The estrogen-like protective effect of *Lycium barbarum* polysaccharides in reducing oxidative stress on myocardial cells from ovariectomized rats

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Abstract. Previous studies have demonstrated that ovariectomy may lead to a reduction in antioxidative biomarkers in the myocardium, thus suggesting that estrogens may serve a protective role in the suppression of oxidative stress. *Lycium barbarum* polysaccharides (LBP) are a well-known antioxidant Chinese traditional medicine, which appear to have a similar function to estrogens with regards to the regulation of cardiac function. In the present study, 30 Sprague-Dawley rats were randomly divided into the following groups: Sham operation group, ovariectomized (OVX) group, estradiol valerate group, high-dose LBP (LBP-H) group and low-dose LBP (LBP-L) group. All of the rats were provided tap water, estradiol valerate or LBP for 12 weeks. In addition, all rats were ovariectomized, with the exception of rats in the sham operation group, which underwent fat removal only. Reactive oxygen species (ROS), malondialdehyde (MDA), glutathione peroxidase (GSH-px), catalase (CAT) and superoxide dismutase activities were subsequently examined. The protein expression levels of cleaved caspase-9, cleaved caspase-3 and phosphorylated-protein kinase B (p-Akt) were also assessed. The results demonstrated that high-dose LBP decreased the enhanced levels of ROS and MDA in OVX rats, whereas GSH-px and CAT activities were increased in the LBP-H group compared with in OVX rats. Furthermore, the expression levels of cleaved caspase-9 and cleaved caspase-3 were significantly upregulated in the OVX group, whereas high-dose LBP exerted protective effects on OVX rats by decreasing the expression of apoptotic proteins. Conversely, p-Akt expression was decreased in the OVX group and was increased in the LBP-H group. These results indicated that LBP is essentially involved in cardiac protection by inhibiting apoptosis in response to oxidative stress. In addition, improvement of antioxidant status by LBP is associated with the Akt signaling pathway in the myocardium of OVX rats.

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Introduction

Cardiovascular diseases are associated with a high rate of mortality in humans. In addition, cardiovascular diseases are less prevalent in women aged between 20 and 50 years compared with the corresponding male population. However, in individuals >50 years old, the incidence of cardiovascular disease is equivalent in both sexes (1,2). Previous studies have reported that the presence of estrogens serves an important role in protection against cardiac injury, thus suggesting that menopause may be a risk factor for numerous cardiovascular diseases (3,4). Studies in animal models have demonstrated that a lack of ovarian hormones, in particular estrogens, has detrimental effects on various organs, including the cardiovascular system (5,6). Hormone replacement therapy (HRT) has therefore been recommended to postmenopausal women; however, the controversies regarding the safety of HRT have drawn attention to novel therapies for postmenopausal women (3,7-9).

Lycium barbarum polysaccharides (LBP) may be used in TCM to prevent postmenopausal symptoms. Clinical research has indicated that polysaccharides extracted from *Lycium barbarum* may serve an important biological role. LBP is composed of arabinose, glucose, galactose, mannose, xylose and rhamnose monosaccharide, etc., and contains various trace elements and amino acids (10). A previous study demonstrated that LBP is the main active ingredient of the TCM medlar, which can regulate immunity and improve age-associated symptoms, including fatigue, loss of appetite and blurred vision, and may reduce blood lipid levels and fatty liver disease, and exert anti-aging effects (11).

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and the ability of the body to readily detoxify reactive intermediates or to repair the resulting damage (12). Numerous studies

have demonstrated that oxidative stress is an important factor underlying abnormal cardiovascular system structure (13-15). In addition, various cardiovascular diseases, including hypertension, atherosclerosis, myocardial ischemia, ischemia-reperfusion injury, myocardial hypertrophy and heart failure, are associated with an increase in ROS generation. Furthermore, a previous study revealed that estrogen exerts antioxidative effects, which may serve a role in cardiac protection (16).

Increased cardiovascular risk in postmenopausal women may be due to the postmenopausal reduction in estrogen levels; therefore, the antioxidative effects are weakened and cardiovascular disease may be initiated. In the present study, LBP exerted a protective effect against heart failure in rats. In addition, LBP has been reported to reduce isopropyl adrenaline-induced heart failure and rat heart mass/weight ratio, reduce myocardial injury and significantly improve cardiac function in rats; the underlying mechanism may be associated with an improvement in antioxidant enzyme activity and a reduction in lipid peroxide formation (17,18). The present study investigated whether LBP effects the oxidative stress state and induces antioxidative effects in the myocardium of ovariectomized (OVX) rats. In addition, the expression levels of apoptotic proteins and Akt pathway proteins were also detected in the myocardium. The present study aimed to explore whether LBP exerts protective effects against oxidative insult in OVX rats.

Materials and methods

Animals. A total of 30 female Sprague-Dawley rats (aged 10-12 weeks, weight 200±10 g) were purchased from Vital River Laboratories Co., Ltd., (Beijing, China). The rats were acclimated for 7 days, prior to use in subsequent experiments, and were housed in specific pathogen-free conditions (temperature 22±1˚C, humidity 50±5%) under a 12-h light/dark cycle. Tap water and chow were provided *ad libitum*. All efforts were made to minimize suffering, and procedures were performed under chloral hydrate (300 mg/kg, i.p.) anesthesia when necessary. The present study was approved by and followed the guidelines of the Animal Ethics Committee of Liaoning University of Traditional Chinese Medicine (Shenyang, China; permit no. 2011-167).

Experimental protocol. The adult female Sprague-Dawley rats were randomly divided into the following five groups (n=6/group): i) Sham operation group, in which a small region of fat was removed via bilateral paraspinal incisions, and rats were fed with tap water for 12 weeks; ii) OVX group, in which the ovaries of the rats were exteriorized and removed via bilateral paraspinal incisions, and rats were fed tap water for 12 weeks; ii) estradiol valerate group (Est), in which rats were fed estradiol valerate (0.105 mg/kg) for 12 weeks following OVX; iv) high-dose LBP group (LBP-H), in which rats were fed LBP (250 mg/kg) for 12 weeks following OVX; v) low-dose LBP group (LBP-L), in which rats were fed LBP (125 mg/kg) following OVX. All procedures were performed under 10% chloral hydrate anesthesia (300 mg/kg). Following surgery, all rats received prophylactic antibiotic therapy (penicillin G procaine; 4,000 IU/kg i.m.). Daily vaginal smears were collected from all rats, as previously described (19). This procedure allowed for the phase of the estrus cycle to be determined by daily analysis of the types of cells that sloughed off the vaginal epithelium. With this approach, four different stages can be observed, as follows: Proestrus (nucleated epithelial cells), estrus (cornified cells), metestrus (some cornified cells in addition to nucleated cells and a large number of leukocytes) and diestrus (leukocyte infiltration). Collected vaginal fluid was placed on glass slides and examined by light microscopy. In the Sham group, estrous cycle regularity was confirmed by the presence of vaginal epithelial cells characteristic of each of the four aforementioned stages. In the remaining groups, the absence of the estrous cycle was confirmed by a permanent diestrus phase.

Hormone assays. The level of 17β-Estradiol (E2) in serum was assessed using an enzyme-linked immunosorbent assay kit (cat. no. 10006315; MultiSciences Biotech Co., Ltd., Hangzhou, China) according to manufacturer's protocol. Absorbance was measured at 450 nm within 15 min (Multiskan FC; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Measurement of ROS, malondialdehyde (MDA), glutathione peroxidase (GSH-px), superoxide dismutase (SOD) and catalase (CAT) activities. The rats were anaesthetized with 10% chloral hydrate, and sacrificed by decapitation after 13 weeks. A 1-2 cm skin incision was first made, 0.5 cm below the rib and 1 cm next to the spine. Then the subcutaneous tissue, muscle and peritoneum were cut in turn. Following the opening of the peritoneal cavity, the rat kidney was identified and a white cellulite beneath it, exposing the soybean-size glandular spheres (the ovaries) which were removed by wire. Finally, following the confirmation that there was no intra-abdominal peritoneal bleeding, the peritoneum and skin were closed layer by layer, and the operating area cleaned. Following the surgery, in a warm environment, the rats were released into cages on wakening and given intraperitoneal injections of penicillin 160,000 units/rat three days after the surgery to prevent infection. The myocardium (100 mg) was homogenized in cold saline. The homogenate was then centrifuged at 900 x g for 15 min. The activities of ROS (cat. no. E004), MDA (cat. no. A003-2), GSH-px (cat. no. A005), SOD (cat. no. A001-1) and CAT (cat. no. A007-2; all from Nanjing Jiancheng Biological Engineering Institute, Nanjing, China) were determined using these assay kits according to the manufacturer's protocols.

Hematoxylin and eosin (H&E) staining of myocardium. Myocardium samples were fixed in 4% paraformaldehyde for 24 h at room temperature and stained with H&E, according to standard techniques. Briefly, the samples were embedded in paraffin and sections (5-μm) were obtained. The samples were then dewaxed with xylene, rehydrated through an alcohol gradient and were stained with H&E for light microscopy. Images were captured using a light microscope linked to a digital charge-coupled device camera (Olympus Corporation, Tokyo, Japan).

Protein extraction and western blotting. Total cellular proteins were extracted from heart tissues using Radioimmunoprecipitation Lysis Buffer (Beyotime Institute of

Biotechnology, Shanghai, China). Protein concentration was measured using a Bicinchoninic Acid Protein Assay kit (Beijing Dingguo Changsheng Biotechnology Co. Ltd., Beijing, China). Western blotting was conducted to assess the protein expression levels of B cell lymphoma-2 (Bcl2), Bcl2-associated X protein (Bax), cleaved caspase-9, cleaved caspase-3 and phosphorylated (p)-protein kinase B (Akt). Briefly, 40 μg protein samples were separated by 10% SDS-PAGE, after which the proteins were transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were then incubated overnight (4˚C) with antibodies against β-actin (cat. no. Sc-130300; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Bax (cat. no. Sc-4239; Santa Cruz Biotechnology, Inc.), Bcl2 (cat. no. Sc-509; Santa Cruz Biotechnology, Inc.), caspase-9/cleaved caspase-9 (cat. no. 9504; Cell Signaling Technology, Inc., Danvers, MA, USA), caspase-3/cleaved caspase-3 (cat. no. 9509; Cell Signaling Technology, Inc.), Akt (cat. no. 9662, Cell Signaling Technology, Inc.) and p-Akt (cat. no. 9667, Cell Signaling Technology, Inc.). The dilution of β-actin, Bax and Bcl2 antibodies was 1:500, and for caspase-9/cleaved caspase-9, caspase-3/cleaved caspase-3, Akt and p-Akt antibodies 1:1,000. The membranes were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (cat. no. Sc-2004; 1:3,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.). Protein expression was normalized to β-actin. ImageJ software version 1.45 (National Institutes of Health, Bethesda, MD, USA) was used to perform densitometric analysis.

Statistical analysis. All experiments were carried out at least in duplicate. Data are presented as the mean ± standard deviation. Statistical analysis was performed with one-way analysis of variance followed by Kruskal Wallis test using the GraphPad Prism 5 software package (GraphPad, La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

LBP increases the serum levels of E2 and modifies the OVX-induced alterations in cardiac tissue. In order to confirm that OVX was successful, serum E2 levels were measured. A significant reduction in serum E2 levels was observed in the OVX group compared with in the Sham group ($P<0.01$), thus indicating that OVX was successful. Treatment with high-dose LBP and estradiol valerate increased serum E2 levels compared with in OVX rats ($P<0.01$). However, there was no significant difference in E2 levels in the serum between the LBP-L and OVX groups (Fig. 1A). In the Sham group, myocardial fibers were arranged regularly with clear striations, without any damage or necrosis in the tissue (Fig. 1B). Histopathological sections of the OVX group displayed disorganized fibers and increased dilatation of intercellular spaces. Conversely, high-dose LBP and estradiol valerate modified the OVX-induced alterations in cardiac tissue (Fig. 1C-F).

LBP recovers the antioxidant status in OVX rats. As illustrated in Fig. 2, various parameters associated with oxidative stress were detected. Enhanced ROS and MDA activity was detected in the myocardium of the OVX group ($P<0.01$). Administration of a high-dose of LBP exerted a significant protective effect on OVX rats ($P<0.01$). In addition, administration of estradiol valerate exerted a significant protective effect on MDA only in OVX rats ($P<0.01$). GSH-px CAT and SOD are markers of the antioxidant defense system. As presented in Fig. 2, OVX resulted in a decrease in GSH-px and CAT activity ($P<0.01$), whereas in the LBP-H and Est groups GSH-px and CAT were significantly increased in the myocardium compared with in OVX rats ($P<0.05$). No alterations in SOD were detected among the various groups. These findings indicated that administration of LBP may recover the antioxidant status in OVX rats.

LBP alleviates apoptosis in OVX rats. Bax, as a proapoptotic protein, Bcl2, as an anti-apoptotic protein, and caspase-9 and caspase-3 are important indicators of apoptosis. As demonstrated in Fig. 3, OVX resulted in an increase in Bax, cleaved caspase-9 and cleaved caspase-3 expression in the myocardium ($P<0.01$). Administration of high-dose LBP and estradiol valerate exerted a significant protective effect on OVX rats, as evidenced by the decreased expression of apoptotic proteins ($P<0.05$). In addition, OVX resulted in a decrease in Bcl2 expression in the myocardium ($P<0.01$). However, Bcl2 expression was enhanced in the myocardium of the LBP-H and Est groups compared with in OVX rats ($P<0.01$).

LBP alleviates apoptosis via the Akt signaling pathway in OVX rats. The Akt signaling pathway is involved in apoptosis. As illustrated in Fig. 4, OVX resulted in a decrease in the phosphorylation of Akt in the myocardium ($P<0.01$). Administration of high-dose LBP resulted in a significant increase in the expression of p-Akt ($P<0.01$).

Discussion

The present study demonstrated that LBP can improve antioxidant status, ameliorate oxidative stress-induced cell apoptosis and increase phosphorylation of Akt in the myocardium of OVX rats. OVX-induced cardiac injury suggests that estrogens may be associated with the maintenance of normal cardiac function. This relationship has already been suggested by other studies (20,21). Estrogen has also been reported to provide cardiac protection in various models of cardiac disease (22,23).

Oxidative stress represents an imbalance between the production and manifestation of ROS and their detoxification. Oxidative stress may serve an important pathophysiological role during the menopause, and is considered one of the main causative factors of various cardiovascular disorders, including postmenopausal cardiovascular disorders (24,25). A number of studies have demonstrated that OVX may result in a decrease in antioxidative biomarkers and an increase in MDA content (oxidative biomarker) in the myocardium (26,27). SOD, CAT and GSH comprise the antioxidant defense system; SOD and CAT are important components of this system. SOD is considered the most important antioxidant enzyme that provides defense against oxidative stress, particularly oxygen radicals. SOD scavenges superoxide by converting it to peroxide, which

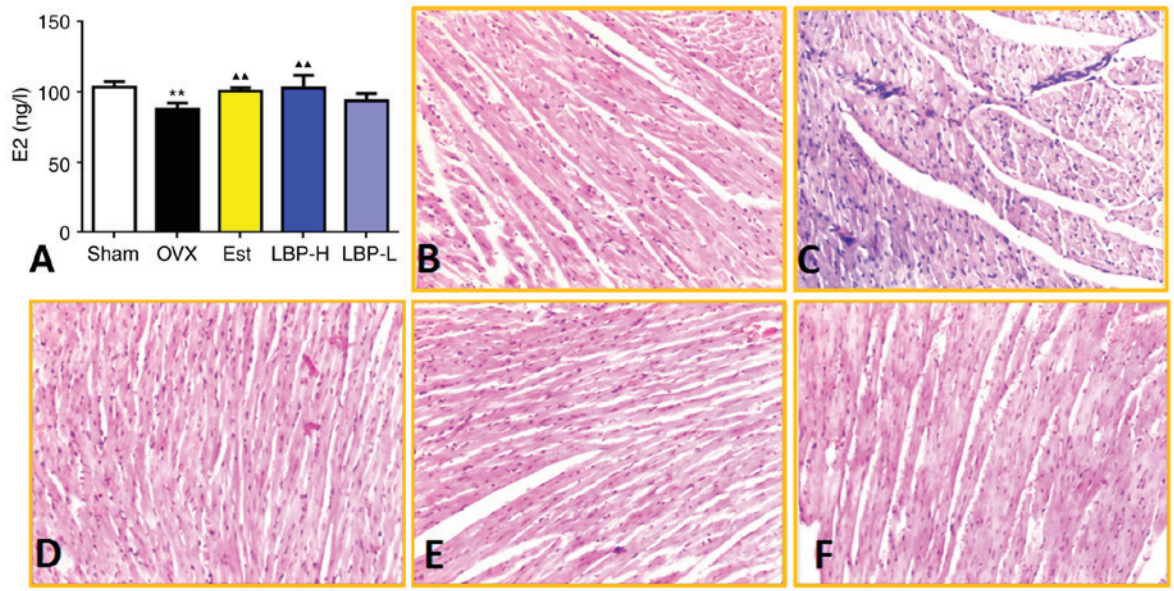

Figure 1. Effects of LBP on serum E2 levels and histopathological examination of cardiac tissue. (A) Effects of LBP therapy on serum E2 levels. A significant reduction in serum E2 levels was observed in OVX rats, whereas E2 levels were increased in the LBP-H group compared with in the OVX group (n=6/group). **P<0.01 vs. the Sham group; ▲▲P<0.01 vs. the OVX group. (B-F) Representative photomicrographs of cardiac tissue from the Sham, OVX, Est, LBP-H and LBP-L groups, respectively. Histopathological sections of the OVX group displayed disorganized fibers and increased dilatation of intercellular spaces, whereas these alterations were modified in the LBP-H and Est groups. Hematoxylin and eosin staining; magnification, x20. E2, 17β-estradiol; Est, estradiol valerate group; LBP, *Lycium barbarum* polysaccharides; LBP-H, high-dose LBP group; LBP-L, low-dose LBP group; OVX, ovariectomy group.

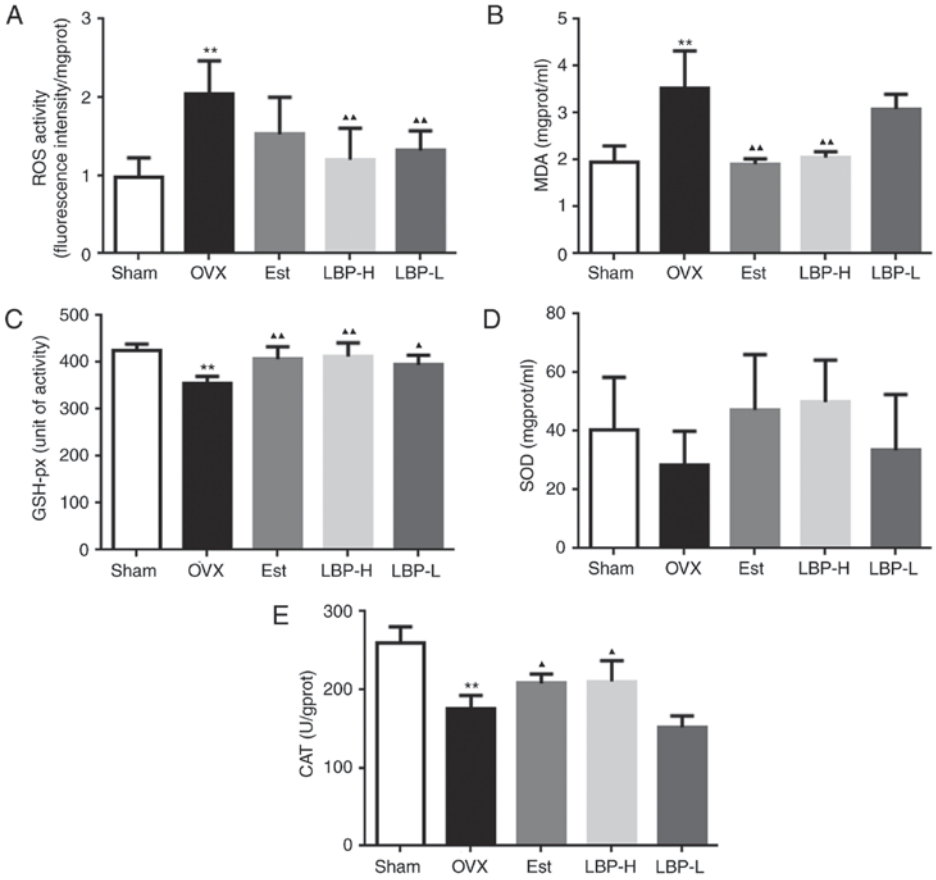

Figure 2. Effects of LBP and OVX on oxidative status in the myocardium of rats. (A and B) Effects of LBP on ROS and MDA activity. A significant increase in ROS and MDA was detected in the myocardium of OVX rats. Conversely, in the LBP-H group ROS and MDA activity was significantly decreased compared with in the OVX group. (C-E) Effects of LBP on GSH-px, SOD and CAT activity. A significant decrease in GSH and CAT was detected in the myocardium of OVX rats. Conversely, in the LBP-H group GSH and CAT activity was significantly increased compared with in the OVX group (n=6/group). **P<0.01 vs. the Sham group; ▲P<0.05, ▲▲P<0.01 vs. the OVX group. CAT, catalase; Est, estradiol valerate group; GSH-px, glutathione peroxidase; LBP, *Lycium barbarum* polysaccharides; LBP-H, high-dose LBP group; LBP-L, low-dose LBP group; MDA, malondialdehyde; OVX, ovariectomy group; ROS, reactive oxygen species; SOD, superoxide dismutase.

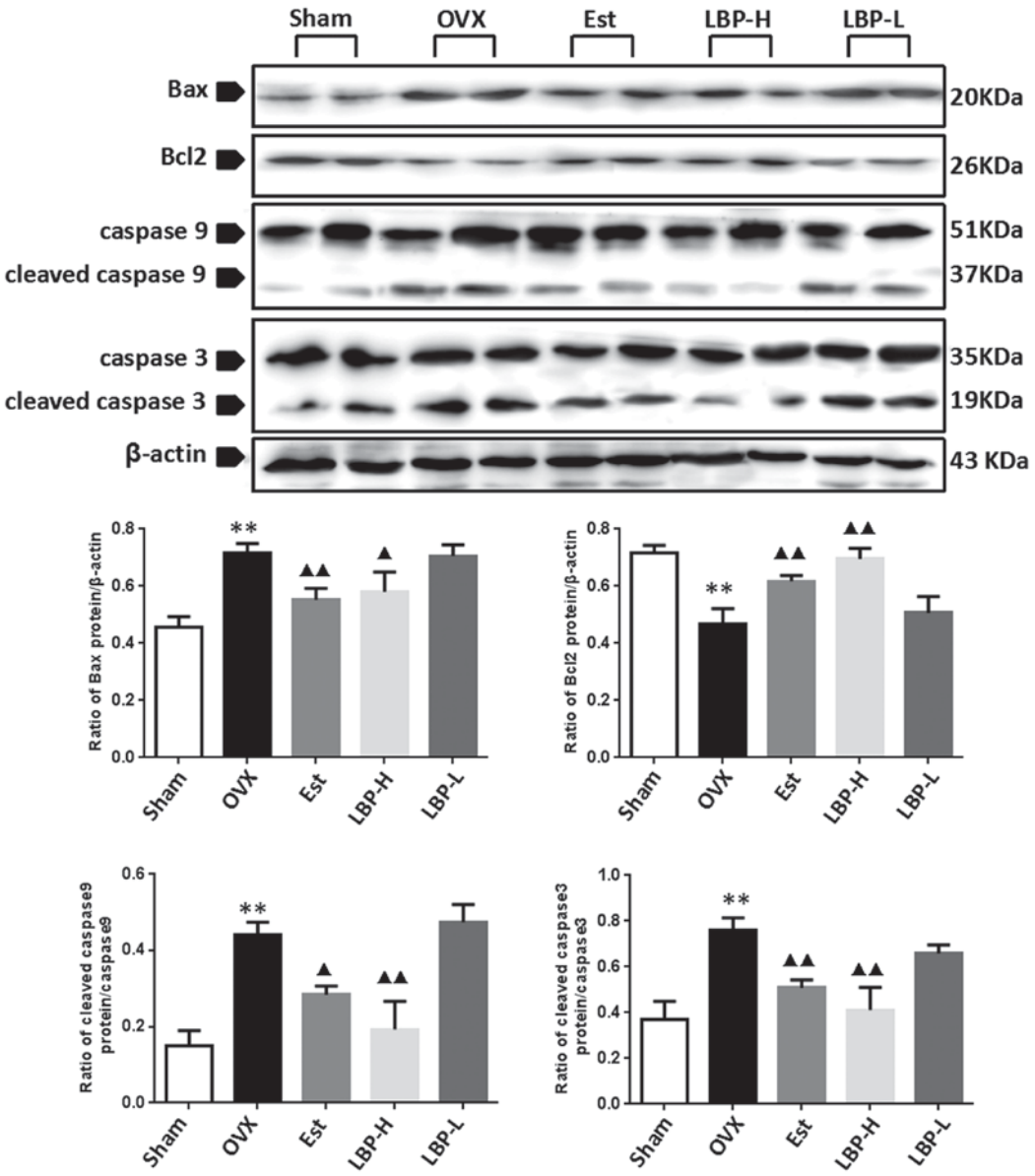

Figure 3. Effects of LBP and OVX on apoptotic protein expression in the myocardium. The expression levels of apoptotic proteins were determined by western blotting. A significant increase in Bax, cleaved caspase-9 and cleaved caspase-3, and a decrease in Bcl2 expression were detected in the myocardium of OVX rats. In the LBP-H group, the expression levels of Bax, cleaved caspase-9 and cleaved caspase-3 were decreased, and Bcl2 expression was increased compared with in the OVX group (n=3/group). **P<0.01 vs. the Sham group; $^{▲}$P<0.05, $^{▲▲}$P<0.01 vs. the OVX group. Bax, Bcl2-associated X protein; Bcl2, B cell lymphoma-2; Est, estradiol valerate group; LBP, *Lycium barbarum* polysaccharides; LBP-H, high-dose LBP group; LBP-L, low-dose LBP group; OVX, ovariectomy group.

in turn is destroyed by CAT. Therefore, SOD and CAT act in a mutually supportive way with antioxidant enzymes to provide a protective defense against ROS. GSH-px catalyzes the reductive action of GSH to H_2O_2, in order to protect the integrity and functions of the plasma membrane (28-30). Ji *et al* (31) demonstrated that estrogens are able to suppress the overproduction of ROS. Numerous studies (32-34) have revealed the protective effects of E2 on GSH synthesis. Therefore, OVX may significantly alter the stimulatory effects of E2 on GSH synthesis.

In the present study, a significant reduction in serum E2 levels was observed in OVX rats. Conversely, 12-week treatment with 250 mg/kg LBP, which has estrogen-like effects, following OVX improved serum E2 levels. Furthermore, OVX induced an imbalance in oxidative stress status: ROS and MDA activity was increased, whereas GSH-px and CAT activity was decreased in cardiac tissues. LBP treatment decreased ROS and MDA activity, and increased GSH-px and CAT activity in cardiac tissues. These results indicated that an important association exists between oxidative stress and LBP-induced cardioprotective effects.

Lycium barbarum L is a well-known antioxidant traditional Chinese medicine. The aqueous extract of *Lycium barbarum* L has been reported to exert marked antioxidant activity *in vivo* and *in vitro* (35,36). LBP is considered the main active component in *Lycium barbarum* L, which possesses numerous bioactivities, including anti-aging, anticancer, immunomodulatory and antioxidative effects (37). Li demonstrated that LBP (20-50 mg/kg) protects liver and kidney tissue from oxidative damage in streptozotocin-induced diabetic rats (38).

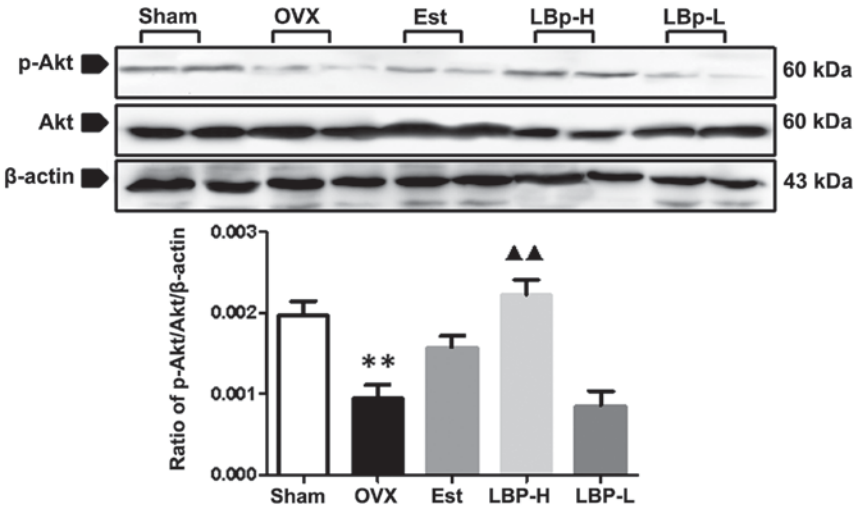

Figure 4. Effects of LBP and OVX on phosphorylation of Akt in the myocardium. The levels of p-Akt were determined by western blotting. A significant decrease in p-Akt protein expression was detected in the OVX rats. However, in the LBP-H group, the protein expression levels of p-Akt were significantly increased compared with in the OVX group (n=3/group). **P<0.01 vs. the Sham group; $^{▲▲}$P<0.01 vs. the OVX group. Akt, protein kinase B; Est, estradiol valerate group; LBP, *Lycium barbarum* polysaccharides; LBP-H, high-dose LBP group; LBP-L, low-dose LBP group; OVX, ovariectomy group; p-Akt, phosphorylated-Akt.

Furthermore, Luo *et al* (39) indicated that LBP could alleviate heat-induced damage of rat testes, and H_2O_2-induced DNA damage in mouse testicular cells, by increasing their resistance to oxidative stress-induced injury. The results of the present study demonstrated that in rats treated with LBP (250 mg/kg), OVX-induced oxidative injury in cardiac tissue was less apparent compared with in the OVX group; LBP was able to decrease ROS and MDA activity, thus improving OVX-induced abnormalities. In addition, LBP significantly (P<0.05) increased CAT and GSH-px activity in the heart tissues of OVX rats. Taken together, LBP exerted indirect effects to alleviate OVX-induced cardiomyocyte damage.

Oxidative stress can activate cell apoptosis signaling, resulting in the induction of apoptosis in various cell types (40). Bax, as a proapoptotic protein, and Bcl2, as an anti-apoptotic protein, and caspase-9 and caspase-3, are important indicators of apoptosis. In the OVX group, increased Bax and decreased Bcl2 protein expression was detected in the cardiac muscle, thus indicating that the cardiomyoctes in these rats were undergoing apoptosis. In addition, there were significant differences in Bax and Bcl2 protein expression between the OVX and LBP-H groups. These results suggested that the balance between anti-apoptotic and proapoptotic factors was disrupted by OVX. Compared with the OVX group, cardiomyocyte apoptosis was alleviated by LBP (250 mg/kg). In addition to Bcl2 and Bax, caspase-3 and caspase-9 deactivation also contributed to LBP-mediated cardiac protection. These findings indicated that estrogen and LBP may serve a similar role in regulating cardiomyocyte function by inhibiting apoptosis.

Previous studies have reported that overproduction of ROS is associated with three pathways: Extracellular auto-oxidation, intracellular metabolism by monoamine oxidase and direct inhibition of the mitochondrial respiratory chain (41,42). Furthermore, the generation of intracellular ROS suppresses Akt phosphorylation, which induces activation of caspase-9 and caspase-3, which finally leads to cell apoptosis (42). In the present study, in the OVX group, cleaved caspase-3 and caspase-9 protein expression was increased, indicating the occurrence of cell apoptosis, whereas in the LBP group, cleaved caspase-3 and caspase-9 protein expression was decreased in cardiac tissues. In addition, the phosphorylation of Akt was decreased in the cardiac tissues of the OVX group; however, following treatment with a high dose of LBP, the phosphorylation of Akt was improved.

In conclusion, LBP may increase activity levels of the antioxidative markers CAT and GSH-px, and decrease activity levels of the oxidative markers MDA and ROS in the myocardium of OVX rats. Therefore, LBP may ameliorate oxidative stress-induced cardiac damage in OVX rats. LBP is associated with cardiac protection by inhibiting the apoptotic signaling pathway in response to oxidative stress; this finding is associated with the Akt signaling pathway in the myocardium.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NY performed the animal breeding, the detection of experimental indicators and the statistical analysis. NS conducted the detection of experimental indicators. CL was mainly responsible for the statistical analysis. GY designed the study. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by and followed the guidelines of the Animal Ethics Committee of Liaoning University of Traditional Chinese Medicine (Shenyang, China; permit no. 2011-167).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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