

Effects of Nesfatin-1 and Wnt / β -Catenin Pathway on OGCs in PCOS

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Research

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Abstract

Background Polycystic ovary syndrome is a complex disease related to the endocrine and metabolism. Its specific cause and pathogenesis have not been clear. Nesfatin-1 could not only regulate energy balance and glucose metabolism, but also affect the reproductive system. The Wnt/ β -catenin signaling pathway affects follicle development, ovulation, corpus luteum formation, and steroid hormone production.

Results Here, we studied the roles of nesfatin-1 and Wnt/ β -catenin signaling pathway in the pathogenesis of polycystic ovary syndrome. Firstly, the human primary ovarian granulosa cells in vitro was cultured. The results showed that the apoptosis rate of ovarian granulosa cells in polycystic ovary syndrome patients was significantly higher than that of granular cells in normal people. Moreover, nesfatin-1 and Wnt/ β -catenin pathway inhibitor IWR-1 could inhibit the expressions of ovarian granulosa cells apoptosis genes and promote their proliferation, as well as nesfatin-1 affected the expressions of foxo3a and its downstream factors. Then, an in vitro culture system for ovarian granulosa cells (OGCs) was established by employing a rat model. The results are the same with those mentioned above.

Conclusion This strongly proves that the nesfatin-1 participates in regulating the apoptosis and proliferation of granulosa cells by the Wnt/ β -catenin pathway. According to the role of nesfatin-1 and IWR in polycystic ovary syndrome, nesfatin-1 and Wnt/ β -catenin pathway can provide a guideline for the diagnosis and treatment of Polycystic ovary syndrome (PCOS).

Introduction

PCOS is a complex and heterogeneous endocrine disorder syndrome. It can cause endocrine and metabolic disorders, obesity, etc. According to the statistics, the women suffering from non-ovulatory infertility in childbearing age accounts for 75%^[1]. The researches on PCOS are much more, but until now, the specific etiology and pathogenesis are still not clear. Hence, there is a lack of targeted and precise treatment.

As a potent anorexic peptide, nesfatin-1 is involved in the regulation of energy homeostasis as well as is expressed in the central nervous system and peripheral tissues. Simultaneously, it can play pleiotropic role in some systems such as cardiovascular system, digestive system, endocrine system, stress response, sleep and reproduction of the organism.

At present, there are four kinds of Wnt pathways in cells, among them, the canonical Wnt/ β -catenin pathway is closely related to reproduction^[2]. Furthermore, the interruption of Wnt/ β -catenin signal level would be harmful for the normal sexual development. Currently, the Wnt/ β -catenin pathway is an important way to regulate ovarian steroid production, and moreover, it may be one of the pathways of gonadotropin signal transduction.

Afterward, it is thought that foxo3a is the signal control point of the Wnt/ β -catenin pathway. The Wnt/ β -catenin pathway chain signaling can promote OGCs apoptosis and inhibit its proliferation by activating

foxo3a and its downstream effectors.

In literature^[3], the results showed that the inhibitor IWR-1 of Wnt/ β -catenin pathway

can make follicles develop better and increase the steroid production. Additionally, it is illustrated in some researches that nesfatin-1 and foxo3a are all expressed in ovarian follicle intimal cells, granulosa cells, and so on. The reproductive system, especially, reproductive endocrine, has significantly physiological changes in various stages of development and interacts with other system. Nesfatin-1 is closely related to endocrine. Based on the influence of Wnt/ β -catenin pathway on the reproductive system, it is suggested that nesfatin-1 may be related to the pathway.

In this paper, we investigate the relationship between nesfatin-1 and Wnt/ β -catenin on OGCs. Our findings would provide new ideas for female infertility and some relevant diseases.

Materials And Methods

Human OGCs in vitro experiment

The OGCs were from the Embryo Laboratory of the Reproductive Medicine Center of Binzhou Medical College Affiliated Hospital. The subjects of this study were randomly selected from the patients who received in vitro fertilization-embryo transfer (IVF-ET) at the Reproductive Center of the Affiliated Hospital of Binzhou Medical College. Moreover, the OGCs are from patients with PCOS. The control group consisted of OGCs from 30 infertile patients with simple fallopian tube factors and normal ovarian reserve.

Materials

The OGCs were obtained on the day of oocyte retrieval in the Embryo Laboratory of the Reproductive Center of the Affiliated Hospital of Binzhou Medical College. Simultaneously, the red blood cell lysis method was used to obtain high-purity OGCs. Then, the OGCs were cultured for 3 days to establish an in vitro culture system of human primary OGCs. On the 3rd day, nesfatin-1 and IWR-1 with different concentrations were added. After 24 hours, the granulosa cells were collected and observed. The apoptosis of each group was determined by flow cytometry, as well as the mRNA level was analyzed by qRT-PCR expression of Foxo3a-1, downstream target gene P27, apoptosis-related genes Bax, Caspase-3, Bim, and Bcl-2. In addition, the effect of CCK-8 on the proliferation of OGCs was observed. This study was approved by the Medical Ethics Committee of Binzhou Medical College Hospital and all patients.

OGCs culture in vitro

The granule cell mixture was centrifuged after oocyte retrieval at 1600r/min for 6 minutes. Then, the supernatant was removed. The red blood cell lysate was added with about three times the volume. They are mixed well, and thus reacting in the dark for 6 minutes. Next, in order to further purify OGCs, they were centrifuged again, and the supernatant was discarded. The remaining part were seeded in a 24-well plate,

and OGCs were cultured in DMEM/F12 medium at 37 °C in a humidified atmosphere with 5% CO₂ and 95% air. The medium contains 10% FBS (FBS00717-1, Aus Gene X), 100 U/ml penicillin and 100 µg/ml streptomycin. Furthermore, the cell culture medium should be changed every 24 hours. After the OGCs were cultured in vitro for 48 hours, the serum-free culture media, containing 0, 1*10⁻⁷M, 1*10⁻⁹M and 1*10⁻¹¹M nesfatin-1, respectively, were added to the experimental group and they were cultivated for 24 hours.

Apoptosis detection by flow cytometry

After drug treatment, the OGCs apoptosis was measured by an Annexin V-FITC / PI apoptosis detection kit. Apoptotic cells, which stained positive for Annexin V-FITC, negative for PI, or double-positive, were counted. The data were represented as a percentage of the total cell count.

Granulosa cells viability measurements by cck-8

The viability of OGCs was measured by Cell Counting Kit-8 (CCK-8). Briefly, the OGCs were treated for 24 h by IWR-1 and nesfatin-1 with the with various concentrations of 1*10⁻⁷M, 1*10⁻⁹M, and 1*10⁻¹¹M respectively. Then, the OGCs viability was examined by CCK-8 assay, simultaneously, the absorbance was read at 450 nm.

Real-Time Quantitative Reverse-Transcriptase Polymerase Chain

MiniBEST Universal RNA Extraction Kit (TaKaRa, Kusatsu, Japan) was used to extract total RNA, and RevertAid First Strand cDNA Synthesis Kit (TaKaRa) was used to perform reverse transcription into complementary deoxyribonucleic acid according to the manufacturer's instructions. QRT-PCR was conducted on CFX96™ Real-time PCR detection system C1000 using specific primers (Biotech, Shanghai, China). The gene expression of each set of samples was determined in triplicate, and the results were analyzed with the 2^{-ΔΔt} formula.

Table 1
qRT-PCR primers in patients

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	ACAAC TTTGGTATCGTGG AAGG	GCCATCACGCCACAGTTTC
Foxo3a	TCACGCACCAATTCTAACGC	CACGGCTTGCTTACTGAAGG
Bcl-2	GGTGGGGTCATGTGTGTGG	CGGTTCAGGTA CT CAGTCATCC
Bax	CCCGAGAGGTCTTTTTCCGAG	CCAGCCCATGATGGTTCTGAT
Bim	TAAGTTCTGAGTGTGACCGAGA	GCTCTGTCTGTAGGGAGGTAGG
P27	CGGCTCATGGGCGACTATC	TGTCTTGGAGGAGGATCGTCC
Caspase-3	CATGGAAGCGAATCAATGGACT	CTGTACCAGACCGAGATGTCA

Materials of rat

Firstly, we purchased thirty six-week-old SD female rats from Jinan Pengyue CO, Ltd. And theirs' weight was between 140g~160 g. Then, they were randomly divided into two groups, and each group with 15 rats. One was the PCOS experimental group, and the other one was the control group. All rats were weighted with an electronic scale every three days at 8 am, and they can have food and water freely.

Specimen collection

For the rats in the PCOS experimental group, letrozole (1 mg/kg) with 1% carboxymethyl cellulose (CMC, 2 ml/kg) was given every day by gavage for twenty-one days. At the same time, the rats in the control group should be given 1% CMC with the same volume every day^[4]. The rats in each group start fasting 20:00 on the 21st day. Then the rats were injected intraperitoneally with Pregnant Mare Serum Gonadotropin (PMSG, 10 IU). After 48h^[5], all rats were sacrificed by cervical dislocation. The heart blood was collected for 4~5 ml at once, and then placed in the refrigerator at 4°C overnight. Next, one side of the ovary was taken away and washed in cold PBS. The other side of the ovary was fixed with paraformaldehyde solution, embedded in paraffin and sectioned.

To measure the estrous cycle of rats

The vaginal discharge of rats on the swab was smeared on a glass slide, and then dried naturally at room temperature. It is essential to add a Ruishi-Jimosa solution A to the glass slide with one minute's standing under the room temperature. Then, the Ruishi-Jimosa solution B was added dropwise on top of the solution A. Next, they were fully mixed, as well as stained for 7 minutes at room temperature. Finally, the glass slide was rinsed slowly with tap water. Subsequently, the cell morphological changes of vaginal smears were observed with a microscope so as to determine the different stages of the estrous cycle^[6].

Determination of serum sex hormones in rat

For the heart blood, the serum sex hormone was determined with the upper serum after centrifugation. The follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E₂), progesterone (P), testosterone (T), nesfatin-1 were measured by the Enzyme-linked immunosorbent assay kit (ELISA kit), offered by Shandong Lingjin Biological Technology Co. LTD (Jinan, China)^[7].

Observation of ovarian morphology

The paraffin section of ovarian tissue was taken for hematoxylin-eosin (HE) staining. At last, the ovarian morphology and structure of slices were observed under the optical microscope.

Nesfatin-1 and Foxo3a immunohistochemistry

The prepared paraffin sections of ovarian tissue were permeabilized with 1% Triton X-100 in phosphate buffered saline (PBS) for 30 minutes at room temperature, boiled in 100 mm sodium citrate (pH 6.0) three times for 6 minutes each at 5 minutes intervals for antigen retrieval, and then incubated with 3% hydrogen peroxide for 30 minutes to remove endogenous peroxidase followed by blocking in 5% bovine

serum albumin at room temperature for 1 hour. The sections were then incubated overnight at 4 °C with the diluted primary antibody of nesfatin-1 (bs-10068R) (1:300) or foxo3a (10849-1-AP) (1:400) in the blocking solution. Following three washes with 0.1% Tween-20 in PBS, the samples were incubated with goat anti-rabbit IgG (PV-9001) in the blocking solution at room temperature for 45 minutes. The stained sections were evaluated under a light microscope.

Isolation and Identification of OGCs

Firstly, the separated ovarian tissue in PBS was transferred to Dulbecco's modified Eagle's medium (DMEM). Then, a syringe needle was used to puncture the mature follicles after development. The released oocytes and OGCs were collected. As well as 1 g/L hyaluronidase was added to digest the OGCs and oocytes for separation. After being filtered and centrifuged, and at last, a pre-placed cell slide 24-well cell plate at 2×10^5 /ml was inoculated.

The OGCs were cultured on cover slips. After the specimen was fixed and blocked, the cells were incubated with anti-FSH receptor antibody (bs-0895R) (1:120) at 4 °C overnight. Then, the cells were incubated with goat anti-rabbit IgG for 30 minutes at 37 °C and stained with DAB for 5 minutes at room temperature. The OGCs were washed with PBS for three times for 5 minutes, and finally, they were observed under light microscopy^[6].

Real-time fluorescence polymerase chain reaction

After drug intervention, all the RNA in each group was extracted by an RNAiso Plus (Takara, Beijing, China), strictly following the manufacturer's instructions, and then reverse-transcribed into complementary DNA. A high capacity complementary DNA (cDNA) reverse transcription kit (Takara, Beijing, China) was used to convert RNA into cDNA. Moreover, the SYBR® Premix Ex Taq™ (Tli RNaseH Plus) was used to perform the PCR reactions. According to the gene sequence in the Genebank, we designed the following primers through Primer 5.0 (Qingdao MedSci Biological Technology Co., Ltd.)

Table 2
RT-qPCR primers in rat

Gene	Forward(5'-3')	Reverse(5'-3')
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA
Foxo3a	TGCTAAGCAGGCCTCATCTCAA	AGATGGCGTGGGAGTCACAA
Bcl-2	GACTGAGTACCTGAACCGGCATC	CTGAGCAGCGTCTTCAGAGACA
Bax	TGGCGATGAACTGGACAACAA	GGGAGTCTGTATCCACATCAGCA
Bim	TGTTGACATCCCTATCCCTGACAC	GCAGCAGAATCCAAGCACTGAA
P27	CGAATGCTGGCACTGTGGA	CATTCAATGGAGTCAGCGATATGTA

The OGCs of rat viability were measured by cck-8, and the apoptosis of rat was detected by flow cytometry.

Statistical analysis

Data were analyzed by Graphpad Prism5 software. The experimental data are presented as the mean \pm standard deviation. The unpaired Student's t-test was used to analyze the comparison between the two groups. One-way ANOVA followed by multiple comparison tests was used for comparison among multiple groups. Here, P value is less than 0.05, with a statistical significance.

Results

Comparison of the model between the PCOS and control group

The changes in estrous cycle of rats

The normal SD rats have an estrous cycle for about 4 ~ 5 days. The vaginal smear at the early stage of proestrus is mainly composed of swollen oval flat nuclear epithelial cells, as well as mainly composed of keratinized epithelial cells at the estrous phase. Under the microscope, they look like many fallen leaves stacked together. Furthermore, white blood cells and nuclear epithelial cells are the main components at metestrus, occasionally, with keratinocytes. At the diestrus phase, the vaginal smear is almost full of the white blood cells.

In our study, it is found that the control group has a complete estrous cycle (Fig. 1a). In the PCOS group, the estrous cycle disorder successively appears on the 10th day after the letrozole intragastric administration, almost during the whole diestrus (Fig. 1b).

The body weights of rats

There was no significant difference in body weight before the induction of PCOS ($P > 0.05$) (Fig. 2a). The body weight of rats in PCOS group is (157.68 ± 10.04)g, and the body weight of rats in control group is (159.17 ± 9.18)g. After the induction of PCOS in the rats, the body weight of the rats in the PCOS group remarkably increases (Fig. 2b), about (220.41 ± 46.21)g, while the body weight of rats in the control group is (204.67 ± 28.55)g. Hence, there exists a significant difference in the body weight of rats between PCOS and control groups. Then, this difference is statistically significant ($P < 0.01$).

The hormonal values of serum sex hormone

Results of ELISA assays indicate that the serum T and LH levels in rats are significantly higher in the PCOS group than those in the control group (all $p < 0.05$) (Fig. 3). However, the serum P levels in rats are significantly higher in the PCOS group than those in the control group (all $p < 0.05$), and the serum E_2 and FSH were no significant difference and between the two groups ($p > 0.05$, Table 3).

The serum nesfatin-1 levels in rats are significantly lower in the PCOS group than those in the control group ($p < 0.05$, Table 3).

Table 3
Difference of serum sex hormone levels in rats between the PCOS group and the control group ($\bar{x} \pm s$)

Indicators	PCOS	Control	P
E ₂ (pmol/l)	41.89 ± 1.19	44.48 ± 0.75	0.0756
P(ng/mL)	6.45 ± 0.15	5.61 ± 0.13	0.0002
FSH(IU/L)	9.82 ± 0.30	9.77 ± 0.13	0.8925
LH(mIU/mL)	30.64 ± 1.30	29.43 ± 1.31	0.5183
T(pg/mL)	176.2 ± 7.11	144.4 ± 7.026	0.0039
Nesfatin-1(pg/mL)	3524 ± 199.3	4085 ± 133.1	0.0266

Significance was determined by unpaired Student's t-test. Asterisks directly above bars and lines represent statistical significance (* $P < 0.05$, ** $P < 0.01$).

Observation of ovarian morphology

The ovarian morphology is observed by HE staining. Compared with the control group (Fig. 4a), there appear multiple follicles with cystic expansion in the PCOS group. They are vacuolated and disorganized in structure with occasional corpus luteum and increasing atresia (Fig. 4b). The number of granulosa cells decreases, and the granulosa cells even disappear. Simultaneously, some oocytes and radiating crowns appear.

The locations of nesfatin-1 and foxo3a in rats

Immunohistochemical detection results reveal that nesfatin-1 is expressed in the corpus luteum, follicular inner membrane cells, granulosa cells, and ovarian stroma of rat ovarian tissue. Foxo3a is expressed in the nucleus of oocytes. Furthermore, at the primordial and primary follicle stages, foxo3a appears in both the nucleus and cytoplasm of oocytes. While at the stages of secondary and mature follicles, foxo3a only appears in the cytoplasm of oocytes.

The identification of OGCs

The OGCs of human are round and partially aggregated under the microscope. After 24 h, the cells adhered to the well and grow mostly fused (Fig. 6a). After 48 h, the cells rapidly divided and proliferate (Fig. 6b). The cells basically covered the bottom of the well after 96 h. Most OGCs follow this growth pattern, whether in a rat or a human. However, the OGCs of rats are smaller than those of humans.

The rat cells are stained with hematoxylin and eosin, and the cell morphology is observed under a microscope. The cells are well-formed and uniform in size, as well as the nucleus is located at the center of the cell, with an oval or irregular shape (Fig. 6c). To determine rat cell purity, immunohistochemical analysis of FSHR is performed. The results show that FSHR-positive staining is located in the cytoplasm of the cells (Fig. 6d).

The viability of OGCs

In order to determine whether nesfatin-1 and IWR-1 can protect the rat OGCs, cell absorbance is investigated by CCK-8 assays. As shown in Fig. 7, the OGCs are treated with IWR-1 and different concentrations of nesfatin-1, that is, 1×10^{-7} M, 1×10^{-9} M, and 1×10^{-11} M, respectively, and IWR-1 for 24 h. Both in rats and humans, after intervention for 24 h, the nesfatin-1 and IWR-1 significantly promoted OGCs viability, compared with that of the control and PCOS groups ($P < 0.01$).

After adding different concentrations of nesfatin-1, there exist significant differences in the viability of rats in each intervention group, compared with that of rats in the PCOS group. Therefore, this is statistically significant ($P < 0.01$). However, the proliferation trend significantly increases ($P < 0.05$) when IWR-1 is used to intervene humans. Anyway, the cell survival rate of rats in the normal group is higher than that in the PCOS group. The nesfatin-1 and IWR-1 significantly promote the viability of OGCs.

The apoptosis of rat OGCs

The apoptosis rate was analyzed by flow cytometry, and the aim is to determine whether the decrease in viability of OGCs is caused by apoptosis. As can be seen in Fig. 8, for rats, the apoptosis rate of the control group is 1.67% (Fig. 8a), while the apoptosis rate of the PCOS group was 9.61% (Fig. 8b). In addition, the apoptosis rate of the PCOS + Nesfatin-1 (1×10^{-9}) group decreases to 1.05% (Fig. 8c). However, there appears a decline (2.36%) in the apoptosis rate of the IWR-1 group (Fig. 8d). The apoptosis of the group treated with nesfatin-1 and IWR-1 significantly decreases.

In patients, the apoptotic rate of the PCOS group is significantly higher than that of the control group ($P < 0.01$), and thus the difference is statistically significant (Fig. 10). The apoptotic rate of OGCs in the group of PCOS + nesfatin-1 is significantly lower than that of the non-intervention group ($P < 0.01$), so the difference has a significant statistical significance. Compared with the non-intervention group, the number of OGCs apoptosis in the PCOS + IWR-1 significantly decreases ($P < 0.01$), and then the difference is statistically significant. Therefore, the nesfatin-1 and IWR-1 can significantly restrain OGCs apoptosis.

The apoptosis rate of each group is detected by Flow Cytometry. The OGCs are stained with Annexin V / PI. Annexin V⁻ / PI⁻ in the lower left quadrant, representing normal OGCs; Annexin V⁺ / PI⁻ in the lower right quadrant, representing early apoptotic OGCs; Annexin V⁺ / PI⁺ in the upper right quadrant, representing late apoptotic OGCs; The upper left quadrant shows Annexin V⁻ / PI⁺, representing dead OGCs.

The relative protein expression in RT-PCR

The expression of apoptosis-related genes in different groups of primary granulosa cells is detected by qRT-PCR technology. The results are shown in Fig. 9, and they are analyzed as follows:

After adding different concentrations of nesfatin-1 to the experimental group, the expressions of pro-apoptotic protein Bax mRNA, and Bim mRNA all decreased ($P < 0.05$), while the expression of anti-apoptotic protein Bcl-2 Mrna increases ($P < 0.05$). As well as the anti-apoptotic protein significantly increases in patients ($P < 0.01$). Hence, the difference is also statistically significant.

The difference in protein is more obvious in patients than that in rat after treated with IWR-1. Furthermore, foxo3a is the signal control point of the Wnt/ β -catenin pathway, and P27 is the downstream target gene of foxo3a. Thus, after Wnt/ β -catenin pathway antagonist IWR-1 and nesfatin-1 are added to the experimental group of human, the expressions of foxo3a mRNA and P27 mRNA are significantly lower than those of the experimental group. And the caspase-3 is lower in the control group.

While for rat, the expression of foxo3a is higher in the PCOS + IWR-1 group than that in PCOS group, while is lower after adding nesfatin-1 to the PCOS. And the P27 is higher in the PCOS group. Thus, it is suggested that nesfatin-1 could activate foxo3a and downstream effectors by the Wnt/ β -catenin pathway to affect OGCs apoptosis. And corresponding to each apoptosis-related protein, nesfatin-1 has its optimal regulatory concentration.

Discussion

At present, most of the patients with infertility caused by PCOS can only rely on IVF – ET for a baby. The infertility is still caused by the diseases related to the ovary. If the pregnancy with ovary can be improved from the origin, a new treatment method for infertility may be discovered. This would, make it possible for patients with poor ovarian reserve, premature ovarian failure, and other reproductive diseases to conceive again.

Nesfatin-1 was firstly proposed by Oh-I and others of Gunma University in Japan. Namely, it is a precursor neuropeptide derived from nuclear histone2 (NUCB2). The gene is encoded as nesfatin-1. The enzyme is cleaved into three different small molecule fragments, that is nesfatin-1, nesfatin-2, and nesfatin-3^[7]. Among them, nesfatin-1 is expressed in the central nervous system^[7], digestive system^[8], adipose tissue^[9], cardiovascular system^[10], etc. When it comes to the expression of nesfatin-1 in the reproductive system, mainly located in mouse OGCs, follicular membrane cells, endometrial epithelial cells and glands^[11], as well as testicular stromal cells^[12]. Nesfatin-1 is closely related to female reproduction in regulating the proliferation and apoptosis of OGCs. Ciccimarra et al, have pointed out that the expression of the precursor protein NUCB2 gene in OGCs is higher than that in granulosa cells of medium, small

follicles, nesfatin-1 stimulates cell proliferation, and corpus luteum by modifying the NO production of large granulosa cells. The production of ketone interferes with the redox reaction^[13].

In this experiment, we first successfully establish OGCs in vitro culture system both in rats and humans. Then, the apoptosis of OGCs in normal and experimental groups are observed and compared with each other. The experimental group is intervened with nesfatin-1. Compared with the non-intervention group, the nesfatin-1 intervention group has a decrease in the rate of granulosa cell apoptosis. The expressions of pro-apoptotic genes Bax mRNA, Caspase-3 mRNA, and Bim mRNA significantly decreased, while the expressions of anti-apoptotic genes Bcl-2 mRNA significantly increase after adding drugs to the PCOS. Simultaneously, the expressions of foxo3a mRNA and P27 mRNA obviously decrease, so the difference was statistically significant. This strongly proves that nesfatin-1 can both affect the apoptosis of granulosa cells and proliferation.

Wnt signaling pathway plays an important role in tumor development, gene expression, cell apoptosis, and other life processes. The important regulatory role is also closely related to the development of follicles^[14]. The classic Wnt/ β -catenin signaling pathway can regulate the target gene in the nucleus. Two receptors bind to Wnt. One of which is a member of the seven-pass transmembrane frizzled protein family, and the gene encodes a secreted glycoprotein interaction with the cysteine-rich extracellular domain on the Fz7 transmembrane receptor, and thus affecting the reproductive function^[15]. The other one is LRP5/6 in the low-density lipoprotein receptor-related protein family^[16].

As studying the effect of the Wnt/ β -catenin pathway on granulosa cell apoptosis, Wnt/ β -catenin pathway inhibitors were used to observe the state of OGCs. IWR-1 is taken as an inhibitor of the Wnt/ β -catenin signaling pathway. Likewise, it affects the regulation of cell proliferation, differentiation, cell cycle, and apoptosis. Foxo3a is regulated by Wnt/ β -catenin signal. Liu et al^[17] have found that foxo3a activation can prevent follicle development to the next stage, indicating that foxo3a may play a decisive role in inhibiting follicle development. Moreover, foxo3a may play an important role in activating primordial follicles. In our study, we detect the expressions of two important foxo3a downstream target genes, including cyclin-dependent kinase inhibitor P27 and apoptosis regulator Bim to prove how foxo3a affects OGCs apoptosis. The Wnt/ β -catenin pathway inhibitor IWR-1 is added to the experimental group, and the apoptosis rate is significantly lower than that of the non-intervened group. Additionally, the expressions of pro-apoptotic genes Bax mRNA, Caspase-3 mRNA, and Bim mRNA significantly decreased, while the expressions of anti-apoptotic genes BCL-2 mRNA was significantly increase in patients. As well as, the expressions of foxo3a mRNA and P27 mRNA decreased. Thus, the difference is statistically significant, and the proliferation of OGCs also significantly increases.

The results indicate that nesfatin-1 can activate Foxo3a and its downstream effectors through Wnt/ β -catenin signaling pathway to affect OGCs, and nesfatin-1 can inhibit the Wnt/ β -catenin pathway to prevent OGCs from initiating apoptosis.

Conclusion

As can be seen from the analysis of the above results, both nesfatin-1 and Wnt/ β -catenin pathway antagonist IWR-1 may can inhibit the apoptosis of OGCs and affect the proliferation. Nesfatin-1 can also affect the expressions of foxo3a and its downstream factors, which is able to prove that nesfatin-1 activates foxo3a and its downstream effectors through the Wnt/ β -catenin signaling pathway to promote OGCs apoptosis and inhibit its proliferation. Therefore, much more researches are needed to explore reproduction-related channels. And IWR-1 may cooperate with a variety of inhibitors to regulate the Wnt/ β -catenin pathway. This also provides useful information for IWR-1 with a role in early follicular development. However, there are still several problems to be solved, such as the specific role of nesfatin-1 in the Wnt/ β -catenin pathway, how to activated β -catenin to regulates the activation of foxo3a, and the complex regulatory mechanisms involved in various signaling pathways.

Abbreviations

PCOS: Polycystic ovary syndrome; OGCs: Ovarian granulosa cells; IVF-ET: In vitro fertilization-embryo transfer; CMC: carboxymethyl cellulose; PMSG : Pregnant Mare Serum Gonadotropin; FSH: Follicle stimulating hormone; LH: luteinizing hormone; E2: estradiol; P: progesterone; T: testosterone; ELISA: Enzyme-linked immunosorbent assay; HE: forhematoxylin-eosin; DMEM: Dulbecco's modified Eagle's medium; cDNA: complementary DNA.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

DPH, ADD and LKX are the co-first authors, conceptualized, designed and carried out the study. DPH drafted the initial manuscript, and reviewed and revised the manuscript. HY, ZY, and LQC designed the data collection instruments, collected data, carried out the initial analyses, and reviewed and revised the manuscript. WYL coordinated and supervised data collection, and critically reviewed the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Availability of data and materials

We declared that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

Ethics approval and consent to participate

Not applicable.

Consent for publication

The consent for publication was obtained from every individual whose data were included in the manuscript.

Competing interests

All authors have significantly contributed to the manuscript, and declare that the work is original and has not been submitted or published elsewhere. None of the authors have any financial disclosure or conflict of interest.

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Figures

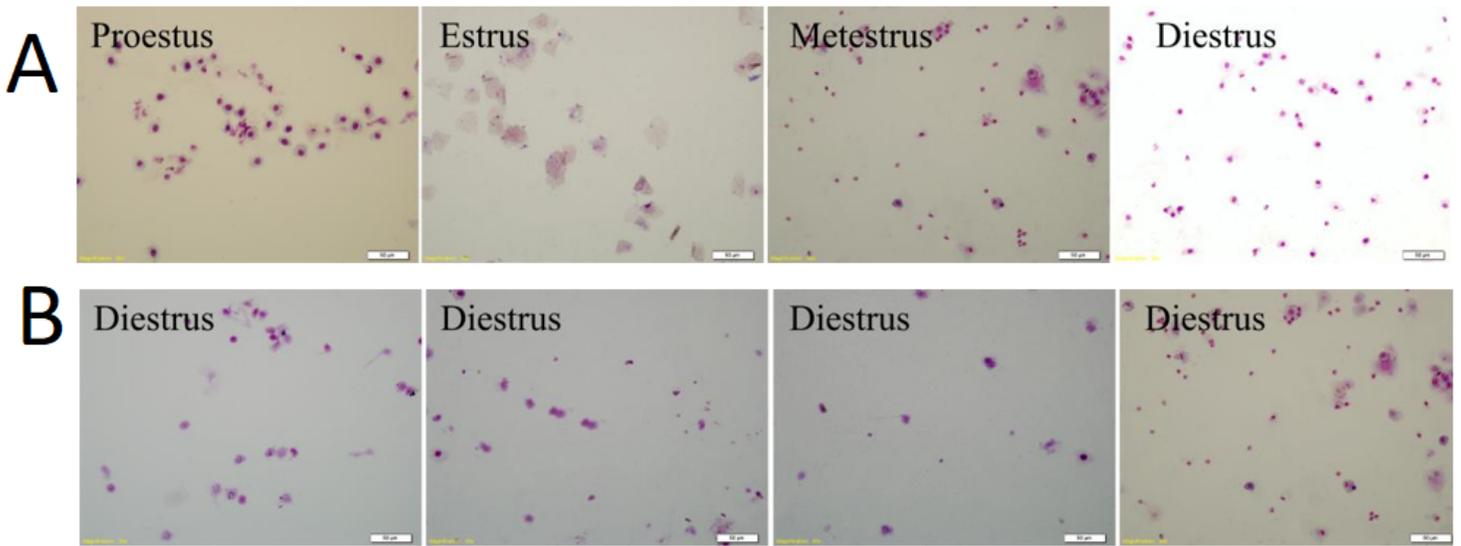


Figure 1

a The estrous cycle of the Control rats. Scale bar = 50 μm. Magnification×200. b The estrous cycle of the PCOS rats. Scale bar = 50 μm. Magnification×200.

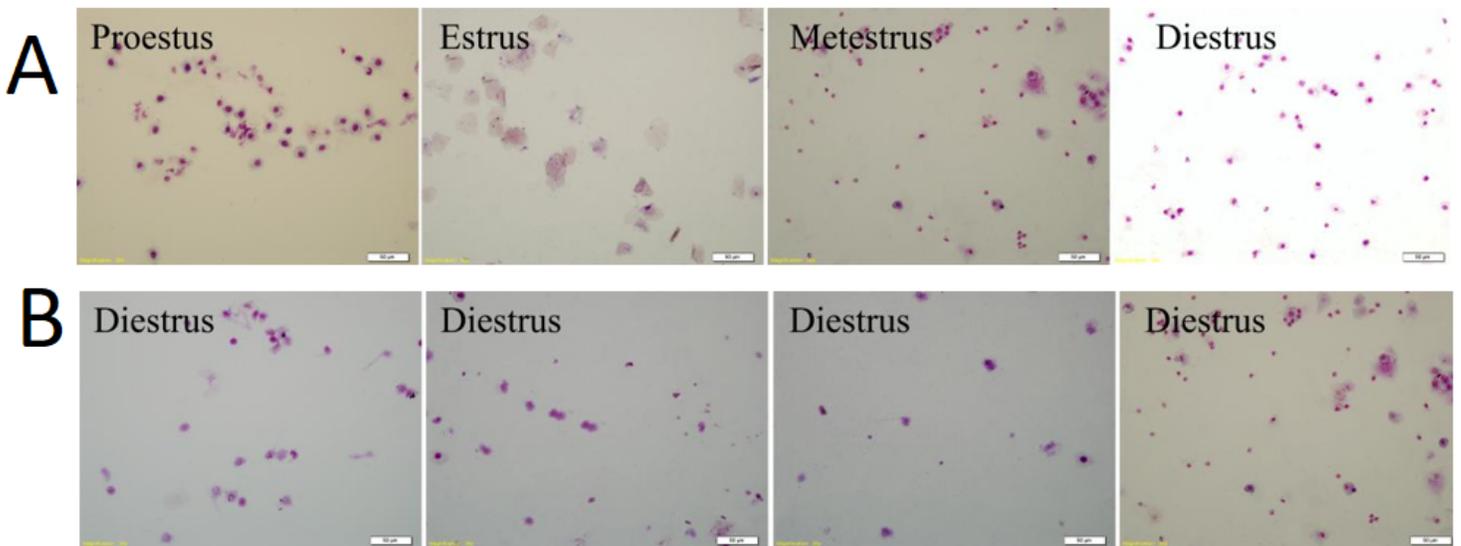


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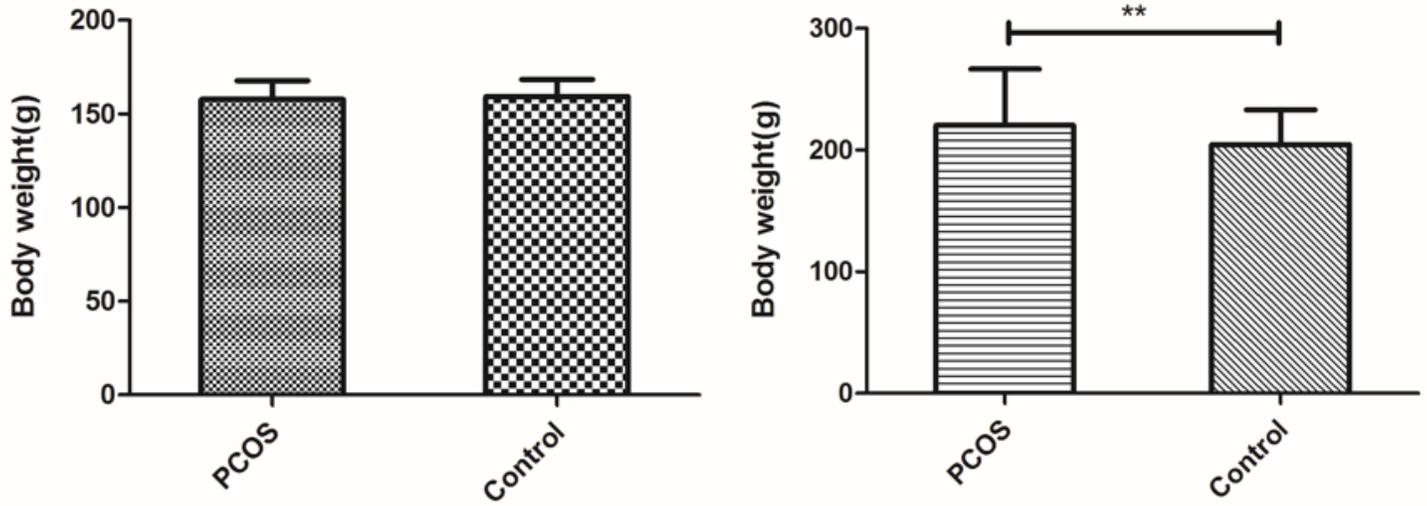


Figure 2

Significance was determined by unpaired Student's t-test. Asterisks directly above bars and lines represent statistical significance (* P < 0.05, ** P < 0.01).

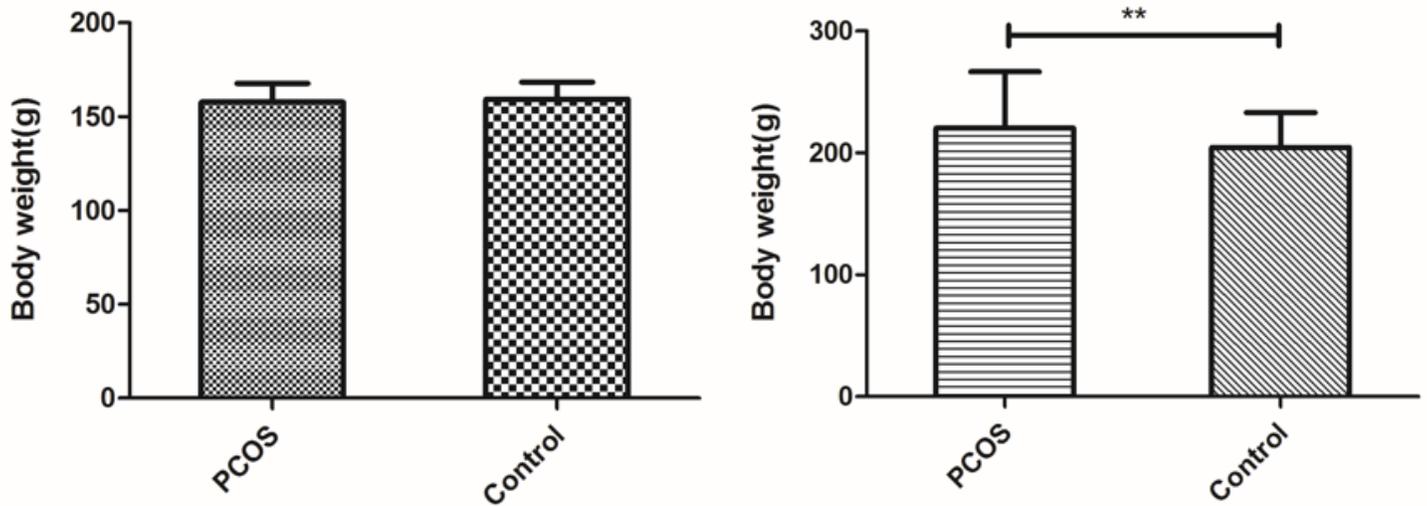


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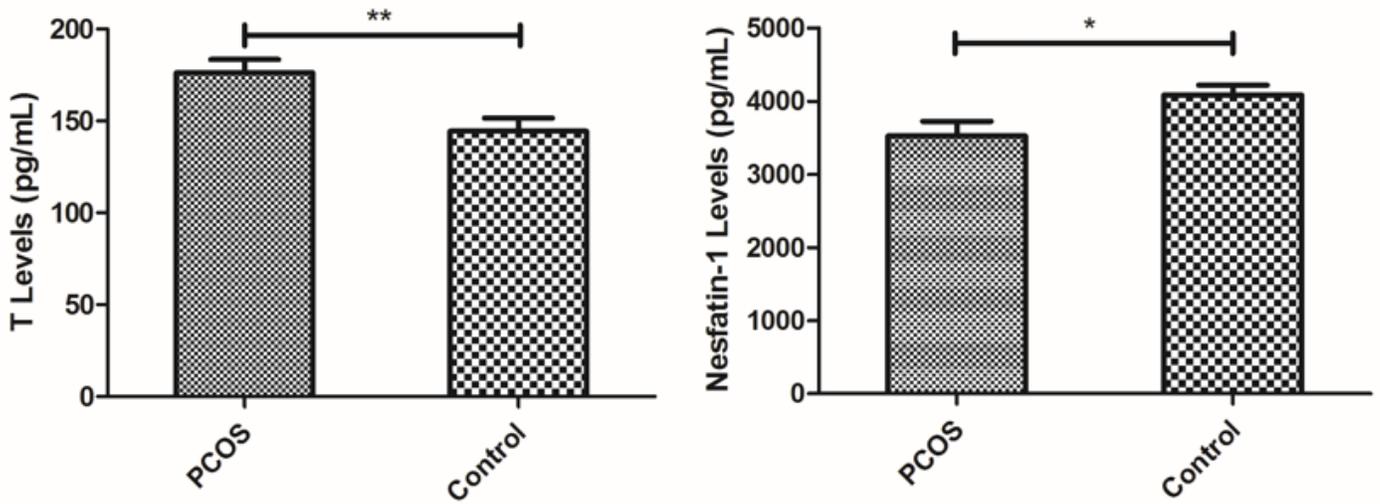


Figure 3

The serum T and LH levels

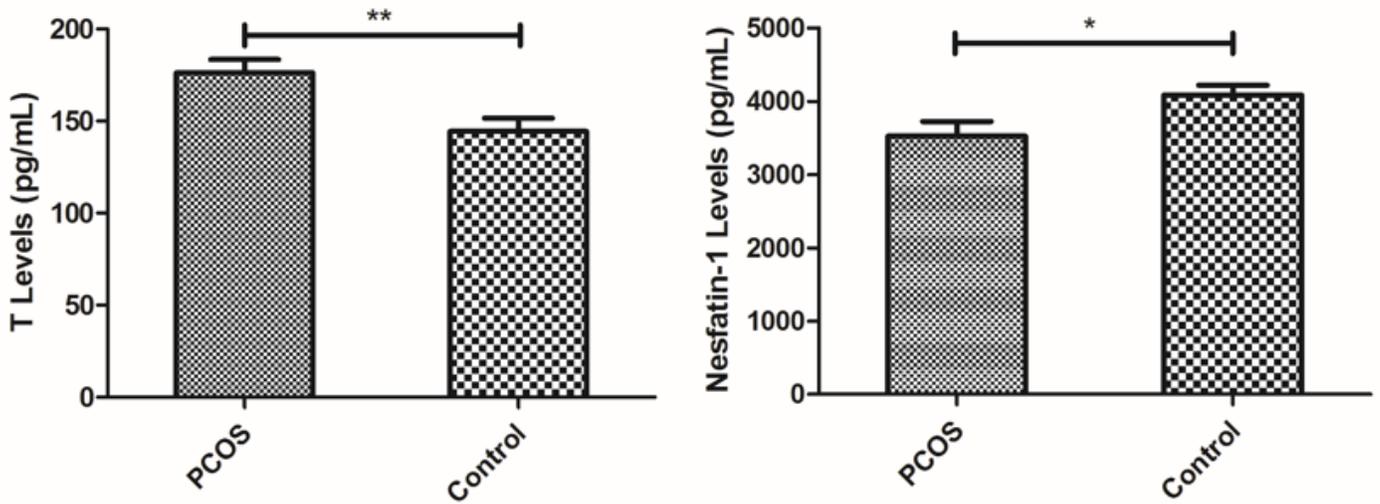


Figure 3

The serum T and LH levels

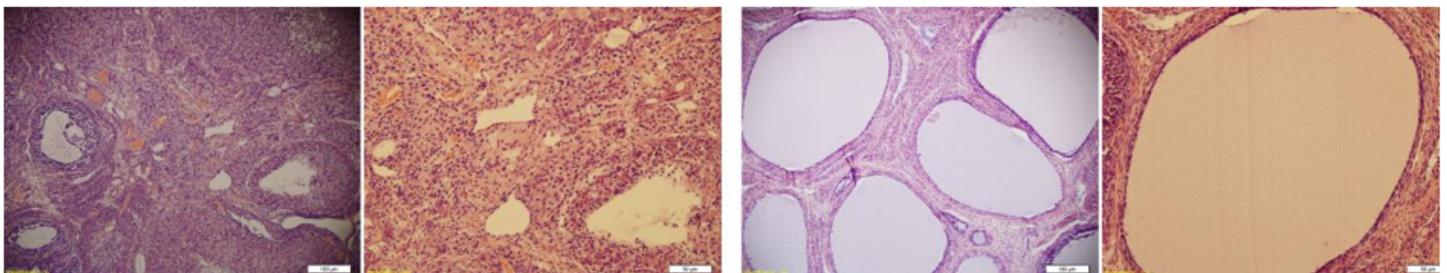


Figure 4

Control group Fig4bPCOSgroup Scale bar = 100 um. Magnification×200.

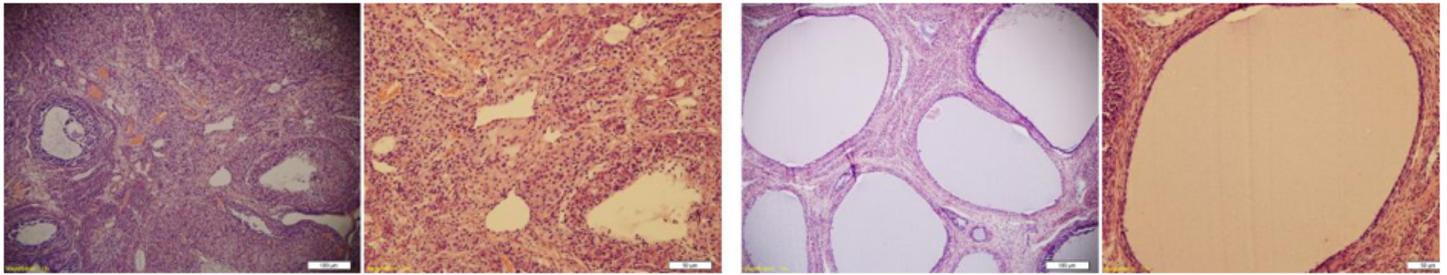


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Control group Fig4bPCOSgroup Scale bar = 100 um. Magnification×200.

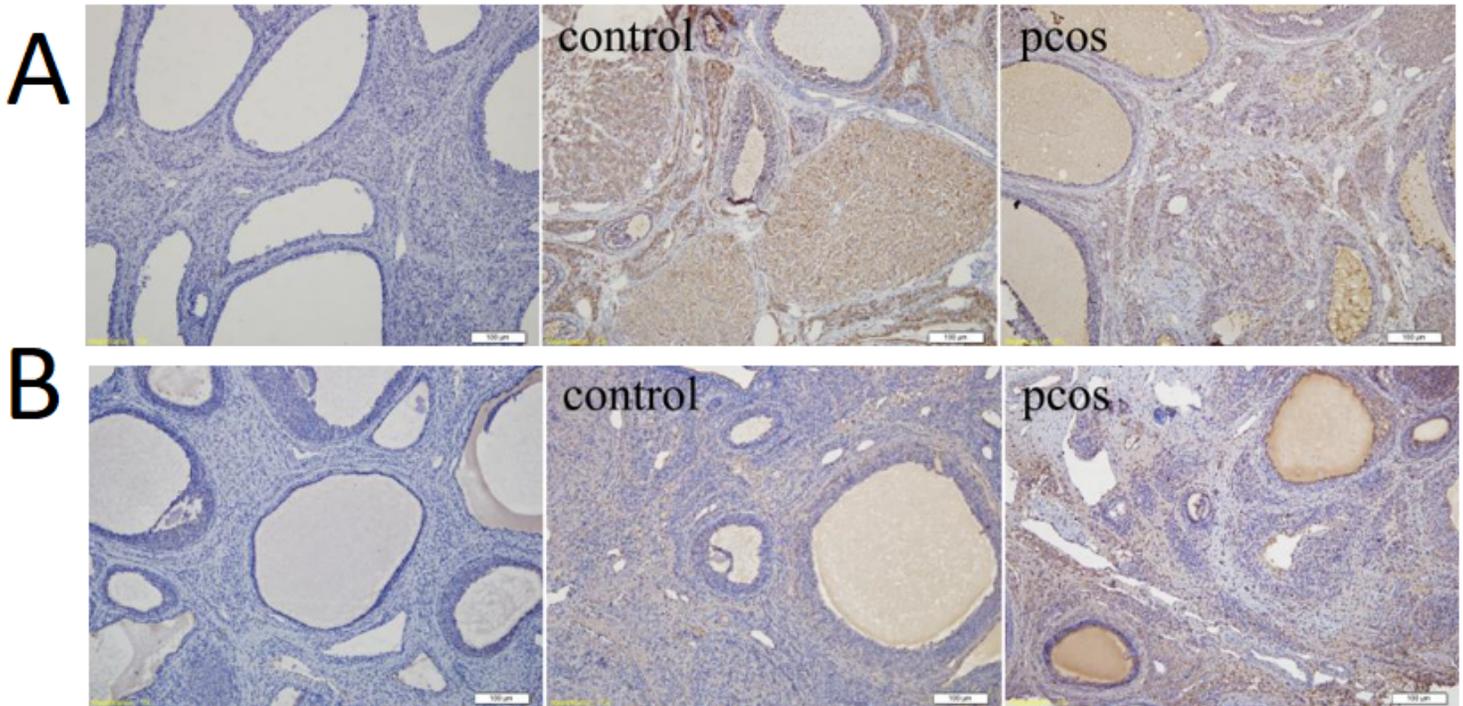


Figure 5

a The location of nesfatin-1. Scale bar = 100 um. Magnification×100. b The location of Foxo3a. Scale bar = 100 um. Magnification×100.

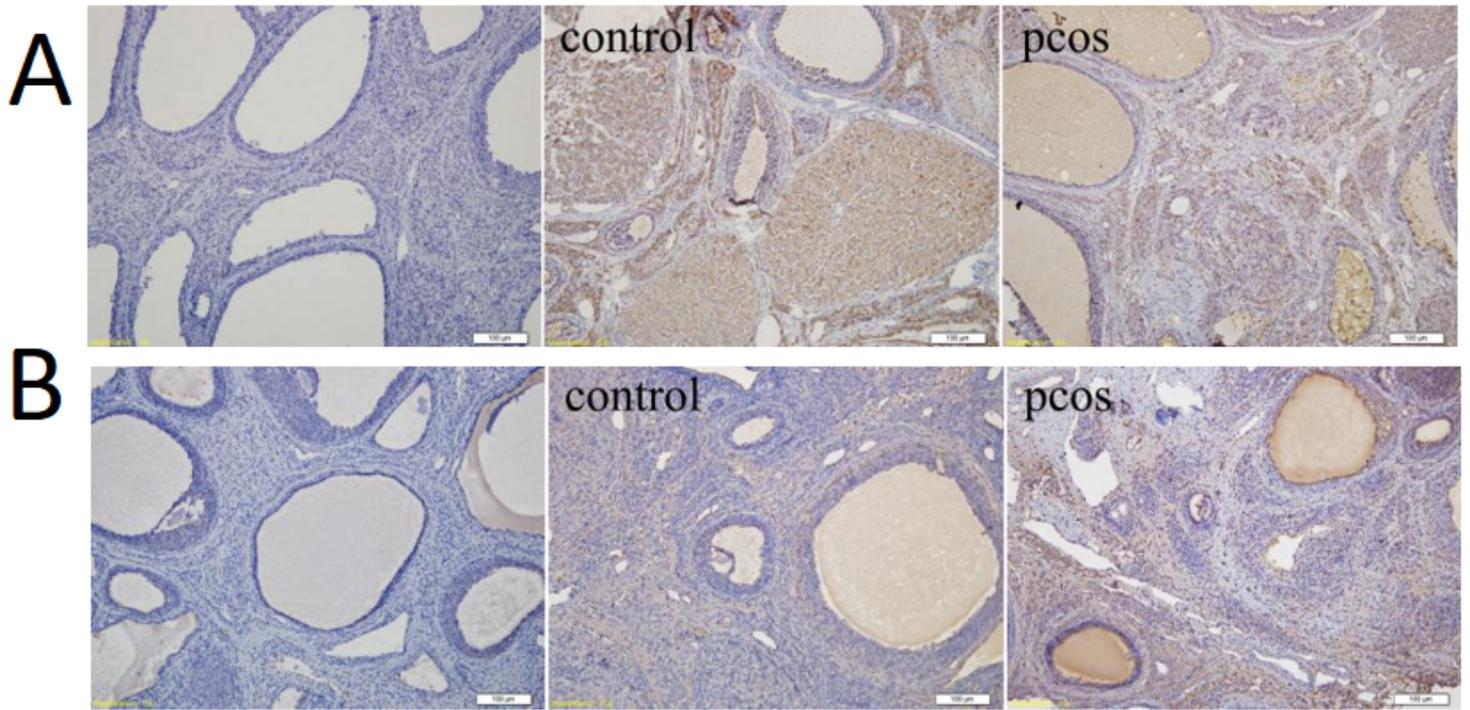


Figure 5

a The location of nesfatin-1. Scale bar = 100 um. Magnification×100. b The location of Foxo3a. Scale bar = 100 um. Magnification×100.

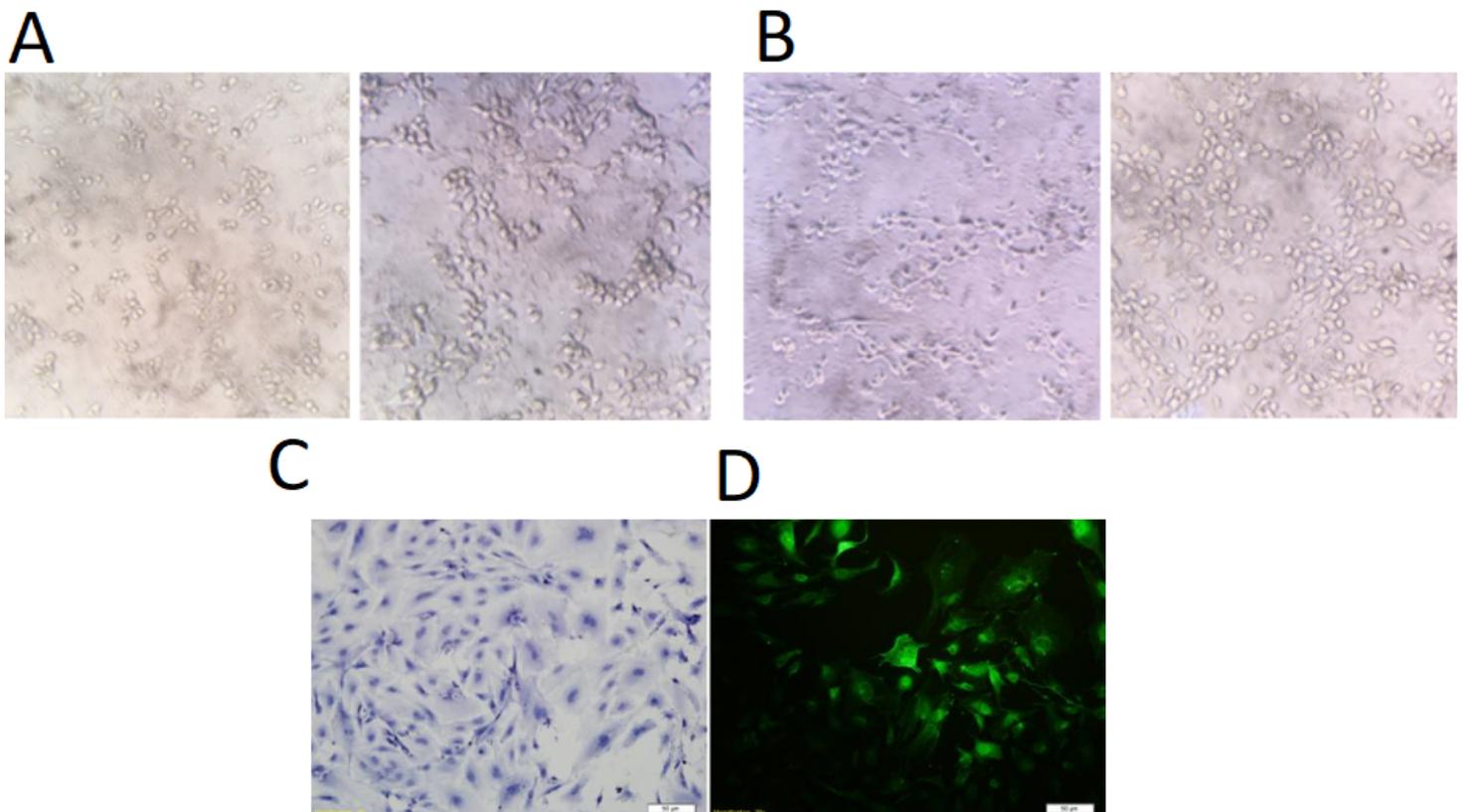


Figure 6

The OGCs in 24h The OGCs in 48h HE staining Immunofluorescence staining Scale bar = 50 μ m.
Magnification \times 200.

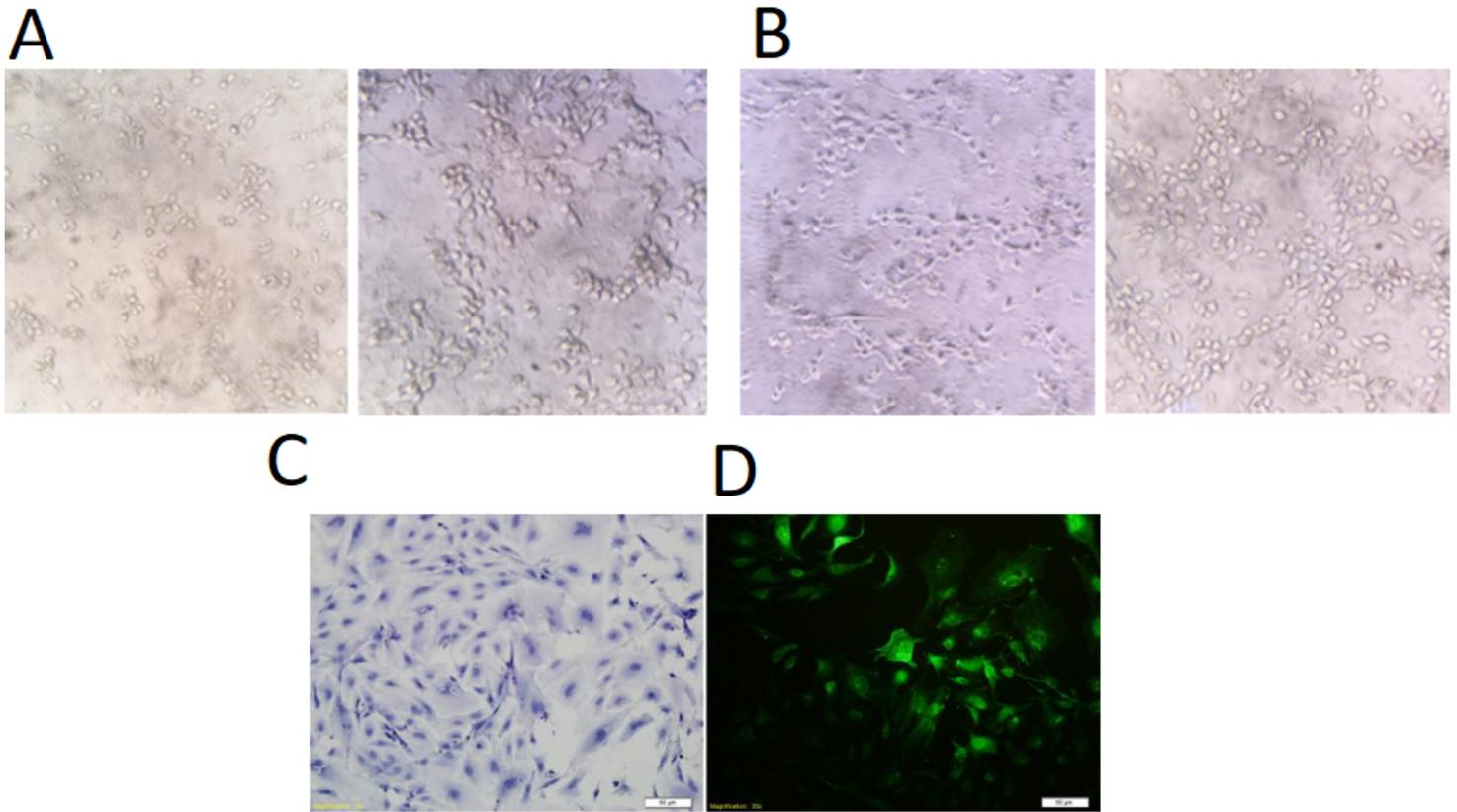


Figure 6

The OGCs in 24h The OGCs in 48h HE staining Immunofluorescence staining Scale bar = 50 μ m.
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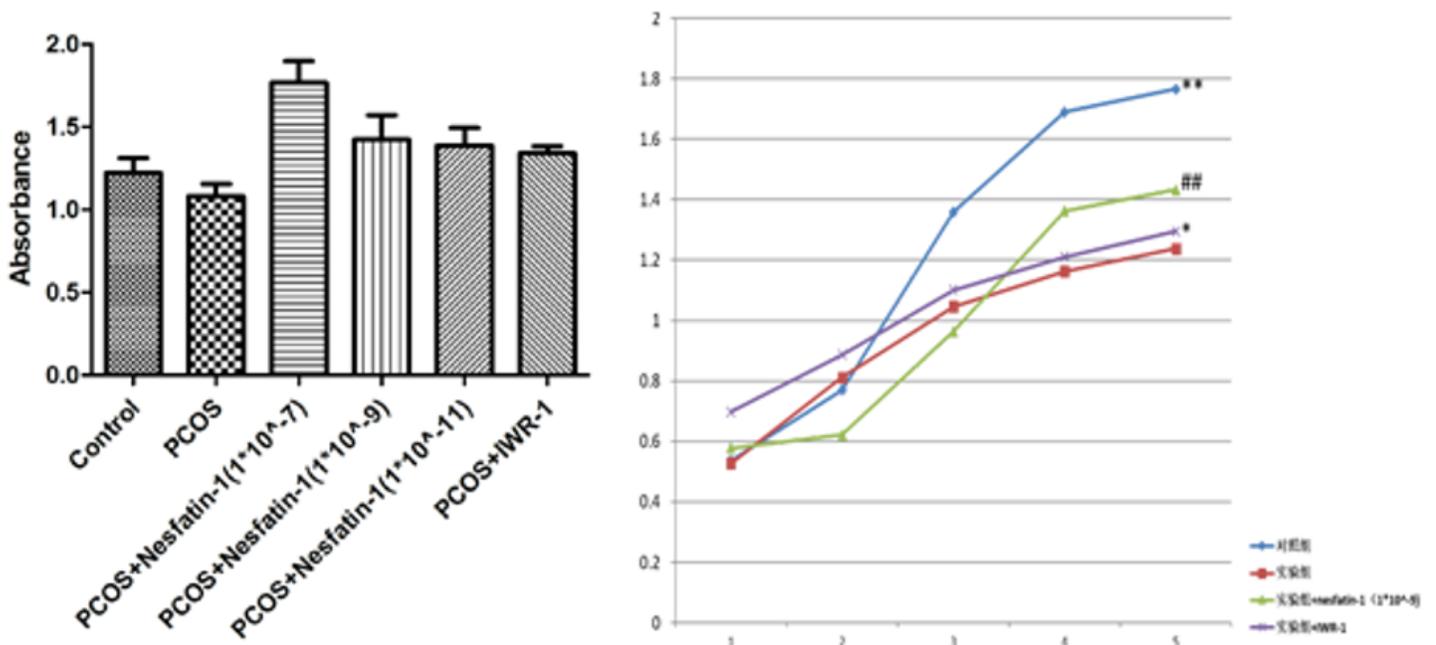


Figure 7

Proliferation trend of human primary granulosa cells in different groups

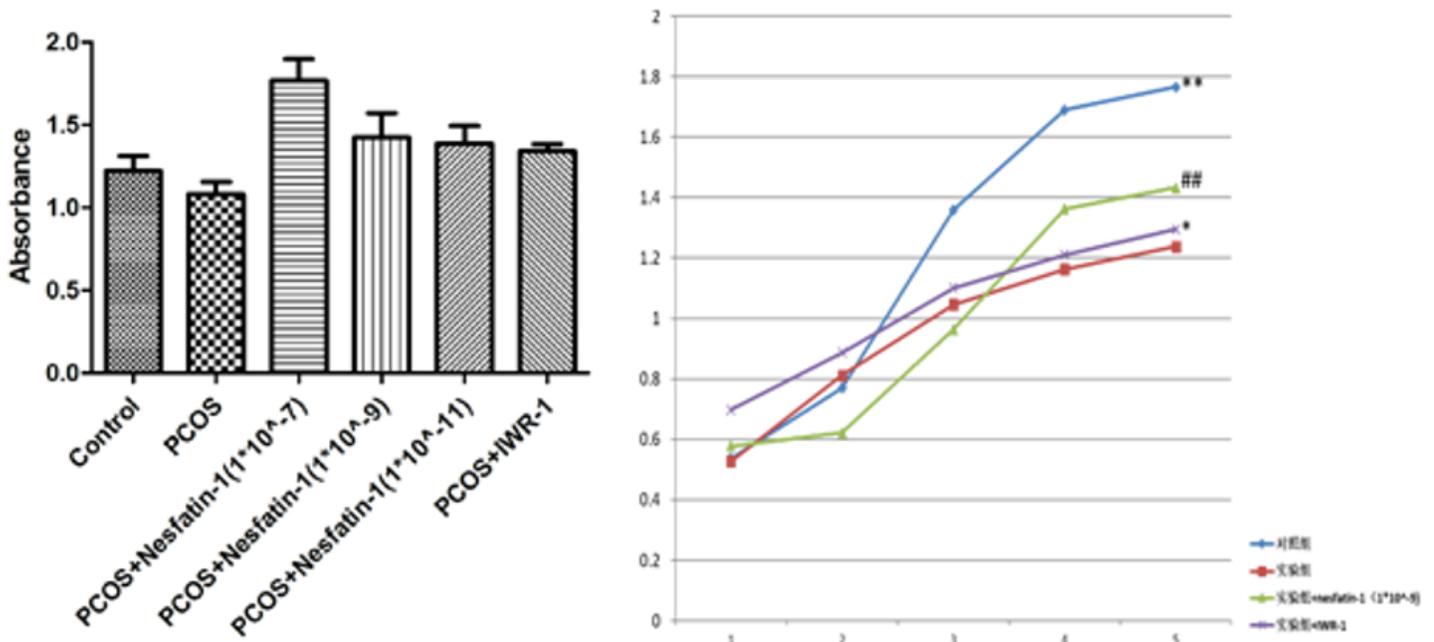


Figure 7

Proliferation trend of human primary granulosa cells in different groups

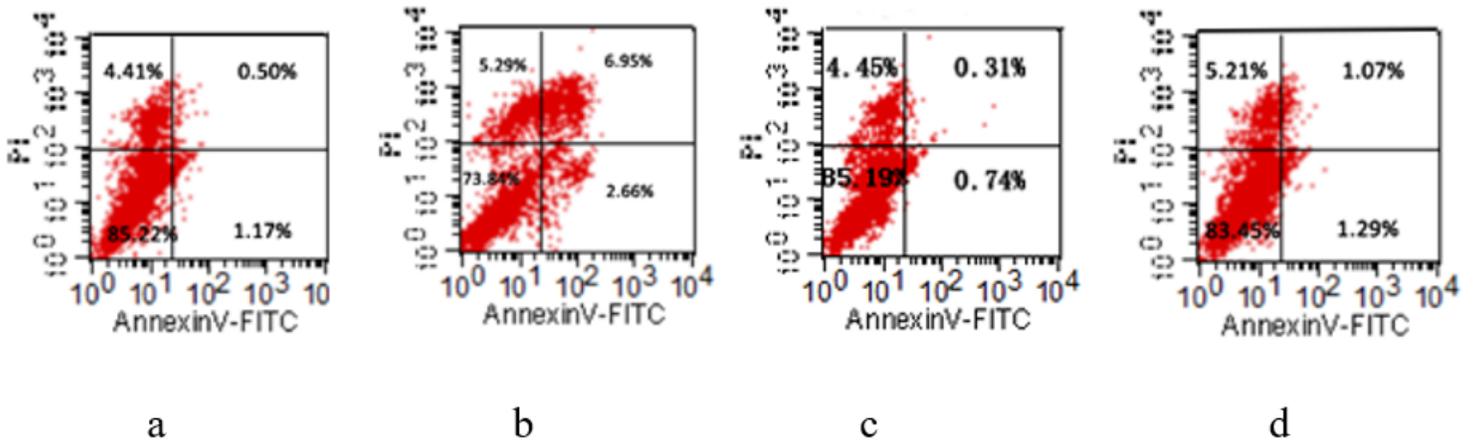


Figure 8

The effect of nesfatin-1 and IWR-1 on apoptosis in OGCs for rat.

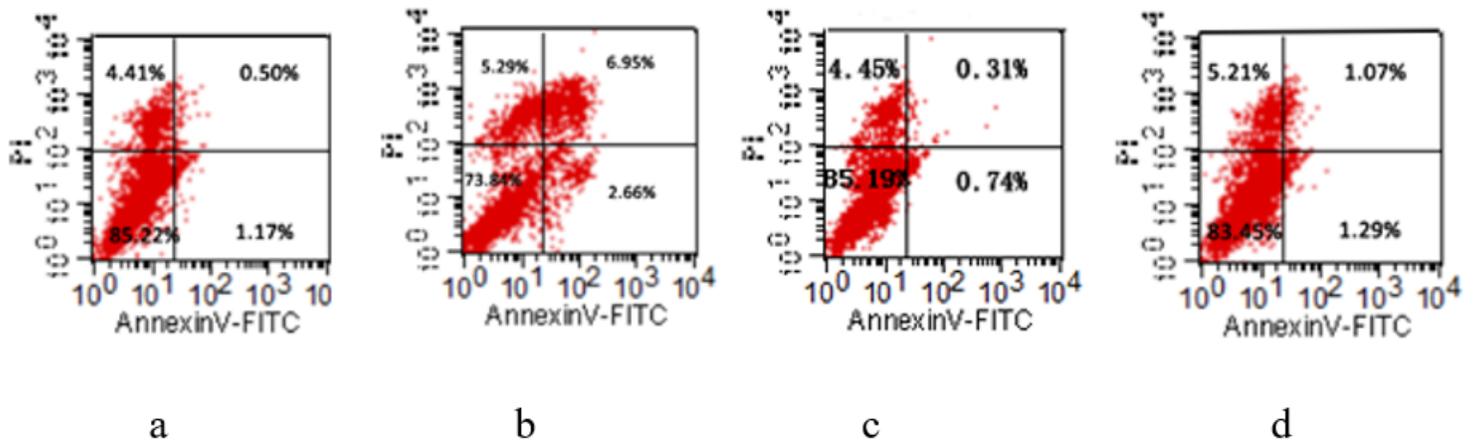


Figure 8

The effect of nesfatin-1 and IWR-1 on apoptosis in OGCs for rat.

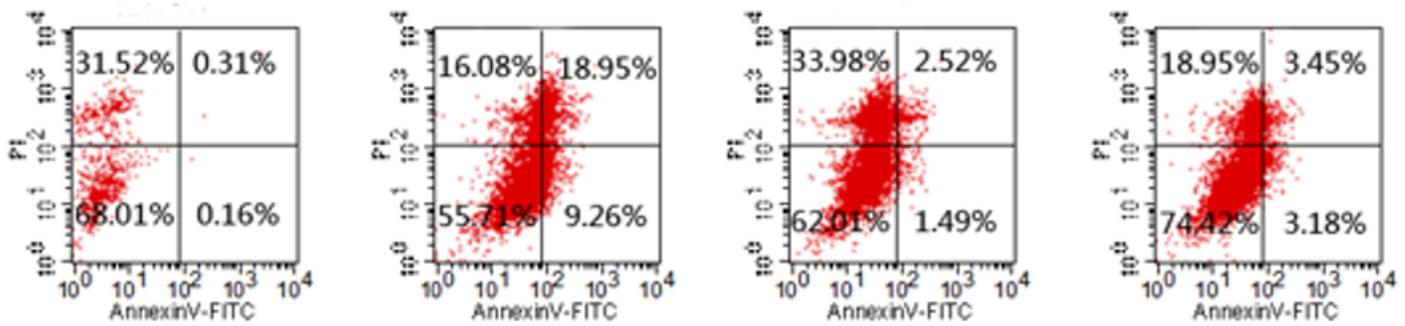


Figure 9

The effect of nesfatin-1 and IWR-1 on apoptosis in OGCs for human. (a) the control group, (b) the PCOS group, (c) the PCOS + nesfatin-1 (1×10^{-9} μ g group), (d) the PCOS + IWR-1 group.

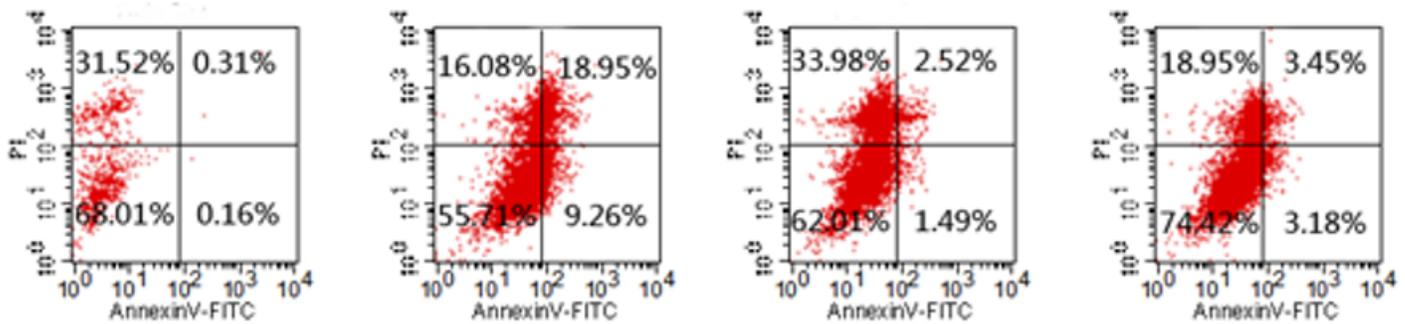


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The effect of nesfatin-1 and IWR-1 on apoptosis in OGCs for human. (a) the control group, (b) the PCOS group, (c) the PCOS + nesfatin-1 (1×10^{-9} μ g group), (d) the PCOS + IWR-1 group.

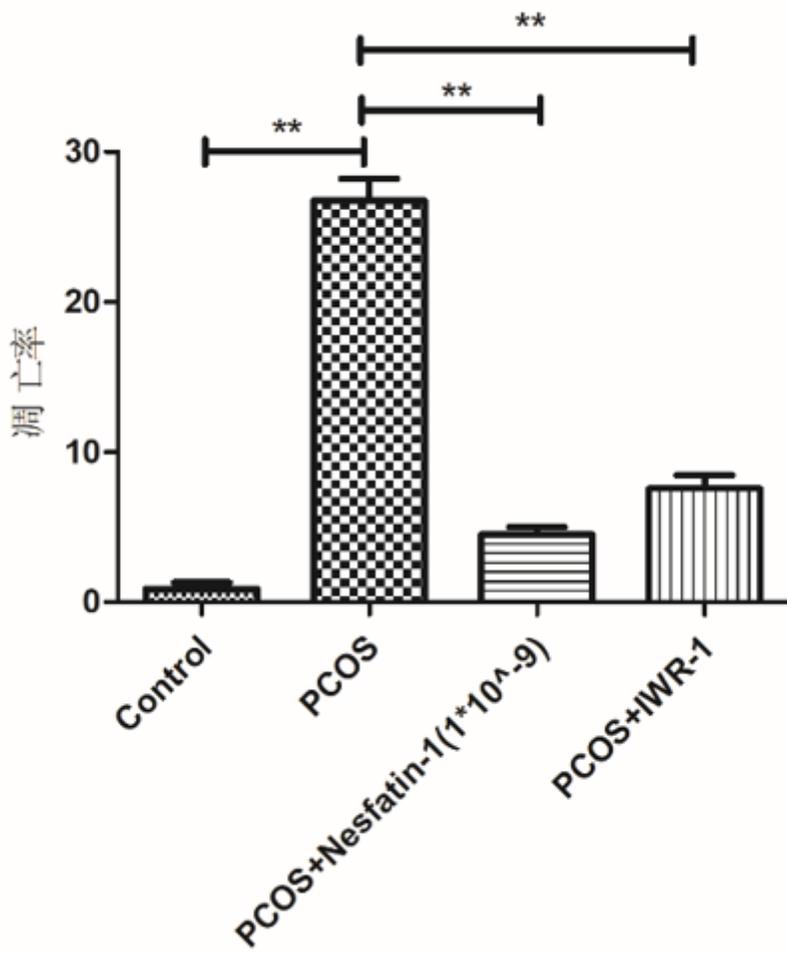


Figure 10

Analysis of Apoptosis Rate of Different Groups of Human Primary Granulosa Cells

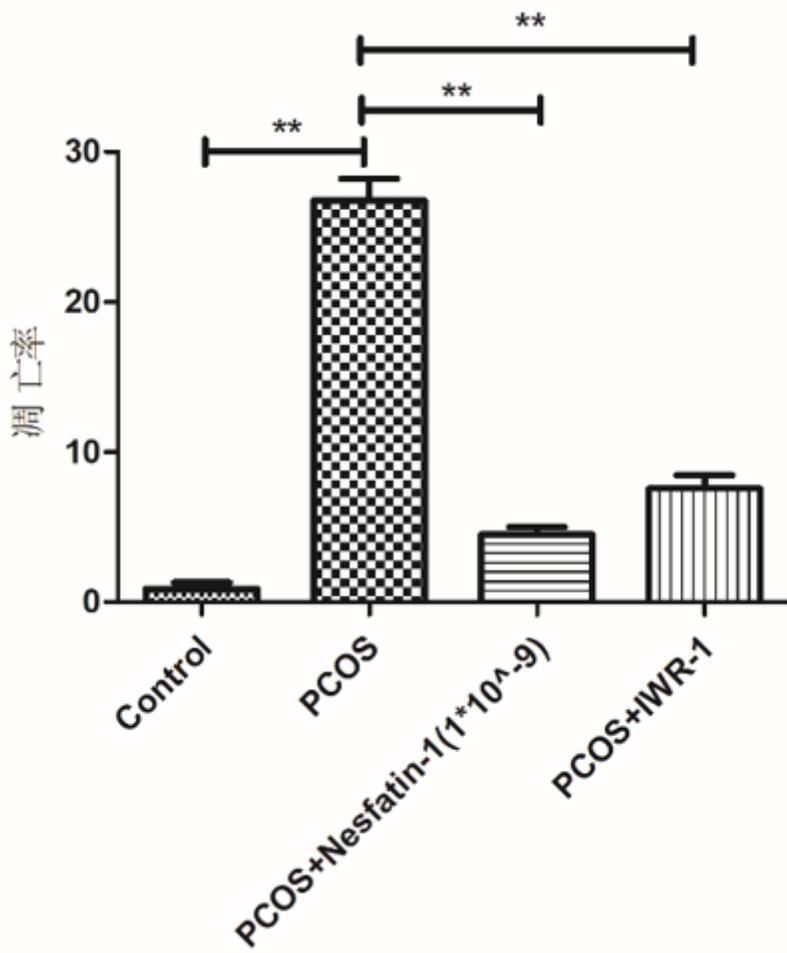


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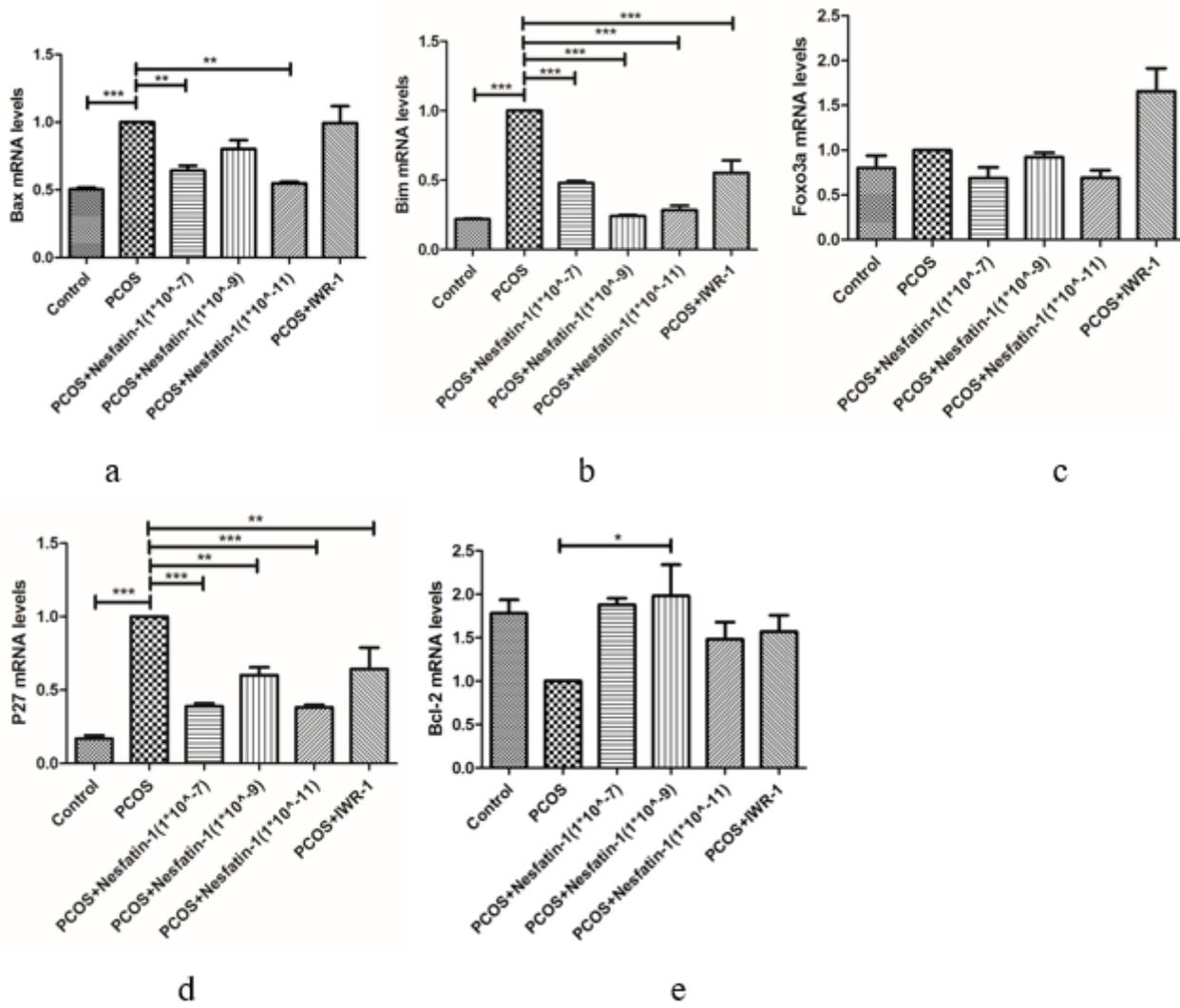


Figure 11

The expression of apoptosis-related genes mRNA

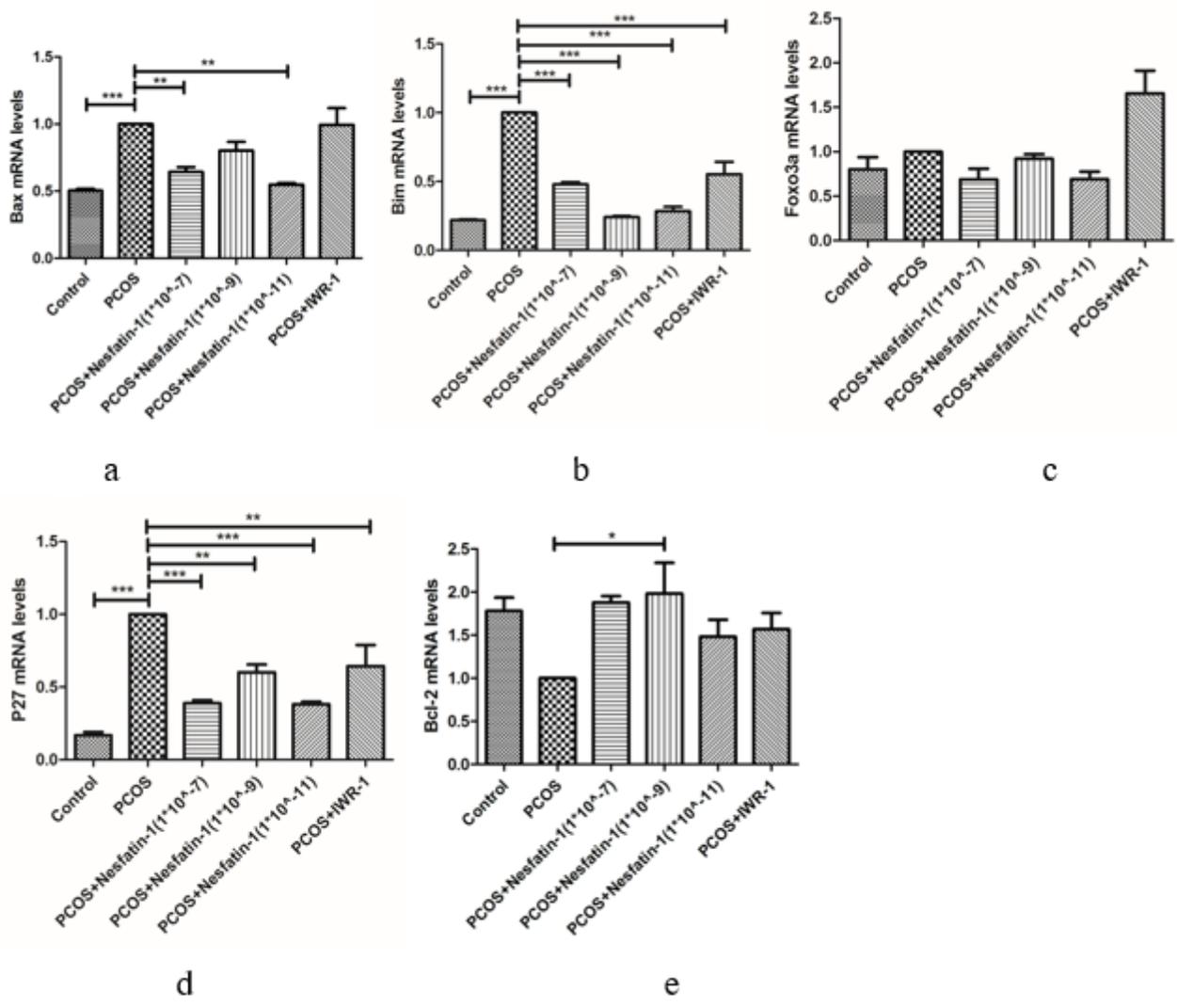


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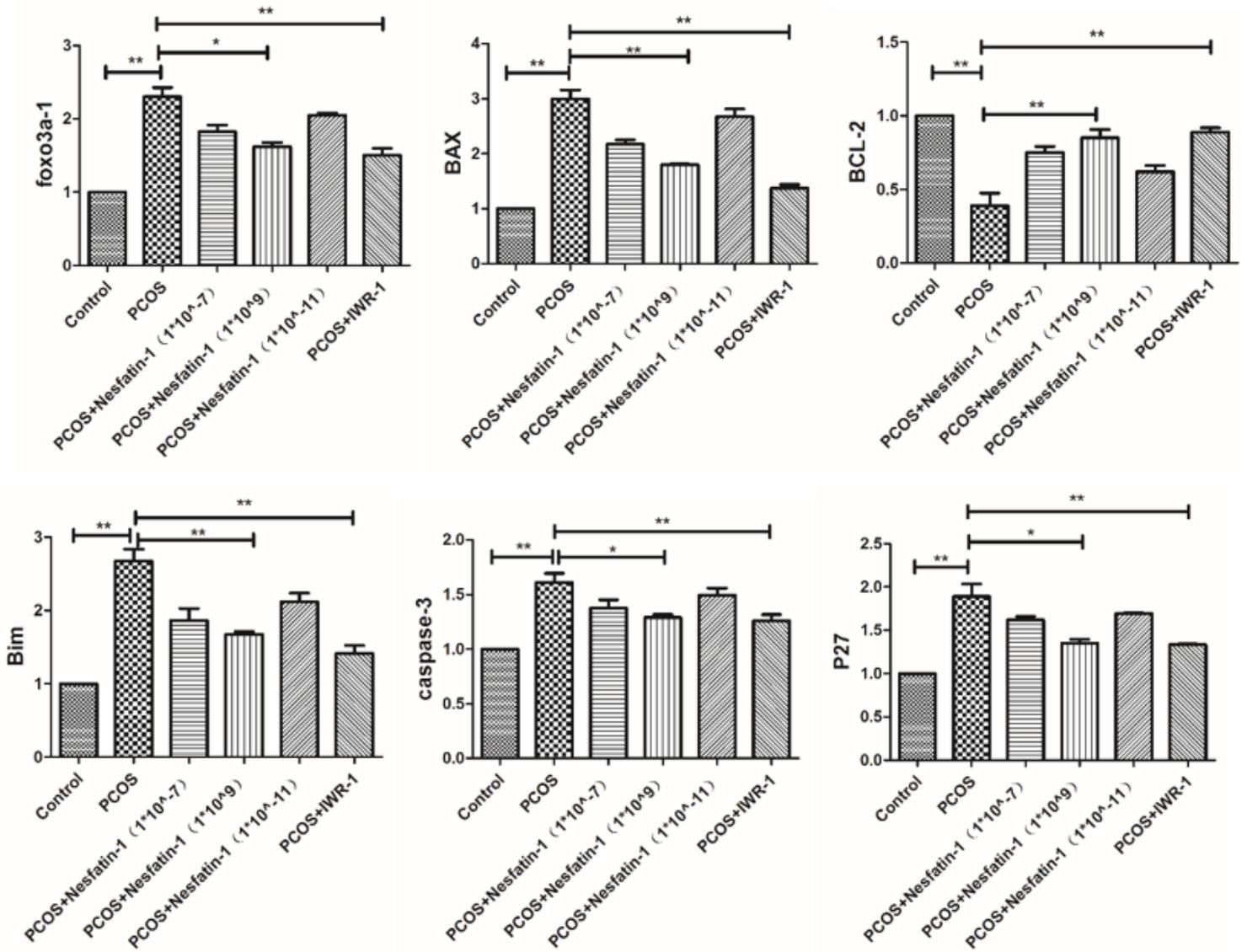


Figure 12

The expression levels of BAX mRNA, Casapase-3 mRNA, Bim mRNA, Foxo3a-1 mRNA and P27 mRNA in different groups of human primary granular cells.

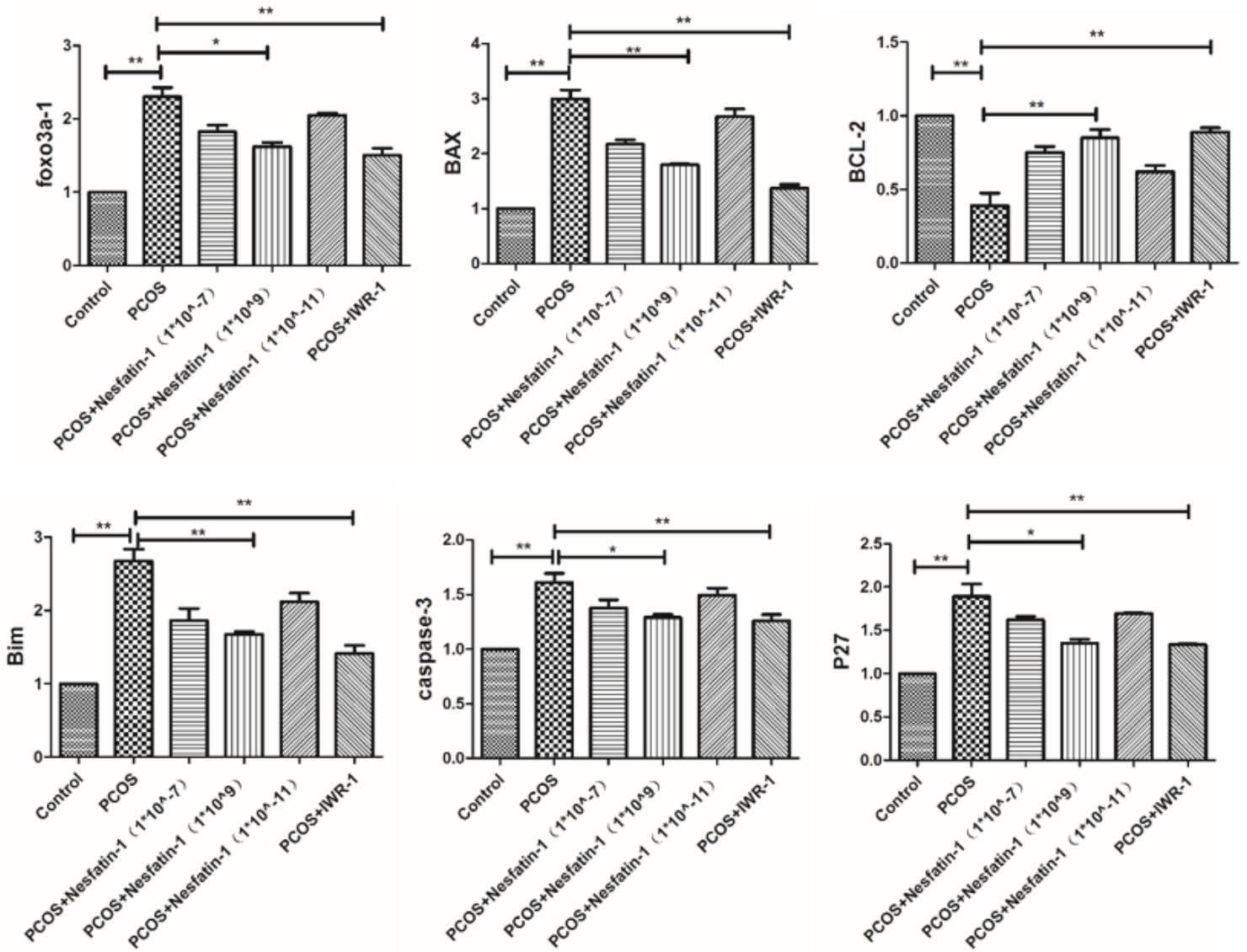


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