Original Article
Assessment of *Lycium barbarum* L. fruits’ effect on premature ovarian failure in rats

Liju Huang¹, Wei Tian², Fujun Qu¹, Zhimin Ren³

¹Department of Pharmacy, The Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, P. R. China; ²Liaoning Koncepnutru Co. Ltd, Benxi, Liaoning, P. R. China; ³Department of Pediatrics, The Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, P. R. China

Received April 13, 2016; Accepted September 5, 2016; Epub October 15, 2016; Published October 30, 2016

Abstract: Premature ovarian failure (POF) is defined as the loss of physiological ovarian function before 40 years old in women by the WHO. The effect of *Lycium barbarum* L. fruits ethanol extracts (Barbary Wolfberry Fruit, BWFE) and its possible mechanisms in treating POF was studied in female Sprague-Dawley rats whose POF was induced by cyclophosphamide. Rat behavior, serum hormone levels, ovarian and uterine size, body weight, pathological features, ovarian tissue expression of Bcl-2/Bax and phosphorylation of PI3K/AKT signal pathway were assessed in controls, untreated POF model rats, and POF model rats feed with low- (200 mg/kg), medium- (400 mg/kg), and high-dose (800 mg/kg) BWFE. Compared with the untreated POF model rats, the rats treated with 200, 400 and 800 mg/kg BWFE show more stable behavior and better coat appearance. The serum hormone levels in BWFE treated group were closer to those in control rats. In addition, treatment with 200, 400 and 800 mg/kg BWFE increased ovarian and uterine size. Hematoxylin and eosin (HE) staining exhibited mature follicles and endometrium with an alternating concave/convex surface structure with visible capillaries and glands in BWFE-treated POF rats. BWFE also regulated the Bcl-2/Bax expression and PI3K/AKT signaling in POF rats. Our results demonstrated that BWFE may effectively prevent POF and arbitrate POF symptoms by regulating serum hormone levels, altering the expression levels of Bcl-2/Bax, and regulating PI3K/AKT signaling pathway related to POF in ovarian tissue.

Keywords: POF, barbary bolfberry fruit, rat, PI3K/AKT

Introduction

Premature ovarian failure (POF) is considered as the loss of physiological ovarian function before the age of 40 by the World Health Organization (WHO) [WHO Scientific Group, 1996] [1]. The clinical symptoms of POF include amenorrhea, infertility, perimenopausal syndrome, hypoestrogenism, and abnormally high levels of luteinizing (LH) and follicle stimulating hormone (FSH) [1-3]. The incidence of POF is associated with cell apoptosis in ovarian granular cells closely [4, 5]. Cyclophosphamide can promote atresia, reduce immature and mature ovarian follicles and suppress the phosphatidylinositol-3 kinase (PI3K) pathway in follicles leading to ovarian failure in mice and rats. PI3K/AKT as an important signal transduction pathway regulates cell proliferation, apoptosis, differentiation, angiogenesis and autophagy. Bcl-2/Bax play crucial role in apoptosis of ovarian granular cells and abnormal expression of Bcl-2/Bax also give rise of cell apoptosis. In recent years, the incidence of POF is increasing, which is associated with tumor chemotherapy in women. Chemotherapy displays serious damage to ovarian, and the use of alkylating agent tends to give rise of POF in women [6-10]. A sharp drop in the number of follicles is observed in patients suffered with POF, which result to poor ovarian function [11-14]. Supplement of oestrogen can alleviate symptoms of POF, while the hormone replacement therapy is limited by its side effects. Therefore, there is a critical need to develop effective therapeutics for the prevention and treatment of POF.

Fruit of *Lycium barbarum* L. is an important Chinese materia medica (CMM) and nowadays has been widely used as a popular functional food with vital biological activities [15, 16]. The
main chemical components were L. barbarum polysaccharides (LBP), zeaxanthin, and other constituents with small molecules, such as betaine, cerebroside, β-sitosterol, p-coumaric acid, and various vitamins. Many beneficial health effects of LBPs, such as immune regulation, anti-stress, anti-colon cancer, neuroprotective, and anti-diabetic effects, have been reported [17-20].

At present, we demonstrated whether Lycium barbarum L. fruits (Barbary Wolfberry Fruit, BWFE) could be effective in treating and preventing POF in a rat model of POF which was induced by treatment with cyclophosphamide [21, 22] and identified the changes in hormone levels in POF-like rats with and without BWFE treatment compared with the healthy control rats. Bcl-2/Bax play crucial role in apoptosis of ovarian granular cells, which is one of the causes to POF. We also estimate the Bcl-2/Bax expression in the ovarian granular cells of five groups by quantitive real time-PCR. The phosphorylation of phosphatidylinositol-3 kinase/protein kinase B (PI3K/AKT) signaling associated with cell apoptois was also identified by western blot.

Materials and methods

Animals

Adult female wistar rats were obtained from China Medical University (Shenyang, China) and housed in an air conditioned facility with a room temperature of 25 ± 1°C, humidity of 50 ± 5%, and a 12-h light/dark cycle, and supplied with food and water ad libitum. All rats were allowed to acclimate to the animal facilities for 1 week prior to experiments, and then they were randomly divided into the following five groups (n = 10 in each): untreated control rats, POF model rats, POF model rats treated with low-BWFE (200 mg/kg), medium BWFE (400 mg/kg), and high-dose BWFE (800 mg/kg). The study was approved by the ethics committee of The Second Affiliated Hospital of Harbin Medical University.

Extract of BWFE preparation

The dried Lycium barbarum L. fruits collected from Hua Yu Chinese Herbs Co., Ltd., were identified by Prof. Fujun Qu, Department of pharmacy, the Second Affiliated Hospital of Harbin Medical University. The Lycium barbarum L. fruits were extracted with 95% ethanol by reflux twice, and each time for 2 hours. The extract was concentrated under reduced pressure, and then dissolved in distilled water.

POF induction and treatment

Except for the control group, rats were intraperitoneally injected with cyclophosphamide (50 mg/kg) on the first day, and then intraperitoneally injected with cyclophosphamide (8 mg/kg) every day for 14 days. Rats were then were given by gavage with the same dose of normal saline daily for 4 weeks. After induction of POF, different doses of BWFE were administered to rats in the treatment groups by gavage for 4 weeks [23]. The clinical BWFE concentration and dose range were converted into the administered rat doses. General condition and the status of the rats was recorded daily, such as the weight.

Pathological evaluation

Pathological evaluation was carried out in accordance with the previous report [24]. Briefly, after the final administration of BWFE or saline, rats were fasted for 12 h and then anesthetized with 10% chloral hydrate. After weighing, specimens were fixed in formalin for subsequent paraffin embedding. Ovary and uterus specimens were sectioned at 7 μm and then stained with hematoxylin and eosin (H&E). Ovary samples for RNA analysis were frozen in liquid nitrogen. Serum and ovary samples were stored at -80°C. After blood collection, ovary and uterus specimens were removed and weighed. Ovarian and uterine indices were calculated by dividing the weight of each organ by the animal’s total body weight.

Cell isolation and apoptosis analysis

All the rats were sacrificed for bilateral ovaries excised in sterile conditions after 48 h, antral follicles were cut-out by eye scissors under the anatomical microscope with 40 times, the pressure caused oocytes and granulosa cells to diffuse out of antral follicles, released granular cells was add into pre-cooling midium DMEM (HyClone corporation, Utah, USA). Granular cells were filtered with stainless steel cellcribble (200-mesh sieve). The granular cells were collected by brief centrifugation, and then sup-
Assessment of *Lycium barbarum* L. fruits’ effect on POF

Assessment of *Lycium barbarum* L. fruits’ effect on POF.

ELISA

Then 5-ml blood samples were collected from an artery in the rat abdomen. Blood samples were centrifuged at 3000 r/min and 4°C for 15 min to obtain serum samples. Commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Quantikine, R&D Systems Inc., Minneapolis, MN, USA) were used to determine serum hormone levels of estrogen (E2), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) according to the manufacturer’s instructions.

RNA isolation

Ovarian tissue samples were thawed and homogenized. Total RNA from ovaries was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. In addition, the concentration and purity of RNA were assessed using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and RNA integrity was assessed via denatured agarose gel electrophoresis to ensure the presence single bands at the predicted sizes.

Real time-PCR (RT-PCR)

Total RNA isolated from each sample was reverse transcribed to generate cDNA using Trizol reagent (Gibco® life technology, Carlsbad, CA, USA) according to the manufacturer’s instructions. RT-PCR was performed in duplicate using AccuPower GreenStar qPCR PreMix (Bioneer) following the manufacturer’s protocols. The primers for each gene were listed as following: 5'-GACCGACCGGATGAGAT-3’ and 5'-TCAGGATGAGGTCGATGAGT-3’ for Bax; 5'-CA-GATGCAGTTAATGCCCA-3’ and 5'-CCTGCTGCTGTTGATTCCTTT-3’ for Bcl-2; 5'-ATACACTGCCACCCAGAAG-3’ and 5'-TCCACGAGGCAGACATTG-3’ for GAPDH (product: 191 bps). ABI 7300 system (Applied Biosystem, Foster City, CA, USA) was programmed to initially incubate the samples at 95°C for 10 min, and then denature at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. The data were normalized to expression levels of GAPDH, and the relative expression levels of relevant genes were quantified using the 2^−ΔΔCt method.

Western blot

Tissue samples were first washed with ice-cold PBS, and the homogenates were lysed in RIPA buffer (Sigma). The protein concentrations of all samples were determined using the bicinchoninic acid assay (Pierce Chemical, Rockford, IL, USA). After which, the samples were stored at -80°C until further use. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Each membrane was blocked with 5% bovine serum albumin in a PBS/Tween-20 solution. The blots were incubated overnight with Bcl-2, Bax, phosphorylation of PI3K, PI3K, phosphorylation of AKT, AKT antibodies (Abcam Inc., Cambridge, MA, USA). After washing with the PBS/Tween-20 solution, the blots were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences), which were then exposed to Biomax L films (Kodak).

Statistical analysis

In this study, all the experiments were conducted at least in triplicate and the results were expressed as mean ± SD. Significance in the difference among different treatments was analyzed by one-way ANOVA analysis at *P* < 0.05 (*) or *P* < 0.01 (**) level.

Results

Changes in behavior, state and oestrous cycle in rats

Rats in POF group exhibited dropping spirit, roach back, curled up, less activity, slow action, hair loose, serious loss, and weight loss. POF rats in BWFE treatment group exhibited weight gaining, sensitive and hair moist bright. Rats in the control group have no obvious abnormal behavior. As for the estrus cycle, rats in the control group rats show normal oestrous cycle: 4 to 5 days. The disorder was existed in oestrous cycle of POF rats, which is 7-9 days. The oestrous cycle of BWFE (200 and 400 mg/kg)
Assessment of *Lycium barbarum* L. fruits’ effect on POF

19366


treated rats were 6-8 days. After BWFE (800 mg/kg) treatment for four weeks, we observed that the oestrous cycle of rats’ changes remarkably close to normal: 5-6 days.

**Effect of BWFE on ovarian tissue and body weight of POF rats**

We further examined the pathological observation of rats’ ovarian tissue in five groups by H&E staining. As shown in Figure 1A, in histopathologically stained sections of ovaries from control rats, large numbers of visible, mature follicles were seen at various stages of development. On the contrary, fewer or no follicles were observed in ovarian sections from untreated POF rats, which showed larger follicle atresia (Figure 1B).

As for the body weight, body weight of POF rats was significantly decreased in the 1 and 2 weeks (Table 1). After BWFE treated for 1 week, the body weight of rats (BWFE 800 g/kg) was bigger than that of the POF rats. In addition, rats of moderate- and high-dose of BWFE showed obvious increase of body weight compared with the untreated POF rats (Table 1).

**BWFE protects preserves ovarian and uterine size and weight**

Ovarian and uterine indices were calculated for organs harvested at the end of the 30-day study period (Figure 2A, 2B). Compared with

---

**Table 1. Effect of BWFE on body weight of rats**

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>0 week (g)</th>
<th>1 week (g)</th>
<th>2 weeks (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>177.37 ± 18.56</td>
<td>197 ± 15.28</td>
<td>215 ± 12.81</td>
</tr>
<tr>
<td>POF</td>
<td>10</td>
<td>179.97 ± 14.24</td>
<td>170 ± 14.83**</td>
<td>162 ± 16.29**</td>
</tr>
<tr>
<td>BWFE (200 mg/kg)</td>
<td>10</td>
<td>180.18 ± 17.36</td>
<td>172 ± 11.69</td>
<td>160 ± 17.24</td>
</tr>
<tr>
<td>BWFE (400 mg/kg)</td>
<td>10</td>
<td>179.13 ± 17.81</td>
<td>175 ± 17.31</td>
<td>174 ± 14.95*</td>
</tr>
<tr>
<td>BWFE (800 mg/kg)</td>
<td>10</td>
<td>182.57 ± 15.96</td>
<td>180 ± 15.87**</td>
<td>176 ± 14.21**</td>
</tr>
</tbody>
</table>

**P** < 0.01, compared with the control group; *P* < 0.05, compared with the POF group; **P** < 0.01, compared with the POF group. POF: Premature ovarian failure; BWFE: Barbary Wolfberry Fruit Extracts.
Assessment of *Lycium barbarum* L. fruits’ effect on POF

The ovaries and uteri from control rats, ovaries of untreated POF model rats displayed significant atrophy (*P* < 0.001), and the uteri of untreated POF model rats were smaller at the end of the study period (*P* < 0.01). However, the ovarian and uterine indices were greater in POF rats treated with medium-dose (*P* < 0.01 for both indices) and high-dose (*P* < 0.01 for ovarian index and *P* < 0.05 for uterine index) BWFE than those in untreated POF rats. Interestingly, the medium dose of BWFE had a greater effect on ovarian and uterine indices than the high dose. Treatment with low-dose BWFE had no effect on these indices (*P* > 0.05).

**Effect of BWFE on E2, FSH, and LH in serum**

Levels of E2, FSH, and LH in serum were measured by ELISA in the five groups at the end of the 30-day experimental period. The E2, FSH, and LH levels in untreated POF model rats were increased obviously compared with the control group (*Table 2, P* < 0.05). Treatment of POF rats with low, medium and high-dose BWFE can lead to different levels of increase in the serum levels of E2, FSH, and LH in a dose-dependent manner (*Table 2, P* < 0.05).

**BWFE reduced cell apoptosis of granulosa cells**

We then isolated ovarian granulosa cells as previously described and cell apoptosis were identified by FCM. As shown in **Figure 3A**, cell apoptosis of POF rats was increased to 28.3 ± 5.38% compared the control group (*P* < 0.01). Moderate and high concentration of BWFE can reduce the apoptosis rate to 11.8 ± 2.36% and 8.1 ± 2.34%, respectively (*P* < 0.01).

**BWFE regulated Bax/Bcl-2 expression and PI3K-AKT in ovarian granular cells**

Bax/Bcl-2 expression plays a crucial role in apoptosis of ovarian granular cells. RT-PCR and western blot were employed to identify the mRNA and protein level of Bax/Bcl-2 expression, respectively. As shown in **Figure 3B-D**, Bcl-2 expression was decreased and Bax expression was increased significantly in untreated POF group compared with the control group. With BMF treatment for 30 d, we can see that Bcl-2/Bax expression was increased obviously both in mRNA and protein level.

**Table 2. Effect of BWFE on E2, FSH, and LH in serum**

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>E2 (pg/ml)</th>
<th>FSH (mIU/ml)</th>
<th>LH (mIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>95.23 ± 8.17</td>
<td>4.58 ± 0.95</td>
<td>3.59 ± 0.35</td>
</tr>
<tr>
<td>POF</td>
<td>10</td>
<td>70.15 ± 5.89</td>
<td>6.87 ± 0.64</td>
<td>5.41 ± 0.75</td>
</tr>
<tr>
<td>BWFE (200 mg/kg)</td>
<td>10</td>
<td>80.18 ± 8.97</td>
<td>6.89 ± 0.77</td>
<td>4.57 ± 1.24</td>
</tr>
<tr>
<td>BWFE (400 mg/kg)</td>
<td>10</td>
<td>88.45 ± 7.84</td>
<td>6.87 ±0.64</td>
<td>4.48 ± 0.68</td>
</tr>
<tr>
<td>BWFE (800 mg/kg)</td>
<td>10</td>
<td>91.89 ± 9.43</td>
<td>5.14 ± 0.57</td>
<td>4.12 ± 0.74</td>
</tr>
</tbody>
</table>

**P** < 0.01, compared with the control group; *P* < 0.05, compared with the POF group; **P** < 0.01, compared with untreated POF model group.

*POF: Premature ovarian failure; BWFE: Barbary Wolfberry Fruit Extracts.

---

**Figure 2.** Ovarian (A) and uterine (B) indices calculated based on weights of harvested organs. Ovarian index = ovarian weight/body weight; Uterine index = uterine weight/body weight. Data are expressed as mean ± SD, *n* = 10. **p** < 0.01, compared with the control group; *p* < 0.05, **p** < 0.01 compared with the untreated POF model group.
Assessment of *Lycium barbarum* L. fruits’ effect on POF

Figure 3. Ovarian granular cells apoptosis and Bcl-2/Bax expression in ovarian granular cells. A. Ovarian granular cell apoptosis was identified by FCM. B and C. mRNA expression of Bax and Bcl-2 in ovarian granular cells was identified by RT-PCR. Data are expressed as mean ± SD, n = 10. **P < 0.01, compared with the control group; *P < 0.05, **P < 0.01 compared with the untreated POF model group. D and E. Protein level of Bax and Bcl-2 in ovarian granular cells was identified by western blot. GAPDH was used as loading control. Data are expressed as mean ± SD, n = 10. **P < 0.01 compared with the control group; ***P < 0.01 compared with the untreated POF model group.
Assessment of *Lycium barbarum* L. fruits’ effect on POF

As shown in Figure 4A and 4B, phosphorylation of AKT and PI3K was decreased in the POF group compared with the control group, while BWFE can effectively increased the phosphorylation of AKT and PI3K in a dose-dependent manner compared with the untreated POF group.

**Discussion**

Cyclophosphamide can promote atresia, reduce immature and mature ovarian follicles and suppress the phosphatidylinositol-3 kinase (PI3K) pathway in follicles leading to ovarian failure in mice and rats [26]. Therefore, cyclophosphamide is applied in establishing the rat model of POF, which shows well stability and reproducibility. The validity of this model is further confirmed by the pathological results (HE staining in Figure 1) in the present study, which showed that the ovaries of POF rats contained fewer follicles than those in control rats and the endometrium of POF rats contained fewer capillaries and glands than that of control rats. The results obtained in the present study not only showed that rat behavior, appearance, serum E2 and FSH levels, ovarian and uterine size and pathological features, and mRNA expression of Bcl-2/Bax related to POF were altered in the cyclophosphamide-induced POF model as compared to controls, but also that these indicators could be preserved to varying degrees by administration of 200, 400 and 800 mg/kg BWFE. The effects of BWFE on serum E2 and FSH levels and ovarian and uterine size may improve on the symptoms of POF and their influence on behavior. The mature follicles and endometrial capillaries and glands observed in POF model rats treated with BWFE indicate that BWFE effectively prevented POF in this model.

The incidence of POF is associated with cell apoptosis in ovarian granular cells closely [4, 5]. Apoptosis, a programmed cell death, has an essential influence on premature occurrence and development of ovarian failure. It is regulated by a wide variety of factors, especially these belonging to the Bcl-2 family: inhibitors (i.e. Bcl-2 protein), promoters (i.e. Bax protein) and by activation of the apoptotic cascade via caspase-3. In the present study, cell apoptosis was increased in POF rats and can be descended significantly by BWFE treatment. We also found that cyclophosphamide effectively caused a decrease in Bcl-2/Bax expression which indicated that cell apoptosis was occurring in ovarian granular cells. Granulosa cell apoptosis is recognized as the main cause of follicular atresia, which can give rise to POF. Interestingly, BWFE can reverse the trend and BWFE can increase the Bcl-2/Bax expression in a dose-dependent manner compared with the model group. As a result, we demonstrated that BWFE can inhibit the apoptosis of ovarian granular cells in order to prevent the process of POF. Cell apoptosis was regulated by multiple signalings. PI3K/AKT was considered as one of the most important signalings in adjusting cell apoptosis. From the previous study, cyclophos-
Assessment of *Lycium barbarum* L. fruits' effect on POF

Phosphamide promotes atresia, reducing immature and mature ovarian follicles and inhibits the PI3K pathway in follicles leading to ovarian failure in mice and rats. BWFE can remarkably increase the p-PI3K and p-AKT expression in different levels.

In conclusion, our study demonstrated that BWFE effectively prevent and attenuate POF symptoms in rats and explained the possible mechanisms involved. BWFE could protect against the negative effects of cyclophosphamide in rat ovary and uterus, and regulating the expression of Bax/Bcl-2 via PI3K/AKT signaling. As a traditional Chinese medicine for preventing POF, BWFE may offer a treatment strategy that overcomes the limitations of symptomatic treatment.

**Disclosure of conflict of interest**

None.

**Address correspondence to:** Zhimin Ren, Department of Pediatrics, The Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, P. R. China. E-mail: zhimingr11@126.com

**References**


Assessment of *Lycium barbarum* L. fruits’ effect on POF


