huili Zhu participated in sample collection and data analysis. Ping Fan contributed to laboratory instruction and revision of the article. Yujing Li contributed to sample collection. Wei Huang contributed to design and revise the article. All authors read and approved the final manuscript.

Acknowledgements: We thank our colleagues at the Reproductive Medicine Centre for assistance with sample collection. We are grateful to Dr. Hao Tan, Kun Zhang and other colleagues for help with the experimental protocol. We very much appreciate all the patients who participated in this study.

References

1. Li R, Zhang Q, Yang D, Li S, Lu S, Wu X, Wei Z, Song X, Wang X, Fu S, et al. Prevalence of polycystic ovary syndrome in women in China: a large community-based study. Hum Reprod. 2013; 28:2562-2569.
2. Murri M, Luque-Ramirez M, Insenser M, Ojeda-Ojeda M, Escobar-Morreale HF. Circulating markers of oxidative stress and polycystic ovary syndrome (PCOS): a systematic review and meta-analysis. Hum Reprod Update. 2013; 19:268-288.

3. Hyatt HW, Zhang Y, Hood WR, Kavazis AN. Changes in metabolism, mitochondrial function, and oxidative stress between female rats under nonreproductive and 3 reproductive conditions. Reprod Sci. 2019; 26:114-127.
4. Hyderali BN, Mala K. Oxidative stress and cardiovascular complications in polycystic ovarian syndrome. Eur J Obstet Gynecol Reprod Biol. 2015; 191:15-22.
5. Fan P, Liu H, Wang Y, Zhang F, Bai H. Apolipoprotein E-containing HDL-associated platelet-activating factor acetylhydrolase activities and malondialdehyde concentrations in patients with PCOS. Reprod Biomed Online. 2012; 24:197-205.
6. Avila J, Gonzalez-Fernandez R, Rotoli D, Hernandez J, Palumbo A. Oxidative stress in granulosa-lutein cells from in vitro fertilization patients. Reprod Sci. 2016; 23:1656-1661.
7. Lai FN, Liu JC, Li L, Ma JY, Liu XL, Liu YP, Zhang XF, Chen H, De Felici M, Dyce PW, et al. Di (2-ethylhexyl) phthalate impairs steroidogenesis in ovarian follicular cells of prepuberal mice. Arch Toxicol. 2017; 91:1279-1292.
8. Lai Q, Xiang W, Li Q, Zhang H, Li Y, Zhu G, Xiong C, Jin L. Oxidative stress in granulosa cells contributes to poor oocyte quality and IVF-ET outcomes in women with polycystic ovary syndrome. Front Med. 2018; 12:518-524.
9. Chung JY, Kim HJ, Kim M. The protective effect of growth hormone on Cu/Zn superoxide dismutase-mutant motor neurons. BMC Neurosci. 2015; 16:1.
10. Caicedo D, Diaz O, Devesa P, Devesa J. Growth Hormone (GH) and Cardiovascular System. Int J Mol Sci. 2018; 19.
11. Granata R, Trovato L, Gallo MP, Destefanis S, Settanni F, Scarlatti F, Brero A, Ramella R, Volante M, Isgaard J, et al. Growth hormone-releasing hormone promotes survival of cardiac myocytes in vitro and protects against ischaemia-reperfusion injury in rat heart. Cardiovasc Res. 2009; 83:303-312.
12. Perrini S, Laviola L, Carreira MC, Cignarelli A, Natalicchio A, Giorgino F: The GH/IGF1 axis and signaling pathways in the muscle and bone: mechanisms underlying age-related skeletal muscle wasting and osteoporosis. J Endocrinol 2010, 205:201-210.
13. Kolibianakis EM, Venetis CA, Diedrich K, Tarlatzis BC, Griesinger G. Addition of growth hormone to gonadotrophins in ovarian stimulation of poor responders treated by in-vitro fertilization: a systematic review and meta-analysis. Hum Reprod Update. 2009; 15:613-622.
14. Qiao J, Feng HL. Extra- and intra-ovarian factors in polycystic ovary syndrome: impact on oocyte maturation and embryo developmental competence. Hum Reprod Update. 2011; 17:17-33.
15. Wood JR, Nelson VL, Ho C, Jansen E, Wang CY, Urbanek M, McAllister JM, Mosselman S, Strauss JF, 3rd. The molecular phenotype of polycystic ovary syndrome (PCOS) theca cells and new candidate PCOS genes defined by microarray analysis. J Biol Chem. 2003; 278:26380-26390.
16. Zheng W, Nagaraju G, Liu Z, Liu K. Functional roles of the phosphatidylinositol 3-kinases (PI3Ks) signaling in the mammalian ovary. Mol Cell Endocrinol. 2012; 356:24-30.

17. Li T, Mo H, Chen W, Li L, Xiao Y, Zhang J, Li X, Lu Y. Role of the PI3K-Akt Signaling Pathway in the Pathogenesis of Polycystic Ovary Syndrome. Reprod Sci. 2017; 24:646-655.
18. Hu CL, Cowan RG, Harman RM, Quirk SM. Cell cycle progression and activation of Akt kinase are required for insulin-like growth factor I-mediated suppression of apoptosis in granulosa cells. Mol Endocrinol. 2004; 18:326-338.
19. Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov. 2006; 5:493-506.
20. John GB, Shidler MJ, Besmer P, Castrillon DH: Kit signaling via PI3K promotes ovarian follicle maturation but is dispensable for primordial follicle activation. Dev Biol. 2009; 331:292-299.
21. Rotterdam EA-SPcwg: Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod. 2004; 19:41-47.
22. Li Y, Tan J, Wang Q, Duan C, Hu Y, Huang W. Comparing the individual effects of metformin and rosiglitazone and their combination in obese women with polycystic ovary syndrome: a randomized controlled trial. Fertil Steril. 2020; 113:197-204.
23. Zhang X, Tang N, Hadden TJ, Rishi AK. Akt, FoxO and regulation of apoptosis. Biochim Biophys Acta. 2011; 1813:1978-1986.
24. Shen M, Lin F, Zhang J, Tang Y, Chen WK, Liu H. Involvement of the up-regulated FoxO1 expression in follicular granulosa cell apoptosis induced by oxidative stress. J Biol Chem. 2012; 287:25727-25740.
25. Vogel MW. Cell death, Bcl-2, Bax, and the cerebellum. Cerebellum. 2002; 1:277-287.
26. Bianchi L, Gagliardi A, Landi C, Focarelli R, De Leo V, Luddi A, Bini L, Piomboni P. Protein pathways working in human follicular fluid: the future for tailored IVF? Expert Rev Mol Med. 2016; 18:e9.
27. Dimmeler S, Haendeler J, Sause A, Zeiher AM. Nitric oxide inhibits APO-1/Fas-mediated cell death. Cell Growth Differ. 1998; 9:415-422.
28. Karuputhula NB, Chattopadhyay R, Chakravarty B, Chaudhury K. Oxidative status in granulosa cells of infertile women undergoing IVF. Syst Biol Reprod Med. 2013, 59:91-98.
29. Ipsa E, Cruzat VF, Kagize JN, Yovich JL, Keane KN: Growth Hormone and Insulin-Like Growth Factor Action in Reproductive Tissues. Front Endocrinol (Lausanne). 2019; 10:777.
30. Ding Y, Jiang Z, Xia B, Zhang L, Zhang C, Leng J. Mitochondria-targeted antioxidant therapy for an animal model of PCOS-IR. Int J Mol Med. 2019; 43:316-324.
31. Zhu L, Yuan H, Guo C, Lu Y, Deng S, Yang Y, Wei Q, Wen L, He Z. Zearalenone induces apoptosis and necrosis in porcine granulosa cells via a caspase-3- and caspase-9-dependent mitochondrial signaling pathway. J Cell Physiol. 2012; 227:1814-1820.
32. Nekoonam S, Naji M, Nashtaei MS, Mortezaee K, Koruji M, Safdarian L, Amidi F. Expression of AKT1 along with AKT2 in granulosa-lutein cells of hyperandrogenic PCOS patients. Arch Gynecol Obstet. 2017; 295:1041-1050.
33. Villavicencio A, Goyeneche A, Telleria C, Bacallao K, Gabler F, Fuentes A, Vega M. Involvement of Akt, Ras and cell cycle regulators in the potential development of endometrial hyperplasia in women with

polycystic ovarian syndrome. Gynecol Oncol. 2009; 115:102-107.

34. Weall BM, Al-Samerria S, Conceicao J, Yovich JL, Almahbobi G. A direct action for GH in improvement of oocyte quality in poor-responder patients. Reproduction. 2015; 149:147-154.
35. Lanzone A, Villa P, Fulghesu AM, Pavone V, Caruso A, Mancuso S. The growth hormone response to growth hormone-releasing hormone is blunted in polycystic ovary syndrome: relationship with obesity and hyperinsulinaemia. Hum Reprod. 1995; 10:1653-1657.
36. Piaditis GP, Kounadi TG, Rangou DB, Trovas GP, Kaklas NA, Tzonou AJ, Chlouverakis CS. Dysfunction of the growth hormone/insulin-like growth factor-I axis in women with polycystic ovarian syndrome. Clin Endocrinol (Oxf). 1995; 42:635-640.
37. Sai T, Goto Y, Yoshioka R, Maeda A, Matsuda F, Sugimoto M, Wongpanit K, Jin HZ, Li JY, Manabe N. Bid and Bax are involved in granulosa cell apoptosis during follicular atresia in porcine ovaries. J Reprod Dev. 2011; 57:421-427.

Figures

Figure 1

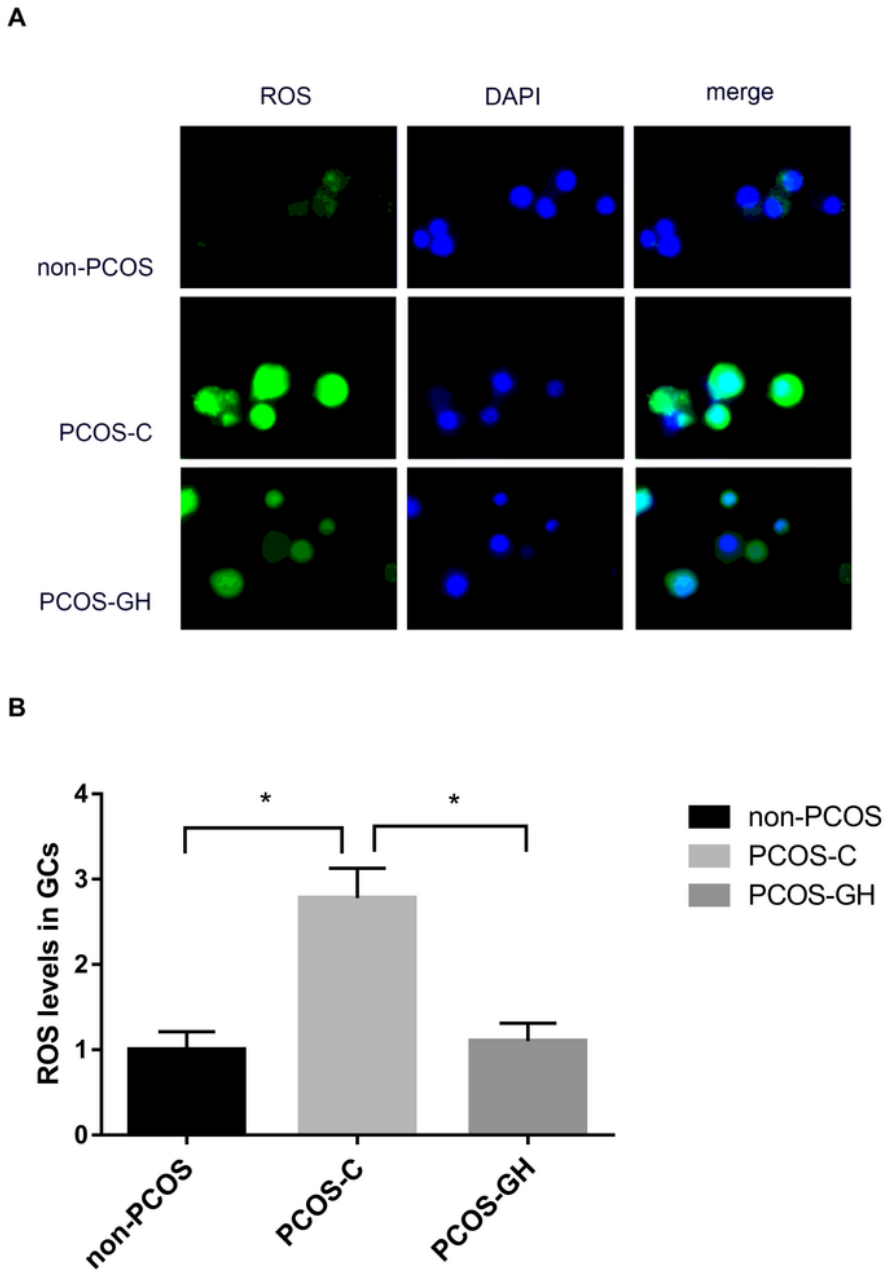

Figure 1

GH inhibits ROS accumulation in GCs of patients with PCOS. A The green fluorescence intensity of ROS in GCs was significantly stronger in the PCOS-C group than in the PCOS-GH and non-PCOS groups, as visualized by fluorescent microscopy. The blue fluorescence signal indicates cell nucleus stained by DAPI. B Quantitative detection by using a spectrophotometer. The fluorescence intensity of ROS is expressed as the fold change relative to the control. ROS intensity was significantly higher in the PCOS-C

group than in the non-PCOS group (2.78 ± 0.35 vs. 1.00 ± 0.21) (P < 0.05). ROS intensity was significantly reduced in the PCOS-GH group compared with that in the PCOS-C group (1.28 ± 0.23 vs. 2.78 ± 0.60) (P < 0.05). *P < 0.05 compared with the PCOS-C group.

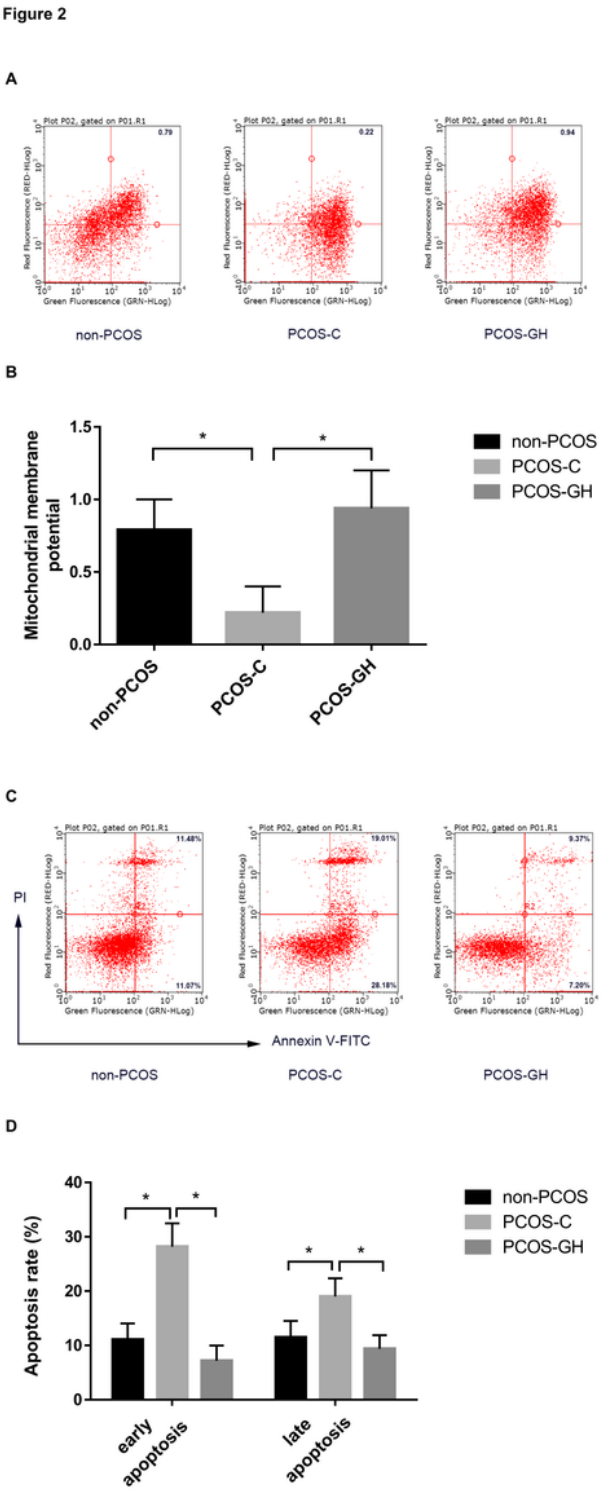

Figure 2

GH improves MMP and inhibits GC apoptosis in patients with PCOS. A Flow cytometric dot plots showed that the ratios of red/green fluorescence are decreased in the PCOS-C group compared with those in the

non-PCOS control and PCOS-GH groups. The ratios of red/green fluorescence were calculated to characterize MMP. B The MMP was significantly lower (0.22 ± 0.18 vs. 0.79 ± 0.21) in the PCOS-C than in the non-PCOS group ($P < 0.05$). Compared with that in the PCOS-C group, MMP was significantly higher in the PCOS-GH group (0.22 ± 0.18 vs. 0.94 ± 0.26) ($P < 0.05$). *$P < 0.05$ compared with the PCOS-C group. C Flow cytometric dot plots showed that the numbers of early and late apoptotic cells are increased in the PCOS-C group compared with those in the non-PCOS control and PCOS-GH groups. PI and FITC are the abbreviations of propidium iodide and fluorescein isothiocyanate, respectively. D The early and late apoptotic rates were significantly higher (28.18% vs. 11.07%, and 19.01% vs. 11.48%, respectively) in the PCOS-C group than in the non-PCOS group ($P < 0.05$). Compared with those in the PCOS-C group, early and late apoptotic rates (28.18% vs. 7.20% and 19.01% vs. 9.37%, respectively) were significantly lower in the PCOS-GH group ($P < 0.05$). *$P < 0.05$ compared with the PCOS-C group.

Figure 3

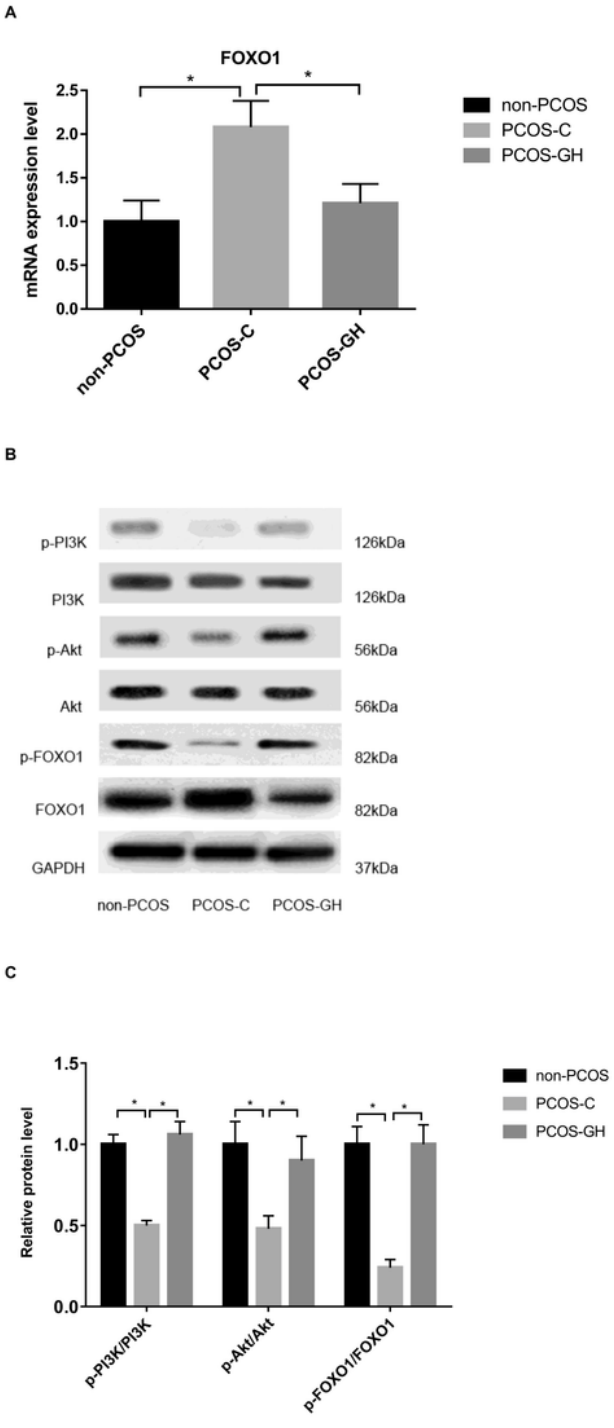

Figure 3

GH enhanced PI3K/Akt signaling in GCs from patients with PCOS. A The mRNA expression of FOXO1 was increased in the GCs of the PCOS-C group compared with the non-PCOS and PCOS-GH groups ($P < 0.05$). B B showed the protein bands of p-PI3K, PI3K, p-Akt, Akt and p-FOXO1 and FOXO1 by western blot. GAPDH was used as a protein-loading control. C The protein level of p-PI3K/PI3K, p-Akt/Akt and p-FOXO1

were decreased, whereas FOXO1 was increased in the GCs of the PCOS-C group compared with the non-PCOS and PCOS-GH groups (P < 0.05). *P < 0.05 compared with the PCOS-C group.

Figure 4

GH regulated apoptosis-related genes and proteins in GCs from patients with PCOS. A: The mRNA expression of Bax, caspase-9, and caspase-3 were increased, whereas that of Bcl-2 was decreased in the GCs of the PCOS-C group compared with the non-PCOS and PCOS-GH groups (P < 0.05). B B showed the protein bands of Bcl-2, Bax, caspase-9, cleaved caspase-9, caspase-3 and cleaved caspase-3 by western blot. GAPDH was used as a protein-loading control. C The protein level of Bcl-2 was decreased, whereas those of Bax, cleaved caspase-9/caspase-9, and cleaved caspase-3/caspase-3 were increased in the GCs of the PCOS-C group compared with those in the non-PCOS and PCOS-GH groups (P < 0.05). D Concentrations of active caspase-9 (22.39 ± 2.79 ng/mL vs. 6.99 ± 1.08 ng/mL) and active caspase-3 (15.88 ± 2.11 ng/ml vs. 5.35 ± 1.06 ng/mL) were significantly higher in the GCs of the PCOS-C group compared with those in the non-PCOS group (P < 0.05). Compared those in the PCOS-C group, the concentration of active caspase-9 (22.39 ± 2.79 ng/mL vs. 7.11 ± 1.31 ng/mL) and active caspase-3 (15.88 ± 2.11 ng/mL vs. 5.90 ± 1.42 ng/mL) were significantly decreased in the PCOS-GH group (P < 0.05). *P < 0.05 compared with the PCOS-C group.