

Protective Effects of Berberine on Rat Model of Polycystic Ovary Syndrome Through the PI3K/AKT Pathway

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Research

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Abstract

Background: Berberine (Ber), a Chinese herbal monomer has been reported to exhibit an array of pharmacological activities relating to the lowering of blood glucose and the treatment of polycystic ovarian syndrome (PCOS). However, the related mechanism underlying these activities is not clear. In the current study, we aimed to elucidate the effect of Ber on rat model of PCOS by PI3K/AKT signaling pathway.

Methods: A letrozole-induced rat PCOS model and primary ovarian granulosa cells model to explore the protective effect and the mechanism of action of Ber. In PCOS rats, the values of fasting blood glucose (FBG) insulin resistance (HOMA-IR), fasting insulin (FINS) values and the serum hormone levels were measured. The ovarian tissues were stained with HE and TUNEL to undergo pathological and apoptosis examination. Moreover, the effect of Ber on proliferation and apoptosis of granulosa cells was detected by CCK-8 and flow cytometry. And then the influence of Ber on granulosa cells was confirmed by blockade of the PI3K/AKT pathway. In addition, the modulatory effect on the expression of related proteins by blockade of PI3K/AKT was demonstrated via western blot.

Results: We found that Ber was able to recover the serum hormone levels and improve insulin resistance (IR) in PCOS rat model. The morphological lesion and apoptosis of the ovary was also restored with the Ber treatment. Blockade of the PI3K/AKT pathway attenuated the influences of Ber on proliferation and apoptosis of granulosa cells. Due to this, we discovered a correlation between the PI3K/AKT signaling pathway and the beneficial effects of Ber, demonstrating that its application could alter the expression levels of key proteins in the PI3K/APT pathway.

Conclusion: The beneficial effects of Ber on PCOS, including alter serum hormone levels, recover ovary morphological lesions, improve IR, improve cell viability and inhibition of apoptosis, which is mediated through the PI3K/AKT pathway.

Introduction

Polycystic ovarian syndrome (PCOS) is an endocrine syndrome characterized by persistent anovulation, hyperandrogenemia or insulin resistance (IR) [1]. It is the most common cause of infertility in women of childbearing age and affects approximately 4% ~18% of all reproductive-aged women in the world [2]. At the same time, PCOS also has been defined as a metabolic syndrome, often associated with metabolic disorders such as obesity, hyperlipidemia, and at a higher risk for type 2 diabetes, hypertension, and other cardiovascular diseases [3, 4]. Clinically, the main cause for reproductive and metabolic abnormalities in women with PCOS are hyperandrogenism and IR [5]. However, the pathogenesis and mechanism of PCOS have not been elucidated.

Ber is an isoquinoline compound derived from many different plants such as *Coptis chinensis Franch.* and *Phellodendron chinensis var. glabriusculum*. It has been used in traditional Chinese medicine for years, and is shown to be effective against IR and obesity, particularly against diabetes type 2 and

hypercholesterolemic [6, 7]. There is recent evidence indicating that Ber offers promise for treating PCOS-associated IR. Several studies indicate that Ber has similar effects as metformin on improving hyperglycemia and is more beneficial for PCOS treatment [8]. And it has additional effects on body composition and hyperlipidemia in women with PCOS when compared with metformin [9].

PI3K/AKT signaling pathway is one of the most important insulin signaling pathways, which is not only closely related to IR, but also plays an important role in cell growth, proliferation, movement, invasion, inhibition of apoptosis and angiogenesis [10–12]. At present, several studies indicated that Ber inhibits the mTOR pathway with abnormally high activity in the state of IR mainly by activating AMPK activity, so as to mediate the insulin signaling pathway and improve IR [13, 14]. However, it remains uncertain whether Ber alter PCOS IR, promotes follicular cell proliferation and anti-apoptotic effects by regulating PI3K/AKT signaling pathway.

In the present study, a rat model of PCOS was constructed by continuous gavage of letrozole solution and coupled with high-fat diet, then the ovarian granulosa cells of the model rats were extracted and cultured. Effects of Ber on IR, serum hormone levels and ovary morphological lesions in PCOS rats were explored, and the direct effects of Ber on proliferation and apoptosis of granulosa cells were also investigated. In addition, LY294002 (a PI3K specific inhibitor) and MK-2206 (an AKT specific inhibitor) were utilized to further verify the actions of PI3K/AKT signaling pathway on granulosa cell proliferation and apoptosis with its influence on the expression of related proteins.

Methods And Materials

Animals

Female Sprague-Dawley (SD) rats (21-23-day-old), were purchased from the Centre of Experimental Animals at the Shanghai Sippr-BK laboratory animal Co. Ltd. (Shanghai, China). All the rats were housed in standard room temperature of $23 \pm 2^{\circ}\text{C}$ and humidity of 55 ~70% under a 12 h light/dark cycle with access to food and water. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal experiments were performed according to the guidelines of laboratory animal care and were authorized by the Ethic Committee of Zhejiang Traditional Chinese Medicine University (Hangzhou, China).

In Vivo Study

PCOS model generation

After a week of adaptive feeding, fifty female SD rats were randomly assigned into 5 groups of 10 rats each: the control, PCOS, PCOS+ L-Ber (95 mg/kg), PCOS+ H-Ber (190 mg/kg) and metformin hydrochloride treated groups (PCOS+MH) (50 mg/kg) [15]. The PCOS, PCOS+H-Ber, PCOS+L-Ber and PCOS+MH groups were administered by a gavage of 1.0 mg/kg (0.4 mL) of letrozole dissolved in 1% carboxymethyl cellulose (CMC) solution once daily for 23 consecutive days. The control group was

administered a gavage of CMC solution. From 24 days, PCOS+H-Ber and PCOS+L-Ber groups were administered by a gavage of Ber, and PCOS+MH group was administered by a gavage of MH for 4 consecutive weeks. As a control, equivalent normal saline was injected subcutaneously into rats in the control and PCOS groups. The control group rats were fed with conventional feed pellets while the PCOS, PCOS+ H-Ber, PCOS+ L-Ber and PCOS+MH groups were fed with high-fat diet for 30 days. From the thirty-one days, vaginal smears were performed on each group, and their estrous cycles were observed for 15 consecutive days (about three sequential cycles). The fasting blood glucose (FBG), serum testosterone (T), insulin (INS) in rats were measured by tail vein blood collection after the model rats lost their emotional cycle. To evaluate the success of model-building in rats with complete emotional cycle and the value of serum T, INS.

Specimen collection

At 24 hours after the last administration, blood from the rat aorta ventralis was centrifuged at 3,000 rpm for 10 min, following supernatant collection, and put the serum in refrigerator at -80°C for subsequent use. Besides, the ovarian tissues collection: vaginal smear was conducted on 6-weeks old rats, and the tissue collection was conducted after fasting for a day. Chloral hydrate (1%, 30 mg/kg) was performed on rats for anesthesia, and then bilateral ovaries were taken out. Further, one side of the ovary was rapidly fixed by 4% paraformaldehyde and another side was kept in refrigerator at -80°C for next use.

Fasting blood glucose test and IR assay

To assess IR in study animals, rats were fasted for 12 h after the last administration, then fasting blood glucose (FBG) and fasting insulin (FINS) were assessed using venous blood samples by ELISA. We followed the homeostasis model assessment of insulin resistance (HOMA-IR) approach. HOMA-IR was calculated using the following formula:

$$\text{HOMA-IR} = \text{FBG (mmol/L)} \times \text{FINS (mU/L)} / 22.5$$

Measurement of hormone level

We used enzyme-linked immunosorbent assay (ELISA) kits (Enzyme Immunity Industry Co., Ltd, Jiangsu, China) to measure the serum concentrations of gonadotropins, including 17β -estradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH), and steroid hormones, progesterone (P), testosterone (T).

Rat ovarian morphology

At study endpoints, the ovaries were quickly removed and dissected on dry ice from all animals using formalin fixed/paraffin embedded the tissue; which was then cut into $4\ \mu\text{m}$ sections and stained using hematoxylin and eosin (H&E). Morphological structure was observed under light microscope and recognized by morphological scoring standard [16]. 0 points: no injury; 1 points: inflammatory cell infiltration was less than 25%, granular cell layer was more than 75%; 2 points: inflammatory cell

infiltration was range from 25% to 50%, granular cell layer was range from 50% to 75%; 3 points: inflammatory cell infiltration was range from 50% to 75%, granular cell layer was range from 25% to 50%; 4 points: inflammatory cell infiltration was more than 75%, granular cell layer was less than 25%.

Ovarian tissue cell apoptosis

Part of ovaries tissue was fixed with 4% paraformaldehyde, routinely dehydrated, paraffin embedded in paraffin, cut into 4-um section, dewaxed and transparent then TUNEL staining. TUNEL was used to evaluate the ovaries. Microscopically, the nuclei of normal ovaries were blue, and the nuclei of apoptotic cell were brown and yellow. The apoptotic index (AI) was expressed as the percentage of apoptotic cell in the total number of ovaries, and expressed as the percentage (%).

***In Vitro* Study**

Preparation of serum containing drugs

Twenty female SD rats were randomly assigned into 2 groups of 10 rats each: L-BER (95 mg/kg) and H-BER (190 mg/kg) group. These groups were administered a gavage of 10 mL/kg of Ber once daily for 7 consecutive days. 1 hour following the last intragastric administration, the abdominal artery blood was drawn from rats in each group under aseptic conditions, placed at 4°C for 1 h, and centrifuged at 900 g and 4°C for 15 min. The drug-containing serum at the same group of rats were mixed, and then serum inactivation was at 56°C water bathing for 30 min. Finally, the samples were sterilized by microporous membrane filtration, and then packed in frozen tubes stored at -70°C for the follow-up experiments.

Isolation, culture and identification of rat ovarian granulosa cells

The *in vitro* experiments were conducted according to the protocol described previously [17, 18]. The successfully modeled rats were injected (50 IU) pregnant mare serum gonadotropin (PMSG, hor-272, prospec) by injected intraperitoneally. The rats were fed routinely for 48 h and then killed. Under sterile conditions, the bilateral ovaries were removed with sterilizing instruments and the adipose tissue wrapped around the ovaries was removed. The ovaries were cleaned thrice with sterile saline solution. Then, the ovaries were transferred to 3 mL serum-free DMEM/F-12 medium (HyClone, Logan, UT, USA), and the follicles were punctured with a 1-mL syringe needle to release granulosa cells. Then granulosa cells were cultured in sterile bottles (2×10^6 cells/bottle) using DMEM/F12 medium supplemented with 10% fetal bovine serum protein (FBS) at 37°C with 5% CO₂. After 24 h of pre-culture, when cells reached ~70% confluence, the media were replaced with fresh media and replaced it every two days. When cells reached ~80%, it can be digested by 0.25% trypsin. The cells were fastened using 4% paraformaldehyde for 10 min; which was then stained using H&E for identification of rat ovarian granulosa cells.

CCK-8 viability assay

A cell counting Kit-8 (CCK-8, MCE, New Jersey, USA) was utilized to determine the granulosa cells viability and proliferation rates. Briefly, following exposure to various concentrations of Ber for various treatment

times (0, 12h, 24h, 48h, 72h), 100 μ L of cell suspension (about 5000 cells/well) was removed and 10 μ L of CCK-8 Solution was added in one well of a 96-well plate. The plate was then incubated in a cell culture incubator. Finally, the optical density (OD value) was calculated with a microplate reader (CMaxPlus, MD, USA), and the absorbances of each experimental group were measured at 450 nm. Each treatment had three replicates. Cell viability = $(A_{\text{Experiment}} - A_{\text{Blank}}) / (A_{\text{Control}} - A_{\text{Blank}}) \times 100\%$.

Apoptosis analysis by flow cytometry

Apoptosis was evaluated using the Annexin V-FITC Apoptosis Detection Kit (556547, BD Pharmingen, China). Briefly, granulosa cells treated as above were collected and washed with ice-cold PBS two times, and resuspended in 500 μ L of binding buffer, the mixture was centrifuged and the supernatant was discarded. Then the granulosa cells were resuspended in 100 μ L Annexin V-FITC binding solution, and stained for 15 minutes with 5 μ L Annexin V-FITC solution and 10 μ L propidium iodide (PI) solution in the dark at room temperature. Finally, the apoptotic rate of granulosa cells was measured by flow cytometer (FC500, Beckman Coulter, Inc., USA).

Western blotting analysis

The granulosa cells were homogenized with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), and centrifugation at 12000 g and 4°C for 5 min. The supernatant was collected, and the protein concentration in each group was detected using the bicinchoninic acid (BCA) protein quantification kit (pc0020, Solarbio, China). An appropriate number of protein samples were taken and added with 2' sodium dodecyl sulphate (SDS) loading buffer (loading buffer: protein samples (v/v)=4:1) to prepare the loading buffer system in an equal concentration, and the protein was inactivated at 95°C for 5 min. 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was prepared for electrophoresis under the constant voltage of 80 V. The protein was transferred onto a polyvinylidene difluoride (PVDF) membrane, the protein band was sealed with freshly-prepared 5% skim milk powder for 2 h and washed. The membranes were then incubated with antibodies solution dilute according to manufacturer's protocol overnight at 4 °C, followed by incubation with a secondary antibody at room temperature for 1~2 h. The primary antibodies including GAPDH antibody (1:1000, 60004-1-1g, Hangzhou Hua'an Biotechnology Co., Ltd, China), Anti-IGF1 Receptor Antibody (1:1000, ab182408, Abcam, USA), Anti-AKT (phospho T308) Antibody (1:1000, Ab32445, Abcam, USA), Anti-pan-AKT Antibody (1:500, ab8805, Abcam, USA), Anti-Bad Antibody (1:2000, Ab32445, Abcam, USA), Anti-Bcl-2 Antibody (1:2000, ab182858, Abcam, USA), Bax Antibody (1:500, AF0120, Affinity, USA), PI3 Kinase p85 Antibody (1:1000, 4292S, CST, USA) and PI3 Kinase p110 α Antibody (1:1000, 4255S, CST, USA). The reactive bands were visualized by use of the ECL-Plus reagent (Solarbio, Beijing, China). The density of band was quantified by the Quantity One analytic software.

Statistical analysis

The statistical analyses were performed using SPSS 16.0 (IBM, Armonk, NY, USA). Values are expressed as $\bar{x} \pm s$. *In vivo* study, group comparisons were processed by using the LSD of One-way-ANOVA test. And

group comparisons *in vitro* study was processed by using the Student's t-test or the Kruskal-Wallis H test. In all cases, $P < 0.05$ was considered as statistical significance.

Result

In Vivo Study

Berberine treatment altered HOMA-IR and insulin sensitivity index values in PCOS model rats

As shown in Figure 1, the values of FPG, FINS and HOMA-IR of PCOS rats had no significant changes compared with control group before administration. After administration 4 weeks, compared with control group, the levels of FPG, FINS and HOMA-IR were significantly increased in PCOS rats. However, compared with PCOS group, the level of HOMA-IR in PCOS+L-Ber group was significantly decrease, and both PCOS+H-Ber and PCOS+MH groups rats showed significant decreases in FPG, FINS and HOMA-IR levels. The results showed that PCOS rats treated with Ber were significantly more glucose tolerant compared to PCOS rats and was similarly to treated with MH.

The level of hormone in serum of rats

Results of ELISA assays indicated that the serum E2, T and LH levels in rats were significantly higher in the PCOS group than those in the control group ($p < 0.01$), whereas the serum P level in rats were significantly lower in the PCOS group than those in the control group ($p < 0.01$; Figure 2). At the same time, we observed the levels of E2 and T were significantly decreased ($p < 0.01$ & $p < 0.05$) while the levels of LH, FSH and P had no significantly change ($p > 0.05$) in PCOS+L-BER group rats compared with PCOS group. In addition, the level of P was increased and the levels of LH, E2 and T were suppressed in PCOS+H-Ber and PCOS+MH group rats compared to PCOS group rats. Furthermore, the level of FSH had no significantly change in all groups ($p > 0.05$).

The influence of letrozole for estrous cycle

The following items were detected under a microscope. Rats in control group had 4-5 days estrous cycles, comprising proestrus, estrus, metestrus and diestrus (Figure 3A-D). The 10 rats in the PCOS group experienced prolonged diestrus after the letrozole gavage. Microscopy of stained smears of vaginal secretion showed the presence of a large number of leukocytes, while few keratinized cells and epithelial cells were visible, suggesting anovulation. It suggested that the cycle of estrous in the model group is irregular due to the gavage of letrozole.

Ovarian morphological changes

Light microscope was applied to observe the rat ovarian structure and shown in Figure 4. Under light microscopy, the rat ovaries demonstrated multiple luteal, preantral, and antral follicles in the control group. The granular cells within the follicles showed multiple layers. Oocytes and corona radiata were visible in the follicles, part of which had been discharged. Compared with the control group, it showed

cystic dilatation follicle, lipid droplets in cytoplasm, a little of corpora lutea, and a thin layer of granulosa cells in the PCOS group. Whereas, in the administration group, it could be seen that follicles were at different developmental stages, corpora lutea was increased, oocyte and corona were radiate in mature follicle, and a thick layer of ordered granulosa cells with complete shape was also observed.

Ber inhibits the apoptosis of ovarian tissue cells

In order to investigate the apoptosis of ovarian tissue cells, we conducted TUNEL staining for ovarian tissues and the results were shown in Figure 5. Compared with the control group, it demonstrated a high number of apoptotic cells in PCOS group. On the other hand, less TUNEL positive cells were observed in the ovarian tissue from rats after Ber post-treatment. At the same time, the number of positive cells in the PCOS+MH group were also decreased significantly. Semi-quantitative assessment confirmed this result showing that the number of TUNEL positive cells significantly increased immediately after letrozole challenge, but decreased by treatment with Ber and MH at the end of the experiment.

***In Vitro* Study**

Ovarian granulosa cells identification

As shown in the Figure 6, the initial stage of ovarian granulosa cells, most of the cells were adherent and presented a rounded shape after 24 h of cell culture in the normal group, and the adherent time of ovarian granulosa cells in PCOS group was slightly later than that in the normal group. After 48 hours of culture, the ovarian granulosa cells showed a pleomorphic or fusiform morphology in normal group, while it showed pleomorphic or similar fibroblast morphology in the PCOS group. At the same time, the cells extended pseudopodia to connect with each other (Figure 6A, B). Then we identified the ovarian granulosa cells by HE staining and the morphological structure was observed under light microscope. The nuclei of normal ovaries were blue, and the cytoplasm were reddish and contains many particles (Figure 6C), as for the PCOS group, it was oval, and some nuclei were enlarged (Figure 6D). were polygonal,

Ber induces proliferative and anti-apoptotic effects on granulosa cells

In order to investigate the effects of Ber exposure on ovarian granulosa cells *in vitro*, primary ovarian granulosa cells were exposed for various lengths of time at various concentrations. The effects of letrozole exposure on cell viability and growth were determined using a CCK-8 assay at four exposure time points (12, 24, 48, 72 h) with the concentration of 0.1 and 0.2 mg/mL. The results suggested that the cell viability was significantly reduced in the letrozole treated groups when compared with that in the control groups after culturing for four exposure time. We found 0.2 mg/mL Ber treated for 48 h significantly increased cell viability ($p < 0.01$). The doses of Ber (0.2 mg/mL) were selected for subsequent experiments.

To further verify the involvement of the PI3K/AKT pathway in the stimulation of growth by ovarian granulosa cells, two pharmacological inhibitors, including a PI3K inhibitor (LY294002, Beyotime Biotech,

Jiangsu, China) and an AKT inhibitor (MK-2206, Sigma–Aldrich Co., LLC, USA), were used. Cell proliferation and cell apoptosis were measured by the CCK-8 assay and flow cytometry, respectively. In granulosa cells treated with 0.2 mg/mL Ber, a significantly stimulatory effect of Ber on cell growth was observed ($P < 0.05$) compared with PCOS group. The inhibitory effects of both 20 mM LY294002 and 20 mM MK-2206 on cell growth were also confirmed. However, simultaneous addition of either LY294002 or MK-2206 in combination with 0.2 mg/mL Ber blocked letrozole induced cell growth but not significantly ($p > 0.05$). CCK-8 analysis further revealed that 0.2 mg/mL Ber treatment significantly stimulated granulosa cell proliferation ($p < 0.01$), while this stimulatory effect was blocked in the presence of either LY294002 or MK-2206 ($p < 0.05$) (Figure 7). In addition, flow cytometry analysis indicated that granulosa cell apoptosis was significantly inhibited by the addition of 0.2 mg/mL Ber ($p < 0.01$), but was elevated in the presence of either LY294002 or MK-2206 ($p < 0.05$). Moreover, the anti-apoptotic effects of Ber were blocked in combination with either LY294002 or MK-2206 (Figure 8). These data confirm that Ber exerts proliferative and anti-apoptotic effects on ovarian granulosa cells in a PI3K/AKT-dependent manner.

Blockade of PI3K/AKT attenuates the modulatory effects of Ber on protein expression

As shown in the western blotting analysis, the expression level of AKT and PI3K were no significant difference in all groups. And the protein expression levels of Bcl-2, p-AKT and p-PI3K were significantly enhanced by 0.2 mg/mL Ber treatment ($P < 0.01$), while pharmacological inhibition of the PI3K/AKT pathway by LY294002 significantly decreased IGF-1, Bcl-2, p-AKT, p-PI3K protein expression ($P < 0.05$ & $p < 0.01$) and MK-2206 significantly decreased p-PI3K, p-AKT, BAD protein expression ($P < 0.05$). In addition, the western blot assay also found that LY294002 promoted the expression of BAD and Bax, while MK-2206 promoted the expression of Bax in ovarian granulosa cells (Figure 9).

Discussion

PCOS is defined as a metabolic syndrome [19, 20]. The infertility that affects women with PCOS is thought to be partly attributable to IR, which affects up to 70% of those with PCOS and is linked to profoundly abnormal insulin activity in the reproductive system [21]. Ber is an isoquinoline alkaloid compound that can be extracted from many different plants, and which has pharmacological activities that make it well-suited to immune modulation, lowering of glucose and cholesterol levels, and combatting cancer or microbial pathogens [22–24]. Multiple recent studies have explored the clinical use of Ber for treating PCOS [25].

IR and the dysregulation of glucose metabolism are common in PCOS patients [26]. Zhang et al [22] found that the level of HOMA-IR and ovarian morphology in PCOS model were restored after treated with Ber. In this study, we elucidated the beneficial effects of Ber for PCOS through the establishment of a disease model *in vivo* and *in vitro*. A major characteristic of PCOS is an elevated serum androgen level [27, 28]. *In vivo* experiment, we found that treated with Ber alleviated the serum hormone imbalance and improved HOMA-IR. And the level of serums E2, LH, P and T were decreased in PCOS treated with Ber and MH by ELISA assay. Meanwhile, this effect was comparable with that of the widely used medicine MH

and thus shows great promise in the application of Ber for PCOS treatment [23, 29]. Ber treatment could reduce the abnormal secretion of androgen and achieve a physiological androgen balance. In addition, by *in vivo* and *in vitro* experiments, we found that Ber positively regulated the ovarian functions by promoting proliferation and inhibiting apoptosis of ovarian granulosa cells.

PI3K signaling cascades are important regulators for a host of cellular activities such as proliferation, differentiation, survival, apoptosis and glucose homeostasis. Previous studies have shown that activation of the PI3K/AKT signaling pathway has important effects on insulin resistance [30]. Long et al [31] created an insulin-resistant *in vivo* model and confirmed that IR was related to the PI3K-AKT signaling pathway. When insulin receptors are activated, they phosphorylate insulin receptor substrate (IRS), which then binds to the PI3K protein [32]. The insulin signaling pathway depends upon signaling through a number of intermediaries including IRS, PI3K, and AKT, thereby regulating cellular glucose intake [33]. Zhang et al [22] found that berberine-mediated effects against IR in a PCOS rat model were associated with its ability to enhance activation of PI3K/AKT signaling in these animals. In line with this result, our result indicated that the levels of FPG, FINS and HOMA-IR were significantly decreased after treated with Ber.

The PI3K/AKT pathway may also be involved in the regulation of ovarian granulosa cells apoptosis and proliferation. Previous works suggested that granulosa cells apoptosis have demonstrated involved in many ovarian physiological processes including the female germ cell cyst breakdown and primordial follicle assembly, follicular atresia, and luteolysis [34]. In particular, ovarian granulosa cells apoptosis is demonstrated to be an important mechanism for follicular atresia [35]. Apoptotic proteins such as Bcl-2, Bax and BAD were detected by western blot. The Bcl-2 protein is antiapoptotic, but Bax and BAD is pro-apoptotic. Both of them play key roles in the regulation of cellular apoptosis and exists a balance among pro and antiapoptotic components [36]. The result showed that high protein expression of pro-apoptotic factors such as Bax and BAD and lower expression of Bcl-2 in granulosa cells of PCOS group after treated with simultaneous addition of either LY294002 or MK-2206 in combination with 0.2 mg/mL BER, with similar values to those found in PCOS group. These results are consistent with the previous finding that PI3K and AKT inhibition has beneficial effects on PCOS [37]. And it confirmed that Ber inhibited the apoptosis of ovarian granulosa cells by PI3K/AKT pathway.

In summary, we reported on the beneficial effects of Ber in a PCOS rat model, including improvements in serum hormone levels, HOMA-IR and the apoptosis and proliferation of ovarian granulosa cells. In addition, one of the important findings was the involvement of the PI3K/AKT pathway in the Ber-induced beneficial effects on PCOS. This work provides experimental evidence to support the potential application of Ber, putting forward a new methodology for PCOS treatment.

Declarations

Acknowledgments

Not applicable.

Conflict of interest

The authors declare that they have no competing interests

Author contributions

Jia Yu designed the experiment. Chenye Wang and Zhoujia Hua performed the experiment and acquisition the data. Jia Yu, Caifei Ding, Xuejuan Jiang and Zhoujia Hua analyzed the data. Jia Yu and Chenye Wang obtained the funding and wrote the manuscript. Chenye Wang revised the manuscript. All authors discussed the results, and approved the final manuscript.

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Ethics approval and consent to participate

All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal experiments were performed according to the guidelines of laboratory animal care and were authorized by the Ethic Committee of Zhejiang Traditional Chinese Medicine University (Hangzhou, China).

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

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Figures

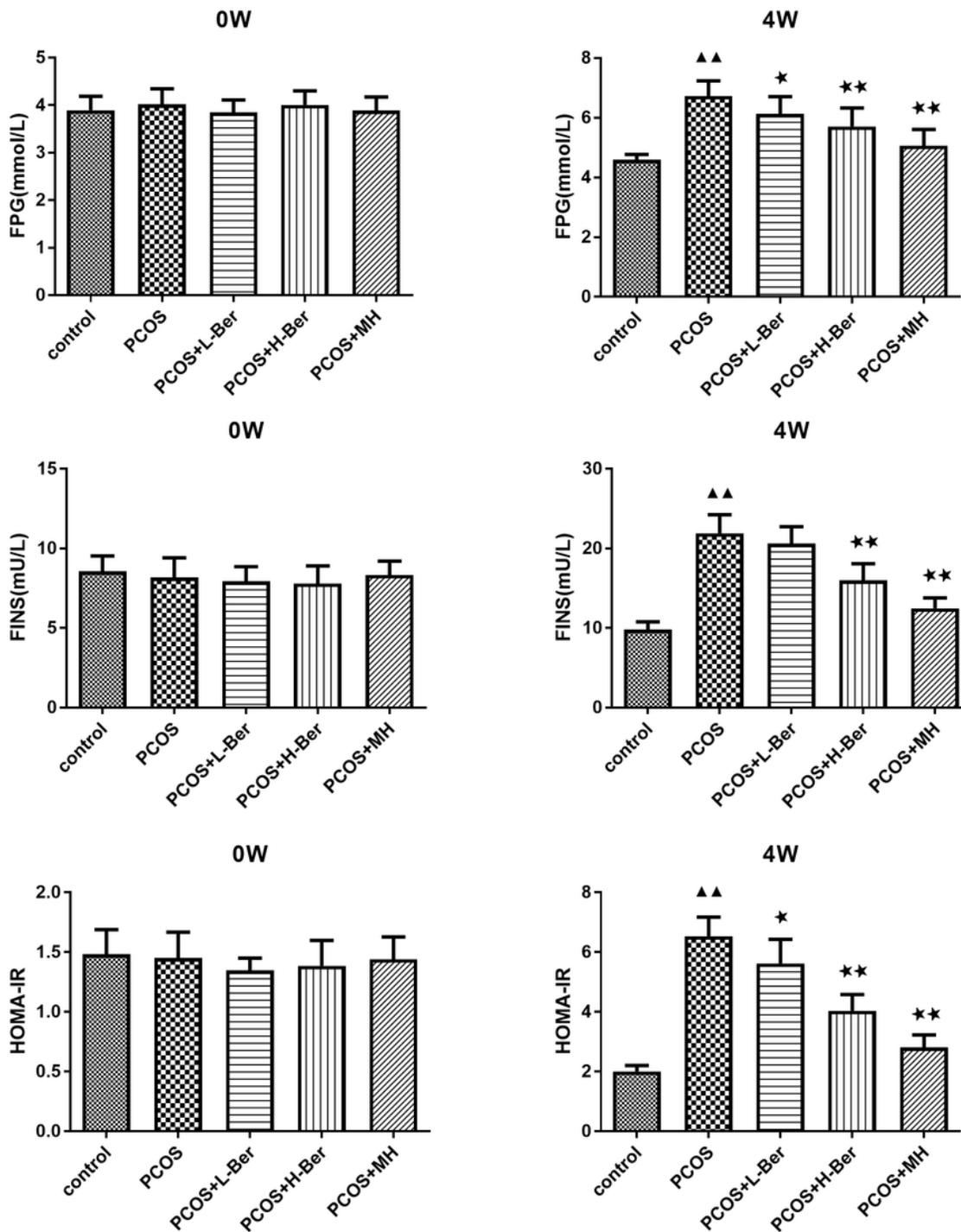


Figure 1

Effects of BER and MH on the level of FPG, FINS and HOMA-IR in PCOS rats. A, The level of FPG, FINS and HOMA-IR in PCOS rats before administration. B, The level of FPG, FINS and HOMA-IR in PCOS rats after administration for 4 weeks ($\bar{x} \pm s$, n=10), ▲P<0.05, ▲▲ P<0.01 vs. control group □P<0.05, □□P<0.01 vs. PCOS group.

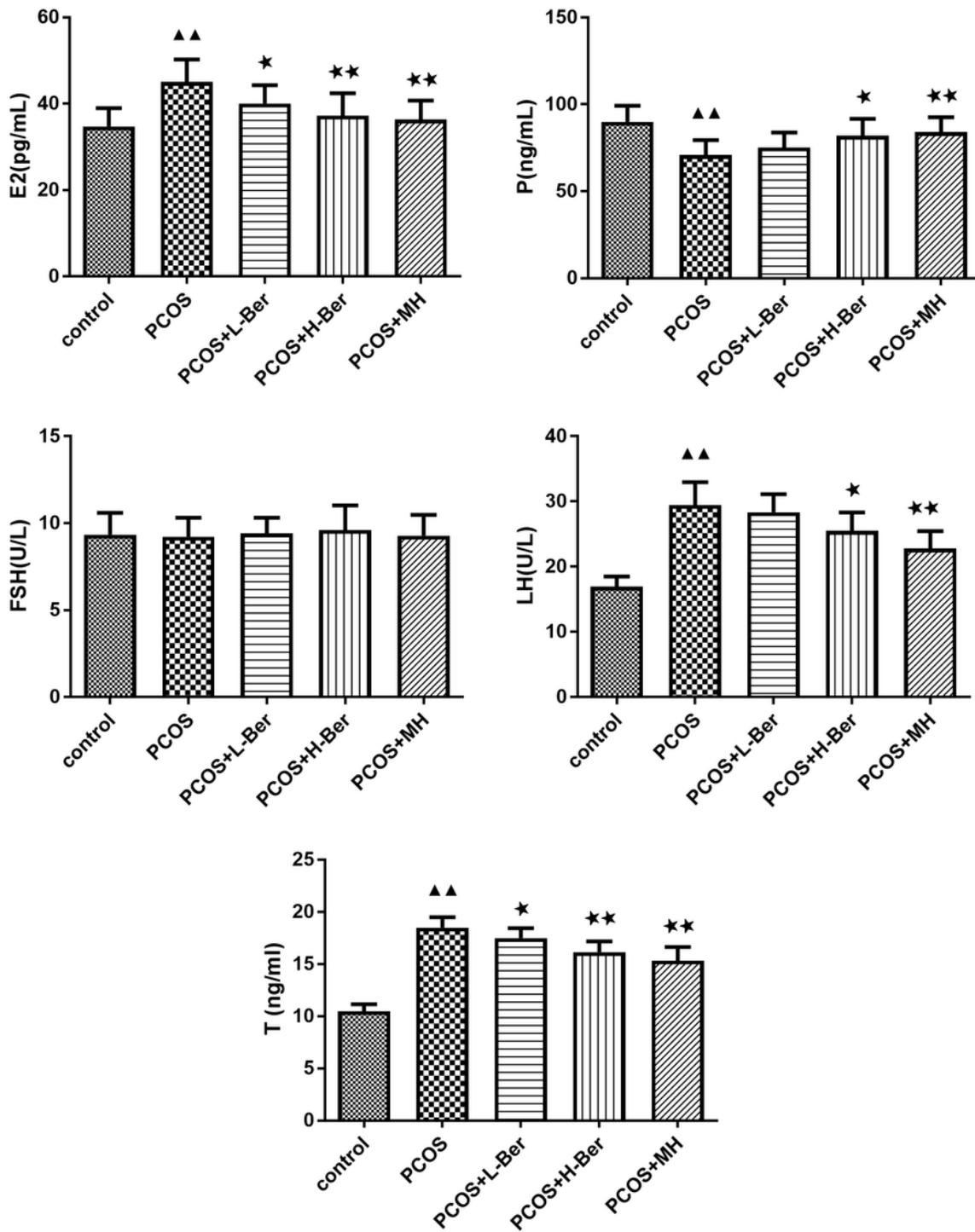


Figure 2

Comparison of hormone level detected by ELISA in serum of rats ($\bar{x} \pm s$, $n=10$), ▲ $P < 0.05$, ▲▲ $P < 0.01$ vs. control group, ⊠ $P < 0.05$, ⊠⊠ $P < 0.01$ vs. PCOS group.

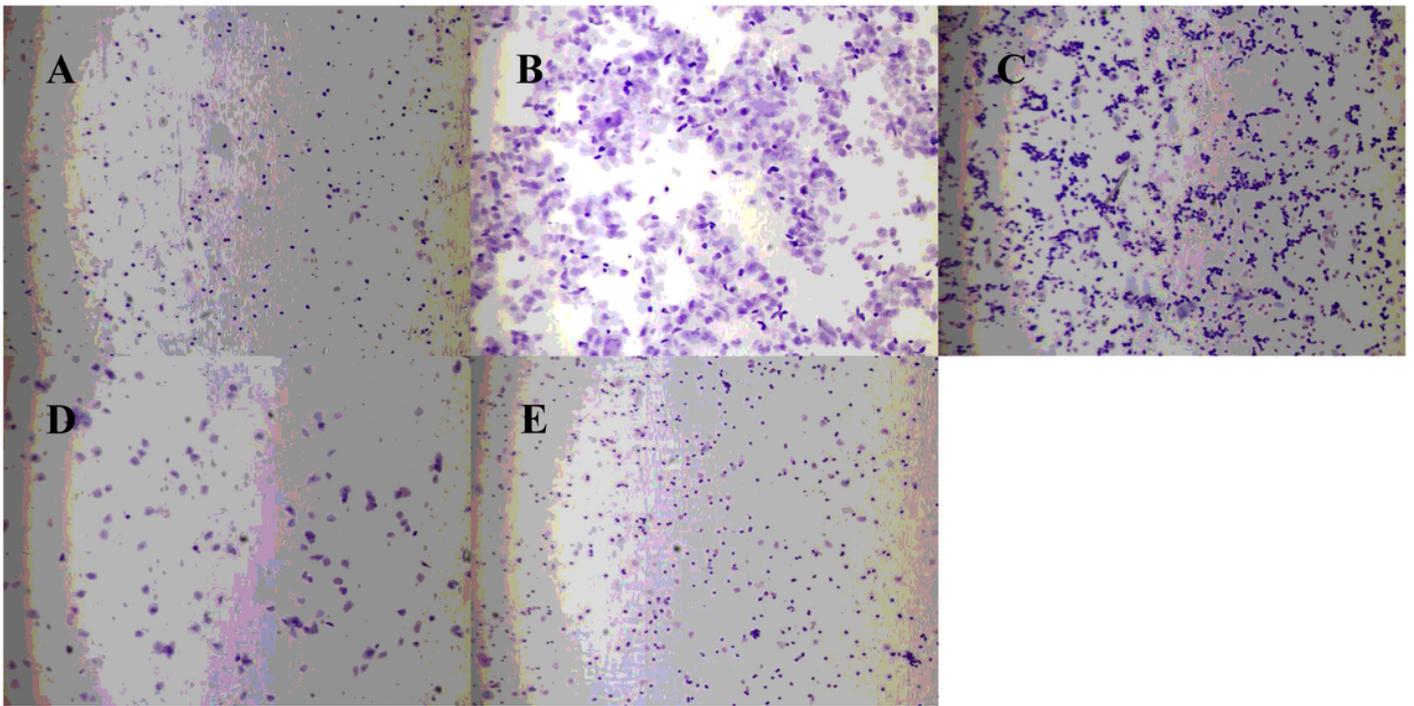


Figure 3

The vaginal smears of rats in the control and PCOS. A, The representative rat's vaginal smears from the control group in diestrus. B, The representative rat's vaginal smears from the control group in estrus. C, The representative rat's vaginal smears from the control group in metestrus. D, The representative rat's vaginal smears from the control group in proestrus. E, The representative rat's vaginal smears from the PCOS group predominantly exhibited leukocytes, the main cell type observed during the diestrus stage (original magnification, $\times 200$).

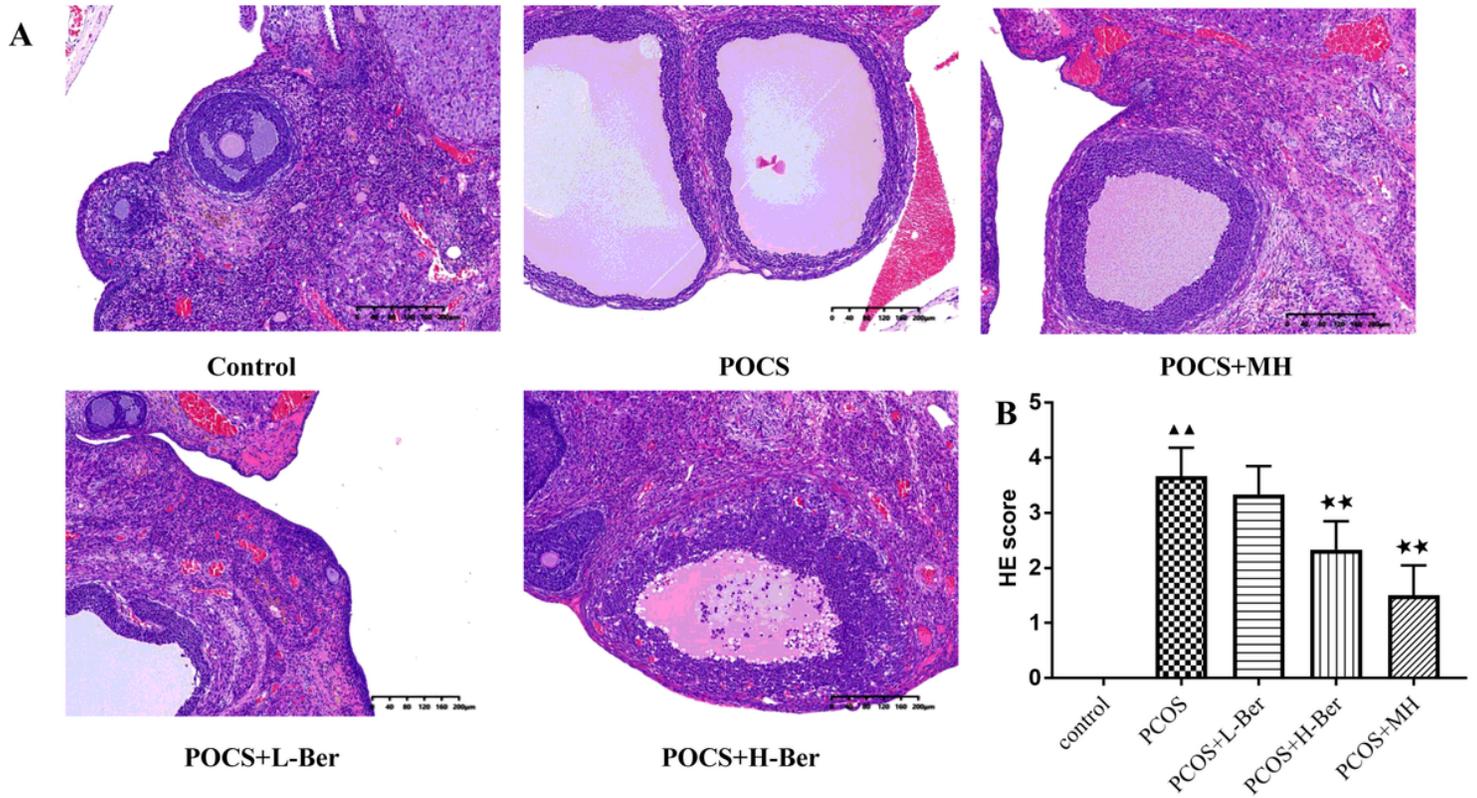


Figure 4

The morphological changes of the rats' ovarian tissues were evaluated by HE staining. A, Representative microphotographs of HE staining, original magnification $\times 100$ ($\chi \pm s$, $n=6$); B, Semi-quantitative assessment of the histological lesions ($\chi \pm s$, $n=6$), ▲▲ $p < 0.01$ vs. control group, $\square \square P < 0.01$ vs. PCOS group.

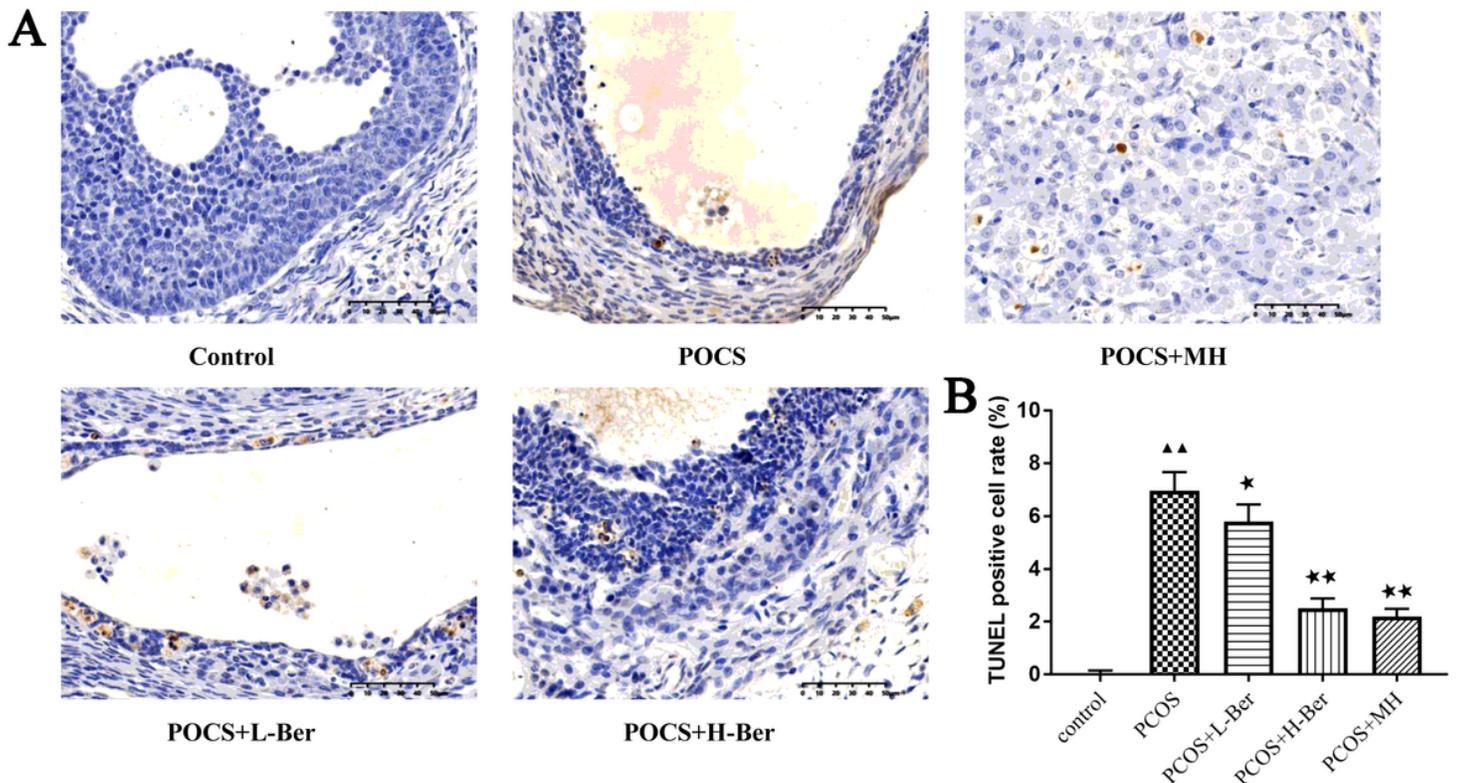


Figure 5

Effect of BER and MH post-treatment on the apoptosis of ovarian tissue cells. A, Representative TUNEL staining images, original magnification $\times 400$. B, Semi-quantitative assessment of the cell apoptosis, ($\chi \pm s$, $n=3$), $\blacktriangle\blacktriangle p < 0.01$ vs. control group, $\boxtimes P \leq 0.01$ vs. PCOS group.

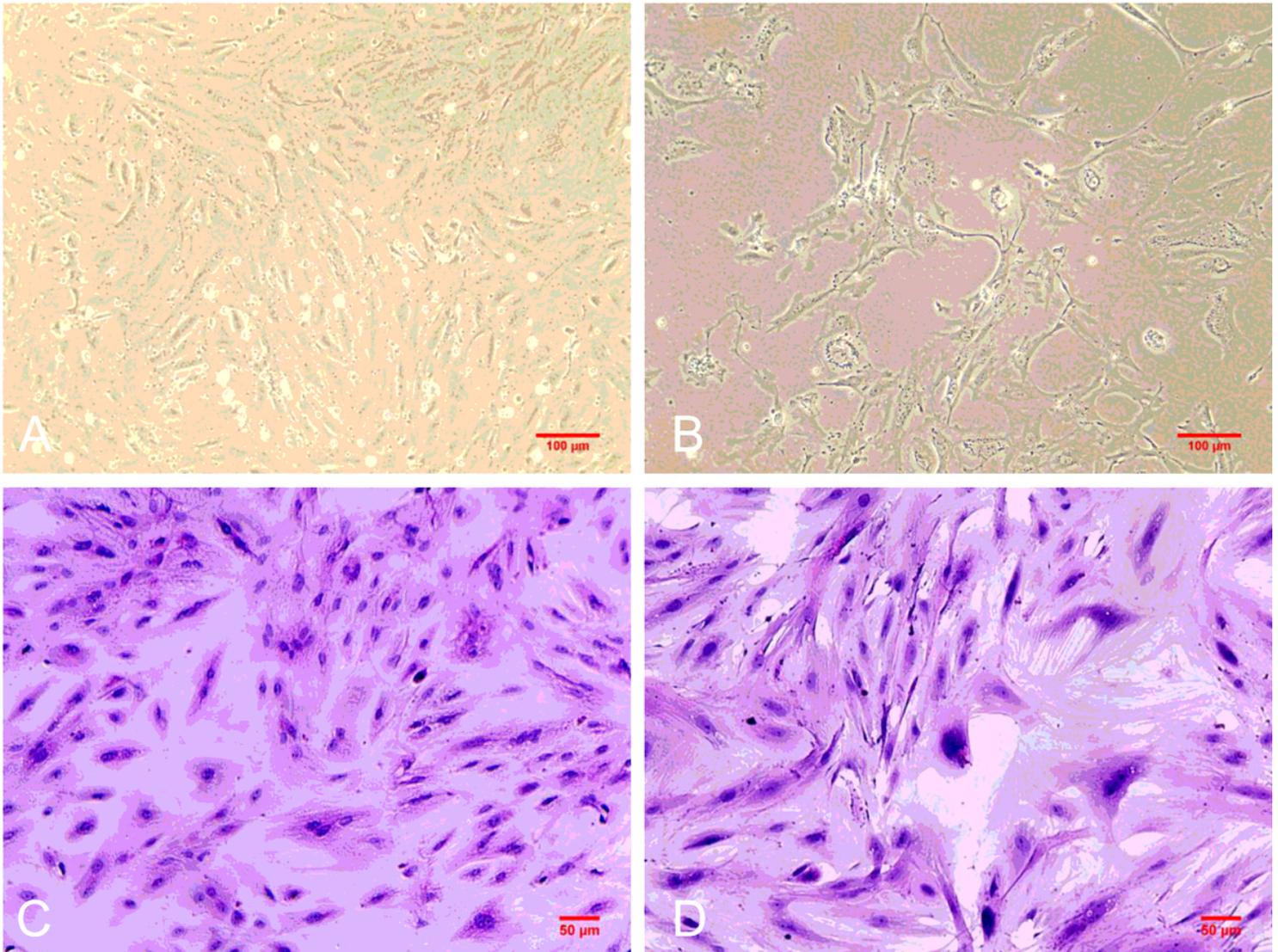


Figure 6

Identification of rat ovarian granulosa cells by HE staining. A, Morphology characteristic of ovarian granulosa cells in control group (A) and PCOS group (B) (original magnification, $\times 100$). HE staining images of ovarian granulosa cells in control group (C) and PCOS group (D) (original magnification, $\times 200$).

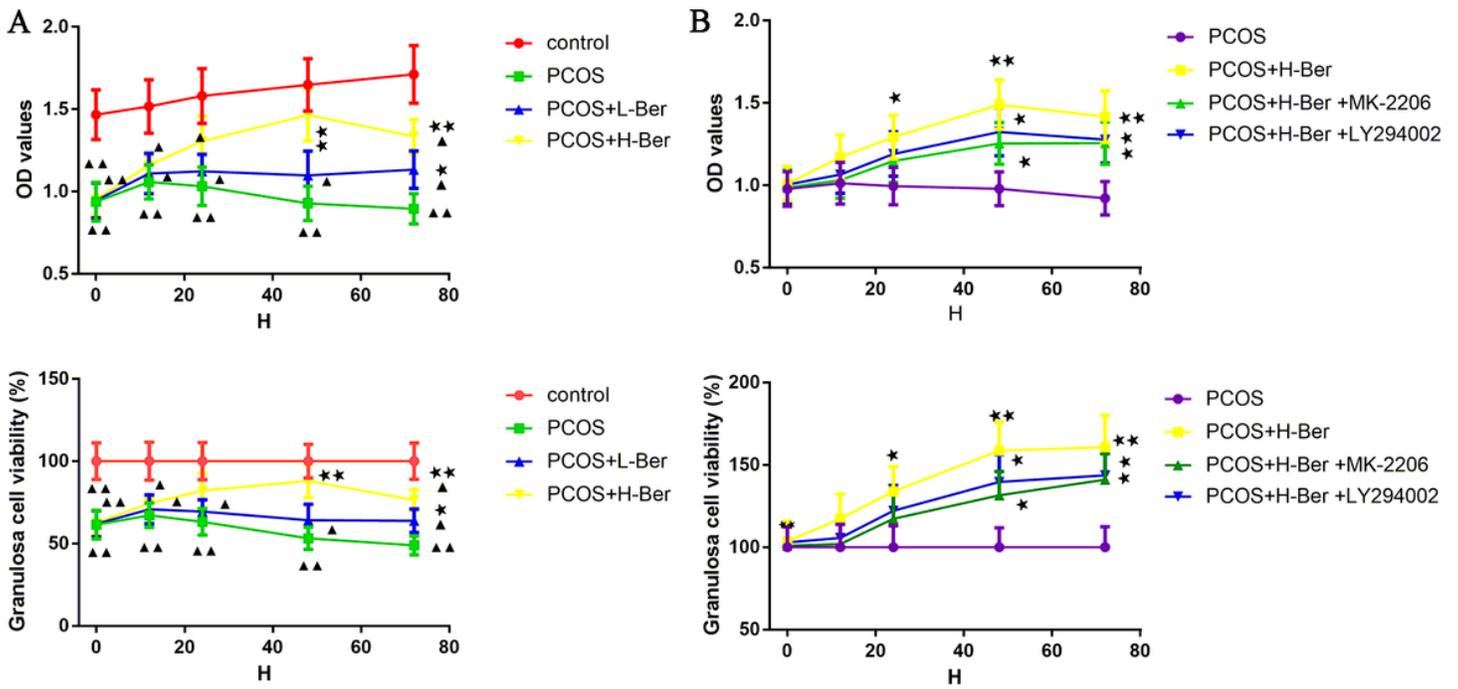


Figure 7

Effects of BER treated on the growth of ovarian granulosa cells in vitro. A, ovarian granulosa cells viability following treated with 0 (control), 0.1, or 0.2 mg/mL of BER for 12, 24, 48, or 72 h. B, ovarian granulosa cells viability following treated with simultaneous addition of either LY294002 or MK-2206 in combination with 0.2 mg/mL BER, ($\bar{x} \pm s, n=3$), $\blacktriangle p < 0.05$, $\blacktriangle\blacktriangle p < 0.01$ vs. control group, $\boxtimes P < 0.05$, $\boxtimes\boxtimes P < 0.01$ vs. PCOS group, $\#P < 0.05$, $\#\#P < 0.01$ vs. PCOS+H-Ber group.

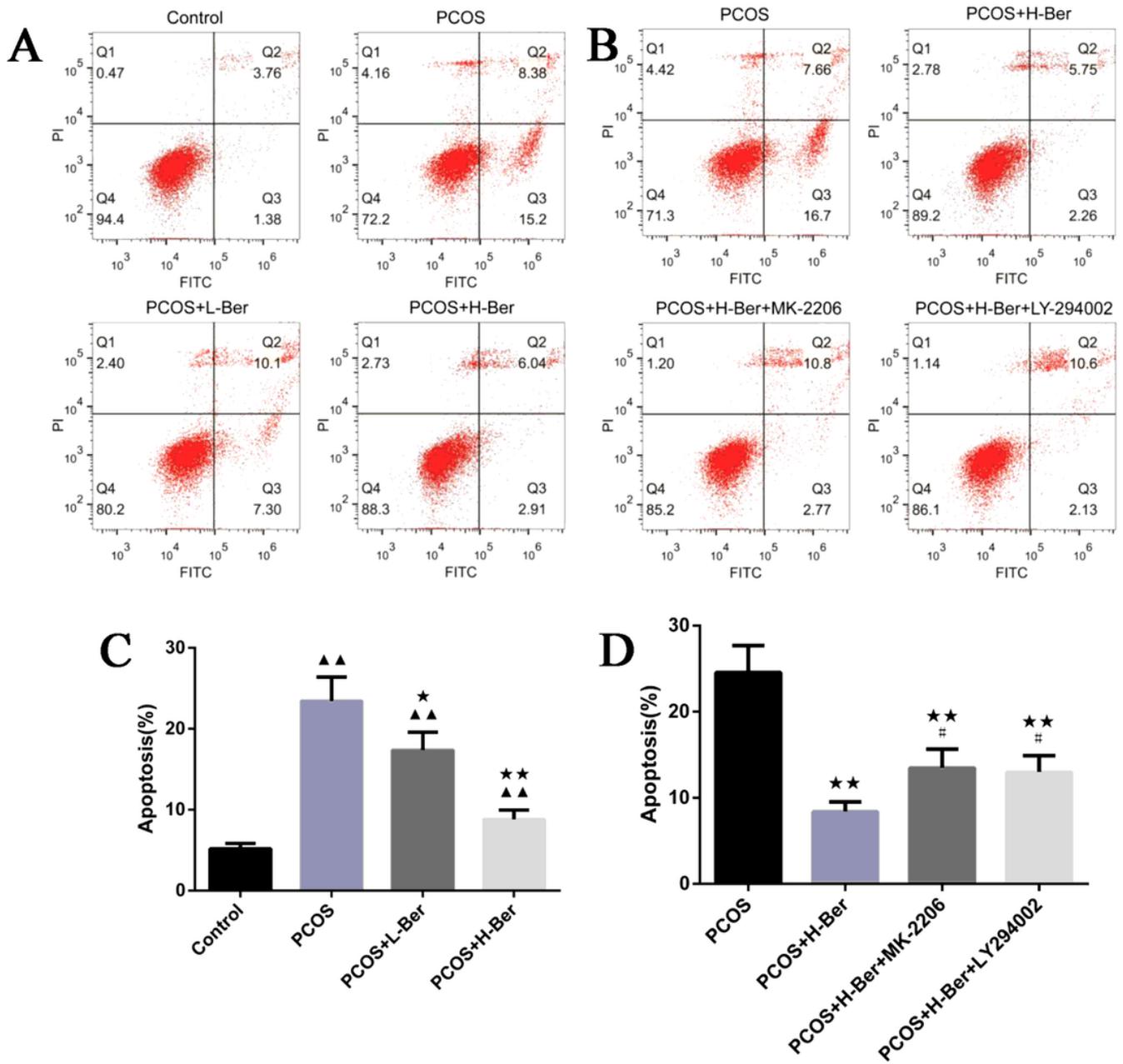


Figure 8

Effect of BER on cell apoptosis after lead acetate induced injury in rat ovarian granulosa cells. A and C, Flow cytometry detection of apoptosis revealed that BER significantly induced ovarian granulosa cell apoptosis. B and D, Flow cytometry detection of the anti-apoptotic effects of BER were blocked in combination with either 20 mM LY294002 or 20 mM MK-2206. ($\chi \pm s$, $n=3$), $\blacktriangle p<0.05$, $\blacktriangle\blacktriangle p<0.01$ vs. control group, $\boxtimes P\leq 0.05$, $\boxtimes\boxtimes P\leq 0.01$ vs. PCOS group, $\#P\leq 0.05$, $\#\#P\leq 0.01$ vs. PCOS+H-Ber group.

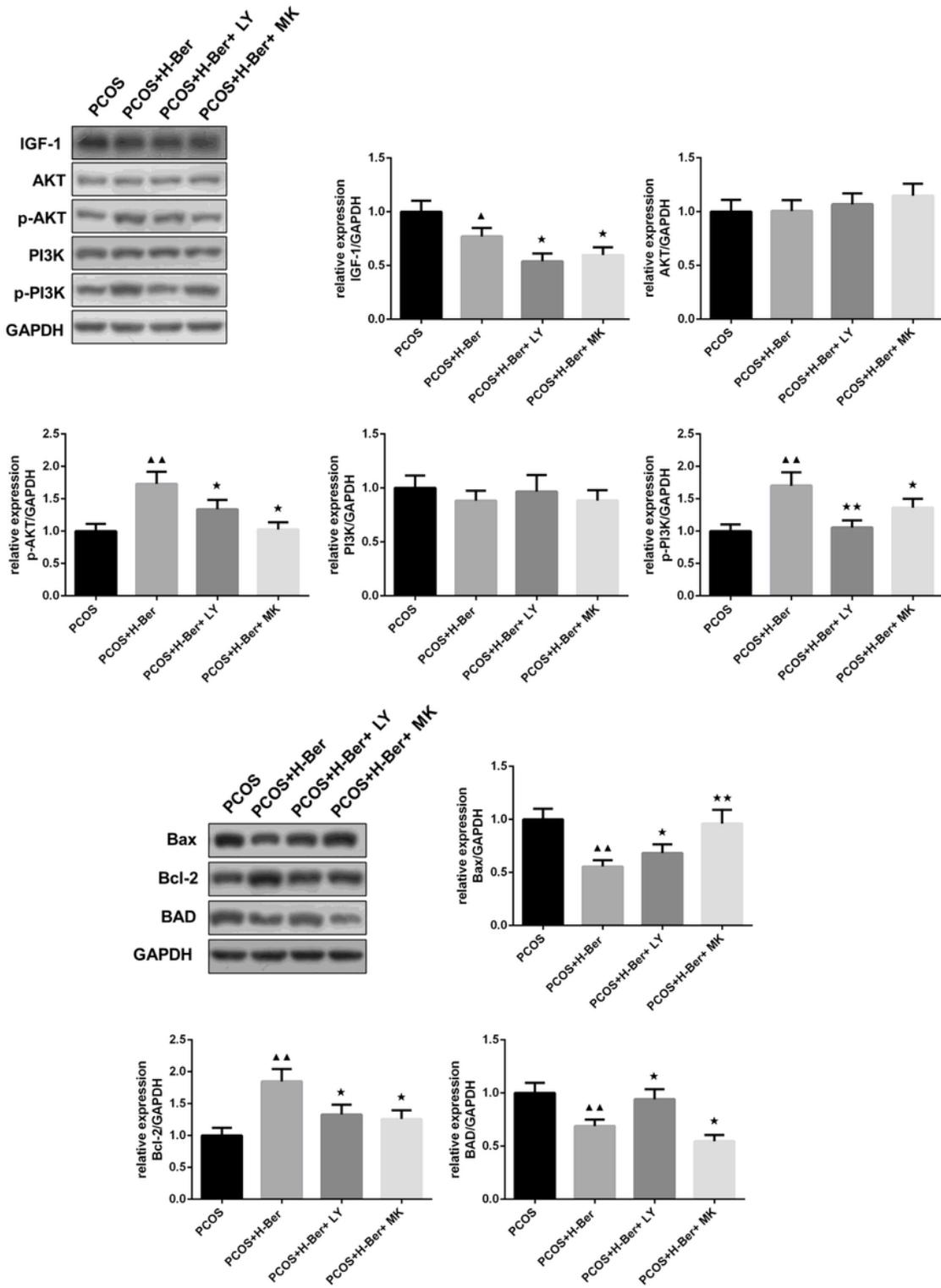


Figure 9

Effects of Ber in combination with either LY294002 or MK-2206 on the protein expression of selected genes associated with cell apoptosis and regulation of the PI3K/Akt pathway. ($\chi \pm s$, $n=3$), ▲ $p<0.05$, ▲▲ $p<0.01$ vs. PCOS group, ⊠ $P\leq 0.05$, ⊠⊠ $P\leq 0.01$ vs. PCOS+H-Ber group.