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Original Research Article

Impact of Lycium barbarum polysaccharide on apoptosis in Mycoplasma-infected splenic lymphocytes

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Abstract

Purpose: *To evaluate the effect of Lycium barbarum polysaccharide (LBP) on apoptosis in Mycoplasma-infected splenic lymphocytes (SLs), and the underlying mechanisms.*
Methods: *SLs isolated from C57BL/6J mice were infected with Mycoplasma. The infected SLs were administered at different concentrations of LBP for 4 h, and the proportions of apoptotic cells and levels of relative reactive oxygen species (ROS) were determined by flow cytometry. The expressions of pro-apoptotic genes and endogenous antioxidant enzymes were investigated by real-time polymerase chain reaction (RT-PCR) and Western blotting.*
Results: *LBP treatment produced dose-dependent reductions in apoptotic ratio and intracellular ROS levels of SLs ($p < 0.05$). In addition, the expressions of pro-apoptotic genes were decreased by LBP treatment with respect to mRNA and protein levels ($p < 0.05$). In contrast, mRNA and protein levels of anti-apoptotic factor Bcl-2 were significantly increased in a dose-dependent manner ($p < 0.05$). Furthermore, RT-PCR and Western blot results demonstrated that the expression levels of mRNA and proteins in Nrf2, HO-1 and NQO1 were up-regulated by Mycoplasma infection ($p < 0.01$), and further increased by LBP treatment ($p < 0.05$).*
Conclusion: *LBP exerts a hyperactive antioxidant response encoded by Nrf2 to protect SLs from apoptosis induced by ROS-related oxidative damage after Mycoplasma infection. These results suggest that LBP may serve as a beneficial and dietary anti-Mycoplasma and anti-apoptotic agent.*

Keywords: *Lycium barbarum polysaccharide, Splenic lymphocytes, ROS, Caspase-3, Bax, Nrf2*

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INTRODUCTION

Mycoplasma has significant impacts on many human diseases [1]. Immune responses are believed to play roles in protection from *Mycoplasma* diseases [1]. In Chinese traditional medicine, the use of *Lycium barbarum* as a functional food for promoting immunity and longevity were documented as far back as 2,800 BC [2]. Studies have shown that *Lycium barbarum* polysaccharide (LBP), a major active component of *Lycium barbarum,* attenuates cytotoxicity, lowers blood glucose, enhances immunity, and also possesses anti-cancer properties [3-5]. Apoptosis is closely associated with lymphocyte development and homeostasis [6]. Enhanced or inhibited apoptosis in lymphocyte can lead to immunodeficiency or autoimmunity, or lymphoma [6]. LBP plays an important role in lymphocyte proliferation and cytokine production [7,8]. A recent study reported that LBP has beneficial effects on spermatogenesis through regulation of apoptosis and antioxidant activity in diabetic male mice [9]. However, little is known about the effect of LBP on apoptosis in lymphocytes.

Reactive oxygen species (ROS) have been considered as messengers of phagocytes involved in defense against pathogen invasion [10]. They are involved in lymphocytes activation, hypo- responsiveness, and apoptosis [10]. Recent studies indicate that LBP has antioxidant properties [9,11]. Thus, this study was aimed at investigating the effects of LBP on apoptotic signaling induced by ROS-related oxidative damage in splenic lymphocytes (SLs) after *Mycoplasma* infection.

EXPERIMENTAL

Mycoplasma species

Mycoplasma (MP) species (ATCC 15488, Ziker Biotech, China) was cultured in modified Friis broth containing 2.5 % CO_2, at 37 °C. The culture medium was centrifuged at 18,000 g for 20 min at 4 °C, to harvest the *Mycoplasma*. The cells were washed thrice in PBS (pH 7.4), and suspended in RPMI 1640 medium. It was previously reported that at 540 nm, an absorbance of 0.2 was equivalent to 10^8 colony-forming units per ml (CFU/mL) of *Mycoplasma* [12]. Thus, the concentration of *Mycoplasma* was determined spectrophotometrically at 540 nm.

LBP isolation

Water extraction and ethanol precipitation were employed to extract LBP from *Lycium barbarum* fruit. Dried fruits of *Lycium barbarum* were ground to fine powder and extracted in water at 4 °C overnight. The *Lycium barbarum* residue was then extracted three times in boiling water. Then, the aqueous extract was concentrated and precipitated using 5 volumes of 100% ethanol. The precipitate was dried to obtain the crude LBP, which was then purified using DEAE-cellulose ion exchange chromatography. Prior to administration, the LBP was dissolved in PBS and filtered through a 0.22 μm filter.

Cell culture and grouping

Splenic lymphocyte cell line (obtained from ATCC, USA) was cultured in RPMI-16 40 medium supplemented with 10 % fetal bovine serum (FBS) at 37 °C in a 5 % CO_2-humidified incubator. The cells were divided into 7 groups: control group, and six *Mycoplasma* (20 cfu/mL) groups, containing different LBP concentrations as follows: 0 (vehicle), 20, 50, 100, 200, and 400 μg/mL (coded MP, LBP 20, LBP 50, LBP 100, LBP 200 and LBP 400, respectively). Cellular apoptosis was determined 4 h after treatment.

Flow cytometry analysis

SLs were harvested and washed twice with PBS. Then, the cell pellet was re-suspended in washing buffer. For the apoptosis analysis, the cells were stained with PI dye at 4 °C for 30 min, followed by examination using a FACSCanto II cytometer (BD Biosciences, Germany). The percentage of apoptotic cells was calculated.

In order to determine the level of ROS, SLs were incubated with fluorescent probe DCFH-DA (10 uM) at 37 °C for 30 min. Then, after washing, intracellular ROS were quantified by FACSCanto II cytometer (BD Biosciences, Germany).

Quantitative real-time PCR

SLs were washed with PBS and RNA was extracted using RNeasy Plus Micro Kit (QIAGEN, USA), followed by cDNA synthesis using SuperScript® IV First-Strand Synthesis System (Invitrogen, USA). PCR primers (with sequences) of the target genes were as follows: β-Actin (internal control): 5′- TGC TGT GTT CCC ATC TAT CG -3′ (forward) and 5′- TTG GTG ACA ATA CCG TGT TCA -3′ (reverse); Caspase-3: 5′- TGG CCC TCT TGA ACT GAA AG -3′ (forward) and 5′- TCC ACT GTC TGC TTC AAT ACC -3′ (reverse); BAX: 5′- TCC TCA TCG CCA TGC TCA T -3′ (forward) and 5′- CCT TGG TCT GGA AGC AGA AGA -3′ (reverse); Bcl-2: 5′- GAT GAC CGA GTA CCT GAA CC -3′ (forward) and 5′- CAG GAG AAA TCG AAC AAA GGC -3′ (reverse); Nrf2: 5′- GAC GGTA TGC AAC AGG ACA TTG AG -3′ (forward) and 5′- AAC TTC TGT CAG TTT GGC TTC TGG A -3′ (reverse); NQO1: 5′- GGA TTG GAC CGA GCT GGA A -3′ (forward) and 5′- AAT TGC AGT GAA GAT GAA GGC AAC -3′ (reverse); HO-1: 5′- ACA TCG ACA GCC CCA CCA AGT TCAA -3′ (forward) and 5′- CTG ACG AAG TGA CGC CAT CTG TGAG -3′ (reverse). The RT-PCR was conducted in a 25-μL reaction volume (7500 Fast Real-time PCR System, ABI, USA) using 20 ng template, Power SYBR® Green PCR Master Mix (Invitrogen, USA), and gene-specific primer pairs for pro-apoptotic genes (Caspase-3 and Bax), anti-apoptosis factor Bcl-2, and antioxidant enzyme genes (Nrf2, HO-1, and NQO1). Amplification conditions were as follows: 95 °C, 2 min; 30 cycles of 95 °C, 30 s, 65 °C, 30 s, 72 °C, 1 min; 72 °C, 5 min. The relative quantification of target genes was calculated by ΔΔCT method.

Western blot analysis

Cells were lysed in an ice-cold hypotonic lysis buffer (containing proteinase inhibitor cocktail and PMSF) and centrifuged at 10,000 rpm at 4

°C for 3 min. The protein concentration was measured using BCA Protein Assay reagent kit (Thermo Fisher Scientific, USA). The cell lysates (20 μg of protein) were subjected to 10 % SDS-PAGE. Then, the gel was electro-transferred onto a PVDF membrane. After blocking with 5 % skimmed milk, the membrane was successively incubated with anti Caspase-3, Bax, Bcl-2, Nrf2, HO-1, and NQO1 primary antibodies (1:1000; Abcam, USA) and goat anti-rabbit IgG-HRP secondary antibody (1:2000; Abcam, USA). Bands were detected by ECL kit (Thermo Fisher Scientific, USA). Target gene expression levels were normalized with an internal control (β-actin).

Statistical analysis

All data are presented as mean ± standard deviation (SD). For multiple group comparisons, one-way analysis of variance (ANOVA) and appropriate post-hoc analysis were performed using a statistical software package (Prism5, USA). $P < 0.05$ and < 0.01 were considered statistically significant.

RESULTS

LBP prevented SLs apoptosis

The LBP-treated groups exhibited dose-dependent reductions in the apoptotic ratio of SLs when compared to the MP-infected group (Figure 1). When the LBP concentration reached 100 μg/mL, the apoptotic ratio of SLs was significantly decreased relative to the MP-infected group ($p < 0.05$). The apoptotic ratio in the 400 μg/mL LBP treated group was decreased to a level similar to the control group. These data indicate that LBP is an effective anti-apoptotic agent in SLs.

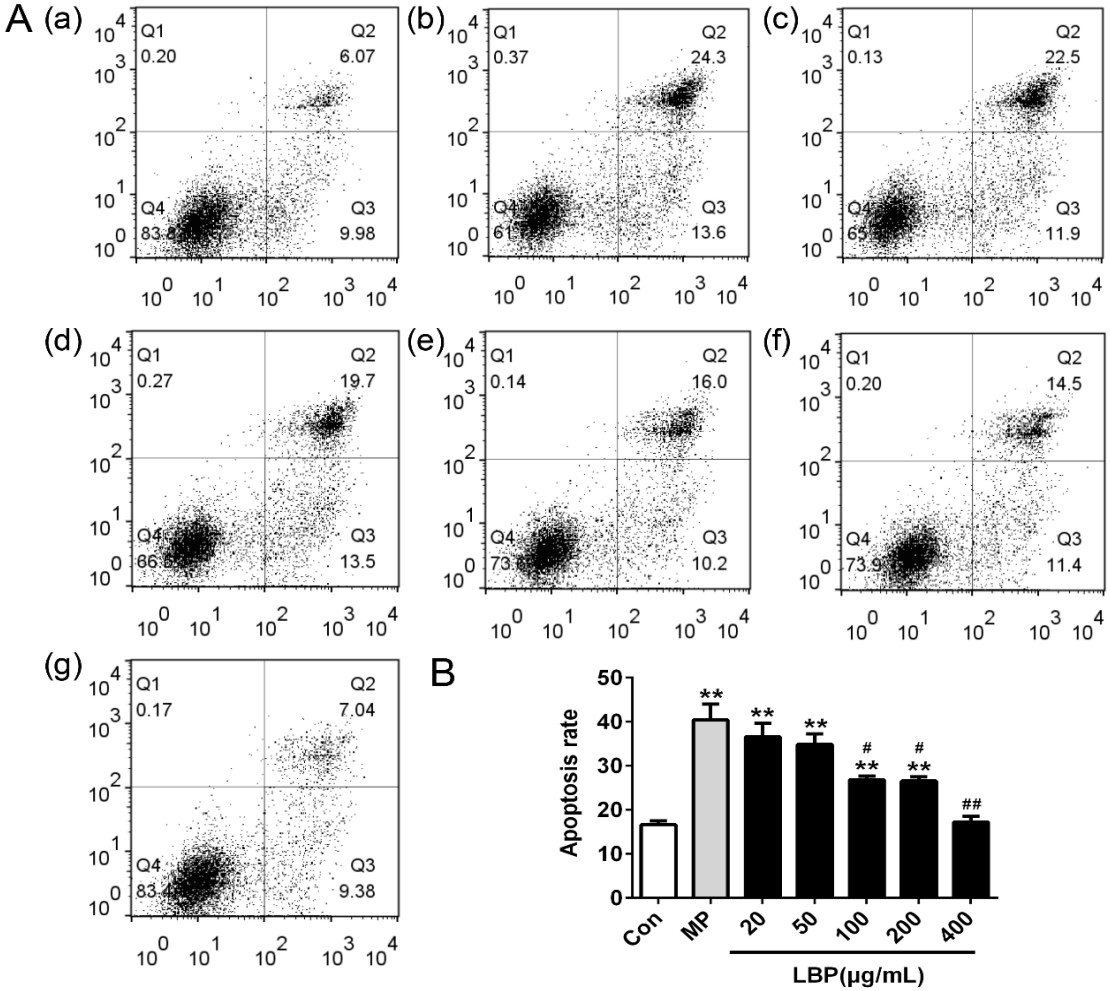

Figure 1: Effect of LBP treatment on apoptosis in SLs. (A) Apoptosis of *Mycoplasma*-infected SLs (b) and SLs treated with vehicle (a), 20 (c), 50 (d), 100 (e), 200 (f), and 400 (g) μg/mL LBP at 4 h was evaluated using PI staining and flow cytometry analysis. (B) Quantitative analysis of the apoptotic cells under different treatment conditions; $^{**}p < 0.01$, versus MP group; $^{\#}p < 0.05$, versus control group; $^{\#\#}p < 0.01$, versus the control group; Results are presented as mean ± SD of three experiments. MP = *Mycoplasma*; LBP = *Lycium barbarum* polysaccharide

LBP prevented accumulation of intracellular ROS

Flow cytometry analysis showed a dose-dependent decrease in intracellular levels of ROS in SLs. When the LBP concentration reached 400 µg/mL, ROS levels were significantly decreased when compared to the MP group ($p < 0.05$, Figure 2). These results indicate that ROS clearance may be a mechanism involved in the protective effect of LBP on the apoptosis of mycoplasma-infected SLs.

LBP regulated the expressions of pro-apoptotic genes

The expressions of pro-apoptotic genes, caspase-3 and Bax, were decreased by LBP treatment at both mRNA and protein levels. In the 200 and 400 µg/mL groups, the relative expressions of these genes decreased significantly when compared with the MP group ($p < 0.01$, Figure 3). In contrast, both mRNA and protein levels of the anti-apoptotic factor Bcl-2 were significantly increased in a dose-dependent manner when compared with the 200 and 400 µg/mL MP groups ($p < 0.01$).

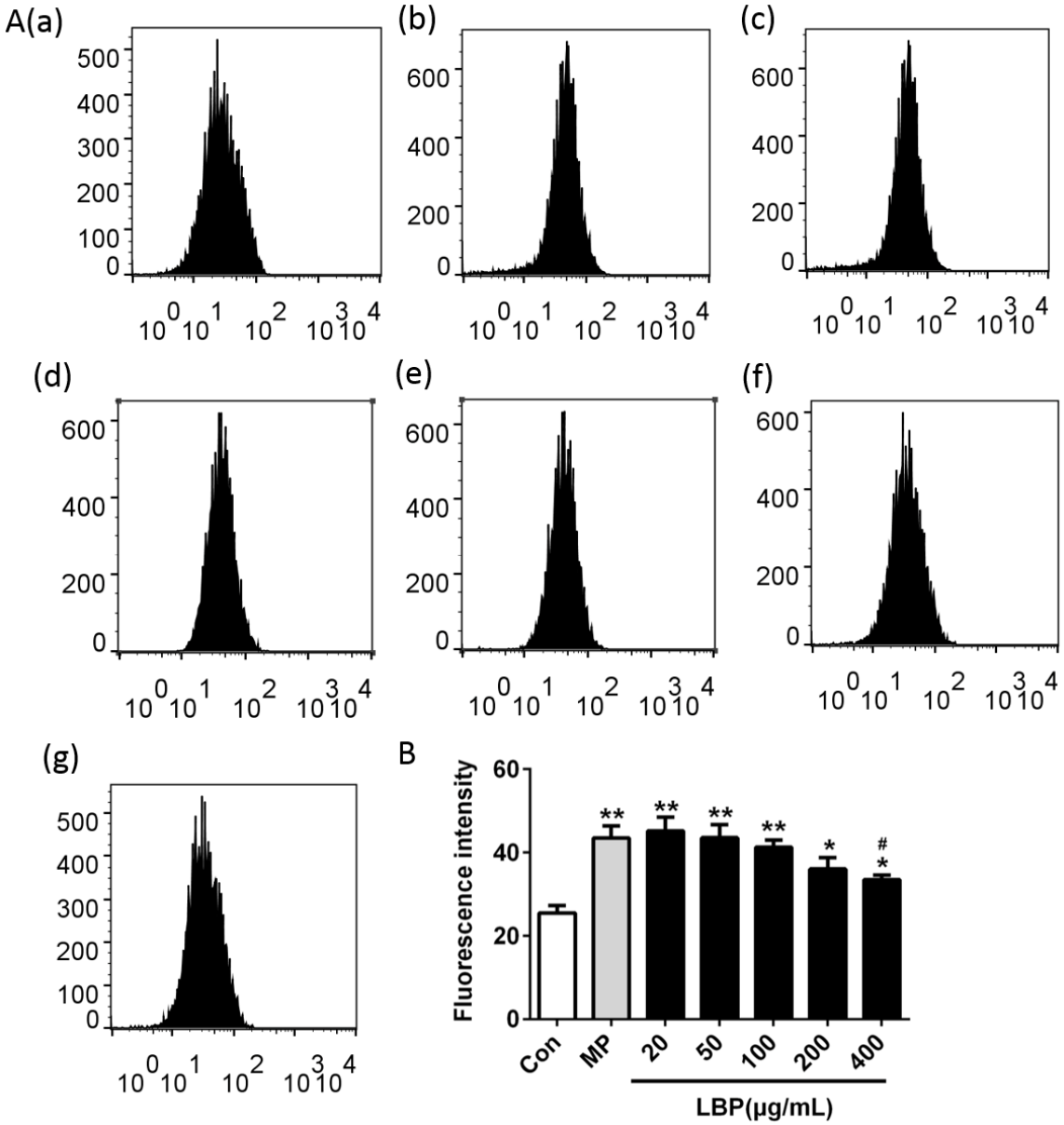

Figure 2: Effect of LBP treatment on ROS production. (A) ROS levels of *Mycoplasm*a infected SLs (b) and SLs treated with 0 (vehicle) (a), 20 (c), 50 (d), 100 (e), 200 (f), and 400 (g) µg/mL LBP at 4 h were evaluated using DCFH-DA fluorescence by flow cytometry. (B) The geometric mean of fluorescence ± SD; $^{**}p < 0.01$, versus the MP group; $^{\#}p < 0.05$, versus the control group; MP = mycoplasma; LBP = *Lycium barbarum* polysaccharide

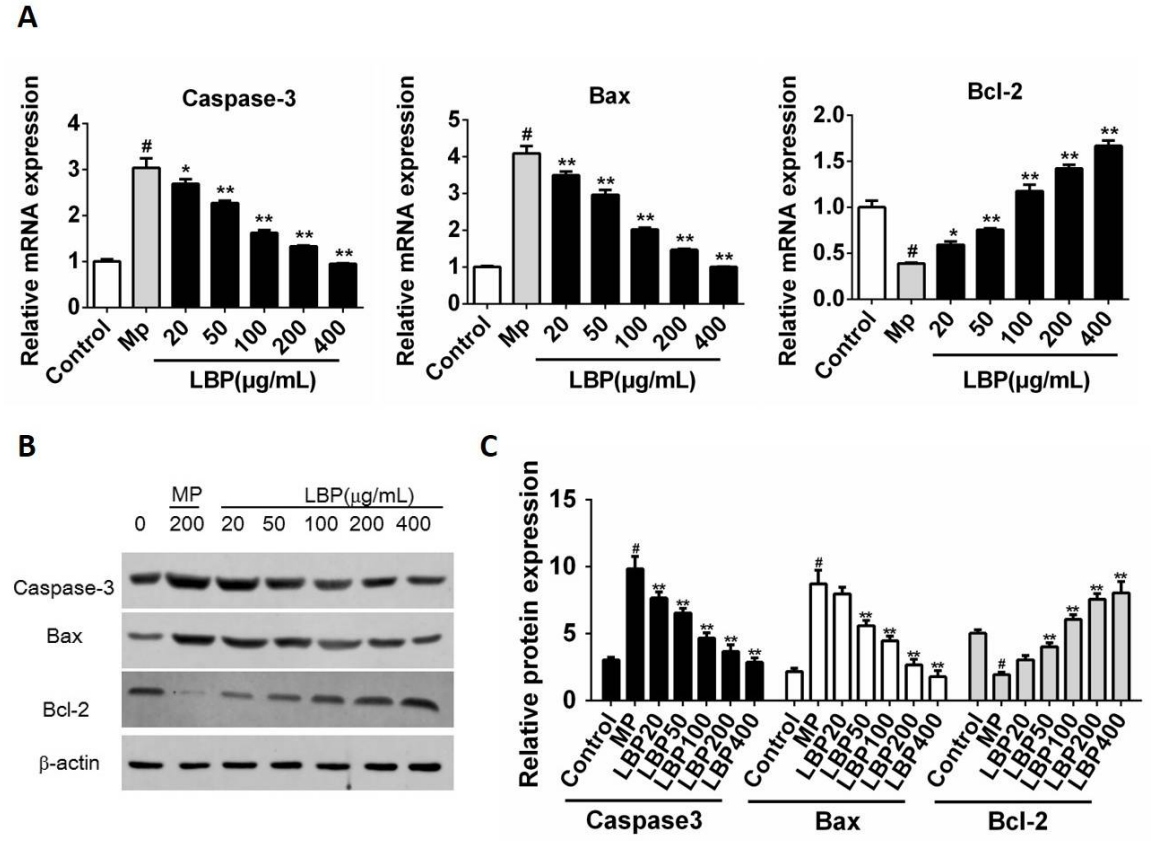

Figure 3: Effect of LBP on the expression of pro-apoptotic genes. (A) Relative mRNA expression of caspase3, Bax, and Bcl-2 were determined by RT-PCR. (B) Bands of caspase3, Bax, and Bcl-2 determined by Western blotting. (C) Quantitative analysis of Western blot result; *$p < 0.05$, versus the MP group; **$p < 0.01$, versus the MP group; #$p < 0.05$, versus the control group; MP = *Mycoplasma*; LBP = *Lycium barbarum* polysaccharide

LBP up-regulated antioxidant enzymes through Nrf2 signaling pathway

Results of qPCR and Western blot show that the mRNA and protein levels of Nrf2 were significantly up-regulated by *Mycoplasma* infection ($p < 0.01$ when compared with the control group. Figure 4). LBP treatment for 4 h further increased the Nrf2 expressions, when compared with the MP group (for mRNA levels: $p < 0.01$ in the 50 to 400 μg/mL groups; for protein levels: $p < 0.01$ in the 400 μg/mL group), HO-1 (for mRNA levels: $p < 0.01$ in the 200 to 400 μg/mL groups; for protein levels: $p < 0.01$ in the 50 to 400 μg/mL groups), and NQO1 (for mRNA levels: $p < 0.01$ in the 50 to 400 μg/mL groups; for protein levels: $p < 0.01$ in the 200 to 400 μg/mL groups; Figure 4). These results support the anti-apoptotic role and ROS-clearing effects of LBP.

DISCUSSION

In this study, *Mycoplasma*-infected SLs were used to investigate the role of LBP in antioxidant defense against immunologic injury. It was found that *Mycoplasma* infection increased apoptosis and ROS accumulation in SLs. However, LBP treatment reduced the ROS levels, increased the activation of Nrf2 pathway, and inhibited apoptotic signals. A growing body of evidence indicate that LBP and *Lycium barbarum* have a variety of immuno-modulatory functions. Some researchers reported that LBP can induce phenotypic and functional maturation of dendritic cells via NF-κB signaling pathways [13,14]. It has been shown that LBP can act as an adjuvant for the generation of Tfh cells to enhance T cell-dependent antibody responses [15]. It has also been demonstrated that LBP can promote cytokine secretion in macrophages, Th1, and Th2 [16]. Researchers have consistently shown that LBP significantly induces proliferation of T lymphocytes [4,17]. However, not much was hitherto know about the anti-apoptotic and antioxidant effects of LBP in lymphocytes. To the best of our knowledge, the present study is the first to demonstrate the anti-apoptotic property of LBP in SLs through the Nrf2 pathway, thereby providing a deeper understanding of its beneficial properties in immunocytes.

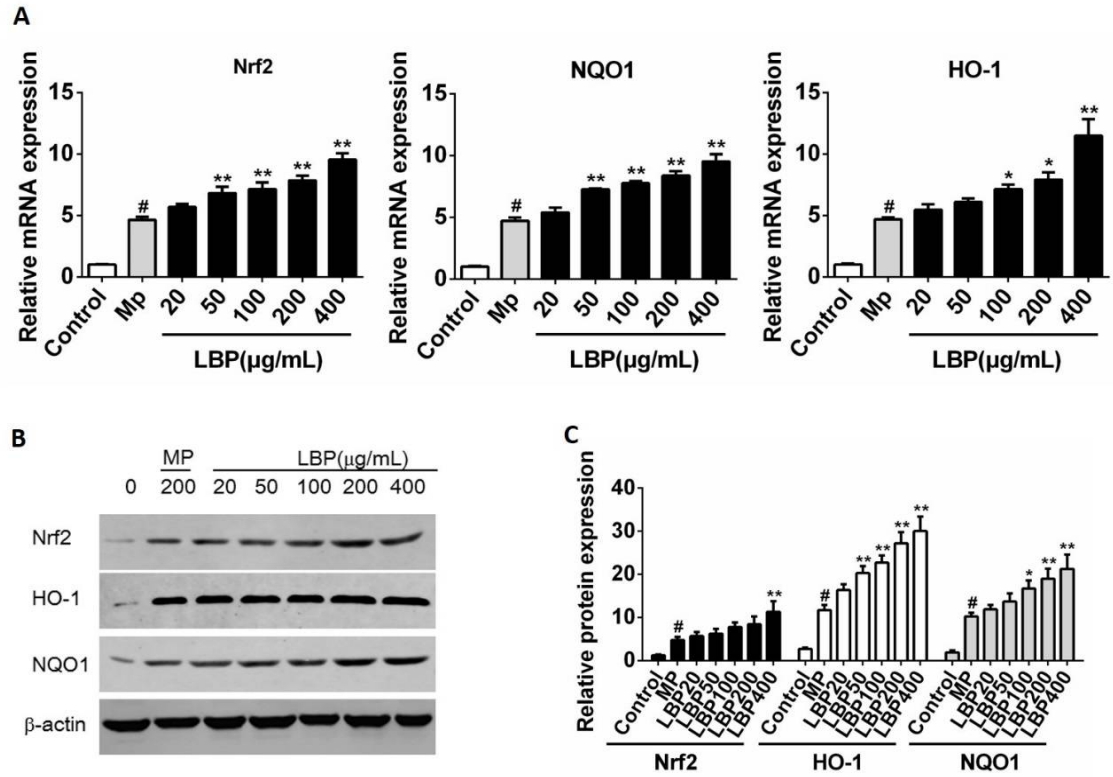

Figure 4: Effect of LBP on expression of antioxidant enzyme genes. (A) Relative mRNA expression of Nrf2, HO-1, and NQO1 were determined by RT-PCR. (B) Bands of Nrf2, HO-1, and NQO1 determined by Western blotting. (C) Quantitative analysis of Western blot results; *$p < 0.05$, versus MP group; **$p < 0.01$, versus MP group; # $p < 0.05$, versus control group; MP = *Mycoplasma*; LBP = *Lycium barbarum* polysaccharide

Numerous studies have reported that the Nrf2 signaling pathway plays a critical role in cellular defense against oxidative stress. Some researchers used Keap1-deficient mice and found that T-cell–specific augmentation of Nrf2 increased antioxidant response and reduced intracellular cytokine production by T-cells [18]. Nrf2-KO mice developed inflammatory lesions and autoimmune syndromes, and decreases in expressions of HO-1 and NQO1 [19]. HO-1 and NQO1 are two well-recognized downstream factors of the Nrf2 signaling pathway. HO-1 is as a potent radical scavenger known to protect cells against oxidative stress [20]. Studies with HO-1-knockout mice which exhibit increased susceptibility to oxidative insults proved that HO-1 is involved in the endogenous defense against oxidative stress [20]. NQO1 is a flavoprotein which prevents the participation of quinones in redox cycling, thereby sequestering ROS [21]. However, there is lack of data on the role of Nrf2 signaling pathway in SLs infected with *Mycoplasma*. Thus, the effect of *Mycoplasma* on activation of Nrf2 and its downstream antioxidant enzymes (HO-1 and NQO1) was investigated in the present study. The results obtained suggest that the Nrf2 pathway is involved in the survival of *Mycoplasma*-infected SLs, and that LBP treatment protects the infected SLs through the clearance of ROS.

CONCLUSION

The results of this study indicate that LBP can protect *Mycoplasma*-infected SLs from ROS-induced apoptosis through a mechanism involving a hyperactive antioxidant response encoded by Nrf2. This finding provides evidence to show that LBP may serve as a beneficial and dietary anti-*Mycoplasma* and anti-apoptotic agent.

DECLARATIONS

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We are grateful to our institutes for their assistance during the preparation of this manuscript.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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