

Mechanism of resveratrol in improving ovarian function in a rat model of premature ovarian insufficiency

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Abstract

Aim: To investigate the mechanism of resveratrol treatment in chemically induced premature ovarian insufficiency (POI) in rats.

Methods: Five-week-old specific pathogen-free healthy Sprague–Dawley female rats ($n = 90$) were randomly divided into five groups ($n = 18$): low-dose resveratrol (group A), moderate-dose resveratrol (group B), high-dose resveratrol (group C), model control group (group D) and blank control group (group E). POI was induced in rats from the resveratrol-treated groups and the model control group according to observed indexes using a previously established model (oral administration of tripterygium wilfordii polyglycoside tablet 50 mg/kg/day for 14 days). Western blot analysis was performed to compare levels of PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, Bax, Bcl-2 and Caspase-3 in ovarian tissues of each group, and expression of p-Akt, p-mTOR, Bax, Bcl-2 and Caspase-3 was also evaluated by immunohistochemistry. Serum levels of malondialdehyde (MDA) and superoxide dismutase (SOD) were determined using enzyme-linked immunosorbent assay.

Results: Compared with controls, we found that serum MDA decreased and SOD increased in resveratrol-treated groups. In addition, we found increased expression of p-PI3K, p-Akt, p-mTOR and Bcl-2, and decreased expression of Bax and Caspase-3 were observed in ovarian tissues of treated rats with POI. There was reduced ovarian function in POI rats compared with rats from the blank control group.

Conclusion: Resveratrol reduced oxidative stress and inhibited apoptosis in granulosa cells by activating the PI3K/Akt/mTOR signaling pathway in a rat model of POI.

Key words: PI3K/Akt/mTOR signaling pathway, premature ovarian insufficiency, rat, resveratrol.

Introduction

Premature ovarian insufficiency (POI) is ovarian follicle depletion or iatrogenic damage to ovarian failure before an age of 40 years. It is characterized by low estrogen and hyperglycemia, and especially, elevated follicle-stimulating hormone.¹ Early onset of ovarian dysfunction features secondary amenorrhea that is often accompanied by perimenopausal symptoms. POI is irreversible, but early detection of ovarian failure allowing for a timely diagnosis in conjunction with early treatment, can delay or possibly improve the

condition. Because a single factor or gene cannot fully explain its pathogenesis, the pathophysiology of POI is thought to be multifactorial, related to genetic factors, autoimmune disorders, radiotherapy and chemotherapy factors, infection, mental, and environmental and idiopathic factors.¹ Recently, the role of signal transduction pathways in POI lesions has become an intense research topic. Many of the molecules in the PI3K/Akt/mTOR signaling pathway are involved in the regulation of oocyte growth as well as development of primordial follicles and proliferation and differentiation of granulosa cells.² Resveratrol is a natural non-

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flavonoid polyphenol compound, obtained mainly from peanuts, grapes, *Polygonum cuspidatum*, veratrum, mulberry and other plants. Studies have confirmed that resveratrol administration not only has protective effects on dementia, osteoporosis and radiation damage, but also exhibits anti-inflammatory, anti-tumor, anti-oxidation and anti-aging properties, protects nerves and confers other effects.^{3,4}

Liu⁵ studied the protective effects of resveratrol against mancozeb-induced apoptotic damage in mouse oocytes, and other studies have shown that resveratrol improves ovarian function in a rat model of POI. Said⁶ reported that resveratrol inhibited inflammatory signaling implicated in ionizing radiation-induced premature ovarian failure through antagonistic crosstalk between silencing information regulator 1 and poly (ADP-ribose) polymerase 1, whereas Kong⁷ demonstrated that resveratrol was an effective regulator of ovarian development and oocyte apoptosis. Based on the findings from these studies, we aimed to determine whether resveratrol modifies the signs of POI via the PI3K/Akt/mTOR signaling pathway using a rat model.

Methods

Rat model of POI and experimental design

All animal study protocols for animal study were approved by and performed at the University of Jinzhou Medical University, China. We obtained 90 healthy 5-week-old female Sprague-Dawley rats (average weight 200 ± 20 g) from the Experimental Animal Center of Jinzhou Medical University (certificate number: SCXK [Liao]2014-0004), China. Rats were housed under specific pathogen-free biosafety protection conditions in the university's animal laboratory. Rats were kept under standard conditions with an artificial light/dark cycle of 12 h, and received litter every other day. Food and water were available ad libitum. All animal experiments were conducted in accordance with principles stated in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996).

After 7 days of adaptive feeding has estrous cycle into the experiment, the 90 rats were housed 3 per cage, and randomly assigned to one of the following experimental groups: low, medium and high resveratrol-treatment groups (groups A–C, $n = 54$), model control group (group D, $n = 18$) or blank control group (group E, $n = 18$). Based on the literature,

tripterygium wilfordii polyglycoside tablets were solubilized in a 0.9% sodium chloride solution to obtain a suspension with a best model concentration of 50 mg/kg, which was administered daily for 14 days to induce an animal model of POI. Prior to administration, 18 rats were randomly selected and comprise the blank control group (group E). The drug-induced POI rats were randomly assigned to one of four experimental groups: groups A–D, which was the model control group. Rats from the other three groups ($n = 18$ /group) received resveratrol at the following daily oral doses for 42 days: group A – low dose (20 mg/kg), group B – moderate dose (40 mg/kg) and group C – high dose (80 mg/kg). Resveratrol was dissolved in an aqueous solution of carboxymethyl cellulose (0.5% w/v) because of its low solubility in water.⁸ The model control group and the blank control group were fed 1 mL of 0.5% sodium carboxymethyl cellulose per 200 g body weight once per day for 42 days. The dosage was adjusted every week according to any change in body weight.

The estrous cycle of rats can reflect the function of the ovary. The estrus cycle of normal rats was 4–5 days, and the estrus period, pre-estrus, estrus and late-estrus emerged alternately. To develop a drug-induced model of POI, we prepared and administered a 50 mg/kg suspension of tripterygium wilfordii polyglycoside tablets in 0.9% sodium chloride solution in rats for 14 days. Then found that rats decreased food and water intake, slow reactions, decreased locomotor activity. The following inclusion criteria were used to assess POI: the previously described changes in mental and active status, as well as smears of vaginal epithelial exfoliative cells showing an estrous cycle disorder. Estrous cycle disorder can possibly be expressed in that the estrus cycle is extended to 6–10 days, continuous estrus or no pre-estrus and estrus. Despite changes in mental and active status, vaginal epithelial cytology smears showing a normal estrous cycle (4–5 days and the estrus period, pre-estrus, estrus and late-estrus emerged alternately) were used as exclusion criteria for POI. Previous studies have shown that disordered estrous cycles indicate successful establishment of a POI model.^{9,10} The optimum concentration of tripterygium wilfordii polyglycoside tablets used in this study was based on the rat model of induced POI by Hao Juan.¹¹

Drug preparation and instruments

The tripterygium wilfordii polyglycoside tablet suspension was formulated at a concentration of 10 mg/

mL in 0.9% sodium chloride solution. Because resveratrol is insoluble in water, it was dissolved in 0.5% sodium carboxymethyl cellulose solution and formulated at a concentration of 10 mg/mL. For sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) and western blot analysis, two plates were used to prepare a 10% separation gel consisting of 6.1 mL double-distilled water, 5.0 mL 30% Acr-Bis, 3.75 mL separation buffer (4×), 150 µL 10% ammonium persulphate (APS) and 6 µL tetramethyl ethylenediamine (TEMED). In addition, two plates were used to prepare a 6% separation gel consisting of 8.1 mL double-distilled water, 3 mL 30% Acr-Bis, 3.75 mL separation buffer (4×), 150 µL 10% APS and 12 µL TEMED. The formulation of a 5% concentrate was 2.33 mL double-distilled water, 0.67 mL 30% Acr-Bis, 1.0 mL concentrated plastic buffer (4×), 40 µL 10% APS and 4 µL TEMED.

We obtained resveratrol (Shaanxi Huike Botan Plant Development Co., Ltd.), tripterygium wilfordii polyglycoside tablet (Grand Pharmaceutical Huangshi Feiyun Pharmaceutical Co., Ltd.), carboxymethylcellulose sodium (Beijing Solarbio Science & Technology Co., Ltd.) and an enzyme-linked immunosorbent assay (ELISA) detection kit from Shanghai Enzyme Biotechnology Co., Ltd.. The primary antibodies of PI3K, phospho (p)-PI3K, Akt, p-Akt, mTOR, p-mTOR, Bax, Caspase-3 and Bcl-2 were obtained from AbSci and β-actin was obtained from TransGen Biotech. The goat anti-rabbit secondary antibodies were purchased from EarthOx Life Sciences. Protease inhibitor phenylmethylsulfonyl fluoride (PMSF), the enhanced chemiluminescence (ECL) reagent kit, radio-immunoprecipitation assay (RIPA) and the butyrylanoacrylate (BCA) protein assay kit were purchased from Beyotime Biotechnology. To perform the various experiments, we used a DJ-1 magnetic stirrer (Jintan Optical Instrument), EG1150C tissue embedding system (Leica), RM2135 rotary microtome (Leica), BX53 upright microscope (Olympus), CT15RE vertical low-temperature high-speed centrifuge (Hitachi Electronics), Infinite M200 multifunctional microplate reader (Tecan), electrophoretic system and 300w film transfer instrument (Bio-Rad), LAS4010 chemiluminescence analysis system (GE Healthcare), and a R104 tissue homogenizer (IKA).

Experimental methods

Determination of serum malondialdehyde and superoxide dismutase by ELISA

On day 70, all 90 rats were euthanized. Blood samples were centrifuged at 3000 rpm for 20 min to separate

components, which were collected and stored at -80°C until further use. Serum levels of malondialdehyde (MDA) and superoxide dismutase (SOD) were spectrophotometrically measured by ELISA. All procedures were strictly performed according to the manufacturers' protocols.

Immunohistochemical detection of p-Akt, p-mTOR, Bax, Bcl-2 and Caspase-3 protein expression in ovarian tissue

Ovaries were harvested after carefully isolating surrounding adipose tissue and fascia, and half were fixed in 4% paraformaldehyde at room temperature for 24 h, then dehydrated through a series of ethanol concentrations, cleared using xylene and embedded in paraffin. Ovarian sections of 4 µm thickness were prepared for immunohistochemistry (IHC), mounted and then observed under a light microscope. All sections were incubated at 60°C for 1 h, deparaffinized in xylene and rehydrated in a graded series of ethanol. Antigen retrieval was performed in sodium citrate buffer (pH 6.0) and high microwave irradiation for 30 min. Endogenous peroxidase activity was eliminated with 3% hydrogen peroxide for 10 min, rinsed three times with phosphate-buffered saline (PBS), followed by blocking of non-specific binding with 10% normal goat serum for 30 min at room temperature. Next, the sections were separately incubated with primary antibodies against Bax (1:100), Bcl-2 (1:100), p-Akt (1:50), p-mTOR (1:50) or Caspase-3 (1:150) overnight at 4°C . The sections were rinsed with PBS, followed by the addition of secondary antibodies for 10 min. After a PBS wash, all sections were incubated with horseradish peroxidase for 10 min at room temperature. Following wash, sections were incubated with 3, 3'-diaminobenzidine to visualize the final product, and stained with hematoxylin. Slides were rinsed in tap water for 10 min, treated with a gradient of alcohol for dehydration, transparent with xylene, dried with filter paper and sealed with neutral gum. Negative controls were prepared by substituting PBS for the primary antibody. Image-Pro Plus 6.0 was used to detect the integral optical density (IOD) value for semi-quantitative analysis.

Western blot analysis of PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, Bax, Bcl-2 and Caspase-3 protein expression in ovarian tissue

The remaining half of harvested ovaries from each group were stored at -80°C until western blot analysis. Ovary protein levels of PI3K, p-PI3K, Akt, p-Akt,

mTOR, p-mTOR, Bax, Bcl-2 and Caspase-3 were measured using western blot analysis. Ovary tissue was homogenized using RIPA and PMSF then incubated for 30 minutes on ice. The supernatants were collected for protein analysis after centrifugation (12 000 rpm for 15 min at 4°C), and total protein concentrations were determined using the BCA protein assay kit. Protein samples (10 µL per lane) were separated by SDS-PAGE (80–120 V) and transferred onto polyvinylidene difluoride membranes. Membranes were blocked using 5% nonfat dry milk for 1 h at room temperature, and then incubated overnight at 4°C with the primary antibodies, PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, Bax, Bcl-2 (1:500), Caspase-3 (1:300) and β-actin (1:5,000), followed by corresponding secondary antibodies for 2 h at room temperature. Protein bands were visualized using an ECL reagent. After development with Image-Pro Plus 6.0 for grayscale determination, β-actin was used as the internal loading control.

Statistics

Statistical calculations were performed with SPSS Statistics for Windows version 19.0 (IBM Corp.). If univariate quantitative data of groups met the normal distribution and homogeneity of variance criterion, one-way analysis of variance (ANOVA) was used between the five groups. Continuous variables are presented as the mean ± standard error (SE). $P < 0.05$ was considered statistically significant.

Results

Based on measurements of serum MDA and SOD levels by ELISA, we found that the level of serum MDA in the model control group was higher than that in any resveratrol-treated group (low, middle and high dose) and the blank control group ($P < 0.05$) (Table 1). In addition, the serum SOD level in rats of the model control group was lower than that found in the resveratrol-treated groups (low, medium and high dose) and the blank control group ($P < 0.05$).

Immunohistochemical detection of p-Akt, p-mTOR, Bax, Bcl-2 and Caspase-3 expression in ovarian tissues is shown in Figure 1. Bax and Caspase-3 were relatively abundant in ovarian tissues of the model control group (Fig. 1A,B, respectively), with expression mainly observed in ovarian granulosa cells. Bax expression was enriched in the cytoplasm, and Caspase-3 expression was localized in the nucleus

Table 1 Comparison of serum MDA and SOD levels in five groups of rats ($\bar{x} \pm s$)

Group	<i>n</i>	MDA (nmol/mL)	SOD (U/mL)
A	18	3.20 ± 0.11	121.63 ± 6.21
B	18	2.99 ± 0.31	141.05 ± 12.09
C	18	2.61 ± 0.35	144.05 ± 18.08
D	18	3.78 ± 0.22	79.33 ± 5.03
E	18	1.70 ± 0.27	176.50 ± 14.23

MDA: A and B compared with the two groups was not statistically significant, $P > 0.05$, the other two groups were statistically significant, $P < 0.05$. SOD: B and C were not statistically significant, $P > 0.05$, the other two groups were statistically significant, $P < 0.05$. and A, low-dose treatment group; B, moderate-dose treatment group; C, high-dose of treatment group; D, model control group; E, blank control group; MDA, malondialdehyde; SOD, superoxide dismutase.

and cytoplasm. In the three resveratrol treatment groups (groups A–C) and the blank control group (group E), there was less expression in ovarian granulosa cells, which exhibited lighter coloring. In the model control group (group D), minimal expression of Bcl-2, p-Akt and p-mTOR was observed in ovarian tissues (Fig. 1C–E, respectively), while maximal expression was detected in ovarian granulosa cells. Bcl-2 was mainly localized in the cytoplasm, while p-Akt and p-mTOR were largely found in the nucleus. In contrast, we found increased expression of p-Akt, p-mTOR and Bcl-2 in resveratrol-treated rats at any dose tested (groups A–C) and rats from the blank control group (group D). These findings demonstrate that resveratrol influenced the expression of factors involved in the activation of the PI3K/Akt/mTOR signaling pathway in the early treatment of POI.

Next, we used western blot analysis to assess protein levels of PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, Bax, Caspase-3, Bcl-2 and β-actin in the five groups studied (summarized in Fig. 2A). Compared with the model control group, there were increased levels of p-PI3K, p-Akt, p-mTOR and Bcl-2 proteins and decreased levels of Bax and Caspase-3 proteins in ovarian tissues (both $P < 0.05$) from the resveratrol-treated group (groups A–C). However, compared with levels found in the blank control group, the protein levels of p-PI3K, p-Akt, p-mTOR and Bcl-2 were decreased and the expression of Bax and Caspase-3 were increased (both $P < 0.05$) in ovarian tissue of resveratrol-treated rats (groups A–C) (Fig. 2B). These findings indicate that resveratrol exerted a pharmacological effect that countered the ovarian dysfunction associated with POI by activating elements of the PI3K/Akt/mTOR signaling pathway, including p-PI3K, p-Akt and p-mTOR.

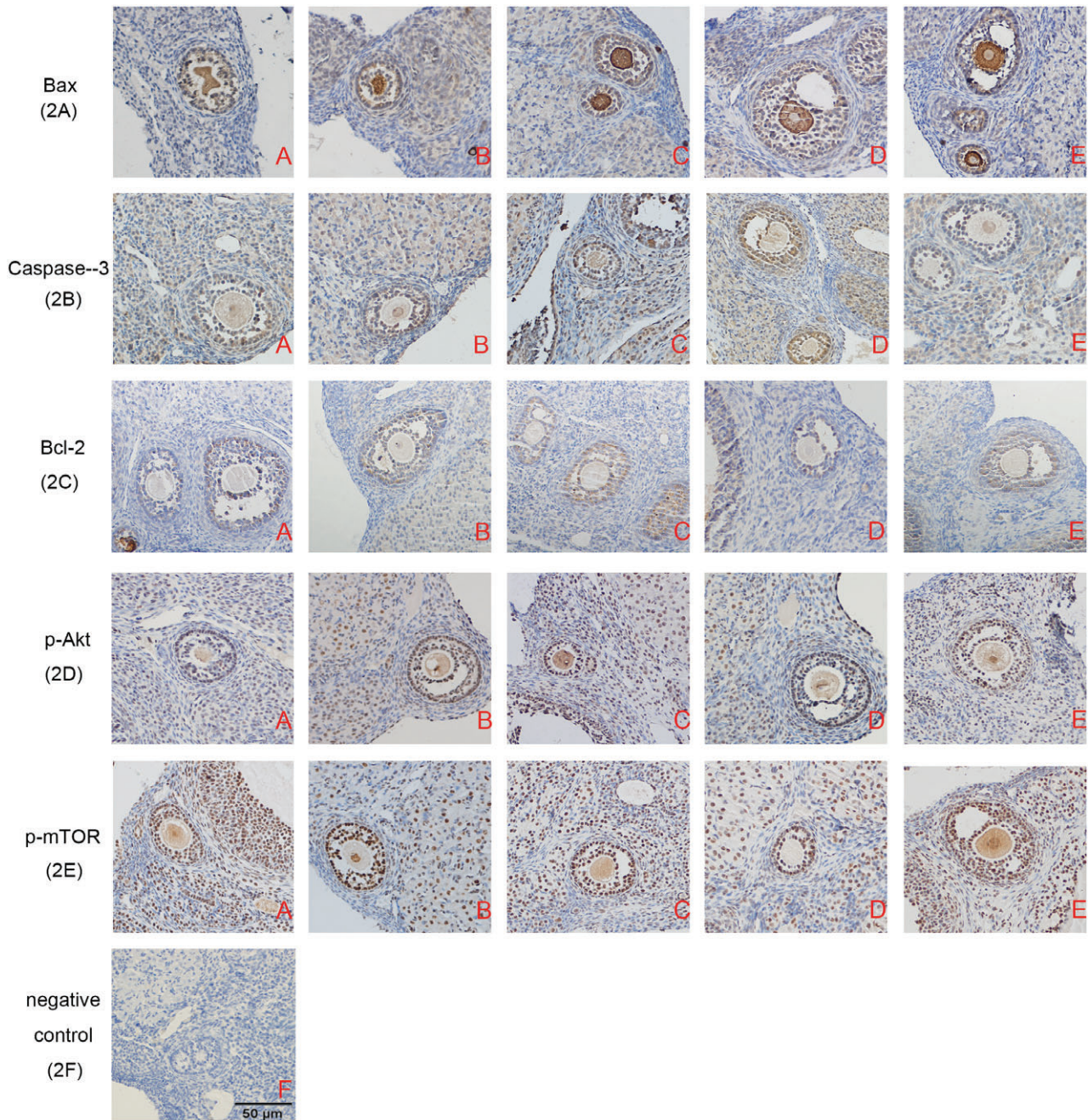


Figure 1 Immunohistochemical comparisons of Bax, Caspase-3, Bcl-2, p-Akt and p-mTOR staining in ovarian tissue of resveratrol-treated, model and blank control rats. A, low-dose treatment group (200 \times); B, moderate-dose treatment group (200 \times); C, high-dose treatment group (200 \times); D, model control group (200 \times); E, blank control group (200 \times); F, antibody negative control group (200 \times). Scale bar: 50 μ m.

Discussion

Early treatment of ovarian dysfunction remains a major area of research and clinical interest in

obstetrics and gynecology. Because its pathogenesis is not clear, and there is no effective treatment regimen, current approaches for POI entail estrogen and progesterone replacement therapy. Resveratrol is

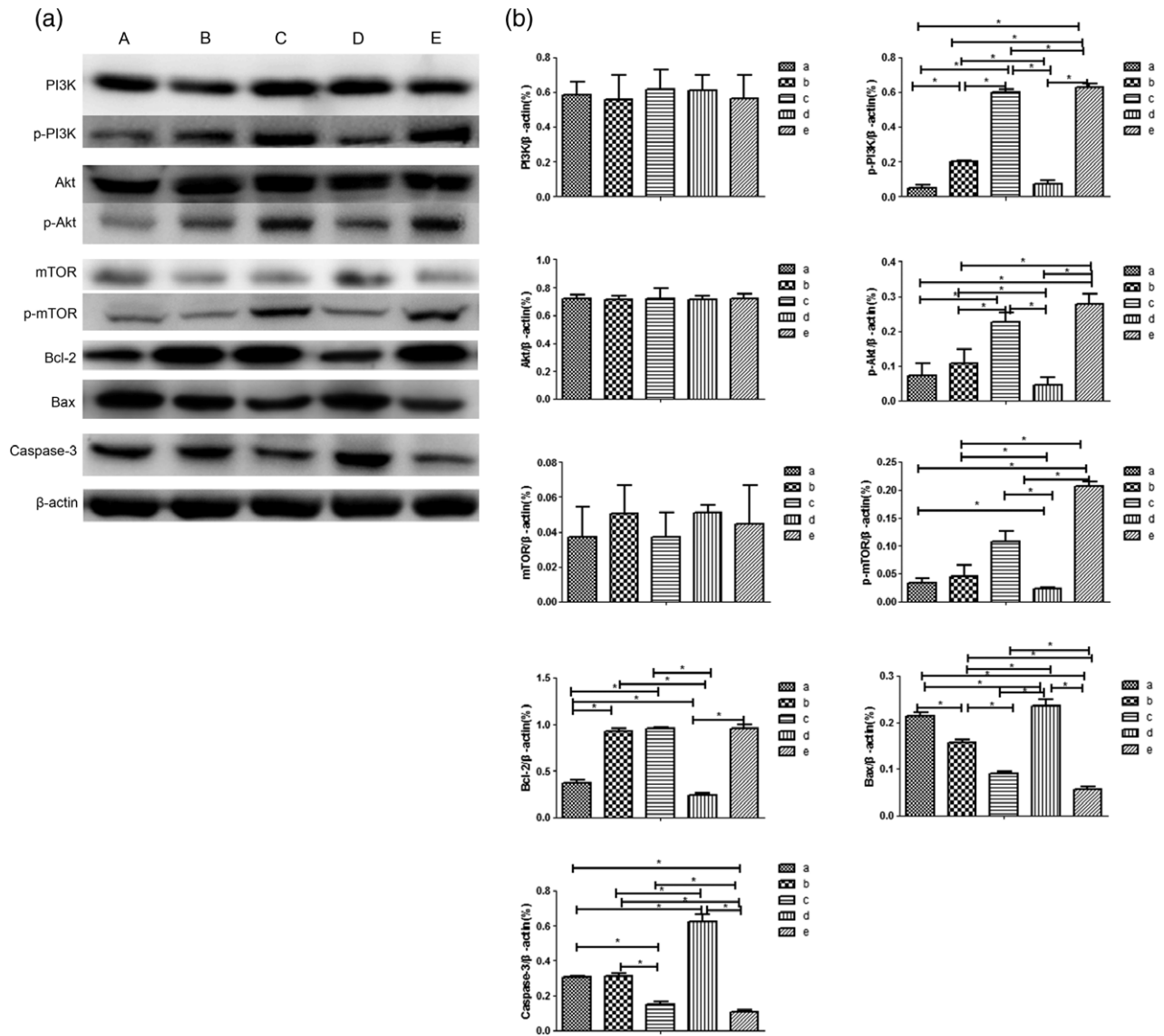


Figure 2 Western blot of PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, Bax, Caspase-3, Bcl-2 and β-actin in ovarian tissue of resveratrol-treated, model and blank control rats. A, low-dose treatment group; B, moderate-dose treatment group; C, high-dose treatment group; D, model control group; E, blank control group. * $P < 0.05$ was considered statistically significant.

derived from plants, and has relatively few adverse reactions, while conferring anti-inflammatory, anti-oxidation and anti-aging effects.³ Oxidative stress can induce cell activation, proliferation, apoptosis and other processes; it is also one of the most important mechanisms leading to ovarian damage. In our study, after treatment with resveratrol, ovarian function in POI rats was improved as indicated by a decreased level of serum MDA and increased level of SOD. Studies have shown that resveratrol may be

effective in treating premature ovarian insufficiency; nevertheless, the mechanism by which resveratrol improves ovarian function is not entirely known. Oxidative stress plays an important role in the process of ovarian aging. Owing to the natural antioxidant properties of resveratrol, it may be an effective factor for protecting ovarian tissue from oxidative damage.¹²

In recent years, researchers investigating the mechanism of early onset of ovarian insufficiency confirmed

from ovarian-related physiological and pathological studies using rat models that the PI3K/Akt/mTOR pathway was not only involved in regulating oocyte growth and regulation, but also had a role in the survival and development of the original follicle, promoted granulocyte proliferation and differentiation, and inhibited apoptosis of granulosa cells.¹³ PI3K is composed of the catalytic and regulatory subunits p110 and p85, respectively. The p110 subunit has both Ser/Thr kinase and phosphatidylinositol kinase activities.¹³

Akt is also known as protein kinase B. When cells are stimulated by extracellular signals, PI3K activation produces PIP3 that results in transcription of Akt, which once fully activated in the cell membrane, causes a cascade of signal transduction pathways.^{14,15} Pdk1 is the primary signal for PI3K regulation, and selective knockout of Pdk1 in mouse oocytes leads to POI,¹⁶ whereas the mTOR pathway promotes follicular proliferation by direct or indirect regulation of upstream Akt.¹⁷

The POI induction model in female Sprague-Dawley rats used in this study was based on a study of POI-induced rats by tripterygium wilfordii polyglycoside exposure described by Hao Juan.¹¹ After treatment with resveratrol, we found that ovary function in POI rats was improved, as indicated by decreased serum MDA and increased SOD. From our IHC results revealed that Bax and Caspase-3 were relatively abundant in ovarian tissues of the model control group localized in granulosa cells. In contrast, there was minimal expression in ovarian granulosa cells in all three resveratrol-treated groups and the blank control group. Moreover, there was minimal expression of Bcl-2, p-Akt and p-mTOR in ovarian tissue from the model control group, but strong expression found in ovarian tissues from the resveratrol low-, moderate-, and high-dose resveratrol treatment groups as well as the blank control group. Western blot analysis results showed that the expression of p-PI3K, p-Akt, p-mTOR and Bcl-2 proteins in the ovarian tissue of rats with POI after intragastric administration of resveratrol was increased, while the expression of Bax and Caspase-3 proteins was decreased. Resveratrol can relieve damage to ovarian function by activating the PI3K/Akt/mTOR signaling pathway, reduce oxidative stress and inhibit apoptosis of granulosa cells. The PI3K/Akt/mTOR signaling pathway plays a decisive role in the maintenance of normal ovarian function, and regulation of the PI3K

signaling pathway has broad implications for the treatment of POI.

In summary, we demonstrated that resveratrol enhances rat ovarian function by activating the PI3K/Akt/mTOR signaling pathway, thereby reducing oxidative stress and inhibiting granulocyte apoptosis. This signaling pathway may be a new therapeutic target for POI, and the early application of resveratrol combined with other drugs may provide an efficient treatment approach for POI.

Disclosure

None declared.

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