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Clomiphene and dexamethasone inhibit apoptosis and autophagy via the ROS-JNK/MAPK-P21 signaling pathway in PCOS

Ruxia Liu, Yuxiang Tang, Xiangjun Chen and Xintong Shang

Department of Obstetrics and Gynecology, Affiliated Hospital of Hangzhou Normal University, Hangzhou City, Zhejiang Province, PR China

Summary. Background. Polycystic ovarian syndrome (PCOS) is a complicated endocrine and metabolic disease, which seriously affects women's health. However, the etiology and genetic basis of PCOS are complex, and the pathogenesis remains unclear. In this study, we aimed to explore the effects of clomiphene and dexamethasone on PCOS and their potential mechanisms.

Methods. Sprague-Dawley (SD) rats were injected with dehydroepiandrosterone (DHEA) to establish a PCOS model. After treatment with clomiphene, dexamethasone, and their combination, ovarian tissue of rats was collected. The morphological changes in the ovary were observed by hematoxylin and eosin (HE) staining and Electron microscopy. The levels of oxidative stress and hormones were determined by ELISA. Apoptosis was assessed by TUNEL assay. The mechanism of clomiphene and dexamethasone effects on PCOS was explored by Immunohistochemical staining, real-time PCR, and western blotting.

Results. Clomiphene and dexamethasone could improve the morphology of the ovary in PCOS. TUNEL assay and ELISA showed that clomiphene, dexamethasone, and their combination could inhibit apoptosis and significantly reverse the levels of ROS, T-SOD, CAT, T, and E2 in the ovary. Immunohistochemical staining revealed that clomiphene and dexamethasone could remarkably reduce the protein levels of Bax, Caspase-3, LC3II, p-JNK, p-P38 MAPK, and P21, and increase P62 and Bcl-2 protein expression. The mRNA levels of Bax, Bcl-2, and Caspase-3 were also modulated in the PCOS model with clomiphene and dexamethasone treatment. Additionally, western blotting indicated that clomiphene and dexamethasone significantly regulated the levels of Bax, Bcl-2, Caspase-3, LC3I, LC3II, P62, p-JNK, JNK, p-P38 MAPK, P38 MAPK, and P21 in PCOS rats.

Conclusions. Clomiphene and dexamethasone can not only reduce oxidative damage, and inhibit apoptosis and autophagy, but they can also regulate the ROS-JNK/MAPK-P21 signaling pathway in PCOS rats. It provides an experimental basis for the clinical application of clomiphene and dexamethasone in PCOS.

Key words: Polycystic ovarian syndrome, Apoptosis, Autophagy, Mechanism, Treatment

Introduction

Polycystic ovarian syndrome (PCOS) is a metabolic disorder involving reproductive and endocrine systems in premenopausal women with an incidence of 9-18% (Zheng et al., 2022). PCOS is characterized by persistent anovulation, menstrual irregularities, insulin resistance, and hyperandrogenism. Chronic PCOS could lead to cardiovascular disorders, obesity, diabetes, endometrial cancer, and amenorrhea/oligomenorrhea (Peng et al., 2022). The etiology of PCOS is complex and is affected by genetic, environmental, and lifestyle factors (Zhou et al., 2021a,b). Currently, there is no specific medication to treat PCOS, it depends on the clinical presentation of each patient. Moreover, long-term medication may cause gastrointestinal discomfort, diarrhea, and other serious side effects that adversely influence the patient's quality of life. Therefore, the development of alternative treatment strategies for women with PCOS is required.

It has been reported that oxidative stress is involved in the pathogenesis of PCOS, and PCOS patients showed higher oxidative stress compared with normal individuals. Oxidative stress can cause abnormal proliferation of endometrial cells, which can lead to PCOS, decreased fertility, and endometriosis. Reactive oxygen species (ROS) is the most important molecule in

Corresponding Author: Yuxiang Tang, Department of Obstetrics and Gynecology, Affiliated Hospital of Hangzhou Normal University, 126 Wenzhou Road, Gongshu District, Hangzhou City, Zhejiang Province, 310000, PR China. e-mail: tang19910802@163.com

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the process of oxidative stress. Previous studies revealed that clomiphene citrate (clomiphene) had antioxidant capacity and effects on oxidative metabolism by regulating ROS production (Costa et al., 2012). Clomiphene is a non-steroidal selective estrogen receptor modulator used to induce ovulation in PCOS patients and to conduct ovarian reserve tests in assisted reproductive therapy (Badawy and Elnashar, 2011). Dexamethasone is a glucocorticoid, and its combination with clomiphene can effectively improve the anti-estrogen effect of the endometrium of patients and increase the pregnancy rate. In a clinical setting, a meta-analysis indicated significant efficacy of clomiphene and dexamethasone in the treatment of PCOS patients (Suzuki et al., 2015). Additionally, ROS has been shown to be a strong signal for the activation of JUN-terminal kinase (JNK), and it may, therefore, regulate several biological events, including autophagy, apoptosis, longevity, and DNA repair (Suzuki et al., 2015). Autophagy is necessary for follicular cells to maintain oocyte development, follicular growth and differentiation, follicular atresia, and the reproductive cycle (Kumariya et al., 2021). p38 mitogen-activated protein kinase (MAPK) could regulate autophagy in response to chemotherapy drugs (Sui et al., 2014). MAPK is also a key link in the transmission of signals from the cell surface to the nucleus to regulate gonadotropin transcription. The MAPK signaling pathway therefore is involved in the pathogenesis of PCOS by regulating the expression of JNK and p38.

In this study, we established a PCOS rat model with dehydroepiandrosterone (DHEA), and the therapeutic effects of clomiphene, dexamethasone, and their combination were investigated. TUNEL staining and ELISA were used to assess the effects of clomiphene and dexamethasone on apoptosis and oxidative stress in the PCOS model. In addition, the potential mechanism of clomiphene and dexamethasone in the treatment of PCOS was evaluated by immunohistochemistry, real-time PCR, and western blotting. These results will improve our understanding of the development of PCOS, and elucidate the molecular mechanism of clomiphene combined with dexamethasone in the treatment of PCOS. It may provide a new strategy for the clinical treatment of PCOS.

Materials and methods

Animal and drug intervention

Female Sprague-Dawley (SD) rats weighing about 120 g were purchased from Shanghai Slack Laboratory Animal Co., Ltd (License NO. SCXK (Hu) 2017-0005, Shanghai, China) and housed at 22±2°C, 50-60% humidity, and 12h light/dark photoperiods. Ten rats were randomly selected as the control group, others were induced as the PCOS model. The model rats were subcutaneously injected with 0.2 ml (6 mg/100 g) of DHEA solution (Shanghai Yuanye Biotechnology Co., Ltd, Shanghai, China) dissolved in sesame oil in the morning and 1.5 IU Human chorionic gonadotropin (HCG, Shanghai Maclean Biochemical Technology Co., Ltd, Shanghai, China) dissolved in 0.4 ml sterile water in the afternoon. After 16 days of modeling, vaginal secretions were collected and observed daily for a week (Jia and Ping, 2015). The rats with keratinized vaginal epithelia and irregular estrous cycles were successfully induced PCOS rats (Jia and Ping, 2015; Teng et al., 2024). Then, PCOS rats were randomly divided into four groups of ten rats: the model group, clomiphene group (4.5 mg/kg/d), dexamethasone group (4.5 mg/kg/d), and the combined treatment group (clomiphene and dexamethasone). Each group was given the corresponding solution by gavage every day for three weeks. This experiment was approved by the Ethical Review Committee (ZJEY-20221107-06).

Histological evaluation and follicle count

Ovarian tissue was fixed in 4% formaldehyde for paraffin section preparation. Every fifth section was stained with hematoxylin and eosin (HE) for morphological evaluation and differential follicle count. Follicular stages were classified according to standard criteria established by Pedersen and Peters. Three experienced gynecologic pathologists performed follicle counts. In addition, the morphological changes in the ovary in each group were assessed by HE semi-quantitative analysis.

Immunohistochemical staining

For immunohistochemistry analysis, paraffin sections of the ovary were deparaffinized in xylene and rehydrated in an ethanol gradient (100-75%). After subsequently incubating with antigen retrieval solution and 3% H_2O_2 for 25 min, the sections were rinsed with PBS and incubated with the primary antibody (P-JNK, 1:50; P-P38 MAPK, 1:100; P21, 1:50; LC3II, 1:100; P62, 1:100; Bax, 1:100; Bcl-2, 1:100; Caspase-3,1:200) overnight at 4°C. The details of primary antibodies are listed in Table 1. Then, the sections were incubated with a secondary antibody (Goat Anti-Rabbit IgG H&L, 1:5000, Abcam, Cambridge, UK) for 1h at room temperature. Finally, the slides were stained with DAB and hematoxylin. After dehydrating and mounting, slides were observed by microscope (Nikon, Tokyo, Japan) and analyzed with the Nikon DS-U3 system.

TUNEL assay

The TUNEL kit was used to detect the apoptosis of ovarian tissue (Servicebio, Wuhan, China). The sections were deparaffinized and rehydrated with graded alcohols, then rinsed in distilled water. After incubation in proteinase K solution for 30 min, the slides were

washed with PBS (pH 7.4) three times. Then, the sections were permeabilized and incubated with the TUNEL reaction mixture at 37°C for 2h. DAPI was used to dye the cell nucleus, and slides were sealed with an anti-fade mounting medium. The images were captured by an inverted fluorescence microscope, and apoptotic cells were counted by Image-Pro Plus software.

Electron microscope

Ovaries were cut into 1 mm^3 strips, fixed with 2.5% glutaraldehyde solution for 3h, rinsed with PBS buffer, and then fixed in 1% osmic acid. After rinsing with PBS three times, the samples were dehydrated in a gradient concentration of ethanol, kept in acetone, and embedded with Araldite. Then, samples were heated overnight at 70°C and stained with both uranyl acetate and lead citrate. The ultrastructural changes in ovary cells were observed by transmission electron microscopy (HITACHI, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA)

Ovarian tissues were homogenized in cold physiological saline and centrifuged at 3500 r/min. After 15 min, the supernatant was collected. According to the manufacturer's protocols, the levels of ROS, T-SOD (total superoxide dismutase), CAT (catalase), T (testosterone), and E2 (estradiol) in serum were determined by ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, China). The optical density was detected at 450 nm with the Microplate Reader (Molecular Devices, California, USA).

Real-time PCR

Total RNA was extracted from the ovary using Trizol reagent (Sangon Biotech, Shanghai, China). Reverse transcription was performed as follows: 42°C, 15 min; 85°C, 5 min. Real-time PCR was performed using SYBR Premix Ex Taq II (Takara, Shiga, Japan) at 95°C, 10 min; 95°C, 15 s; 60°C, 60 s; 40 cycles. The *GAPDH* gene was used as an internal control for the quantification of target genes. Primer sequences are listed as follows: Bax forward 5'- TTGCTTCAGGGT TTCATCC-3', reverse 5'- GACACTCGCTCAG CTTCTTG-3'; Bcl-2 forward 5'- CTTTGAGTTCG GTGGGGTCA-3', reverse 5'- AAACAGAGGTC GCATGCTGG-3'; Caspase-3 forward 5'- TGACTG GAAAGCCGAAACT-3', reverse 5'- GGGTGCGGT AGAGTAAGCAT-3'; GAPDH forward 5'- GATGGT GAAGGTCGGTGTGA-3', reverse 5'- TGAACTTG CCGTGGGTAGAG-3'.

Western blotting

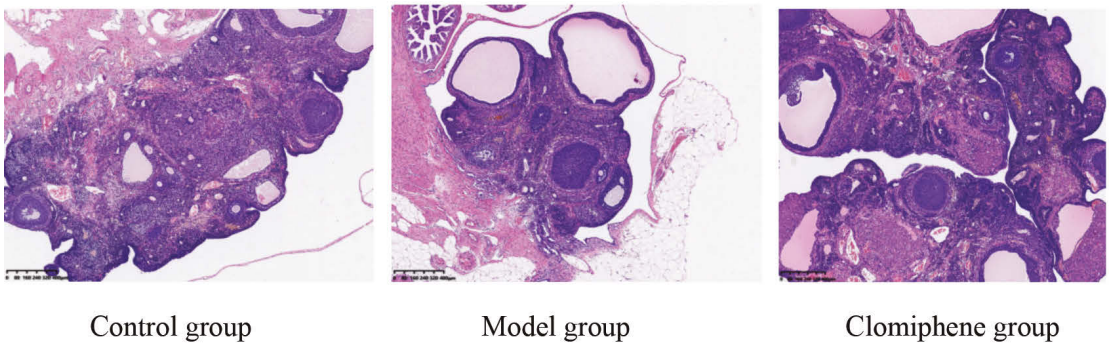
The ovary tissue was lysed and homogenized at 4°C. After centrifuging at 12,000 g for 5 min, the supernatants were collected in a tube. The total protein concentration was determined by the Bicinchoninic acid (BCA) method. The proteins were separated in SDS-PAGE at 100 V and transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare Life, Chalfont, UK) at 200 mA for 120 min. After washing with TBST three times, the PVDF membrane was blocked with 5% skim milk and incubated with primary antibodies (p-JNK: Affinity, AF3318; JNK: Affinity, AF6318; p-P38: Affinity, AF4001; P38: Affinity, AF6456; p21: Affinity, AF6290; Bax: Affinity, AF0120; Bcl-2: Affinity, AF6139; Affinity, Caspase 3: Affinity, AF6311; LC3: CST, 4108s; p62: CST, 8025s; GAPDH: Affinity, AF7021) successively. Then, the membrane was incubated with a secondary antibody (Anti-rabbit IgG, CST, 7074, Massachusetts, USA) for 1.5h at room temperature. Following incubation with Clarity Western ECL Substrate (Bio-Rad, California, USA), the protein band was visualized by the ChemiScope6100 system (CLINX, Shanghai, China) and analyzed by Chemi capture software.

Statistics analysis

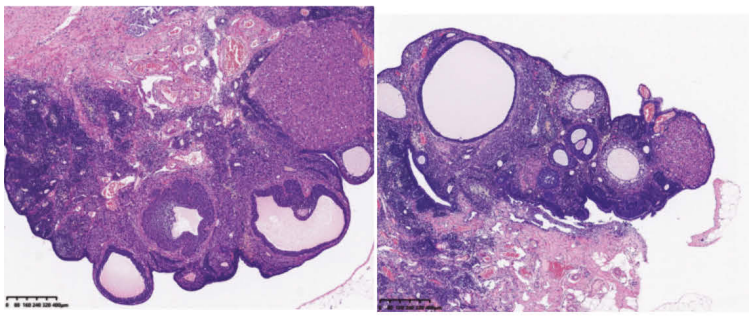
All experimental data are presented as mean ± standard deviation and analyzed using SPSS 20.0 (IBM, New York, USA) and GraphPad Prism 7 (GraphPad, San Diego, USA). Differences between two groups were determined by the SNK test, independent-sample student's t-tests, or Kruskal-Wallis H test. A value of $P<0.05$ was considered significant, and $P<0.01$ was considered statistically significant.

Table 1. The information on primary antibodies in immunohistochemistry.

Primary antibody	Company	Code	Dilution ratio
Bcl-2 Antibody	Affinity	AF6139	1:100
p21 Waf1/Cip1 (12D1) Rabbit mAb	CST	2947T	1:50
Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb	CST	4668T	1:50
Bax Antibody	CST	2774	1:100
LC3A/B Antibody	Affinity	AF5402	1:100
caspase-3	Proteintech	19677-1-AP	1:200
Phospho-p38 MAPK (Thr180/Tyr182) Antibody	Affinity	AF4001	1:100
SQSTM1/p62 Antibody	Proteintech	18420-1-AP	1:100

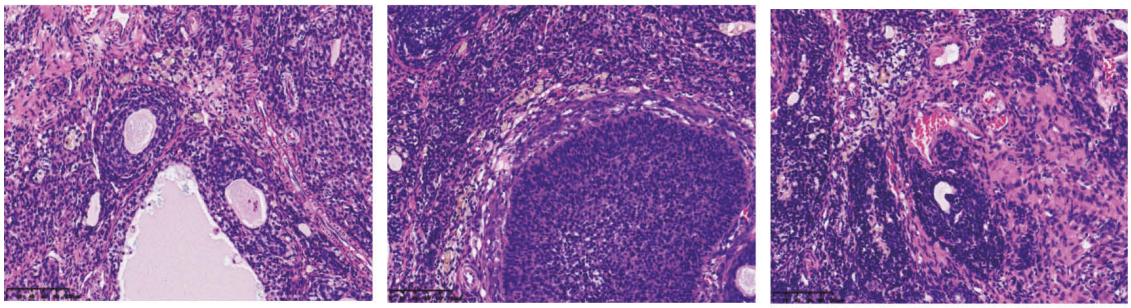


Control group Model group Clomiphene group

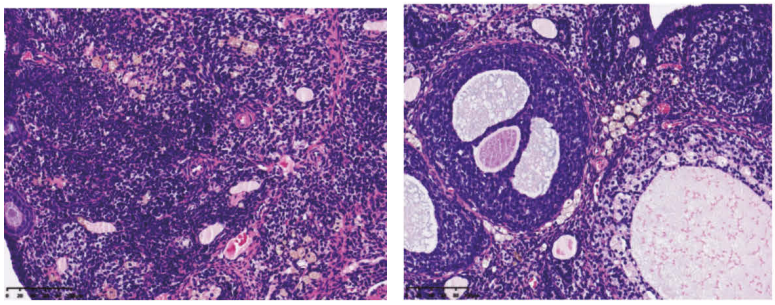


Dexamethasone group Combined treatment group

(a) 40X



Control group Model group Clomiphene group



Dexamethasone group Combined treatment group

(b) 200X

Fig. 1. Hematoxylin and eosin staining of ovarian tissue in rats.

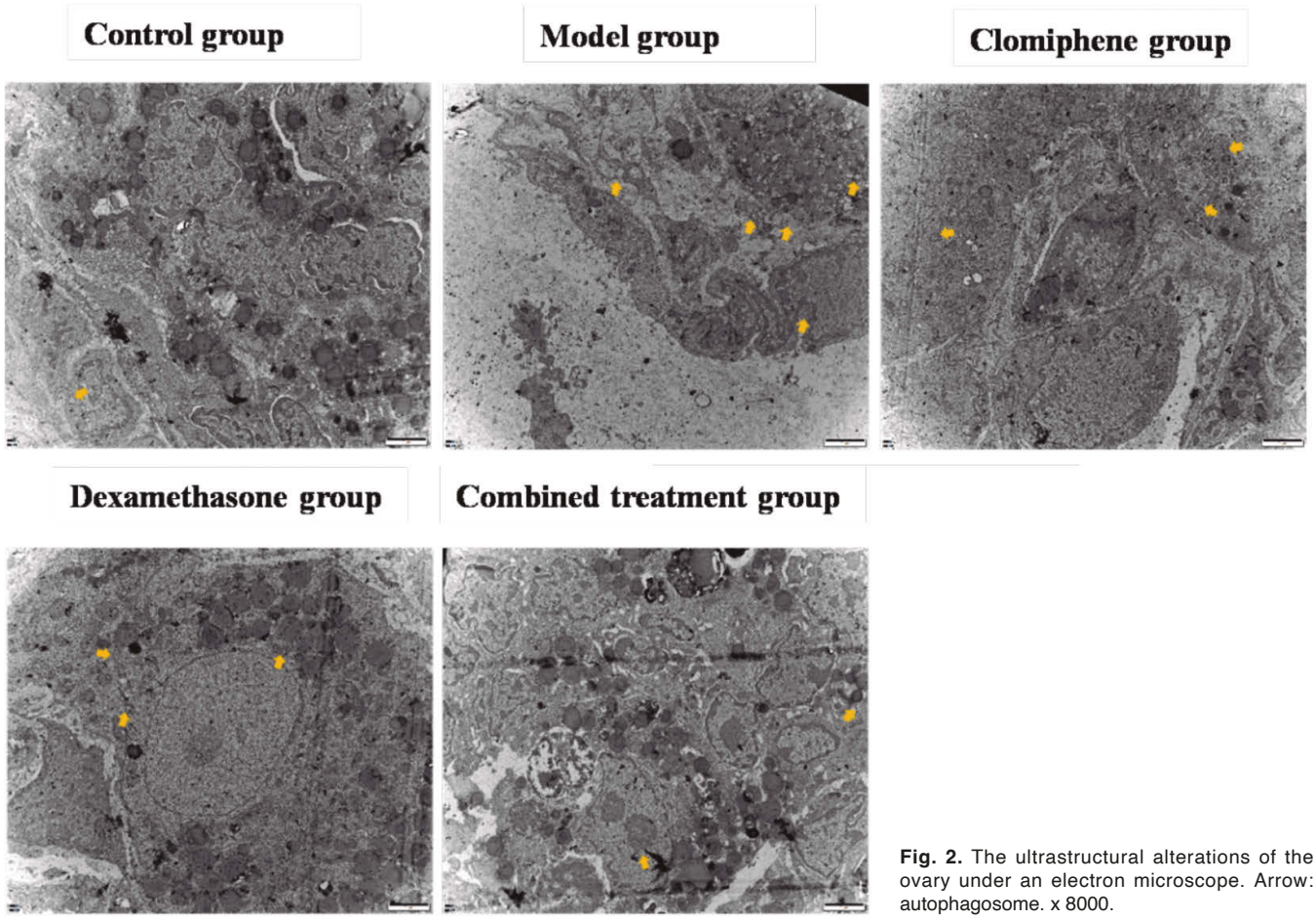


Fig. 2. The ultrastructural alterations of the ovary under an electron microscope. Arrow: autophagosome. x 8000.

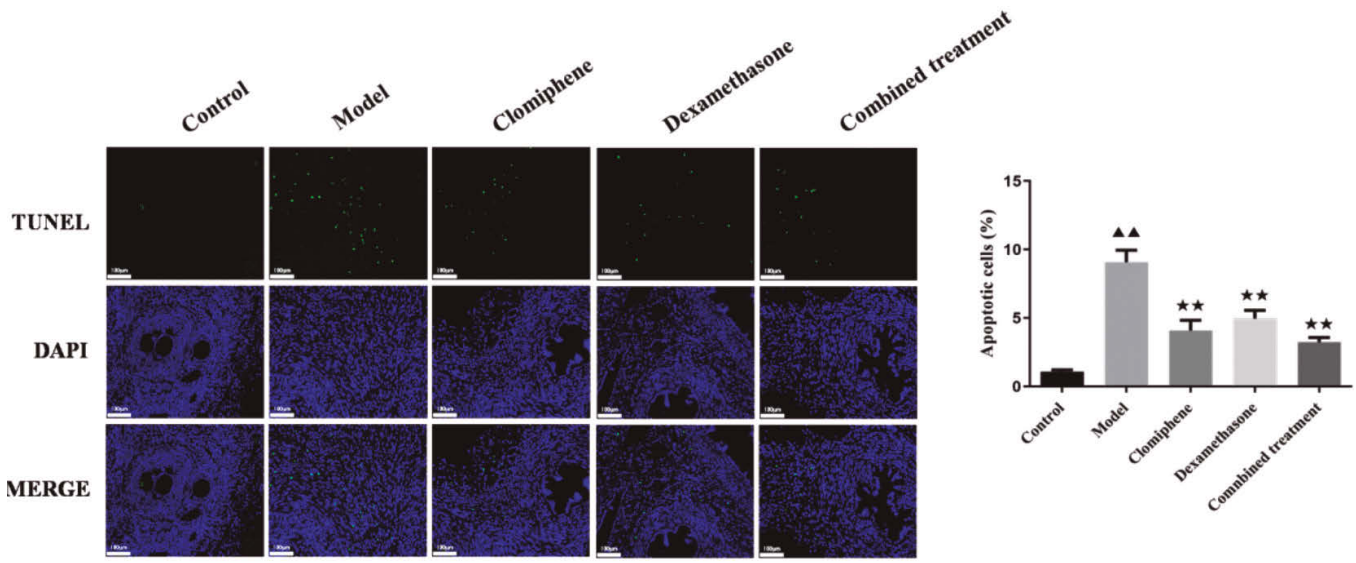


Fig. 3. The TUNEL assay in ovarian tissue. ▲▲P<0.01 vs. control group; ★★P<0.01 vs. model group. x 400.

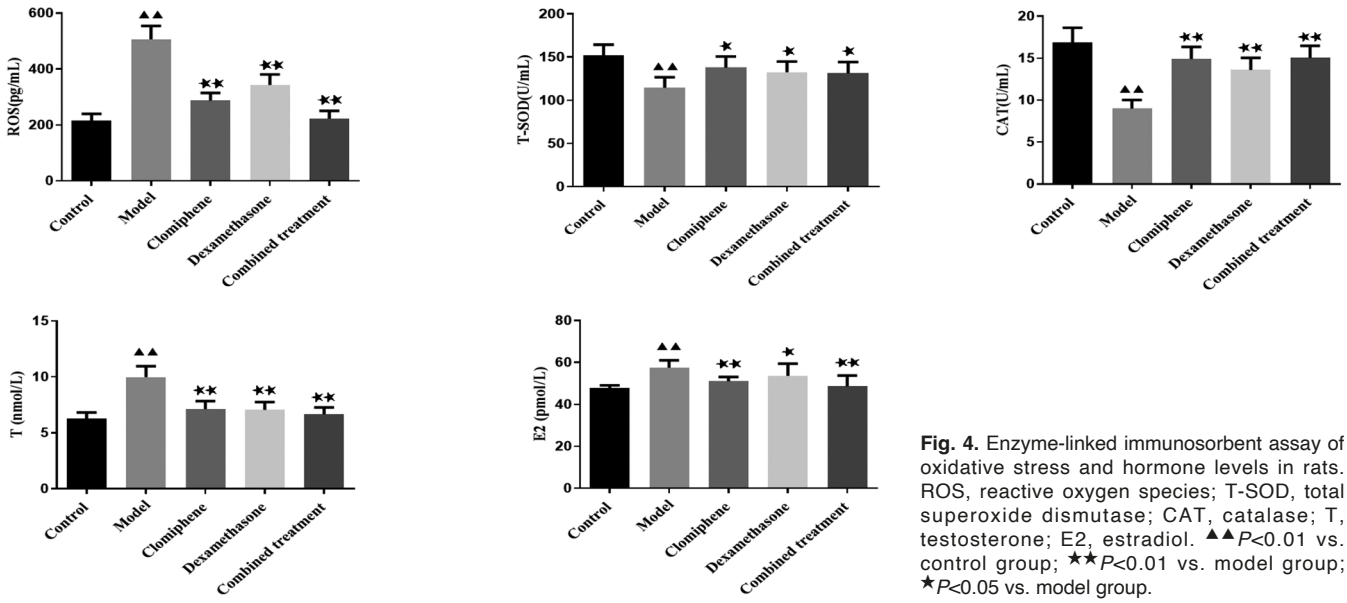


Fig. 4. Enzyme-linked immunosorbent assay of oxidative stress and hormone levels in rats. ROS, reactive oxygen species; T-SOD, total superoxide dismutase; CAT, catalase; T, testosterone; E2, estradiol. ▲▲P<0.01 vs. control group; ★★P<0.01 vs. model group; ★P<0.05 vs. model group.

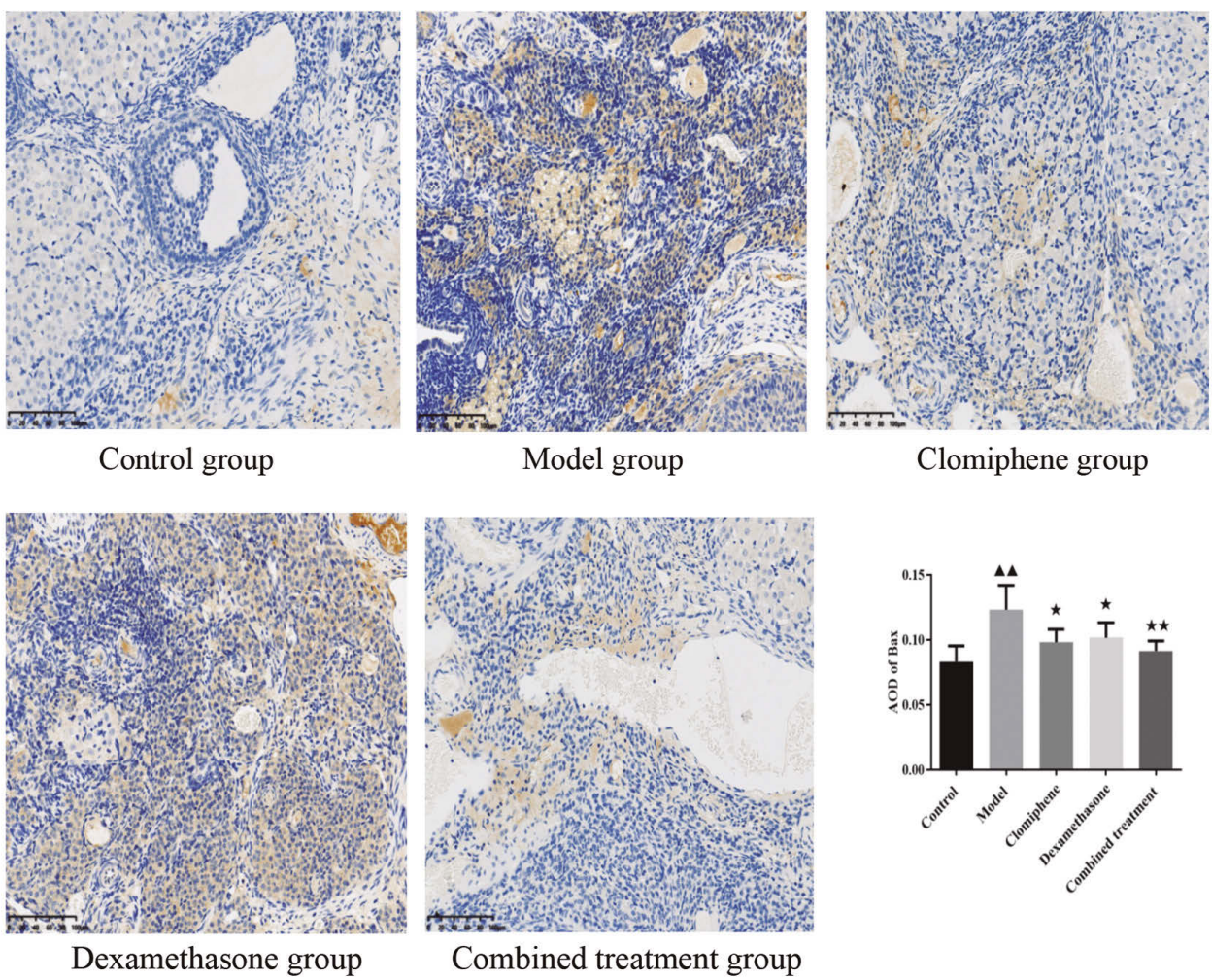


Fig. 5. The immunohistochemical staining for Bax protein in the ovary. ▲▲P<0.01 vs. control group; ★★P<0.01 vs. model group; ★P<0.05 vs. model group.

Results

Histological effects of clomiphene and dexamethasone on PCOS rats

Compared with the control group, PCOS rats showed reduced follicles and significant ovarian fibrosis (Fig. 1). After treatment with clomiphene and dexamethasone, the number of granulosa cells increased and arranged neatly in a radial pattern, and follicles at all levels began to appear. Ovarian tissue was scored semi-quantitatively (grades 0-4) on HE staining. As shown in Table 2, the score was significantly lower in the clomiphene, dexamethasone, and combined treatment group compared with the

Table 2. The HE Semi-quantity score of ovary tissue (χ±s, n=6).

Group	Score
Control group	0.50±0.55
Model group	3.33±0.52▲▲
Clomiphene group	2.33±0.52★★
Dexamethasone group	2.50±0.55★
Combined treatment group	1.83±0.41★★

The grade is from 0 to 4 means the pathological morphology becomes more serious. Compared with control group, ▲P<0.05, ▲▲P<0.01; Compared with model group, ★P<0.05, ★★P<0.01.

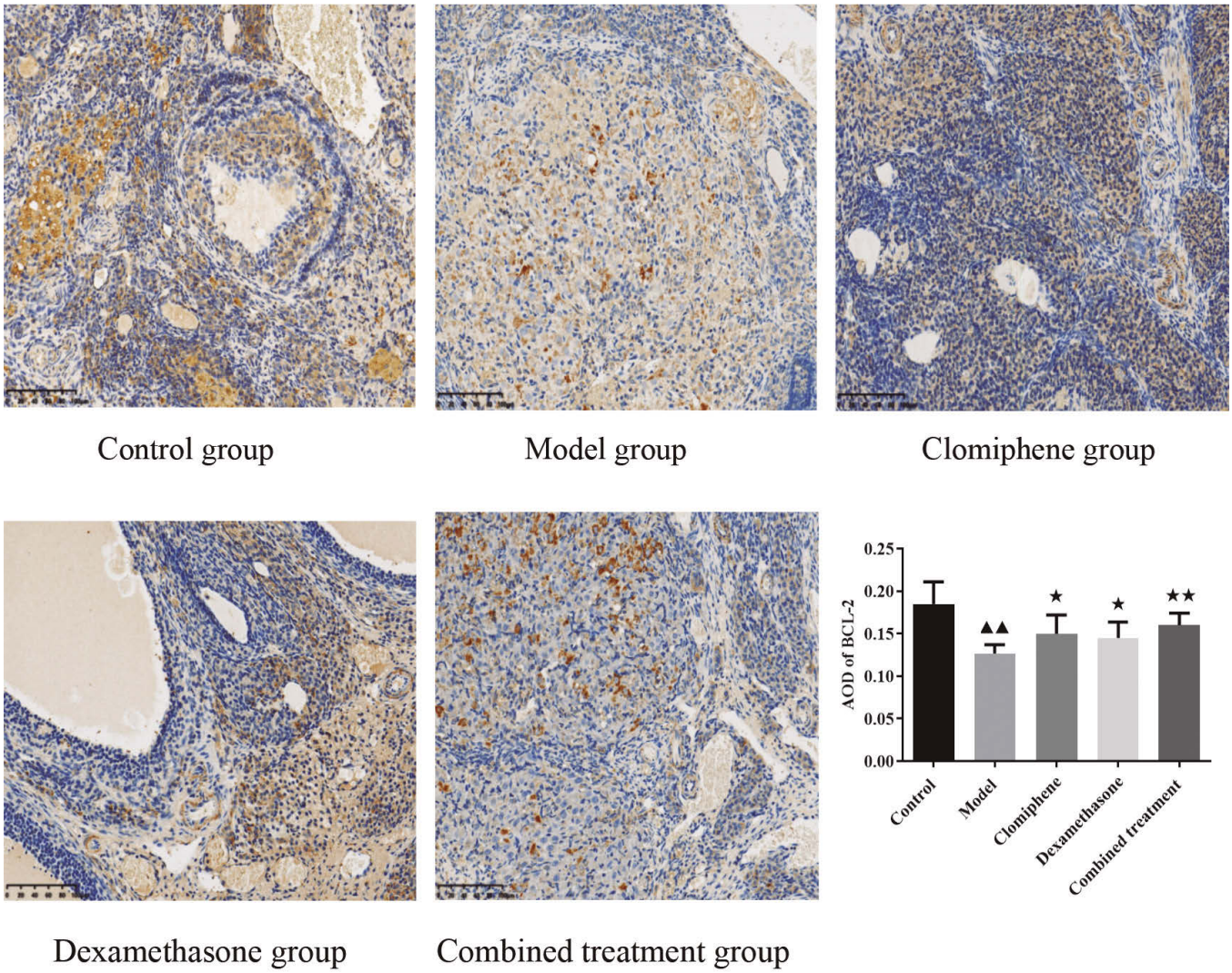


Fig. 6. The immunohistochemical staining for Bcl-2protein in the ovary. ▲▲P<0.01 vs. control group; ★★P<0.01 vs. model group; ★P<0.05 vs. model group.

Table 3. The comparison of follicle counts in rats ovary (χ±s, n=6).

Group	Primordial follicle	Primary follicle	Secondary follicles	Mature follicle	Corpus luteum
Control group	7.67±0.82	7.00±0.89	4.00±0.63	5.33±0.82	6.17±0.75
Model group	4.00±0.89▲▲	2.83±0.41▲▲	2.83±0.41▲▲	3.17±0.41▲▲	3.17±0.41▲▲
Clomiphene group	6.67±0.82★★	5.17±0.75★★	3.83±0.75★	3.83±0.75	4.83±0.98★★
Dexamethasone group	6.00±0.89★★	4.67±0.82★★	3.17±0.41	3.33±0.52	4.17±0.75★
Combined treatment group	7.17±0.98★★	6.17±0.75★★	3.67±0.52★	4.50±0.55★★	5.00±0.63★★

Compared with control group, ▲P<0.05, ▲▲P<0.01; Compared with model group, ★P<0.05, ★★P<0.01.

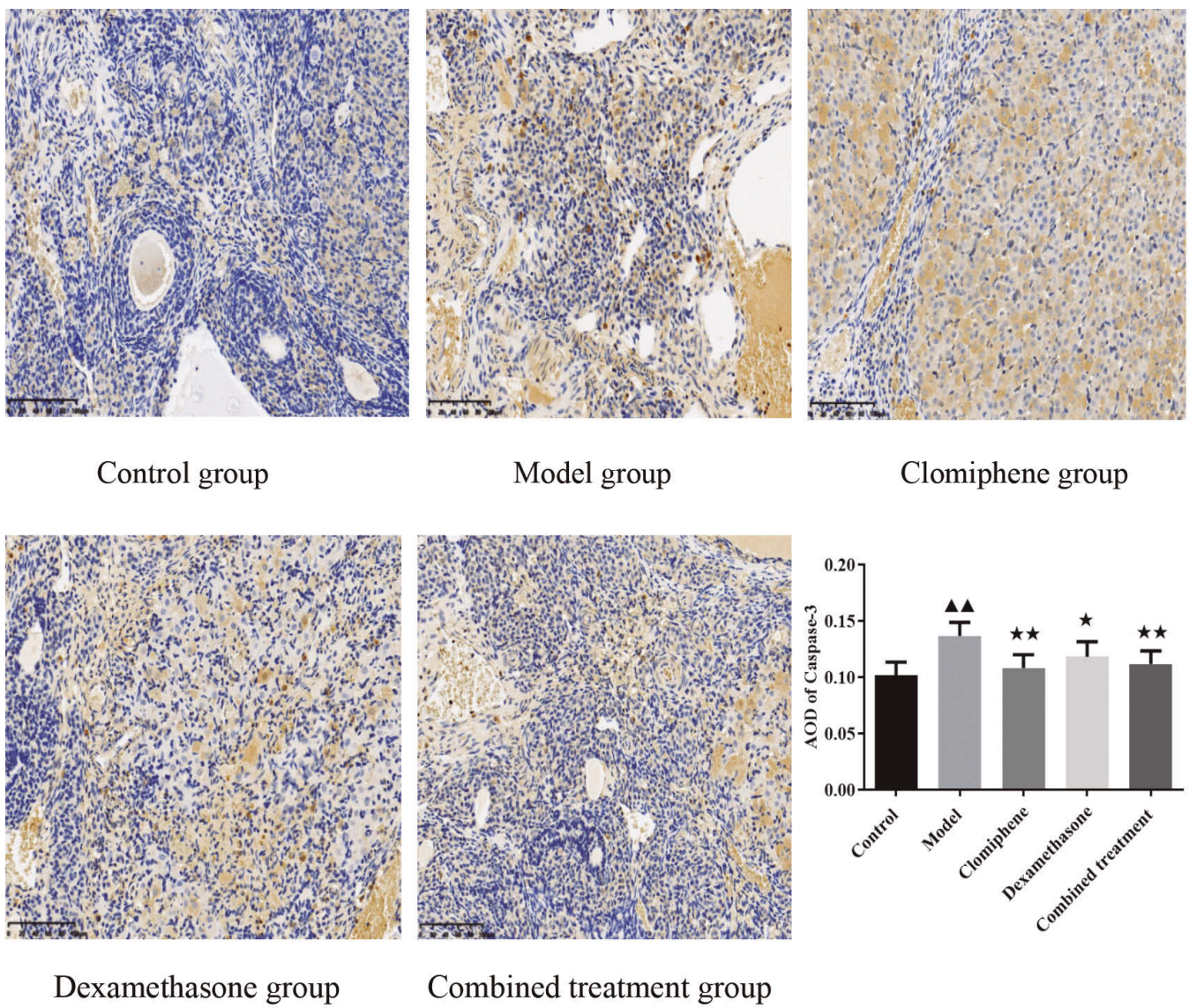


Fig. 7. The immunohistochemical staining for Caspase-3 protein in the ovary. ▲▲P<0.01 vs. control group; ★★P<0.01 vs. model group; ★P<0.05 vs. model group.

model group. In addition, we found that the follicle counts in the model group decreased as compared with the model group (Table 3, $P<0.01$). With the clomiphene combined with dexamethasone treatment, the follicle counts at different developmental stages and corpus luteum were significantly increased than in the model group ($P<0.01$). In addition, we observed the ultrastructure of ovarian granulosa cells by transmission electron microscopy (Fig. 2). This indicated that the autophagosomes of ovarian granulosa cells remarkably increased and the ultrastructural damage is serious in PCOS rats. Treatment with clomiphene, dexamethasone, and their combination could reverse the ultrastructural injury of ovarian granulosa cells.

Inhibitory effects of clomiphene and dexamethasone on apoptosis and oxidative stress in PCOS rats

In this study, we explored the preventive effects of clomiphene and dexamethasone on cell apoptosis in the ovary of PCOS rats (Fig. 3). TUNEL staining showed that the apoptotic cell rate was significantly different between the control and model groups ($P<0.01$). After clomiphene treatment, with or without dexamethasone, the apoptosis of ovarian tissue cells was inhibited compared with the model group. Additionally, we assessed the levels of serum ROS, T-SOD, CAT, T, and E2 by ELISA. Compared with the control group, the levels of ROS, T-SOD, and CAT in the model group were significantly different (Fig. 4, $P<0.01$).

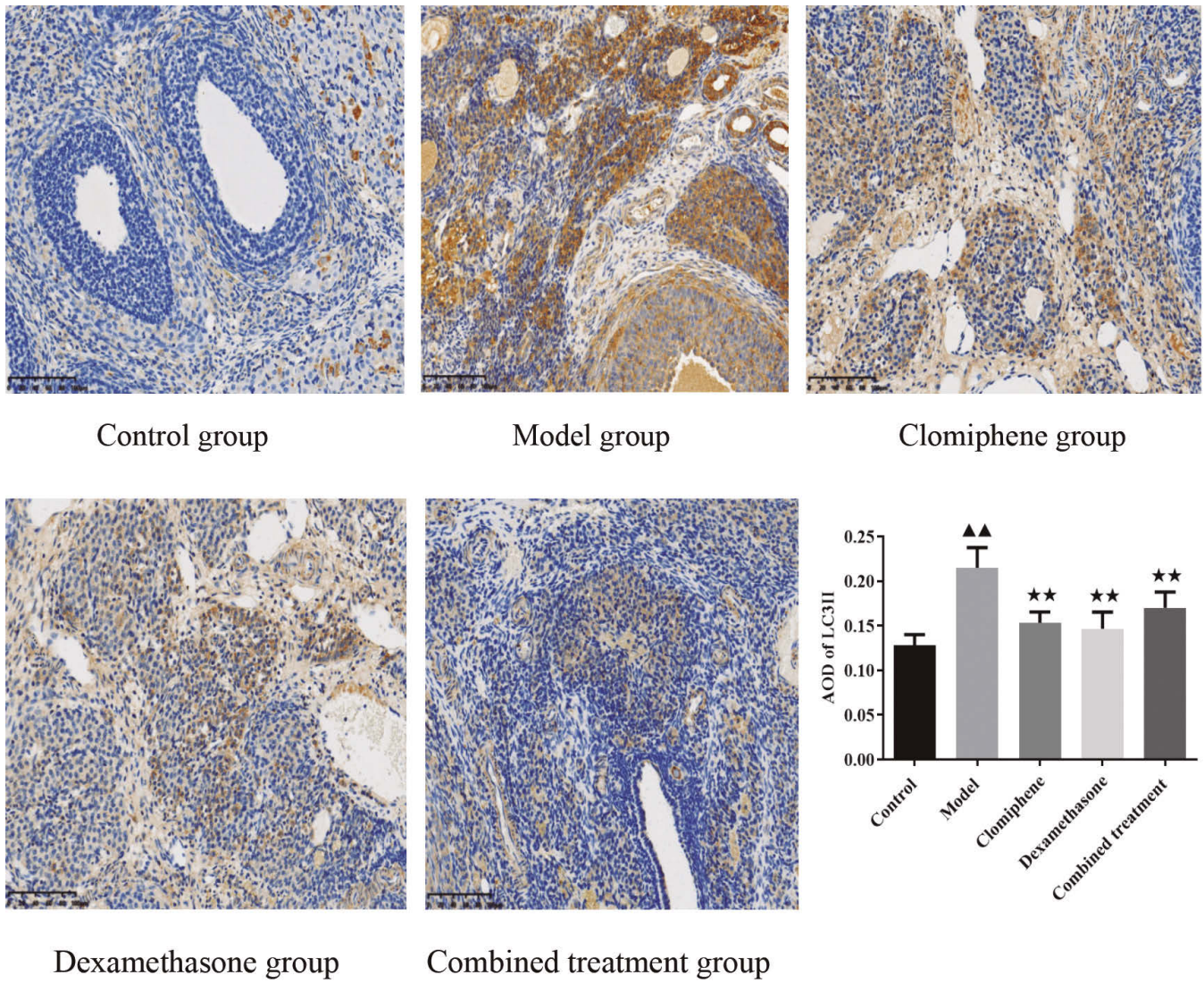


Fig. 8. The immunohistochemical staining for LC3II protein in the ovary. ▲▲$P<0.01$ vs. control group; ★★$P<0.01$ vs. model group; ★$P<0.05$ vs. model group.

Clomiphene combined with dexamethasone could significantly reverse the serum ROS, T-SOD, and CAT levels in PCOS rats ($P<0.05$). Meanwhile, there were significant differences in the levels of T and E2 among the five groups. The results suggested that clomiphene and dexamethasone could regulate the oxidative stress index and also had significant effects on hormones.

The mechanism of clomiphene and dexamethasone on PCOS

To investigate the mechanism of clomiphene and dexamethasone on PCOS rats, we performed immunohistochemistry, real-time PCR, and western blotting in this study. The immunohistochemical results showed that the expression levels of Bax, Caspase-3, LC3II, p-JNK, p-P38 MAPK, and P21 proteins were significantly increased compared with that of the control group, while P62 and Bcl-2 protein expression were significantly decreased (Figs. 5-12, $P<0.05$). The clomiphene and dexamethasone treatment could remarkably reverse the levels of apoptosis-, autophagy- and ROS-JNK/MAPK-P21 signaling pathway-related proteins. As shown in Figure 13a, we evaluated the mRNA levels of apoptosis-related genes by Real-time PCR, including Bax, Bcl-2, and Caspase-3. It showed that the expression of Bax and Caspase-3 in the ovaries of the model group significantly increased, whereas the Bcl-2 level was significantly decreased compared with the control group ($P<0.01$). After clomiphene and dexamethasone treatment, the mRNA levels of Bax, Bcl-2, and Caspase-3 were significantly reversed. We also assessed the expression levels of proteins associated with apoptosis, autophagy, and the ROS-JNK/MAPK-

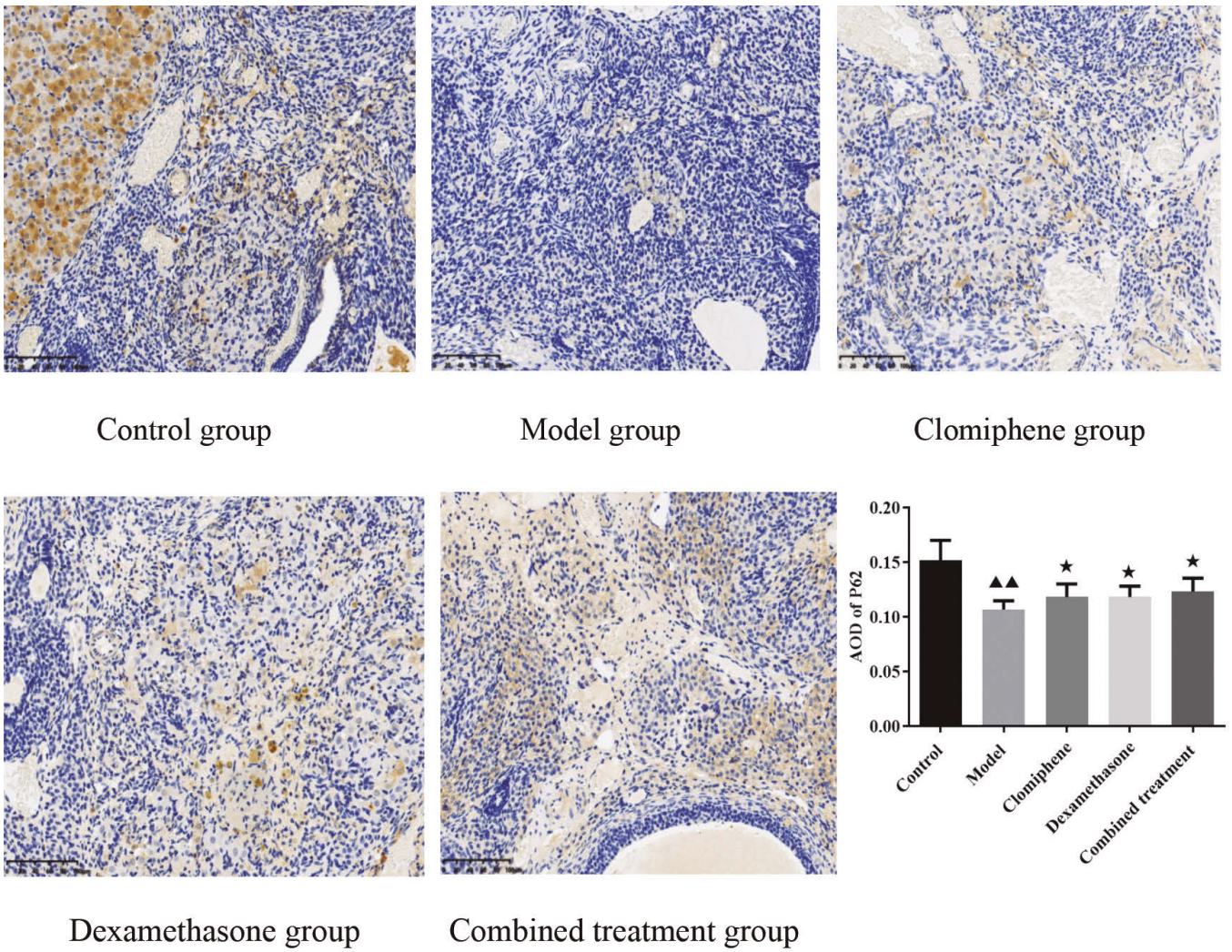


Fig. 9. The immunohistochemical staining for P62 protein in the ovary. ▲▲$P<0.01$ vs. control group; ★★$P<0.01$ vs. model group; ★$P<0.05$ vs. model group.

P21 signaling pathway in five groups. In Figure 13b, there were significant differences in the protein expression of Bax, Bcl-2, Caspase-3, LC3I, LC3II, P62, p-JNK, JNK, p-P38 MAPK, P38 MAPK, and P21 between the groups. After treatment with clomiphene and dexamethasone, the expression of apoptosis-related proteins (Bax, Bcl-2, and Caspase-3) was reversed (P<0.05). In PCOS rats, clomiphene and dexamethasone could reduce LC3 levels and increase P62 levels (P<0.05). Furthermore, clomiphene and dexamethasone significantly inhibited JNK/MAPK phosphorylation (P<0.05).

Discussion

PCOS is a common infertility disorder that affects a large proportion of the global population11. PCOS is a multifactorial and complex disease involving multiple pathways (Khan et al., 2019). Clomiphene is recommended as a first-line drug for PCOS treatment by several guidelines (Brown et al., 2009). Ege et al. observed an 89% ovulation rate in infertile women treated with clomiphene combined with letrozole (Sedigheh et al., 2009). *In vivo*, clomiphene induces growth and maturation of preovulation follicles (Olsson et al., 1986). Leonardo et al. also reported that rats treated with clomiphene had a decrease in the number of cysts, an increase in the number of ovarian follicles, and the presence of corpus luteum (Lombardi et al., 2021). Consistent with previous studies, we found that clomiphene improved the physiological and morphological functions of rat ovaries. However, clomiphene is significantly resistant and is associated with a reduced chance of pregnancy and live birth (Brown and Farquhar, 2016). Therefore, alternative and adjuvant treatments are necessary in the clinic.

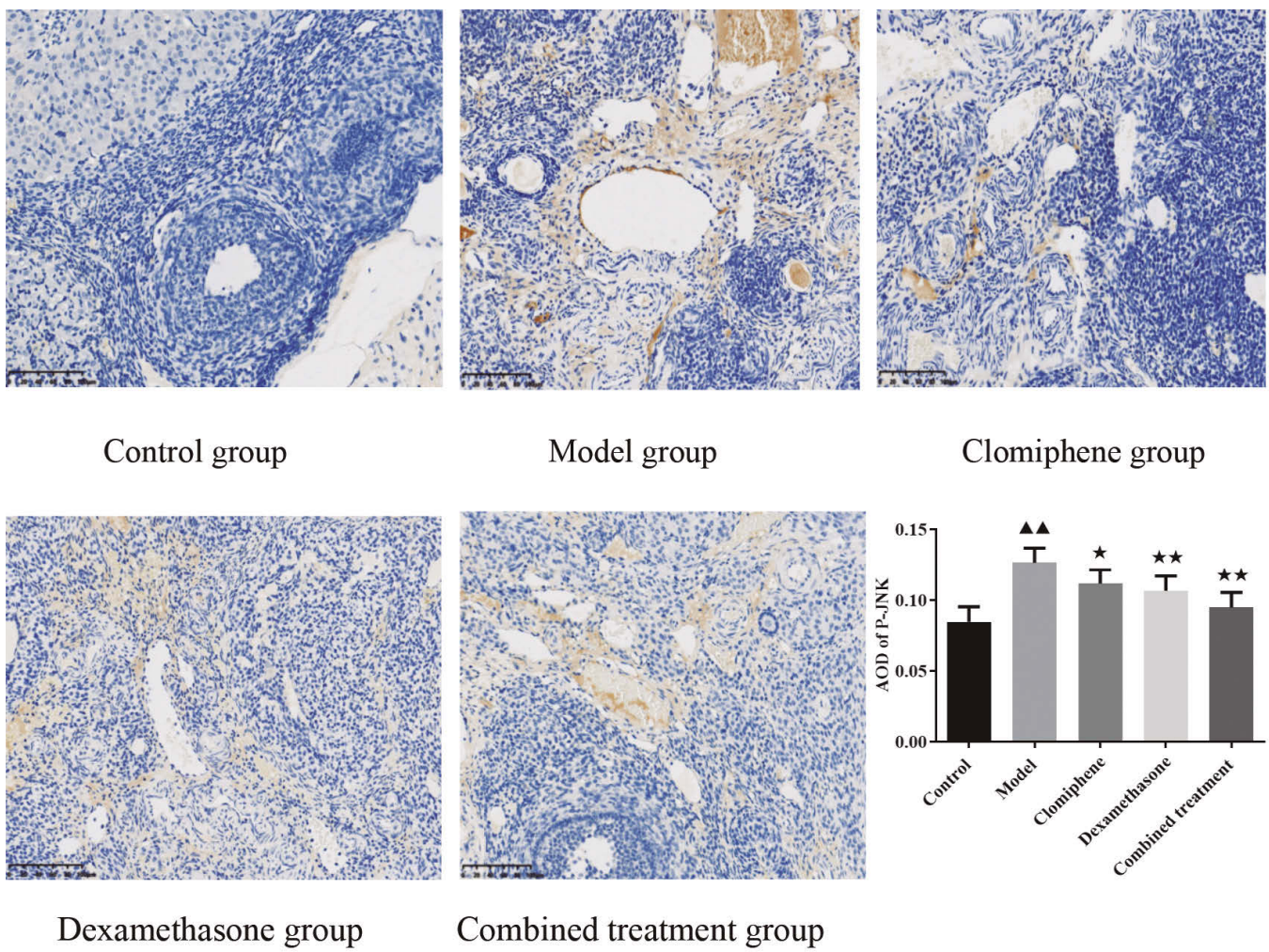


Fig. 10. The immunohistochemical staining for P-JNK protein in the ovary. ▲▲P<0.01 vs. control group; ★★P<0.01 vs. model group; ★P<0.05 vs. model group.

Dexamethasone is a synthetic glucocorticoid that suppresses the immune system, which has been increasingly studied in recent years (Dare et al., 2018). Previous studies reported that dexamethasone improves ovulation and pregnancy rates in women with PCOS (Risti et al., 2021). Joseph et al. found that high-dose dexamethasone caused multiple changes in the histological characteristics of the ovary and uterus, resulting in type I and type II anti-estrogenic effects (Dare et al., 2018). In our study, we compared the effects of clomiphene, dexamethasone, and their combination in rats with PCOS. The results indicated that dexamethasone enhances the therapeutic effect of clomiphene in PCOS. We observed that clomiphene combined with dexamethasone significantly promoted follicle development and reduced the number of autophagosomes, suggesting clomiphene and dexamethasone improve the morphology of the ovary by regulating autophagy.

Autophagy is an important mechanism to maintain the energy balance and function of cells. It has been reported that clomiphene and tamoxifen could regulate the expression of mTOR and LC3II related to autophagy in ovaries with PCOS (Kuşçu et al., 2022). In this study, we evaluated the key regulators of autophagy by immunohistochemistry and western blotting. After clomiphene and dexamethasone treatment, autophagosome levels and the ratio of LC3II to LC3I were

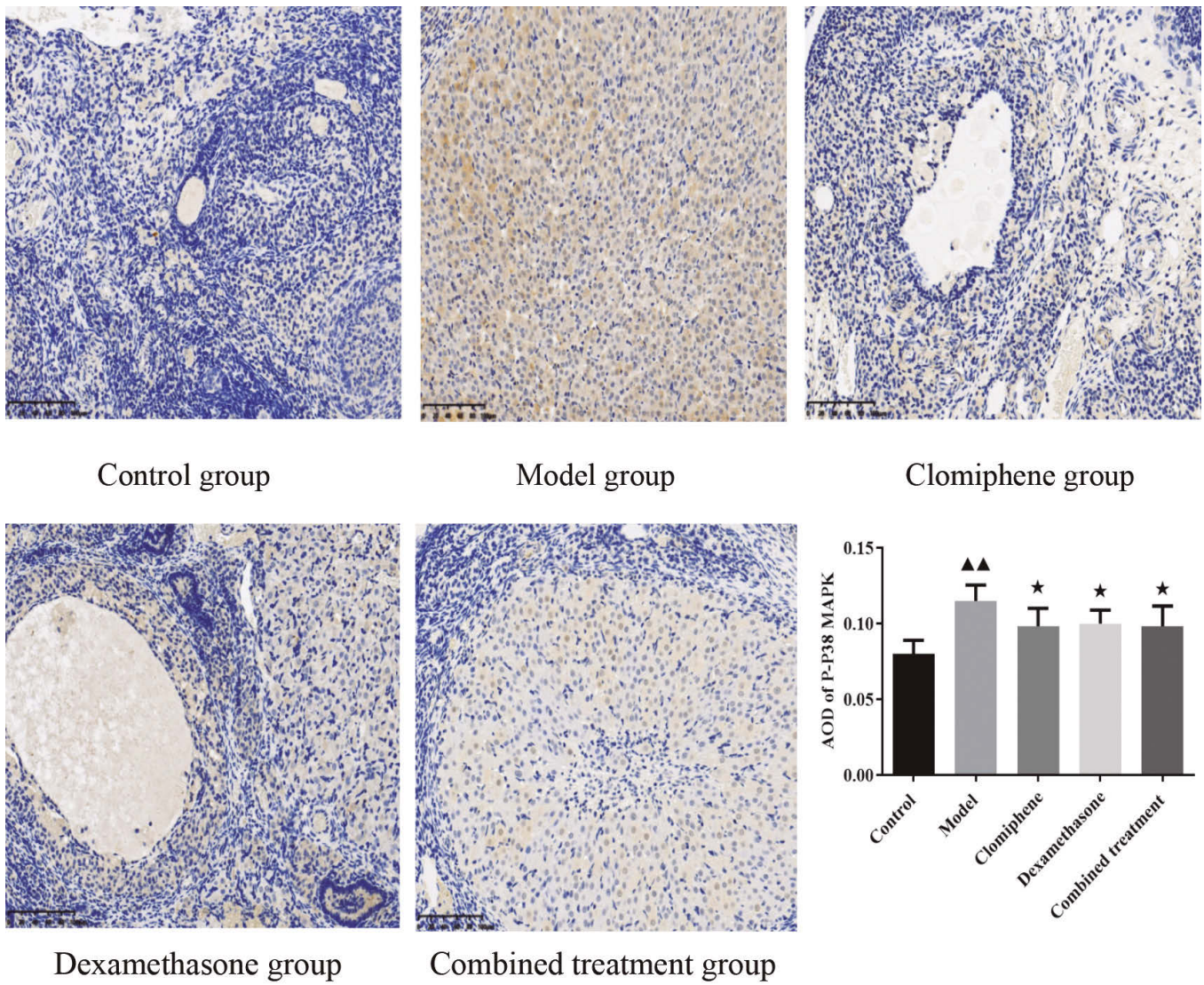


Fig. 11. The immunohistochemical staining for P-P38 MAPK protein in the ovary. ▲▲P<0.01 vs. control group; ★★P<0.01 vs. model group; ★P<0.05 vs. model group.

increased and P62 expression was decreased in PCOS rats. Similar results were observed in hypoxia-induced pulmonary arterial smooth muscle cells (Gui et al., 2017). Autophagy is considered a survival mechanism in most studies and is also believed to play a pro-apoptotic role (Kumariya et al., 2021). Yu et al. found that inhibition of autophagy further increased the percentage of apoptotic cells induced by soft substrates (Chen et al., 2021). In mouse ovarian granulosa cells, autophagy plays a role in lysophosphatidylcholine-induced apoptosis. Wei et al. reported that clomiphene promotes Transcription factor EB (TFEB)'s nuclear translocation and induces apoptosis by enhancing lysosomal membrane permeabilization (Li et al., 2019). We further assessed apoptosis levels in PCOS by the TUNEL assay. It showed that the increased number of ovarian cells in the PCOS group was significantly reduced in the treatment groups. We also evaluated the mRNA and protein expressions of apoptosis-related genes, including Bax, Bcl-2, and Caspase-3. These results revealed that clomiphene and dexamethasone could regulate autophagy and apoptosis in PCOS.

ROS hormone autophagy apoptosis

Ovarian hormone lies upstream of signaling

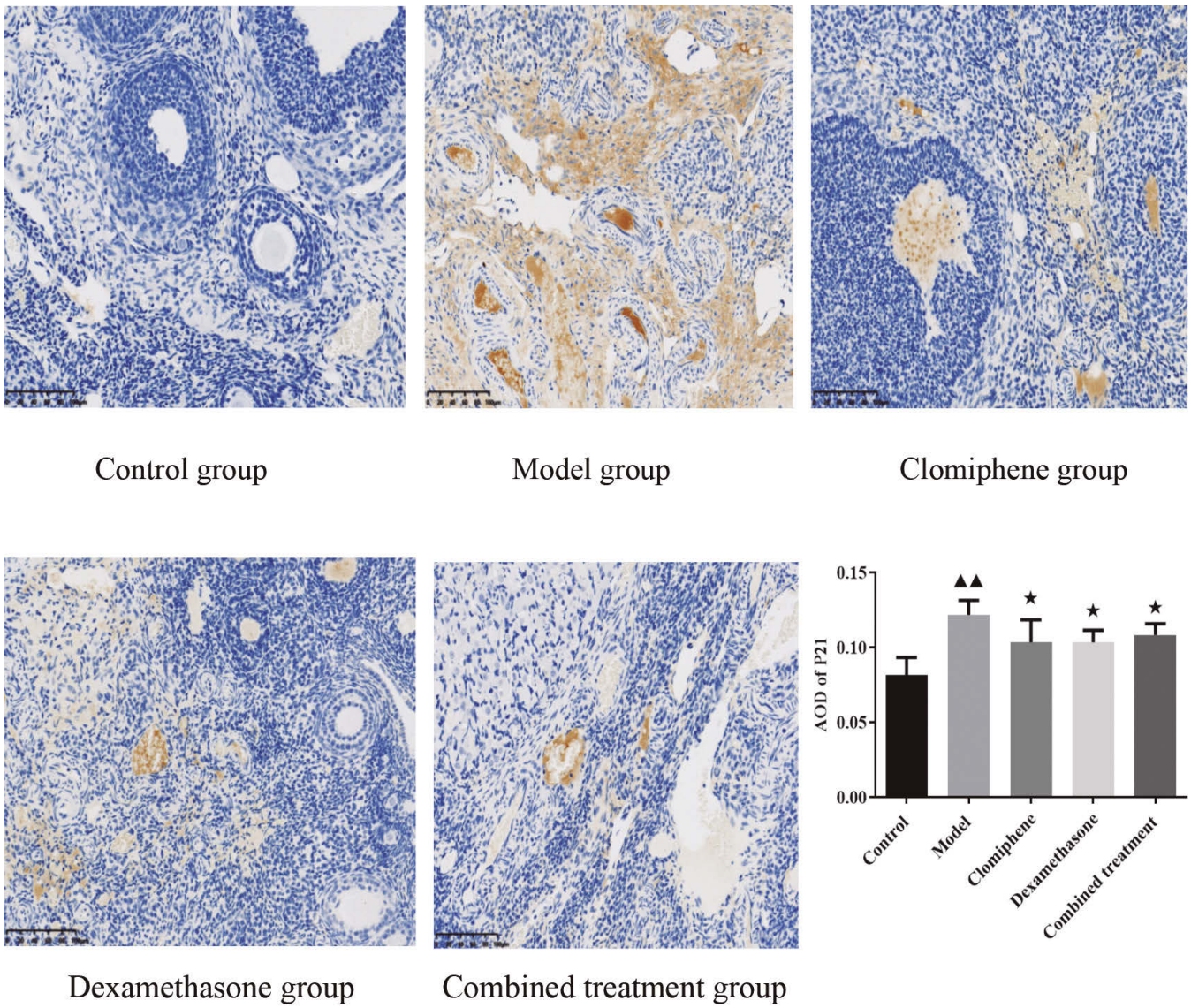
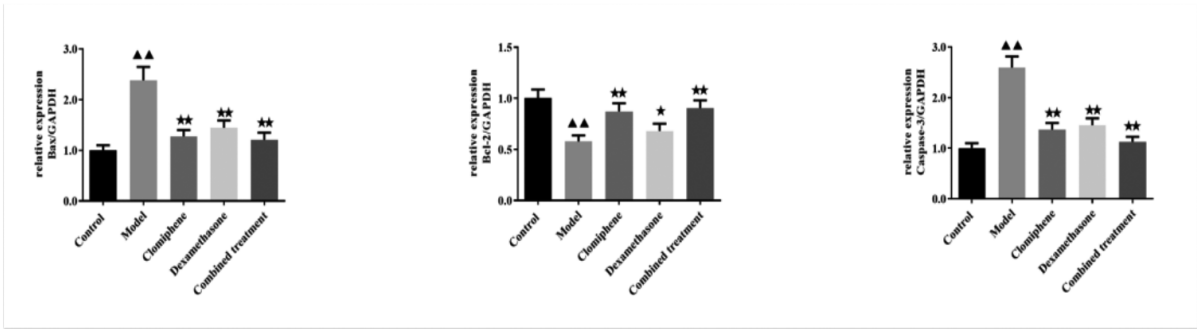


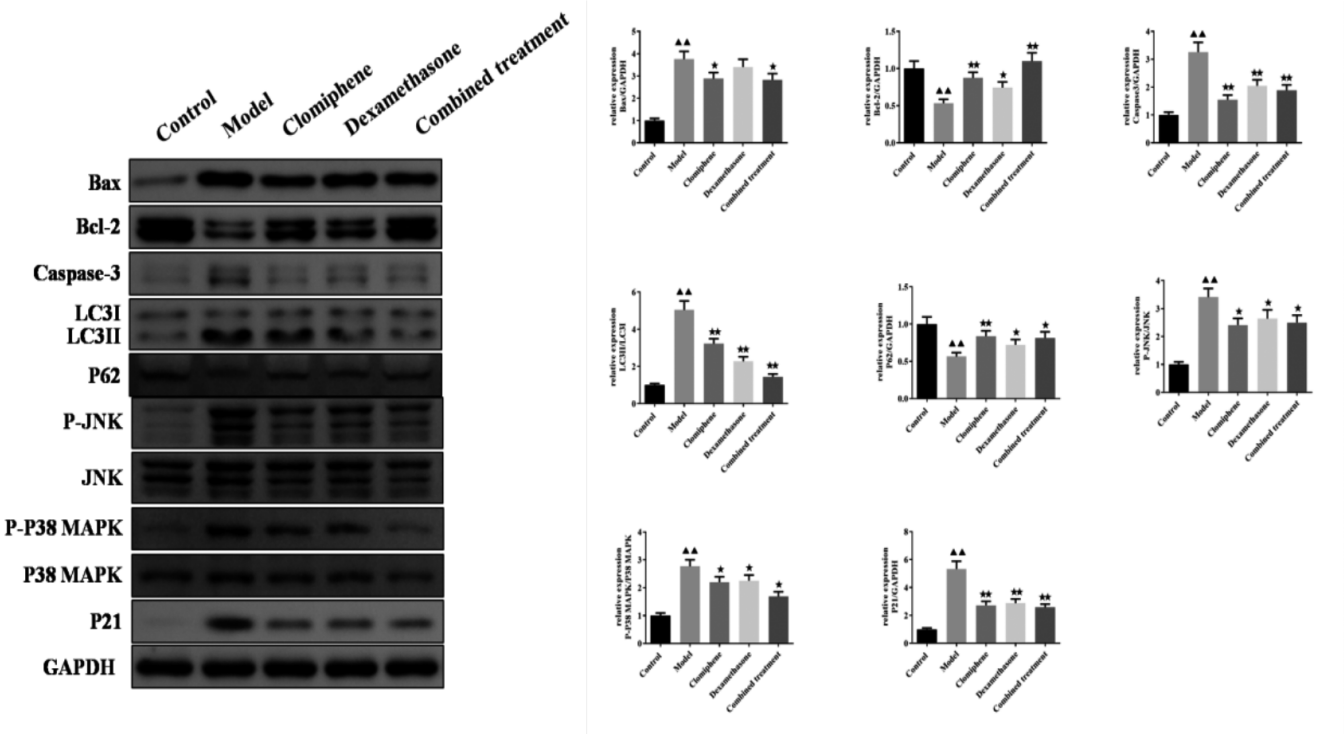
Fig. 12. The immunohistochemical staining for P21 protein in the ovary. ▲▲P<0.01 vs. control group; ★★P<0.01 vs. model group; ★P<0.05 vs. model group.

mediators regulating various aspects of uterine physiology and is also a regulator of autophagy. Previous studies showed that autophagy plays a pivotal role in sex steroid hormone biosynthesis in different animal models and human testis (Jha et al., 2021). Hui et al. found that estrogen could inhibit autophagy through CXCL12/CXCR4 interaction and NF-κB signal activation in endometrial stromal cells (Shen et al., 2021). In addition, the function of ovarian hormone secretion was closely related to apoptosis (Jin et al., 2011). Cui et al. reported that alginic acid displays toxicity in female reproduction by disrupting estrogen production and inducing oxidative, mitochondria-mediated apoptosis and autophagy (Cui et al., 2022). In this study, our results indicated that the treatment of clomiphene and dexamethasone has significant effects on hormone levels, apoptosis, and autophagy in PCOS rats.

The ROS-JNK pathway is considered a key factor in the regulation of autophagy and apoptosis. It has been



(a)



(b)

Fig. 13. The mechanism of clomiphene and dexamethasone on the treatment of polycystic ovarian syndrome. **a.** Real-time PCR; **b.** Western blotting. ▲▲P<0.01 vs. control group; ★★P<0.01 vs. model group; ★P<0.05 vs. model group.

reported that metformin could induce programmed cell death, including apoptosis and autophagy-related cell death, by activating ROS-JNK signaling (Li et al., 2020). Wang et al. showed that the ROS-JNK pathway promotes cell survival by activating pro-survival autophagy (Wang et al, 2017). Meanwhile, the MAPK pathway has been widely studied as the downstream signaling pathway of ROS, which is critically involved in cell proliferation, differentiation, and survival (Gong et al., 2019). In our study, we found that the treatment of clomiphene and dexamethasone on PCOS regulates oxidative stress and sex hormones. Moreover, we explored the potential mechanism of clomiphene and dexamethasone in PCOS rats. Previously, Jihene et al. found that the MAPK signaling pathway was influenced in both ER-positive and ER-negative breast cancer cells treated with clomiphene, which contributes to clomiphene-induced cell cycle arrest and cell death (Elloumi-Mseddi et al., 2015). In ovarian granulosa cells, advanced oxidation protein products may induce cell cycle arrest via the ROS-JNK/p38 MAPK-P21 pathway (Zhou et al., 2021b). Similarly, our results indicated that the therapeutic effects of clomiphene and dexamethasone on PCOS were through modulating the ROS-JNK/MAPK-P21 signaling pathway.

In conclusion, the present study demonstrated that clomiphene and dexamethasone can effectively improve the morphology of the ovary in PCOS rats. The therapeutic effects of clomiphene and dexamethasone in PCOS may be achieved by regulating apoptosis and autophagy through the ROS-JNK/MAPK-P21 signaling pathway. Hence, clomiphene combined with dexamethasone may be an effective therapeutic strategy for PCOS.

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