# *Lycium barbarum* polysaccharide arbitrates palmitate-induced apoptosis in MC3T3-E1 cells through decreasing the activation of ERS-mediated apoptosis pathway

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**Abstract.** Palmitate (PA) has been identified to induce cell apoptosis in osteoblasts. The c-Jun NH2-teminal kinase (JNK) signaling pathway and endoplasmic reticulum stress (ERS) were found to be important contributors. Therefore, natural or synthetic agents may antagonize PA-induced apoptosis in osteoblasts, and demonstrate the potential application to reverse osteoporosis. The present study demonstrated that the *Lycium barbarum* polysaccharide (LBP) is as a major active ingredient of *Lycium barbarum* and that it can reduce the fatty acid toxicity of PA. Furthermore, this study attempted to elucidate the underlying molecular mechanisms of LBP. Firstly, it was demonstrated via a Cell Counting Kit-8 assay, that LBP could significantly increase the viability of MC3T3-E1 cells in a dose-dependent manner. Flow cytometric analysis indicated that LBP inhibits PA-induced apoptosis in osteoblastic cells. Reverse transcription-quantitative polymerase chain reaction and western blotting results showed that the expression levels of glucose-regulated protein 78, C/EBP homologous protein and cysteinyl asparate specific proteinase-3/-9/-12, were increased in MC3T3-E1 cells following PA treatment. The treatment of the cells with PA resulted in an activation of the ERS and the JNK signaling pathway. These pathways were effectively suppressed by co-incubation with LBP. Taken together, PA may cause ERS, in cell apoptosis, and it may further activate the JNK signaling pathway. LBP reversed PA-induced apoptosis in MC3T3-E1 cells through inhibition of the activation of the ERS-mediated JNK signaling pathway.

## Introduction

Decreas bone mineral content and increase in bone fragility that may cause structural variations in bone tissue are typical for osteoporosis (OP), which can easily result in a fractures in the whole body (1,2). In the recent years, the increasing global incidence of OP-assocaited morbidity and mortality have resulted in a severe losses (1-3). More and more researches have verified that obesity is closely associated with the occurrence and development of OP, which might be another risk factor that causes OP (4,5). The content of serum free fatty acids in osteoblasts generally increases because of the accumulation of lipids and the reduction of utilization of fatty acids, which causes lipotoxicity in many cell types including osteoblasts (6-8). In the bone tissue, osteoblasts are the basis of the bone metabolism. The changes of in their functional status and relative amounts can cause bone metabolism abnormalities to ultimately progress to OP (9,10). Therefore, the apoptosis of osteoblasts induced by high fat may be one of the important mechanisms for OP of osteoblasts. Palmitate (PA) is one of the most common saturated fats found in plants and animals, which can induce the apoptosis of osteoblasts due to its lipotoxicity (11,12).

The endoplasmic reticulum (ER) is an important subcellular organelle involved in the synthesis of post-translational modifications, and proper folding of protein. Chemical stimulation can cause change its function in a process known as ER stress (ERS) (13). ERS is conducive to the restoration of cellular homeostasis and the mainteinance of cell survival. The continuous and high-intensity ERS can result in cell apoptosis. The up-regulation of GRP78 is considered to be the most sensitive signal in ERS (14). c-Jun NH2-teminal kinase (JNK) signaling pathway, as one of the important pathways in the mitogen-activated protein kinase pathway, plays an important role in the regulation of programmed cell death. The continuous and high-intensity ERS can activate the JNK signaling pathway by the formation of Irel/TRF2/ASK1 to induce cell apoptosis (15).

*Lycium barbarum* polysaccharide (LBP) is a kind of water-soluble polysaccharide extracted from *Lycium barbarum*, and it possesses important physiological functions in the human body. It consists of six monosaccharides, including arabinose, glucose 6-phosphate, galactose, mannose, xylose and rhamnose (16). Many studies showed that LBP has lipid-decreasing, hypoglycemic, anti-stress, antifatty-liver as well as, immunomodulatory effects (16,17). At present, western medicine is still the main therapy for OP. Long-term medication has produced serious poisonous side effects, once

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has limited its drugs' efficacy better display. Compared to chemicals, Chinese herbal medicine has abundant resources and little side effects during the treatment of various diseases. This has been followed with interest (18,19). Therefore, the aim of the present study was to investigate whether LBP treatment could attenuate apoptosis induced by PA, and to analyse the underlying molecular mechanisms.

## Materials and methods

*Cell culture and drug treatment*. MC3T3-E1 cells were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China), and maintained in α-MEM medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). They were cultured in a humid incubator at 37˚C (95% $O_2$ and 5% $CO_2$).

*Cell viability*. MC3T3-E1 cells were treated with various concentrations of PA (0, 100, 200, 300, 400 and 500 $\mu$g/ml) for 6, 12 and 24 h. The cell viability was then assessed by the Cell Counting Kit (CCK)-8 kit (Tongren, Shanghai, China). Next, MC3T3-E1 cells were pre-treated with various concentrations of LBP (0, 50, 100, 200, 400 and 800 $\mu$g/ml) for 24 h. Afterwards, 500 $\mu$g/ml of PA was added into the culture medium, and incubated for 6 h. The cell viability was assessed by the CCK-8 kit (Tongren). Briefly, $4x10^3$ cells were seeded in each 96-well plate, and further incubated for 24 h. The CCK-8 reagent was added to each well 1 h before the endpoint of the incubation. The optical density (OD) 450 nm values in each well were determined by a microplate reader. Experiments were repeated at least three times and each time in triplicate. The no-treatment group was taken as control.

*Flow cytometric analysis*. The apoptotic rate was assayed with a flow cytometer using an Annexin V-FITC apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. Briefly, MC3T3-E1 cells were pre-treated with 500 $\mu$g/ml of PA for 6 h, and various concentrations of LBP (0, 50, 100, 200, 400 and 800 $\mu$g/ml) were then added into the culture medium, and incubated for 24 h. Then the cells were harvested, washed with ice-cold PBS, resuspended in 500 $\mu$l of binding buffer, followed by addition of 5 $\mu$l of Annexin V stock solution and incubation for 10 min at 4˚C. Propidium iodide (PI) (5 $\mu$l) was added to the cells, and they were immediately analysed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

*RT-PCR analysis*. Total RNA was extracted from cultured MC3T3-E1 cells using the TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China). Then the RNA was reversely transcribed into cDNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.). PCR amplification was executed in an ABI 7500 Fast Thermocycler (Applied Biosystems, Foster City, CA, USA), using a SYBR-Green PCR kit (TransGen Biotech Co., Ltd., Beijing, China). The PCR cycles were 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and an annealing/extension step at 60˚C for 45 sec. The primers were designed by Shanghai Sangon Company (Shanghai, China). The specific primer sequences for each gene were listed as the follows: 5'-AACGATATCGCGGGCCCGAA-3' and 5'-GGAGGTGCCTTGAGCTAATT-3' for Caspase-3 (product: 117 bp); 5'-GATCAGATCGGGAATTGCAA-3' and 5'-AGGTGAGGAATTGGCTCCTT-3' for Caspase-9 (product: 112 bp); 5'-ATCGCCAACGATCAGGGCAA-3' and 5'-GGGTTGGAGGTGAGCTGGTT-3' for GRP78 (product: 107 bp); 5'-TGGAGCTTGTTCCAGCCACT-3' and 5'-GCAGGTCCTCATACCAGGCT-3' for CHOP (product: 144 bp); 5'-ACCGTAACTGCCAGAGTCTGAA-3' and 5'-ACCTTGCAAGAGCCGACCAT-3' for Caspase-12 (product: 140 bp) and 5'-AGCTCACTGGCATGGCCTTC-3' and 5'-CGCCTGCTTCACCACCTTCT-3' for GAPDH (product: 116 bp). GAPDH was used to normalize gene expression. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method. All experiments were performed in triplicate.

*Western blot analysis*. After PA/LBP co-treatment, MC3T3-E1 cells were harvested and washed twice with PBS and lysed in ice-cold radio immunoprecipitation assay buffer (RIPA; Beyotime, Shanghai, China) with freshly added 0.01% protease inhibitor cocktail (Sigma, Shanghai, China) and incubated on ice for 30 min. The cell lysis were s centrifuged at 13,000 x g for 10 min at 4˚C and the supernatants (20-30 $\mu$g of protein) were separated on a 10% SDS-PAGE gel and transferred electrophoretically to a polyvinylidene fluoride membrane (Merck Millipore, Shanghai, China). The blots were blocked with 5% skim milk, followed by the incubation with the antibodies against Caspase-3, Caspase-9, GRP78, CHOP, Caspase-12, p-JNK, JNK and GAPDH (Beyotime). The blots were then incubated with goat anti-mouse or anti-rabbit secondary antibody (Beyotime) and visualized using enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Inc., Shanghai, China).

*Statistical analysis*. In this study, all variables were evaluated using the SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Differences between numerical variables were calculated using the Student's t-test and the results are presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical analysis. All tests performed were two-sided. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Pre-treatment of LBP increased the cell viability of MC3T3-E1 cells with after treatment with PA*. In order to identify the effect of PA on the proliferation of MC3T3-E1 cells, CCK-8 assay was employed for the cell viability analysis. As shown in Fig. 1A, the cells were treated with PA (0, 100, 200, 300, 400 and 500 $\mu$g/ml) for 6, 12 and 24 h, PA inhibited the cell proliferation in a dose- and time-dependent manner. A concentration of PA over 300 $\mu$g/ml could obviously decrease the cell viability of MC3T3-E1 cells at 6, 12 and 24 h. Therefore, 500 $\mu$g/ml of PA was determined as the concentration to be used for further investigations. MC3T3-E1 cells were pre-treated with various concentrations of LBP (0, 50, 100, 200, 400 and 800 $\mu$g/ml) for 24 h. Afterwards, 500 $\mu$g/ml of PA was added into the culture medium and incubated for 6 h. The CCK-8 assay was then employed for the analysis of cell viability. As shown in

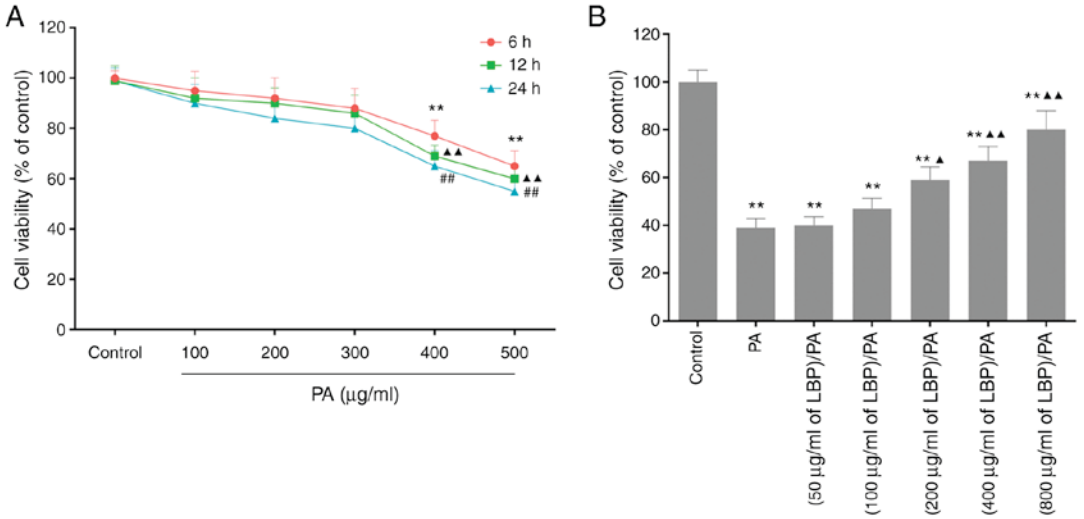


Figure 1. Effects of LBP on cell viability and cell apoptosis in MC3T3-E1 cells with PA pre-treatment. (A) Cells were treated with various concentrations of PA (0, 100, 200, 300, 400 and 500 $\mu$g/ml) for 6, 12 and 24 h, and cell viability was detected by CCK-8 analysis. Data were presented as mean ± standard deviation, n=3, $^{**}$P<0.01 vs. control in 6 h; $^{▲▲}$P<0.01 vs. control in 12 h; $^{\#\#}$P<0.01 vs. control in 24 h. (B) Cells were pre-treated with various concentrations of LBP (0, 50, 100, 200, 400 and 800 $\mu$g/ml) for 24 h, 500 $\mu$g/ml of PA was then added into the culture medium, and incubated for 6 h, and cell viability was detected by CCK-8 analysis. Data were presented as mean ± standard deviation, n=3, $^{**}$P<0.01 vs. control; $^{▲▲}$P<0.01 vs. PA. PA, palmitate; CCK-8, Cell Counting Kit-8.

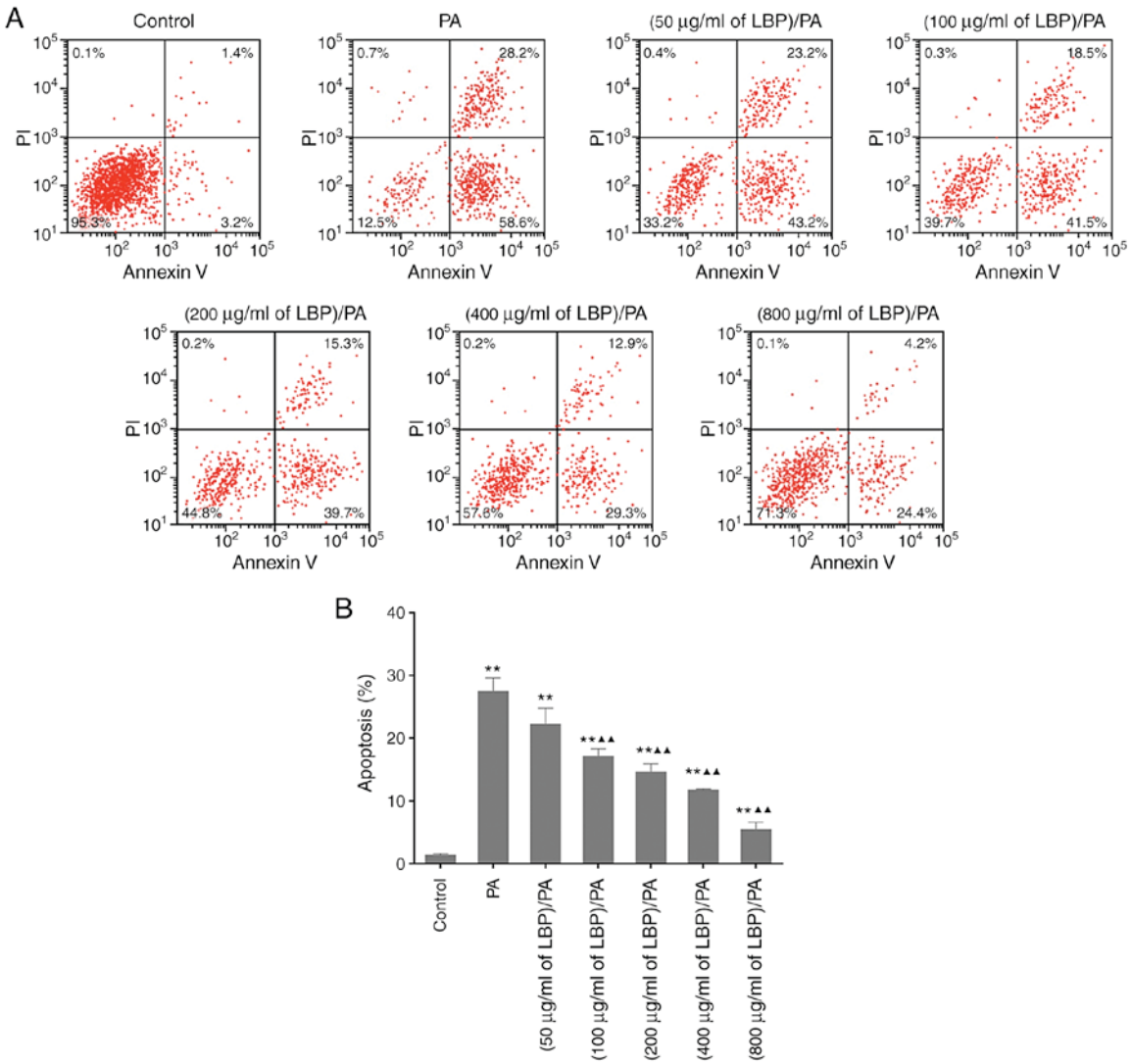


Figure 2. Effects of LBP on PA-induced apoptosis. (A) Cells were pre-treated with various concentrations of LBP (0, 50, 100, 200, 400 and 800 $\mu$g/ml) for 24 h, 500 $\mu$g/ml of PA was then added into the culture medium, and incubated for 6 h. The apoptosis rate was assayed with flow cytometry and (B) quantified. Data were presented as mean ± standard deviation, n=3, $^{**}$P<0.01 vs. control; $^{▲▲}$P<0.01 vs. PA. LBP, *Lycium barbarum* polysaccharide; PA, palmitate.

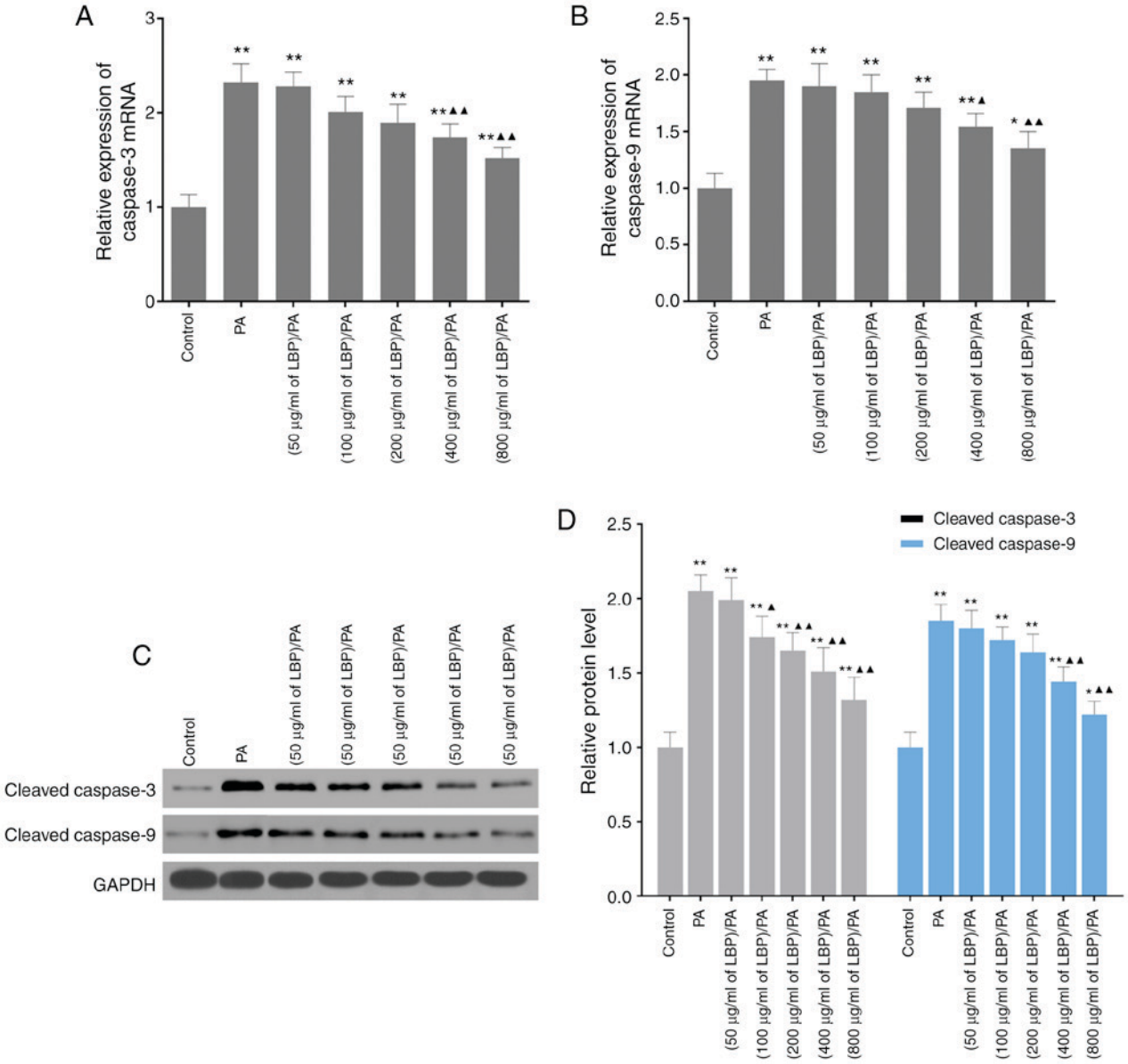


Figure 3. Effects of LBP on the expression levels of apoptosis-related genes. (A and B) Cells were pre-treated with various concentrations of LBP (0, 50, 100, 200, 400 and 800 $\mu$g/ml) for 24 h, 500 $\mu$g/ml of PA was then added into the culture medium, and incubated for 6 h. The mRNA expression levels of Caspase-3/9 were detected by RT-PCR. (C and D) Cells were pre-treated with various concentrations of LBP (0, 50, 100, 200, 400 and 800 $\mu$g/ml) for 24 h, 500 $\mu$g/ml of PA was then added into the culture medium, and incubated for 6 h. The protein expression levels of clevaed-caspase-3/9 were detected by western blotting. Data were presented as mean ± standard deviation, n=3, $^{*}$P<0.05 and $^{**}$P<0.01 vs. control; $^{\blacktriangle}$P<0.05 and $^{\blacktriangle\blacktriangle}$P<0.01 vs. PA. LBP, *Lycium barbarum* polysaccharide; PA, palmitate.

Fig. 1B, LBP could promote the cell proliferation in a dose- and time-dependent manner.

*Pre-treatment of LBP reduced the PA-induced apoptosis in MC3T3-E1 cells*. PA showed cytotoxic effects in MC3T3-E1 cells. Therefore, we explored the protective effect of LBP on PA-treated MC3T3-E1 cells. As shown in Fig. 2A, 500 $\mu$g/ml of PA significantly increased cell apoptosis compared to the control group, while LBP could decrease PA-induced apoptosis in a dose dependent manner. Fig. 2B shows that the cell apoptosis rate of the control as well as PA, (50 $\mu$g/ml of LBP)/PA, (100 $\mu$g/ml of LBP)/PA, (200 $\mu$g/ml of LBP)/PA, (400 $\mu$g/ml of LBP)/PA, and (800 $\mu$g/ml of LBP)/PA. PA resulted in an apoptosis rate of 27.5±2.12% compared to the control group of 1.40±0.15%. However, LBP decreased the apoptosis rate as 22.3±2.5% (50 $\mu$g/ml), 17.2±1.12% (100 $\mu$g/ml), 14.70±1.2% (200 $\mu$g/ml), 11.8±0.09% (400 $\mu$g/ml), and 5.5±1.1% (800 $\mu$g/ml). This shows that LBP decreases the PA-induced apoptosis in MC3T3-E1 cells. In addition, RT-PCR and Western blot analyses were used to detect the levels of apoptosis-related genes, including Caspase-3/9. We found that the expression levels of Caspase-3/9 were significantly up-regulated after PA treatment in MC3T3-E1 cells, and LBP could reduce the expression levels of Caspase-3/9 in MC3T3-E1 cells with PA treatment (Fig. 3)

*LBP inhibits the expression of ERS-associated genes including GRP78, CHOP and Caspase-12 in MC3T3-E1 cells with PA pre-treatment*. Consequently, we examined the

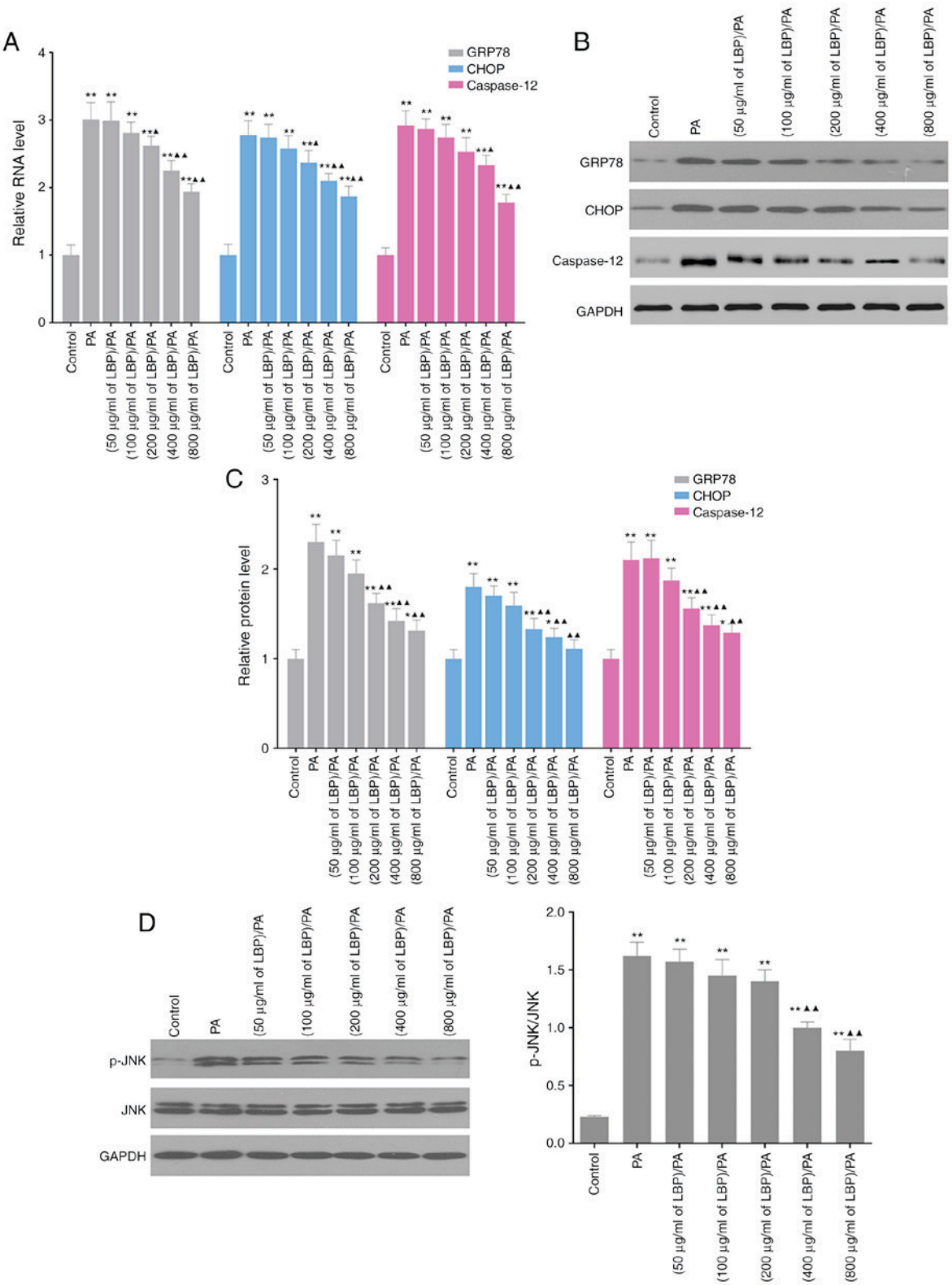


Figure 4. Effects of LBP on the expression levels of ERS-related genes and the activation of JNK signaling pathway in MC3T3-E1 cells with PA pre-treatment. (A-C) Cells were pre-treated with various concentrations of LBP (0, 50, 100, 200, 400 and 800 $\mu$g/ml) for 24 h, 500 $\mu$g/ml of PA was then added into the culture medium, and incubated for 6 h. The expression levels of GRP78, CHOP and Caspase-12 were detected by Western blot and RT-PCR. (D) Cells were pre-treated with various concentrations of LBP (0, 50, 100, 200, 400 and 800 $\mu$g/ml) for 24 h, 500 $\mu$g/ml of PA was then added into the culture medium, and incubated for 6 h. The phosphorylation level of JNK protein was detected by western blotting. Data were presented as mean ± standard deviation, n=3, $^{*}$P<0.05 and $^{**}$P<0.01 vs. control; $^{\blacktriangle}$P<0.05 and $^{\blacktriangle\blacktriangle}$P<0.01 vs. PA. LBP, *Lycium barbarum* polysaccharide; PA, palmitate; ERS, endoplasmic reticulum stress; JNK, c-Jun NH2-teminal kinase.

effects of LBP on the expression levels of GRP78, CHOP and Caspase-12 by RT-PCR and western blot analysis. As shown in Fig. 4A-C, the treatments of the cells with PA resulted in a steep decrease in the activation of GRP78, CHOP and

Caspase-12, which caused ERS. Interestingly, the addition of LBP (50, 100, 200, 400 and 800 $\mu$g/ml) significantly and dose-dependently inhibited the PA-induced activation of GRP78, CHOP and Caspase-12. Collectively, these results indicate that LBP is a potent inhibitor of PA-induced apoptosis in osteoblastic cells.

*LBP suppresses the activation of JNK signaling in MC3T3-E1 cells with PA pre-treatment*. In a Western blot analysis, JNK expression was identified in MC3T3-E1 cells pre-treated with PA. Fig. 4D showed that JNK expression was increased by PA treatment. However, LBP could revise this increase. The treatment of the cells with LBP greatly suppressed the expression level of JNK. Therefore, co-incubation of the cells with LBP significantly inhibited the phosphorylation of JNK. Collectively, these results suggest that LBP could suppress the activation of the JNK pathway caused by the PA treatment.

## Discussion

Normally, bone mechanical integrity is maintained by a dynamic balance between bone resorption by osteoclasts and bone formation by osteoblasts (9,10). The decreased number and the dysfunction of osteoblasts as well as the increased activities and ratio of osteoclasts can result in balanced disturbances of the bone metabolism. When the bone remodelling is disturbed, this leads to OP (5,9,10). Obesity means an over-deposition of fat due to a disequilibrium of the energy metabolism and a too much intake of calories (4,5). Recent studies found that obesity could result in the loss of bone density, the thinning of bone tissue, the enhancement of bone fragility and the increased of the risk of bone fracture and many more (20). The establishment of an animal mode with hyperlipidaemia also confirmed that obesity caused by a high-fat diet could induce OP (21,22). There reported that the increase of apoptosis induced by high fat in osteoblasts was one of the major causes for OP in obese patients (7,11). In our results, PA could significantly suppress cell viability and increase cell apoptosis in MC3T3-E1 cells (Figs. 1 and 2). In addition, the expression levels of apoptosis-related genes were significantly decreased after LBP treatment (Fig. 3). The results were in accordance with other reports, and showed that PA could induce the apoptosis of osteoblasts *in vitro*.

Apoptosis is a programmed cell death regulated by genes in order to maintain homeostasis. The death-receptor, mitochondria and ERS are involved in cell apoptosis. Unfolded proteins accumulate during ERS and ER resident protein GRP78 protects cell via inhibiting protein synthesis and increasing protein degradation (23). However, continued, excessive or aberrant ERS can cause cell apoptosis through activation of the CHOP pathway, the Caspase-12 pathway and the JNK pathway (13). In this study, the expression of GRP78, CHOP and Caspase-12 aas well as the phosphorylation level of JNK were significantly increased after PA treatment (Fig. 4). Some studies showed that Caspase-12 expression was detected during ERS, but Caspase-12 expression was not detected during the death-receptor and mitochondria apoptotic signaling pathways (24). Therefore, PA could significantly increase cell apoptosis caused by ERS via the activation of CHOP, Caspase-12 and JNK pathway. When we pretreated MC3T3-E1 cells with LBP, the apoptosis in osteoblasts was effectively inhibited (Fig. 1) in a dose dependent manner. The expression of GRP78, CHOP and Caspase-12 as well as the phosphorylation level of JNK were effectively decreased by co-incubation of LBP. It showed that LBP could significantly reduce the apoptosis of osteoblasts, which was depended on the concentration of interaction through inhibiting excessive or aberrant ERS.

In conclusion, PA could cause osteoblasts cells apoptosis, and LBP reduces PA-induced apoptosis in osteoblasts cells via inhibition of the activation of ERS caused by the JNK pathway.

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