Original Article
Pretreatment of *Lycium barbarum* polysaccharide reduces H_2O_2-induced myocardial apoptosis

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Abstract: The study is aimed to explore the effects of *Lycium barbarum* Polysaccharide (LBP), the main active compound of *Lycium barbarum*, on H_2O_2-induced oxidative stress in myocardial cells, thereby to investigate the possibility of LBP to be an effective prophylactic agent for cardiac vascular disease (CVD). In the study, rat myocardial cells were randomly divided into control group, H_2O_2 model group and three LBP+H_2O_2 groups. Myocardial viability, cell apoptosis andoxidative stress parameters were detected. Real-time fluorescent quantitative PCR (RT-PCR) and Western blot were applied to investigate the influences of LBP pretreatment on apoptosis regulator proteins B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X (Bax) expression and NF-E2 p45-related factor 2 (Nrf2)/antioxidant responsive element (ARE) signaling pathway. The results revealed that LBP pretreatment significantly attenuated the increased myocardial apoptosis in H_2O_2-induced oxidative stress by activating the Nrf2 signaling pathway and endogenous antioxidants. It suggested that LBP has great potential to be an antioxidant to protect people from oxidative stress induced CVD.

Keywords: *Lycium barbarum* polysaccharide, cardiovascular disease, apoptosis, oxidative stress

Introduction

CVD, including atherosclerosis, hypertension, ischemic heart disease and hyperlipemia etc., are becoming one of the biggest health killers worldwide in recent decades [1]. In China, CVD has been the second highest cause of death only after cancer. In the pathological processes of numerous CVD, excessive generation of oxygen free radicals is generally triggered when the anti-oxidant defenses are restrained, resulting in imbalanced status of oxidation and anti-oxidation in body [2]. As big family members of oxygen free radicals, reactive oxygen species (ROS) play an important part in not only normal signal and physiological mechanisms but also pathological processes, however, when the level of ROS overwhelm the body's capability of regulation, oxidative stress, a condition considered as possible underlying pathogenic mechanism in progression of CVD, ensues [1, 3]. When the stress level exceeds the certain limit, irreversible damages i.e. cell apoptosis and necrosis will be caused [2]. Myocardial apopto-

sis originates from the lost of myocardial cells, with recurrent lost, cellular progressive function subsequently deteriorates due to drop of cell count and functional degradation of the rest cells, resulting in necrosis and entering to the terminal phase of CVD, heart failure [4]. It admits of no delay to research the more in-depth pathogenesis of CVD and discover safer and more effective medicines.

Red-colored fruits of *Lycium barbarum* (family *Solanaceae*), commercially called goji berry, have been used as a traditional Chinese herbal medicine for thousands of years [5]. With a large variety of biological effects and pharmacological functions, *Lycium barbarum* fruits contribute to preventing and treating many chronic diseases, such as diabetes, hyperlipidemia, cancer, hepatitis, hypo-immunity function, thrombosis and male infertility [6]. *Lycium barbarum* polysaccharide (LBP), a soluble polysaccharide extracted from *Lycium barbarum*, is studied to be one of the active ingredients responsible for the biological benefits [7].

The study was aimed to explore whether LBP pretreatment could have protective effect on H_2O_2-induced oxidative stress in myocardial cells. Except for direct oxidative stress, exogenous H_2O_2 is able to penetrate biological membranes and increase formation of ROS, so in this study, treatment of 50 µmol/L of H_2O_2 for 3 h was selected to establish a model of H_2O_2-induced oxidative stress [8]. For the purpose, myocardial viability, lipid levels along with enzymatic antioxidants were detected in three groups of myocardial cells in rats pretreated by H_2O_2 with and without LBP, plus a normal control group. RT-PCR and Western blot were applied to investigate the influences of LBP on apoptosis regulator proteins Bcl-2 and Bax expression and Nrf2/ARE signaling pathway.

Materials and methods

Animals and treatments

Myocardial cell line of neonatal Wistar rats were purchased from Jining Shiye Co. Ltd (Shanghai, China). α-sarcomericactin immuno-cytochemical method was applied to identify the myocardial cells. Normal myocardial cells were cultured for 2-3 days, proliferated to reach the density of 80%-95%. Cells that pulsed at the rate 120-140 times per minute were selected for the use of experimental myocardial cells. Cells were divided into groups: normal control group, H_2O_2 group, and three H_2O_2+LBP experimental groups. Cells in LBP+H_2O_2 groups were pretreated with respectively 100 µg/mL, 200 µg/mL and 400 µg/mL of LBP (Jianglai Biotechnology Co. Ltd., Shanghai, China) for 24 h, then treated with 50 µmol/L H_2O_2 for 3 h for the following experiments. Cells in H_2O_2 group were treated with 50 µmol/L H_2O_2 (final concentration).

Methyl thiazolyl tetrazolium (MTT) assay

The cell metabolic activity was assessed by using MTT formazan powder (Sigma-Aldrich Co. LLC., USA). Briefly, myocardial cells were seeded in 96-well plates at density of $5×10^4$ cells per well, and then were incubated at 37°C for 48 h. Then after different preconditions depending on experimental groups, the cells were washed with phosphate-buffered saline (PBS) (Whiga Technology Co. Ltd., Guangdong, China) 3 times. With addition of 20 µL 5 g/L MTT dye and 100 µL serum free DMEM culture

solution (Whiga Technology Co. Ltd., Guangdong, China) into all wells, the cells were incubated in 5% CO_2 incubator for 4 h, after that, the reaction was stopped by adding 150 µL dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co. LLC., USA). With 10-minute vibration at room temperature, water-insoluble purple formazan was sufficiently dissolved, the optical density (OD) was assessed at 490 nm by a spectrophotometer (Sigma-Aldrich Co. LLC., USA).

Oxidative stress parameters

Myocardial cells were seeded in six-well plate, grouped by different experimental treatments. Detection of the level of ROS, lactate dehydrogenase (LDH), malondialdehyde (MDA) and super oxide dismutase (SOD) were conducted according to their corresponding test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China), the OD values were read at 420 nm on a spectrophotometer (Sigma-Aldrich Co. LLC., USA). The contents of ROS, LDH, MDA and SOD in each sample were calculated. Each test was run three times.

Flow cytometry

Myocardial cells were seeded in 6-well plates at density of $2×10^4$ cells/well, digested and collected with EDTA free trypsin (Whiga Technology Co. Ltd., Guangdong, China), stained by Annexin V-FITC and Propidium iodide (Keygen Biotechnology Co. Ltd., Jiangsu, China), then incubated at room temperature for 5-15 min in dark place. The cultures were analyzed by FACSCalibur flow cytometry (Becton, Dickinson and Company, USA) with excitation wavelength 488 nm and emission wavelength 530 nm. The test was run three times, the proportion of apoptotic and necrotic cells in every group were calculated.

Real-time quantification PCR (RT-PCR)

RT-PCR and SYBR Green I chemistry (TransStart Top Green qPCR, SuperMix, TransGen Biotech Co., Ltd, Beijing, China) were used to evaluate expressions of genes in the study. Cells were seeded into 6-well plates at a density of $2×10^7$ cells/well, collected in optimal condition in 2-3 days and then grouped by different experimental treatments. Total RNA of myocardial cells were extracted with Trizol (Takara bio inc., Japan), purity and concentra-
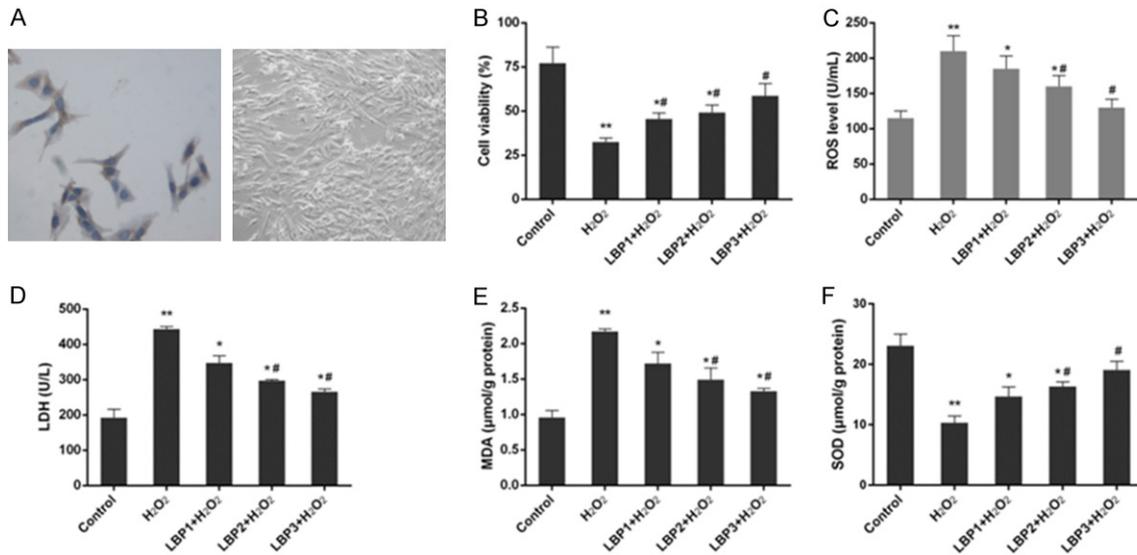
Figure 1. Morphological observation of cells and changes in cell proliferation and oxidative stress parameters in control, H_2O_2 (50 μmol/L) and LBP (100, 200 and 400 μg/mL)+H_2O_2 (50 μmol/L) groups. A: Myocardial cells were identified by α-sarcomeric actin immunocytochemical strain (×400). B: The proliferation of myocardial cells was improved with LBP pretreatment in H_2O_2-induced injury. C: The intracellular ROS level was decreased with LBP pretreatment in H_2O_2-induced injury. D: The content of LDH was reduced with LBP pretreatment in H_2O_2-induced injury. E: The content of MDA was decreased with LBP pretreatment in H_2O_2-induced injury. F: The activity of SOD was increased LBP pretreatment in H_2O_2-induced injury. Data were presented as mean ± SD, n=3, $^*P<0.05$ and $^{**}P<0.01$ vs. control, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. H_2O_2 treatment (50 μmol/L of H_2O_2).

tion of the extracted RNA were measured on a UV spectrophotometer. cDNA was synthesized by reverse transcription, then fluorescence quantitative detection of the target genes was performed afterwards. GAPDH, as the internal control, was used to monitor RT-PCR efficiency. All RT reactions were performed in triplicate. Primers for each gene were designed by Shanghai Sangon Biotech Co. Ltd. (Shanghai, China). The specific primer sequences were listed as the follows: 5' TCATGGGCTGGACAC-TGGAC 3' and 5' CAGCCACCCTGGTCTTGGAT 3' for Bax (product: 67 bp); 5' GCCTGAGAGCA-ACCCAATGC 3' and 5' CGGAGGGTCAGATGGAC-CAC 3' for Bcl-2 (product: 129 bp); 5' AG-CACTCCGTGGAGTCTTCC 3' and 5' GGAGAA-TGTGCTGGCTGTGC 3' for Nrf2 (product: 130 bp); 5' CTACACGGCCCTGGAAGAGG 3' and 5' TGAGGCCCATACCAGAAGGC 3' for HO-1 (product: 144 bp) and 5' TGGCCTTCCGTGTTCCTACC 3' and 5' TTCAGTGGGCCCTCAGATGC 3' for GAPDH (product: 121 bp).

Western blot

Myocardial cells were seeded at a density of $2×10^7$ cells/well in 6-well plates, collected in 2-3 days and then grouped by different experimental treatments. Each group of cells was harvested and washed twice with PBS and protein lysed in ice-cold radio immunoprecipitation assay buffer (Whiga Technology Co. Ltd., Guangdong, China) with freshly added 0.01% protease inhibitor phenylmethanesulfonyl fluoride and incubated on ice for 30 min. Cell lysis was centrifuged at 10,000×g for 5 min at 4°C, and the supernatant (20-30 μg of protein) was run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred electrophoretically to a nitrocellulose membrane (Millipore, Shanghai, People's Republic of China), then detected with Bax, Bcl-2, Nrf2, HO-1 and NQO1 proteins. Protein loading was estimated using mouse anti-glyceraldehyde 3-phosphate dehydrogenase (antiGAPDH) monoclonal antibody. Blots were visualized using enhanced chemiluminescence (Thermo Fisher Scientific Inc, NY, USA).

Statistical analysis

All values were expressed as mean ± S.D. Differences between groups were assessed by means of variance analysis and student's t-test.

Statistical significance was defined as $P<0.05$ and $P<0.01$.

Results

Morphological observation of myocardial cells

Through α-sarcomeric actin immunocytochemical method, stained positive signals-appearance of brown granules in cytoplasm-were noticed in over 95% cultured myocardial cells collected from neonatal rats in the study (**Figure 1A**).

LBP pretreatment improved cell viability in H_2O_2-induced injury

The changes of cell viability under the different conditions of H_2O_2 and LBP pretreatment were detected with MTT assay *in vitro*. In condition of 50 μmol/L of H_2O_2 without any pretreatment, a significant decrease of cell proliferation was observed compared to control group ($P<0.01$). The study found that LBP pretreatment of myocardial cells for 24 h positively influenced on H_2O_2-induced decrease of cell viability, from 21.57%±2.25% in H_2O_2 group to 45.53%± 3.35%, 49.13%±4.22% and 58.70%±6.86% in H_2O_2+LBP groups with respectively 100, 200 and 400 μg/mL of LBP ($P<0.05$), and the effect was concentration dependent (**Figure 1B**).

LBP pretreatment decreased level of ROS, LDH and MDA and increased SOD activity in H_2O_2-induced injury

Treatment of H_2O_2 to myocardial cells obviously increased the content of ROS, LDH and MDA and reduced the level of SOD by over 50% in comparison with control group ($P<0.01$) (**Figure 1C-F**). In the H_2O_2-induced injury, with 100, 200 and 400 μg/mL LBP pretreatment, the activity of SOD was improved while the contents of ROS, LDH and MDA were all reduced ($P<0.05$). The effects of LBP pretreatment on these parameters were positively correlated with the concentration of LBP.

LBP pretreatment reduced myocardial apoptosis in H_2O_2-induced injury

A remarkable increase of apoptosis rate from 6%±1% to 23.4%±1.8% was detected with flow cytometry in myocardial cells under the condition of 50 μmol/L of H_2O_2 as compared with

control group ($P<0.01$). After 100, 200 and 400 μg/mL of LBP pretreatment for 24 h, myocardial apoptosis rate in H_2O_2-induced injury was obviously reduced to 15.1%±1%, 9.5%±0.8% and 8.4%±1% respectively, and the effect of LBP pretreatment in high concentration is the most significant ($P<0.01$) (**Figure 2A**).

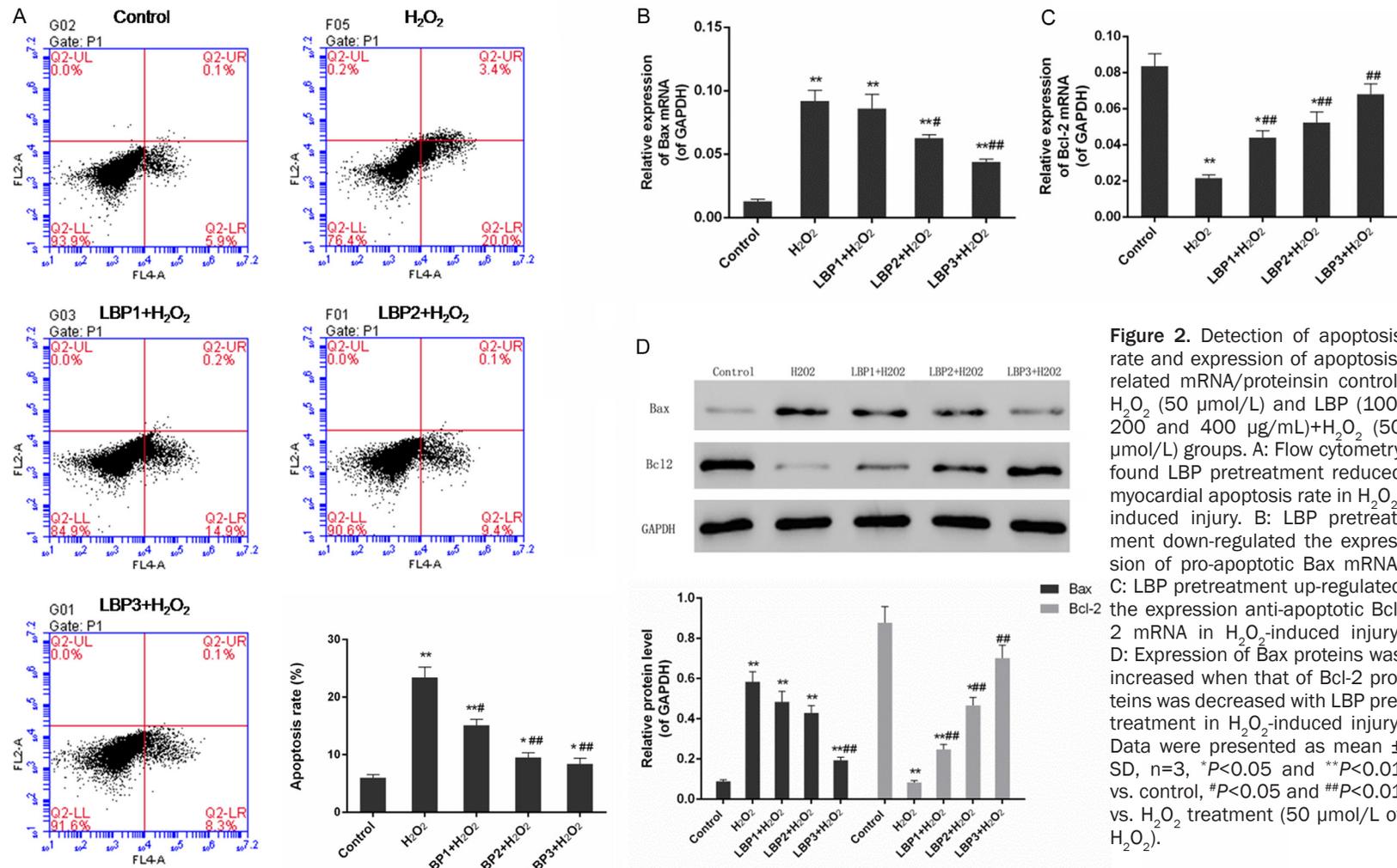*Effect of LBP pretreatment on apoptosis-related protein expression and Nrf2 pathway*

RT-PCR and western blot were applied to detect the expression of apoptosis-related Bax/Bcl-2 protein and Nrf2 pathway in this study. Treatment of 50 μmol/L H_2O_2 to myocardial cells evidently increased the expression of Bax and suppressed the expression of Bcl-2 in comparison with control one ($P<0.01$). Expression of Bax mRNA and protein was down-regulated while that of Bcl-2 was up-regulated by LBP pretreatment in a dose-dependent manner ($P<0.05$) (**Figure 2B-D**). Significant decrease was observed in protein and mRNA expressions of Nrf2, HO-1 and NQO1 under condition of H_2O_2 without LBP pretreatment as compared with control group ($P<0.01$). The research found that LBP pretreatment significantly improved the expression of Nrf2, HO-1 and NQO1 mRNA and proteins through a positive regulatory way in a dose-dependent manner ($P<0.05$) (**Figure 3A-D**).

Discussion

Our study provided evidences of protective effect of LBP pretreatment on cardiomyocyte apoptosis in H_2O_2-induced injury by regulating apoptosis related genes and Nrf2 pathway. In our established model of H_2O_2-induced injury, significant decrease of proliferation, increase of apoptosis rate along with a series of changes in oxidative stress parameters were detected. Myocardial cells pretreated with proper concentration of LBP were rescued from severe H_2O_2-induced oxidative stress by improving cell viability and reducing cell apoptosis.

Based on previous studies, oxidative stress has been reported to be involved in a variety of phases of apoptotic pathway and development of CVD [9]. Excessive ROS distorts the delicate equilibrium between free radical production and antioxidant capability, and triggers a series of peroxidatic reactions of biomacromolecules including nucleic acid, protein and lipids [3].

Figure 2. Detection of apoptosis rate and expression of apoptosis-related mRNA/proteinsin control, H_2O_2 (50 µmol/L) and LBP (100, 200 and 400 µg/mL)+H_2O_2 (50 µmol/L) groups. A: Flow cytometry found LBP pretreatment reduced myocardial apoptosis rate in H_2O_2-induced injury. B: LBP pretreatment down-regulated the expression of pro-apoptotic Bax mRNA. C: LBP pretreatment up-regulated the expression anti-apoptotic Bcl-2 mRNA in H_2O_2-induced injury. D: Expression of Bax proteins was increased when that of Bcl-2 proteins was decreased with LBP pretreatment in H_2O_2-induced injury. Data were presented as mean ± SD, n=3, $*P<0.05$ and $**P<0.01$ vs. control, $\#P<0.05$ and $\#\#P<0.01$ vs. H_2O_2 treatment (50 µmol/L of H_2O_2).
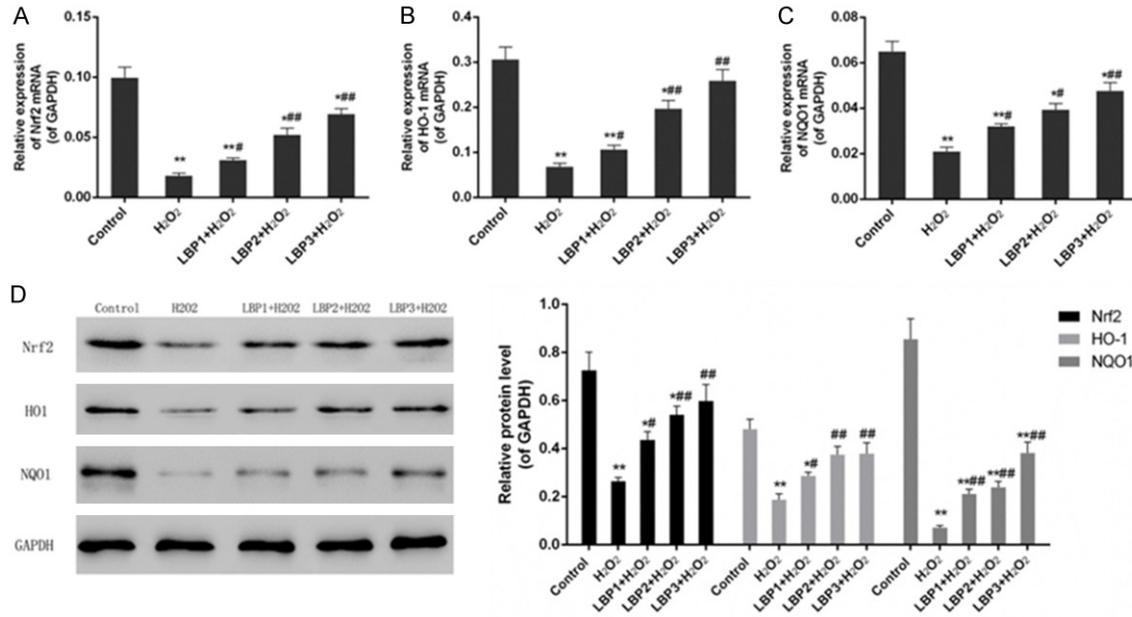
Figure 3. Expression of Nrf2 pathway related mRNA/proteinsin control, H_2O_2 (50 μmol/L) and LBP (100, 200 and 400 μg/mL)+H_2O_2 (50 μmol/L) groups. A: LBP pretreatment up-regulated the expression of Nrf2 mRNA in H_2O_2-induced injury. B: LBP pretreatment up-regulated the expression of HO-1 mRNA in H_2O_2-induced injury. C: LBP pretreatment up-regulated the expression of NQO1 mRNA in H_2O_2-induced injury. D: Increasing expression of proteins in Nrf2 pathway was observed. Data were presented as mean ± SD, n=3, *$P<0.05$ and **$P<0.01$ vs. control, #$P<0.05$ and ##$P<0.01$ vs. H_2O_2 treatment (50 μmol/L of H_2O_2).

Oxidative actions lead to DNA damage, intracellular caspases activation as well as increase of membrane brittleness and decrease of fluidity in cell membrane lipid [10]. In hypoxic condition, structure and function of mitochondria can be also damaged by disturbance of oxidative phosphorylation, increasing exhaust of high energy phosphate compound and reduction of the activity of energy-dependent ionic pump, resulting in accelerate of mitochondria-dependent cell apoptosis [11]. If the condition of myocardial apoptosis persists, progressive loss of myocardial cells are ensued, and even develop into heart failure [4].

A lot of work has been done in the area of exploring an effective antioxidant to eliminate the damage of ROS to myocardial cells and avoid induced CVD. In the treasure house of Chinese herbal medicine, there are a lot of potential natural antioxidants. Previous researches have demonstrated that LBP possesses numerous biological activities and pharmacological functions, such as enhancing exercise endurance capacity, reducing fatigue and exhibiting antioxidant activity in vitro and *in vivo* [6]. In H_2O_2-induced injury of rat myocardial

cells, we found LBP pretreatment increased cell proliferation, and reduced apoptosis rate and ROS content closely to normal level, which suggests that LBP pretreatment is able to improve heart function in oxidative stress and potentially protect against heart failure. Moreover, the result shows LBP pretreatment also inhibited the increase in the level of LDH and MDA, and restrained the reduction in the activity of SOD. As three good indicators of oxidative stress in cells, the level of LDH, MDA and SOD indirectly reflect the peroxidatic reaction in the study. LDH, an enzyme found in almost every living cell, is released extensively during tissue damage including heart failure. According to the leakage rate of it, the integrity of envelope can be determined [12]. As the end-product of polyunsaturated fatty acids oxidation, the content of MDA indicates the level of lipid peroxidation in cells [13]. SOD, a group of enzymes which catalyzes the reaction of superoxide anion clearance, can protect the organism from oxygen radical damage [14]. The amounts of SOD in cells indirectly reveal the capability of cellular oxygen radical clearance and lipid peroxidation resistance. In the condition of H_2O_2-induced oxidative stress, the enhancement of

myocardial membrane permeability makes LDP which is inside of membrane began to be leaked into nutrient solution, resulting in an remarkable increase of LDH level detected in the test.

It is well accepted that apoptosis is regulated by a series of intracellular apoptosis regulation genes including pro-apoptotic genes and anti-apoptotic genes. Bcl-2 family, which includes anti-apoptotic genes like Bcl-2, Bcl-xl, Mcl-1 and Bcl-2 and pro-apoptotic proteins such as Bax, Bad, Bak and Bid, is the first apoptosis regulating gene family ever found. The interaction of anti-apoptotic genes and pro-apoptotic genes can be affected by different levels of ROS, so the relative ratio of pro-apoptotic genes and anti-apoptotic genes of a cell is the key factor to influence cellular survive or perish. As the center of apoptosis regulation in Bcl-2 family, the expression of Bcl-2 and Bax proteins is used as the markers for apoptosis in the study [15]. Our findings suggested that LBP pretreatment obviously increased the expression of Bcl-2 and inhibited the expression of Bax, leading to improvement of the imbalance between Bax and Bcl-2 caused by H_2O_2-induced oxidative stress. The anti-apoptotic functions of LBP pretreatment made contributions, at least in part, to its protective effects on heart failure induced by oxidative stress.

Nrf2 is a key transcription factor of cell anti-oxidative stress system, and Nrf2 nuclear translocation combined with anti-oxidative response element ARE on the nucleic acid sequence is a key link in the process of activation of Nrf2 signaling pathways [16]. The activation of Nrf2 signaling pathway can start the transcription of the downstream phase II detoxification enzymes and antioxidant enzymes such as Heme oxygenase-1 (HO-1), NAD (P) H: quinine oxidoreductase (NQO1) and glutathione-S-transferses (GST) etc. so as to reduce the cell injuries caused by reactive oxygen and electronic materials, keep the cells in a stable state, and maintain the body's redox in dynamic balance [17]. In the study, it was observed that LBP pretreatment activated Nrf2 pathway and up-regulated the expression of Nrf2, HO-1 and NQO1, which explained the protective effects of LBP pretreatment on cardiomyocyte apoptosis in H_2O_2-induced injury.

Conclusion

These results demonstrate that LBP has a protective effect on H_2O_2-induced myocardial apoptosis *in vitro*. LBP pretreatment decreased apoptosis rate through activating Nrf2 signaling pathway and down-regulating the ratio of Bax/Bcl-2. LBP could be a potential prophylactic agent for CVD, but the further *in vitro* and *in vivo* experiments are required to explore the internal mechanism in the near future.

Acknowledgements

Disclosure of conflict of interest

None.

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