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Research

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Effects of Huoxue Xiaoyi Decoction on Apoptosis of Granulosa Cells in Endometriosis Rats

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Abstract

Background: Endometriosis(EM) is a common disease that occurs in reproductive age. 50% endometriosis patients is suffering from infertility. Follicular development is the main cause of endometriosis-associated infertility. Here the study based on apoptosis of granulosa cells during follicular development will explore the effect and the possible mechanism of Huoxue Xiaoyi Decoction (HXXYD) on apoptosis of ovarian granulosa cells in endometriosis model rats.

Methods: Thirty 8-week-old female SD rats were divided into four groups: blank group, sham-operation group, model group and HXXYD group. Blank group, sham-operation group and model group were given double-distilled water, while HXXYD group were given HXXYD for 15 days. After intragastric administration, blood samples from abdominal aorta of rats were collected to detect oxidative and antioxidative indexes including ROS, T-SOD, CAT. The morphology of follicles were observed by H&E staining and every stage of follicles were calculated. The location of granulosa cells and apoptosis related factors including Bax, Bcl-2, caspase-3 were stained by immunohistochemistry staining. The apoptosis of granulosa cells were stained by TUNEL staining and the rate of apoptosis were calculated. Apoptosis related proteins including p-JNK, Bax, Bcl-2, caspase-3 were detected by Western blot.

Results: The level of serum ROS decreased, and the levels of serum T-SOD and CAT increased in the HXXYD group. The number of secondary follicles increased in HXXYD group. The expression of Bax, caspase-3 in ovarian granulosa cells decreased and the expression of Bcl-2 increased in the HXXYD group with immunochemical staining. The apoptosis rate of ovarian granulosa cells in the HXXYD group decreased. The expression of p-JNK, Bax and caspase-3 protein decreased, the expression of Bcl-2 increased in the HXXYD group.

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Conclusions: These results indicate that HXXYD may improve the oxidative stress state, decrease the apoptosis of ovarian granulosa cells, and improve the development of follicles in endometriosis model rats through ROS-JNK signaling pathway.

Keywords: endometriosis, Huoxue Xiaoyi Decoction, granulosa cell, apoptosis.

Background

Endometriosis is an estrogen-dependent disease that occurs in women of reproductive age. The prevalence of endometriosis seems to be 10%-15%[1]. The association between endometriosis and infertility is complex. 50% endometriosis patients is suffering from infertility[2]. The fecundity rate in normal reproductive age couples without infertility is estimated to be around 15%-20%, while fecundity rate in women with untreated endometriosis is about 2% to 10%[3, 4]. Patients who received embryos derived from endometriotic ovaries showed a significantly reduced implantation rate and pregnancy rate. However, endometriosis patients have the same chances of implantation and pregnancy rate as other recipients when the oocytes came from donors without known endometriosis. This research had indicated that infertility in endometriosis patients may be related to alternations in the oocyte. The morphology and amount of ovarian granulosa cells are markers for the development of follicle and the outcome of pregnancy. Nakahara K first reported granulosa cells of endometriosis patients had more apoptotic bodies than infertility patients compared with male factors in fluorescence microscope. Granulosa cells of endometriotic ovary had more apoptotic bodies than that in healthy side. That suggested ovarian endometriosis cysts incurred apoptosis of granulosa cells, lead to atresia of follicle, finally affected the development of follicle[5].

Apoptosis is type I programmed cell death. During apoptosis, cells undergo morphological changes such as shrinking of the nuclei and mitochondria, blebbing of plasma membrane, continuously reduces cell size and functionality[6]. Apoptosis can be triggered by extrinsic and intrinsic pathway. Over ROS can cause oxidative stress and as a stimulation, result in apoptosis. Over ROS can be produced by ectopic endometrium of endometriosis as foreign body[7]. Then ROS-JNK signaling pathway that regulate apoptosis can be activated[8, 9]. Early research by our team have proved that Huoxue Xiaoyi Decoction (HXXYD) can improve the number of ovulation, fertilization, and increase pregnancy rate and live birth rate in endometriosis model rats during IVF-

ET[10, 11]. In addition, HXXYD also have antioxidant stress effect. Referring to apoptosis of follicle, HXXYD take more advantages than Bushen Zhuyun Decoction and Sequential Therapy[12]. So this study will explore the effect and the possible mechanism of HXXYD on apoptosis of ovarian granulosa cells in endometriosis model rats.

Materials and Methods

Reagents and Drugs

The rat reactive oxygen species (ROS) enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems, Inc.(Minneapolis, USA). The catalase (CAT), total superoxide dismutase (T-SOD) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu province, China).Rabbit anti-FSHR receptor polyclonal antibody (bs-20658R) was bought from Beijing Bioss antibodies Bioengineering Institute (Beijing, China). Rabbit anti-Bcl-2(BA0412), anti-Bax(BM3964), anti-caspase-3(BM3957) were purchased from Boster Biological Technology co. Ltd (Wuhan, Hubei province, China). The terminal deoxynucleotidyl transferase dUTP-nick-end labeling (TUNEL) kit (11684817910) was purchased from Roche (Basel, Switzerland). The phosphor-SAPK/JNK rabbit mAb (4668) was purchased from Cell Signaling Technology Inc.(Boston, USA). The Rabbit anti-Bax antibody(ab32503), Bcl-2 antibody(ab59348), caspase-3 antibody(ab13847) were bought from Abcam Biotechnology (Cambridge, USA). BCA protein assay kit (02912E) was bought from Beijing ComWin Biotech Co., Ltd.(Beijing, China). Estradiol valerate (1mg/tablet, J20171038) (Bayer, Germany).

Experimental Animals

Thirty eight-week female Sprague-Dawley rats (body weight 180-200g) were purchased from Beijing Vital River Laboratory Animal Technologies Co. Ltd (Beijing, China). The experiment was approved by the Laboratory Animal Ethics Committee in Guang'anmen Hospital China Academy of Chinese Medical Sciences (Ethics Number: IACUC-6AMH-2018-005). Animals were housed under a controlled environment at 22°C with 12h/12h light/dark cycle and fed with standard chow and water freely.

Preparation of Formula

All of herbs consist of HXXYD that were provided by Guang'anmen Hospital China Academy of Chinese Medical Sciences (Beijing, China). The raw herbs totally 130g were decocted with 10 times double-distilled for 3 times, 1 hour each time, and mix the decoction. Referring to equivalent dose, then the decoction was filtered and concentrated $10.71\text{g}/\text{kg}^{-1}\cdot\text{d}^{-1}$ by heating. The decoction was reserved in 4°C refrigerator.

The Rat Model of Endometriosis and Animal Treatment

Thirty rats were divided into four groups: a group of 6 rats served as the blank, a group of 6 rats as the sham-operation, a group of 3 rats as the donor, the remaining 15 rats as the recipient. The donor and recipient were given estradiol valerate $1\text{mg}/\text{kg}$ by intragastric administration. The method of allogeneic endometrium transplant was used to establish endometriosis model. The donor rats underwent laparotomy by midventral incision to expose the uterus, the uterus were removed, put into a Petri dish containing 0.9% NaCL solution, opened longitudinally and cut into $3\text{mm}\times 4\text{mm}$ pieces. Then donor rats sacrificed. After anesthetization, the recipient rats underwent laparotomy by midventral incision, the pieces uterus of donors were transplanted onto the outer surface of the bilateral abdominal wall and around ovaries with endometrium facing the peritoneal cavity one piece each side. A 5-0 nylon suture line was used to attach the graft. The sham-operation group were subjected to suture a knot onto the outer surface of the bilateral abdominal wall and around ovaries. The midventral abdominal incision was closed by 3-0 nylon suture line. After surgery, penicillin was injected for 3 days at 8 units per rat. Iodophors was used to sterilize incision and estradiol valerate $1\text{mg}/\text{kg}$ by intragastric administration was used to stimulate the development of transplanted endometrium for 5 days.

After 7 days of surgery, two recipient rats were randomly selected and underwent laparotomy to examine if models of experimental endometriosis have been successfully established, which was determined by observation of the volume of transplanted tissue enlarged, cystic formation on implant surfaces and adhesion with other tissues around. Obvious endometrium or mesothelial cells, gland was observed by Hematoxylin-Eosin (H&E) staining under microscope suggested the success of endometriosis model. Then the two recipient rats sacrificed. During the establishment of

endometriosis model, a recipient rat died of intestinal obstruction. After the establishment of endometriosis model successfully, the recipients were randomly divided into two groups as follows: the model groups and the HXXYD group, 6 rats in each group. The HXXYD group were given HXXYD ($10.71\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) intragastric for 15 days, the blank, sham-operation, model group were given an equal volume of double-distilled water intragastric for 15 days.

Specimen Collection

After treatment of 15 days, bilateral ovaries of rats were dissected. The left ovary was immediately fixed in 4% paraformaldehyde solution, and then embedded in paraffin for H&E staining and immunohistochemical staining. The right one was immediately put into liquid nitrogen for western blot. The blood of abdominal aorta of rats in each group were taken for measurement of ROS, T-SOD, CAT.

Measurement of ROS, T-SOD, CAT

Serum level of ROS were assayed by ELISA following the manufacturer's instructions and using Multiskan MK3 Microplate Reader (Thermo, USA) at wavelength of 450nm. Serum level of T-SOD were assayed by Hydroxylamine following the manufacturer's instructions and using UV-2000 Ultraviolet Spectrophotometer (JingHua Instruments, Shanghai, China) at wavelength of 550nm. Serum level of CAT were assayed by Spectrophotometer Detection following the manufacturer's instructions and using UV-2000 Ultraviolet Spectrophotometer at wavelength of 405nm.

Hematoxylin-Eosin Staining

The left ovary were embedded in paraffin along the biggest section of ovary, follicle section outward and sliced into $4\mu\text{m}$ thick serial 6 sections. The first section was used to H&E Staining for histological examination under a light microscope. The number of primordial follicle, primary follicle, secondary follicle, mature follicle and corpus luteum were measured by the VENTANA Image Viewer Software (version 3.1.4, Roche). Every stage of follicles were judged by the criterion of morphology. The primordial follicle is a structure enveloping a small primary oocyte within a single layer of squamous granulosa cells on a basal lamina. The primary follicle is an oocyte

surrounding by a single layer of cuboidal granulosa cells. The secondary follicles contain a fully grown oocyte surrounded by zona pellucida, 5 to 8 layers of granulosa cells, a basal lamina, and a theca interna and externa with associated blood vessels. The mature follicle form a cavity containing follicle fluid, with over 500µm diameter. Corpus luteum are contributed to by the membrana granulosa, theca interna, theca externa, and invading vasculature[13].

Immunohistochemical Staining

The third to sixth section of paraffin-embedded ovary were used to immunohistochemical staining for FSHR, Bax, Bcl-2, caspase-3. The slices were subjected to antigen retrieval with citric acid buffer and were then incubated with diluted primary antibody(FSHR 1:400, Bax 1:20, Bcl-2 1:50, caspase-3 1:20) for 60min 37°C. After washing in PBS 3 times for 3 min each, the sections were incubated with secondary antibody for 20min. Slices were washing in PBS again, and incubated with 0.01% 3,3-diaminobenzidine tetrahydrochloride (DAB) for 5min. Then sections were washed in PBS 3 times for 5 min each, counterstained in haematoxylin for 20s, dehydrated in absolute alcohol, cleared in xylene, and mounted in neutral resin for microscopic examination. Five vision were randomly selected for each slice, and photos were taken in high-power field (× 400 times) under microscope. The outcome was established by two independent observers using a standard light microscope by semi-quantitative evaluation combining the intensity with area of staining.

TUNEL Assay

A standard protocol for TUNEL was provided by the manufacturer. The second paraffin-embedded ovary section were washed in xylene twice for 5min each, followed by hydration with a series of 100%,95%,90%,80% and 70% ethanol solutions twice for 3min each. Then slides were incubated with proteinase K for 15min 37°C. After being washed twice with PBS 3min each. The tissue slides were incubated with 50µL TUNEL reaction mixture for 1h 37°C in humidified dark slide box. Then the slides were washed with PBS three times for 5min each time. Preliminary observation: one drop of PBS was added to observe the apoptosis under the fluorescence microscope. Add 30 µ L converter pod to the sample after the slide is dry, cover the slide and react in humidified dark slide box for 30min 37 ° C. The tissue slides were washed with PBS twice 3min each, add 50 µL DAB to the

tissue and react at room temperature 10 min. The tissue slides were washed with PBS three times 3min each. Haematoxylin staining for 10 min, dehydrated in absolute alcohol, cleared in xylene, and mounted in neutral resin for microscopic examination. Apoptotic cells were observed under light microscope and photos were taken. The apoptotic rate of follicle was calculated as the number of apoptotic follicles divide the total number of follicles \times 100%.

Western Blot Analysis

Proteins were extracted from right ovary. The protein concentration was determined using bicinchoninic acid (BCA) method according to the manufacturer's instructions. 27 μ g proteins from each sample were loaded onto SDS- PAGE. The primary antibody dilutions were 1 : 1000 for antibodies of p-JNK, Bax, Bcl-2, caspase-3, GAPDH. The relative protein levels were semi-quantitatively determined by the ratio with GAPDH using Tanon Image Software(version 4.0, Gel Image Systems, Inc., America). Protein levels of six animals per group were measured, respectively.

Statistical Analysis

All data were analyzed with SPSS 23.0 statistics software. Data conforming to the normal distribution and the homogeneity test of variance, comparisons of variables among groups were performed by one-way analysis of variance (ANOVA) and followed by least squares difference (LSD) tests. Data not conforming to the normal distribution, comparisons of variables among groups were performed by nonparametric test and followed by Kruskal-Wallis test. Data are presented as mean \pm standard deviation (SD). A level of $p < 0.05$ was considered statistically significant.

Results

Effects of HXXYD on the Serum Levels of ROS, T-SOD and CAT

The serum levels of ROS in the model group were higher than that of the blank group and the sham-operation group ($p < 0.05$). The serum levels of T-SOD and CAT in the model group were lower than that of the blank group and the sham-operation group ($p < 0.05$). Compared with the model group, the serum levels of ROS were significantly reduced and the levels of T-SOD, CAT increased in the HXXYD group ($p < 0.05$) (Figure 1).

Effects of HXXYD on Development of Follicle

There was every stage of follicles in the blank group, sham-operation group, model group and HXXYD group. The number of secondary follicles in the model group were less than that in the blank group and sham-operation group ($p < 0.05$). Compared with the model group, the number of secondary follicles were significantly increased ($p < 0.05$). There was no significant difference in the number of primordial follicle, primary follicle, mature follicle and corpus luteum in four groups ($p > 0.05$) (Figure 2 a,c).

Effects of HXXYD Immunohistochemical Staining of Bax, Bcl-2 and caspase-3

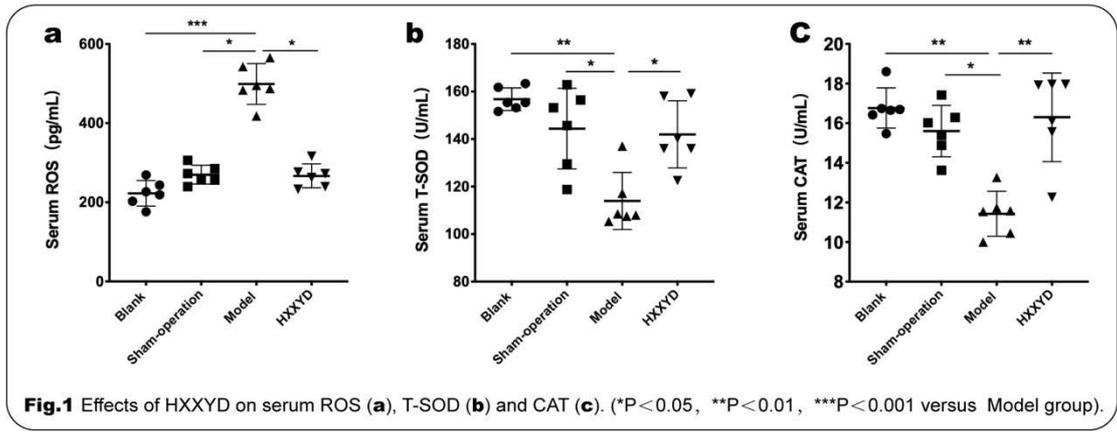
Immunochemical staining of rabbit anti-FSHR receptor polyclonal antibody was used to locate ovarian granulosa cells. Granulosa cells were around oocyte, with yellowish-brown staining (Figure 3a). The expression of Bax and caspase-3 in the model group were higher than that of the blank group and the sham-operation group ($p < 0.01$). The expression of Bcl-2 in the model group were lower than that of the blank group and the sham-operation group ($p < 0.001$). Compared with the model group, the expression of Bax and caspase-3 were significantly reduced and the expression of Bcl-2 increased in the HXXYD group ($p < 0.01$) (Figure 3b,c).

Effects of HXXYD on the Apoptosis of Ovarian Granulosa Cells

The rate of apoptosis in the blank group was lowest, next was the sham-operation group and the HXXYD group, the model group is highest. The rate of apoptosis of granulosa cells in the model group was higher than that of the blank group and sham-operation group ($p < 0.001$). Compared with the model group, the rate of apoptosis of granulosa cells was reduced ($p < 0.001$) (Figure 2b,d).

Effects of HXXYD on Expression of Protein of p-JNK, Bax, Bcl-2 and caspase-3

The expression of p-JNK, Bax and caspase-3 protein in the model group were higher than that of the blank group and the sham-operation group ($p < 0.05$). The expression of Bcl-2 in the model group were lower than that of the blank group and the sham-operation group ($p < 0.05$). Compared with the model group, the expression of p-JNK, Bax and caspase-3 protein were significantly reduced and the expression of Bcl-2 increased in the HXXYD group ($p < 0.05$) (Figure 4).



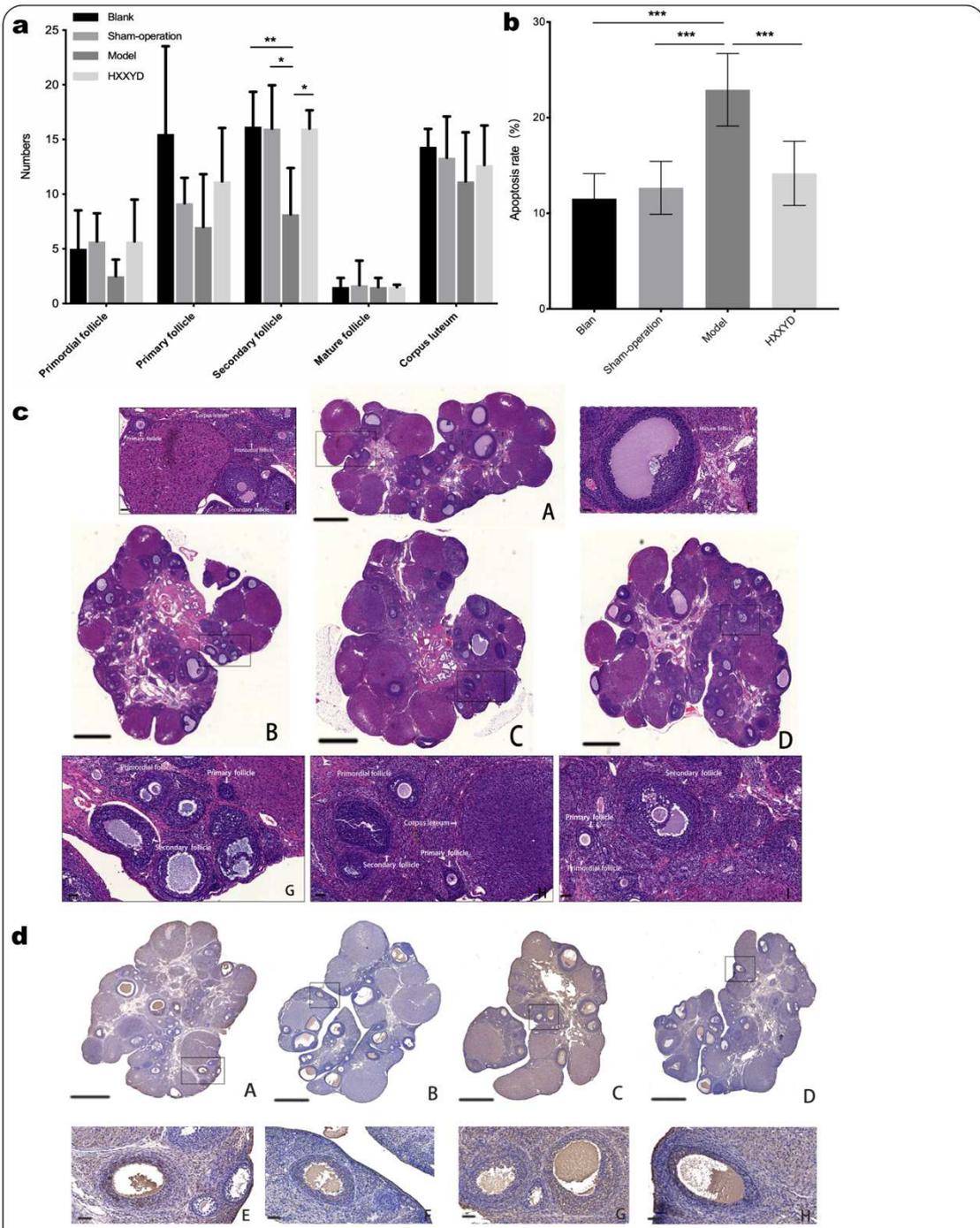


Fig.2 Effects of HXXYD on development of follicles and corpus luteum (a), and apoptosis of granulosa cells (b) (n=6). (*P<0.05, **P<0.01, ***P<0.001 versus Model group). H&E staining: the morphology of follicles and corpus luteum (c). Blank group (A), Sham-operation group (B), Model group (C), HXXYD (D). Scale bar=1cm. The morphology of primordial follicle, primary follicle, secondary follicle, mature follicle and corpus luteum (E-I). Scale bar=50µm. TUNEL staining: apoptosis of granulosa cell (d). Blank group (A), Sham-operation group (B), Model group (C), HXXYD (D). Scale bar=1cm. Apoptotic follicle (E-H). Scale=50µm.

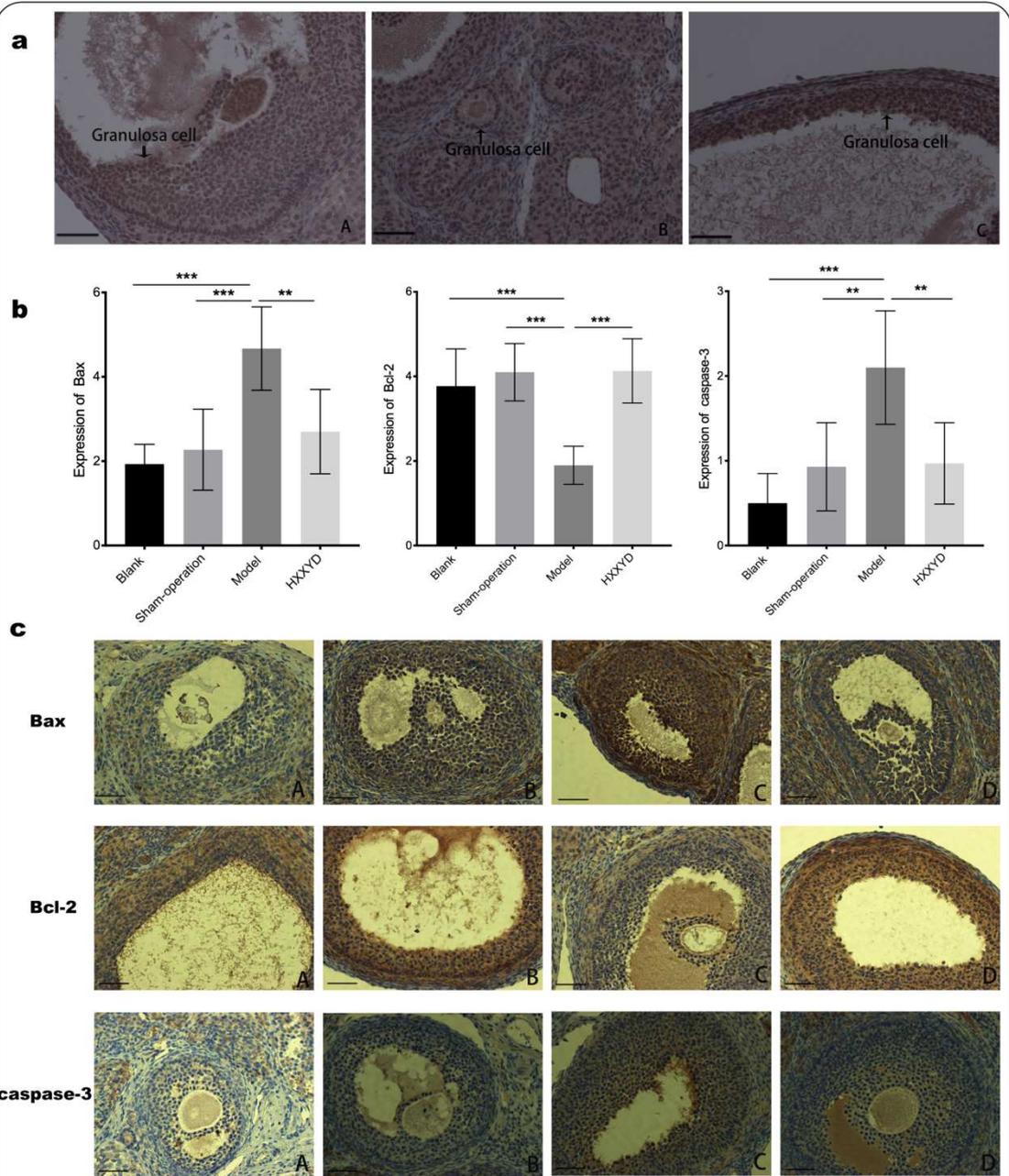
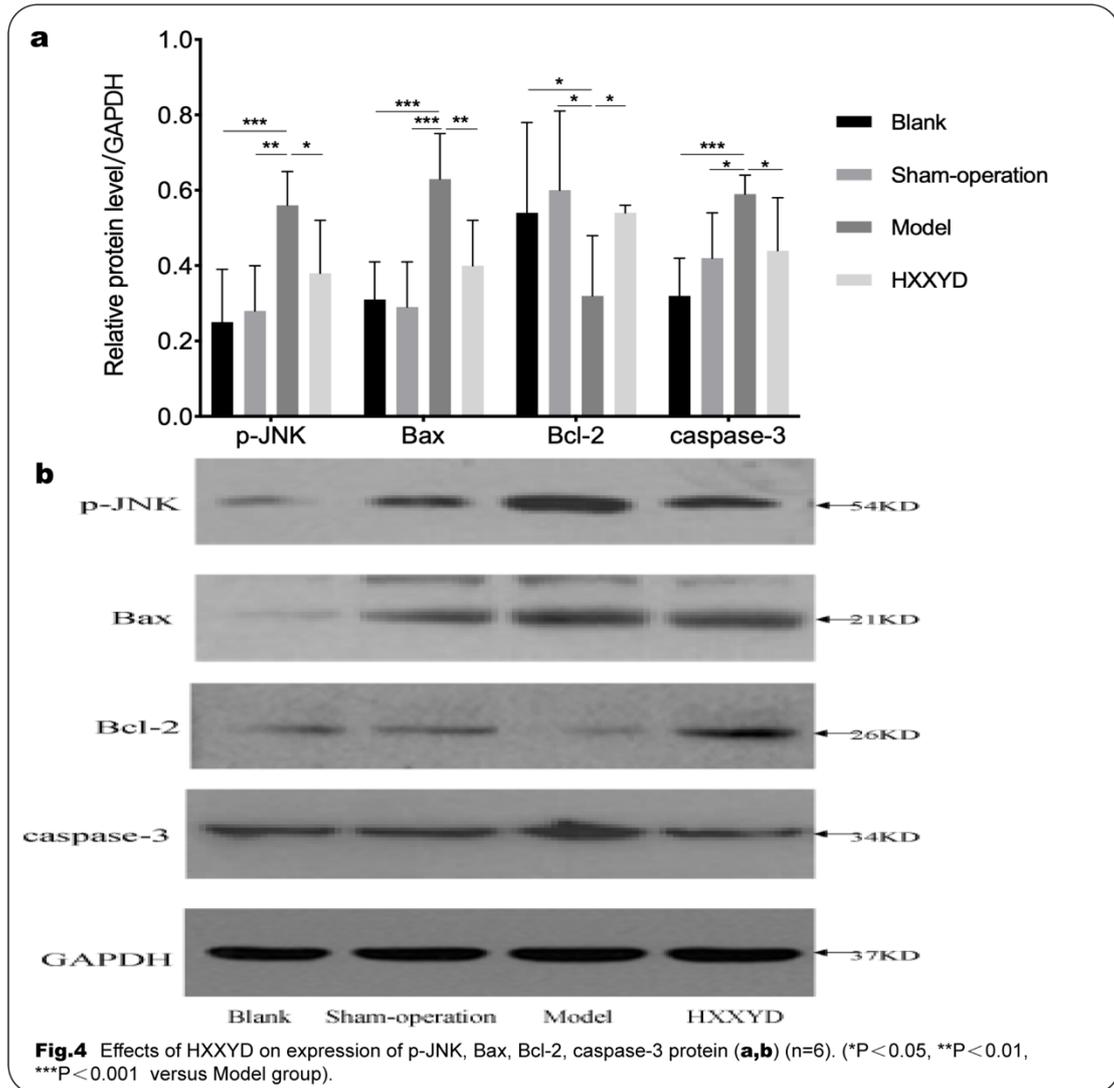


Fig.3 Effects of HXXYD on expression of Bax, Bcl-2, caspase-3 of granulosa cells (**b**) (n=6). (**P<0.01, ***P<0.001 versus Model group). The location of granulosa cells (**a**). Scale bar =50 μ m. Immunohistochemical staining of Bax, Bcl-2 and caspase-3(**c**). Blank group (**A**), Sham-operation group(**B**), Model group(**C**), HXXYD(**D**).Scale bar =50 μ m.



Discussion

Endometriosis is defined as the presence of endometrial tissue exterior to the uterine cavity. The pathophysiology of endometriosis is unclear. To elucidate the pathophysiology of endometriosis, several theories have been suggested. The theory of Sampson first described in 1927 is the most prevalent theory. The theory suggests the existence of retrograde menstruation. Retrograde menstruation, that is ectopic endometrium, as “foreign matter” exist in pelvic and hemorrhage with menstrual cycle. Cyclic hemorrhage of ectopic endometrium cause inflammatory reaction, immune cell infiltration, pro-inflammatory factor production, iron overload, and increase metabolism of blood cell, these can produce more ROS and deplete more antioxidant enzymes, finally result in oxidative stress (OS)[14]. The elevated OS in endometriosis may either be a cause or a consequence

of the pathophysiology of endometriosis[15]. Compared with non-endometriosis patients, the level of serum ROS was higher, the activity of plasma SOD reduced for endometriosis patients[16, 17]. The imbalance of oxidation and antioxidation in peritoneal fluid of endometriosis patients cause abnormal development of follicle, damage of DNA, cytoskeleton and cell membrane, low quality of follicle, and even infertility[17, 18]. Oxidative stress may be targeted as the goal of endometriosis treatment with both a relief of symptoms and improved fertility[15]. It has been shown that the model rats of endometriosis exist the imbalance between oxidation and antioxidation in this study. Moreover, the reaction of oxidative stress in HXXYD treatment has alleviated. The study proved HXXYD have the function of antioxidant.

HXXYD was created by Professor Zhao inheriting the three types of syndrome differentiation of endometriosis from Professor Li Guangrong for treating the syndrome of qi stagnation and blood stasis of endometriosis. The main medicines in HXXYD include *Salvia Miltiorrhizae*, *Radix Bupleuri*, *Cyperus*, *Rhizoma Curcuma*, *Radix Paeoniae Rubra*, *Endothelium Corneum*, etc. The main chemical component of *Salvia Miltiorrhizae* is water-soluble salvianolic acids. The water extract has obvious antioxidant capacity in vivo, and can clear superoxide anion, hydroxyl radicals, inhibit lipid peroxidation, improve the SOD activity and the level of GSH, reduce MDA in liver tissue of oxidative stress mice[19, 20]. Saikosaponin is the effective component of *Radix Bupleuri*. Saikosaponin have the functions of anti-oxidation, anti-inflammatory, anti-apoptosis[21, 22]. It can reduce the level of MDA, enhance SOD activity in liver tissue and inhibit liver damage caused by oxidative stress through anti-oxidant stress[23]. Flavonoids of *Cyperus* are the effective components of *Cyperus*, which have been proved to have strong antioxidant activity in vitro and in vivo to reduce the oxidative damage of tissues and cells[24]. *Curcuma polysaccharide* and *Endothelium Corneum polysaccharide* have the function of anti-oxidation. Therefore, the whole prescription have a strong antioxidant effect.

This study established endometriosis rat model and proved the existent of oxidative stress. The production of ROS in endometriosis model rats is excessive. ROS, as the upstream of JNK signaling pathway, is the target of mitochondrial apoptosis[25]. Excessive ROS can active JNK signaling pathway, and phosphorylation of Bcl-2 family through ROS-JNK signaling pathway[26,27]. Then phosphorylation of Bcl-2 family can inhibit the expression of anti-apoptotic Bcl-2, promote the

expression of pro-apoptotic Bax, release cytochrome C to the cytoplasm, and activate the important apoptotic executioner caspase-3[28,29], finally induce apoptosis of granulosa cell, atresia of follicle, affect quality of follicle and development of follicle. HXXYD can reduce the production of ROS through the function of anti-oxidation, block ROS -JNK signaling pathway, promote the expression of Bcl-2, inhibit the expression of Bax, reduce the activation of caspase-3, therefore reduce the apoptosis of granulosa cell and improve follicular development.

Conclusion

In summary, the present study has shown that HXXYD may improve oxidative stress state, decrease the apoptosis of ovarian granulosa cells, and improve the development of follicles in endometriosis model rats through ROS-JNK signaling pathway.

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Authors' Contributions

Professor Rui-hua Zhao designed the experiment. Guang Shi and Yong Liu preformed the experiment and data analysis, contributed equally to this work and should be considered as co-first authors. Guang Shi wrote the manuscript. Wei-wei Sun was responsible for giving guidance for experiment. Qian Han, Yong-jia Zhang, Jin-hao Qu, Xin-chun Yang, Ting Xiong give help for specimen collection.

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Availability of data and materials

The data used or analyzed during the study can be available on journal request from corresponding author.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Figures

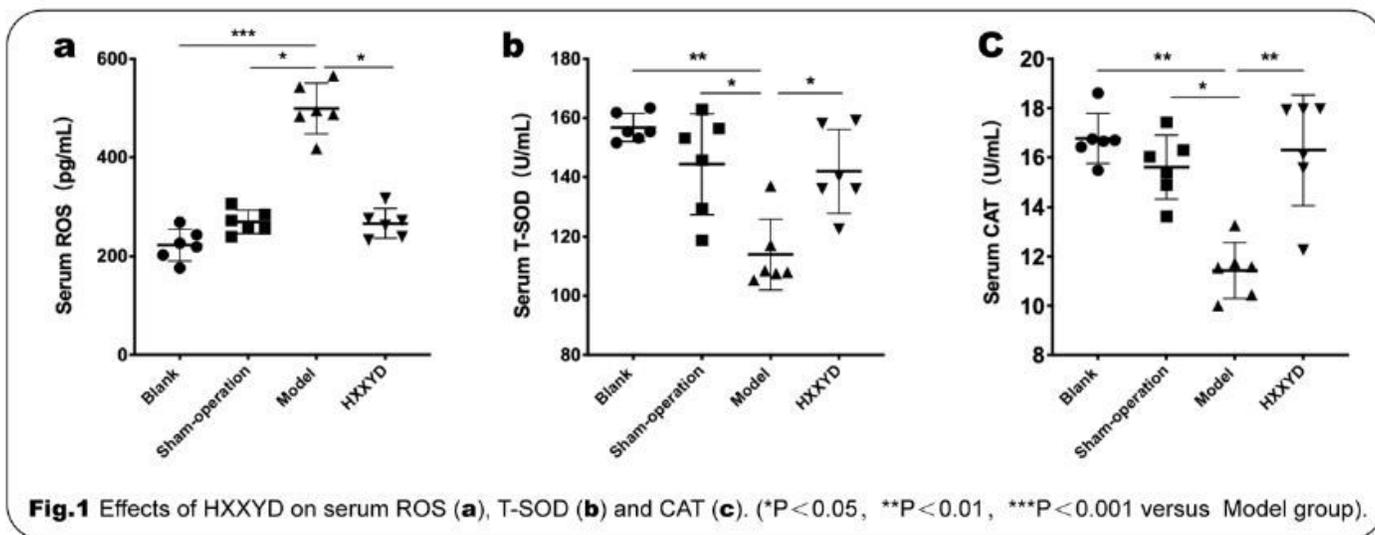


Figure 1

Figure 1

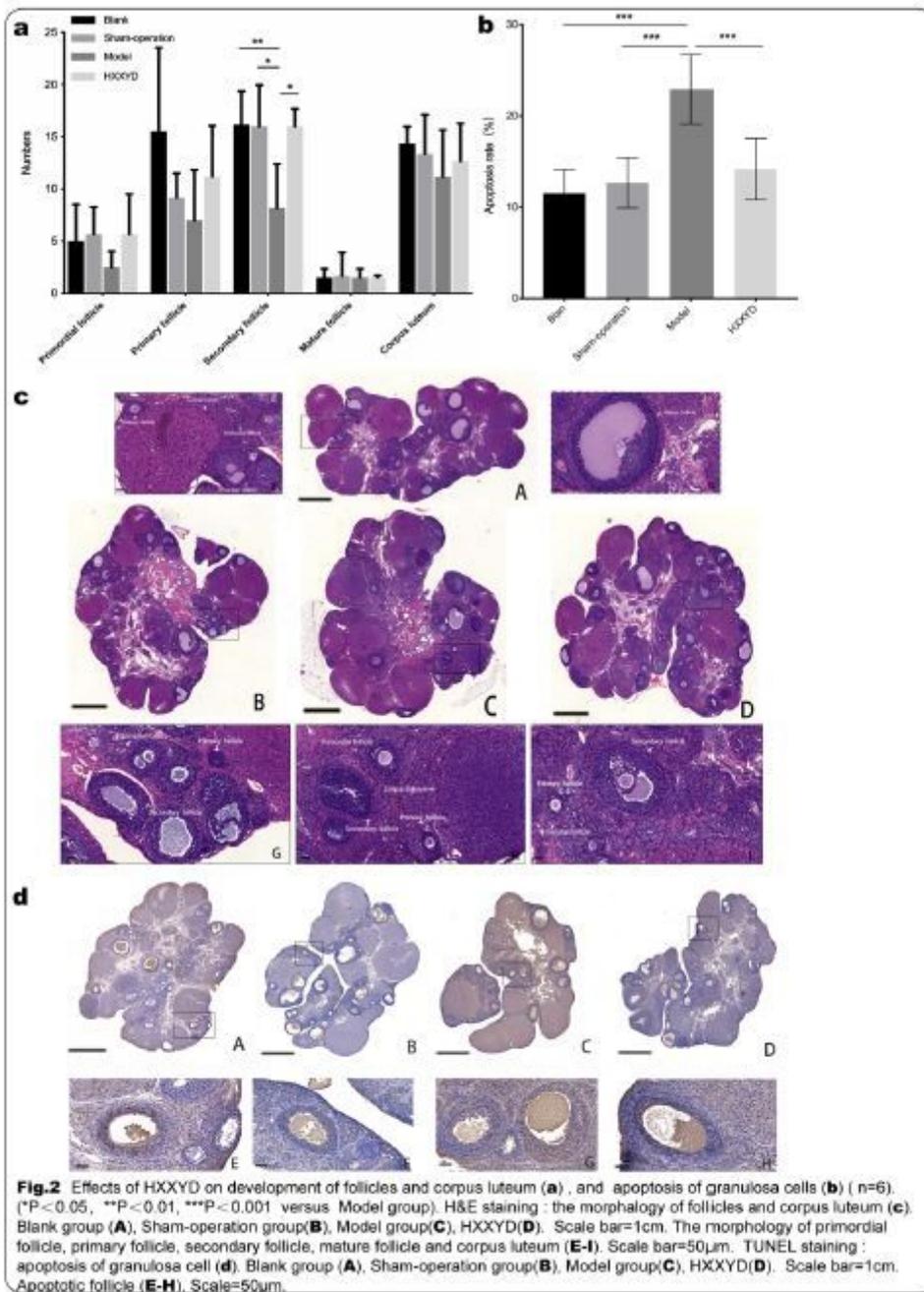


Figure 2

Figure 2

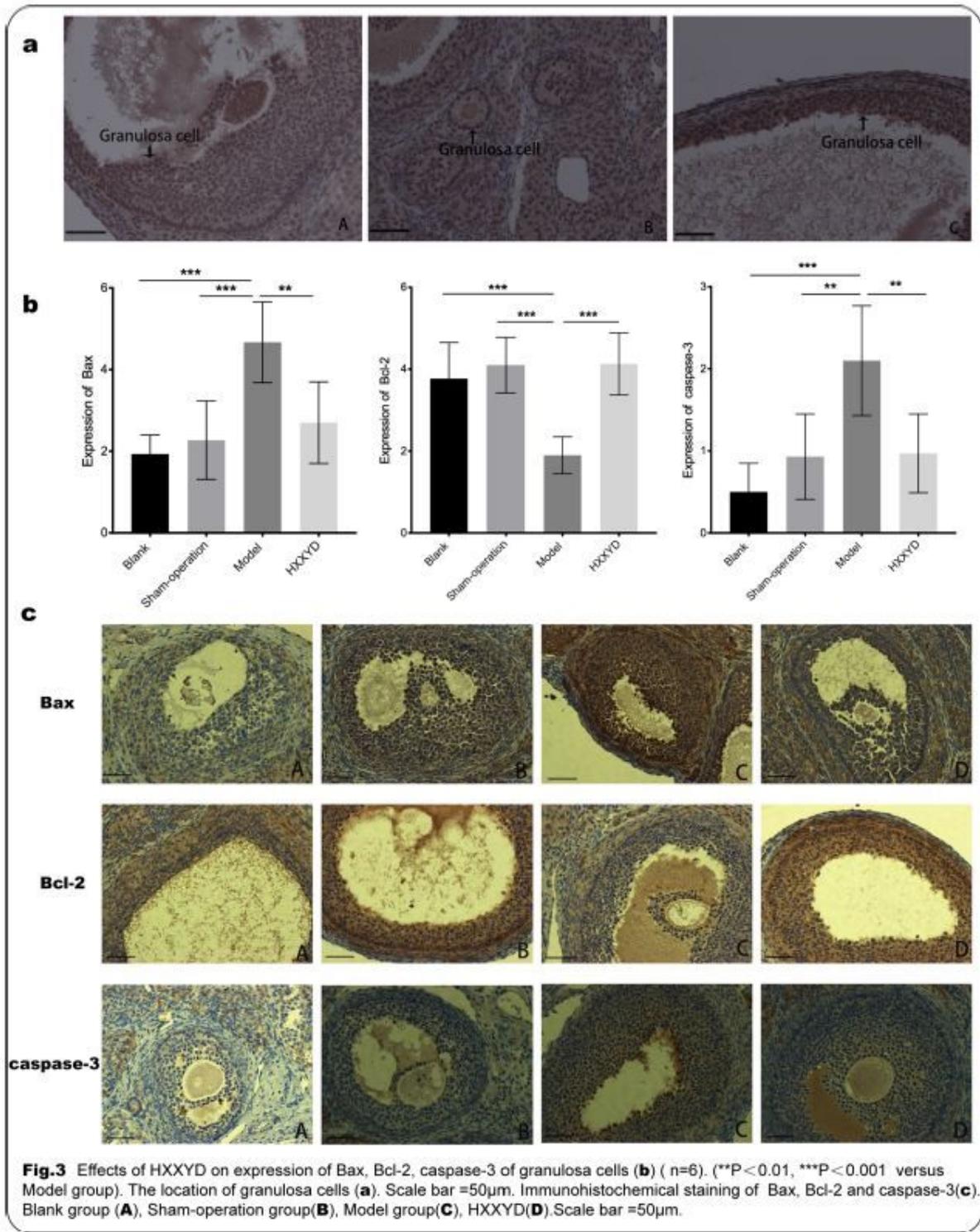


Figure 3

Figure 3

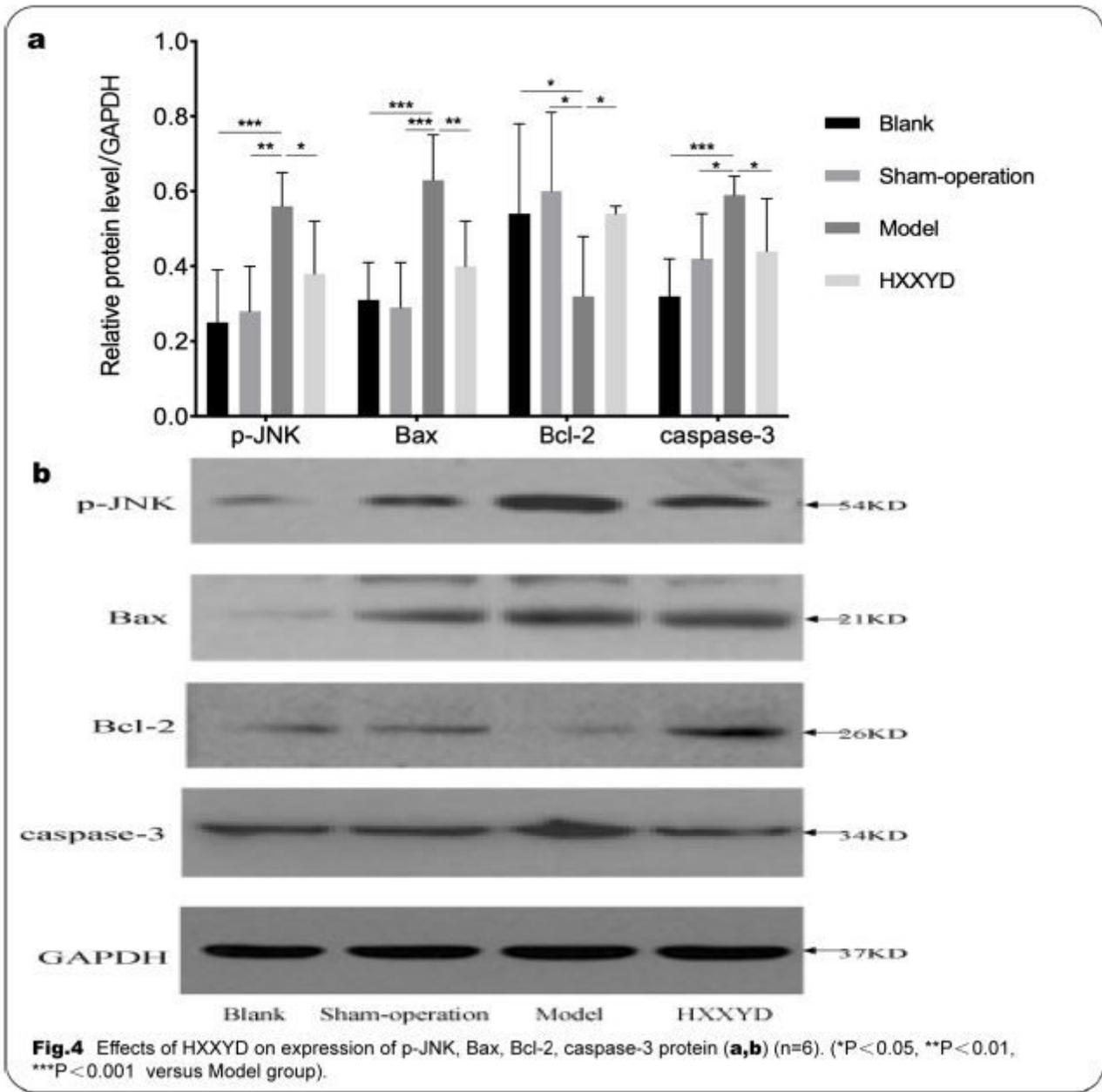


Figure 4

Figure 4