

Salvianolic acid B attenuates lipopolysaccharide-induced acute lung injury in rats through inhibition of apoptosis, oxidative stress and inflammation

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Abstract. The present study was performed to assess the protective effect of salvianolic acid B on lipopolysaccharide (LPS)-induced acute lung injury (ALI). Sprague Dawley rats were injected with 100 μ g/kg LPS through a 24-gauge catheter. One group of rats was pre-treated with salvianolic acid B (1 mg/ml; 20 ml/kg body weight) 1 h prior to LPS challenge, then 20 ml/kg salvianolic acid B every 2 days for 4 weeks thereafter. Salvianolic acid B attenuated LPS-induced increases in the lung wet/dry weight rate and lung tissue injury in ALI model rats. LPS-induced changes in the content of caspase-3, malondialdehyde, superoxide dismutase, catalase, glutathione peroxidase, tumor necrosis factor- α and interleukin-6 in ALI model rats were attenuated by treatment with salvianolic acid B. Furthermore, treatment with salvianolic acid B inhibited the protein expression of type I collagen I, endogenous transforming growth factor- β 1 production and α -smooth muscle actin in ALI model rats. These findings indicated that salvianolic acid B attenuates LPS-induced ALI through inhibition of apoptosis, oxidative stress and inflammation in rats and therefore exerts a protective effect against ALI.

Introduction

Acute lung injury (ALI) is defined as pulmonary dysfunction caused by pathogenic factors. It may aggravate to develop into more severe acute respiratory distress syndrome (ARDS) (1). In the clinic, affected patients present with intractable hypoxemia and progressive respiratory distress. The major associated pathologic changes are extensive injury

of pulmonary capillary endothelial cells and alveolar epithelial cells (2). Subsequently, pneumonema develops and the membrane becomes transparent (3). Major pathophysiological changes associated with ALI and ARDS are a decrease in lung compliance, diffusion disturbance and the disproportionality of ventilation/perfusion. The pathogenesis of ALI is complex and has remained to be fully elucidated (4). When ALI occurs, inflammatory cells, including neutrophils and macrophages, adhere, accumulate and can be activated in the lung (5). Afterwards, inflammatory cells produce large amounts of active oxygen and oxidase through respiratory burst and degranulation. Consequently, the pulmonary vasculature and alveolar epithelial cells are seriously damaged (6).

Apoptosis, also known as programmed cell death, refers to an active type of death in which cells themselves terminate their lives under certain physiological or pathological conditions. It is an essential process for organisms to maintain a dynamic equilibrium, which affects lesions and recovery of lung tissues (7). Excessive apoptosis of alveolar epithelial cells and vascular endothelial cells may result in structural distortion and destruction of pulmonary tissues as well as fibroblast proliferation. The association between apoptosis and ALI remains a hot topic (8).

In recent years, molecular biological mechanisms have received continuous attention. The pathway associated with biological damage of the lung is the induction of a lung inflammatory reaction mediated by cells and inflammation mediators (9). Biological damage refers to an inflammatory injury in which inflammatory mediators, cytokines and inflammatory cells participate. Mechanical and biological damage are connected with each other (7). ALI causes biological damage, while biological damage may exacerbate mechanical damage (10).

As a traditional Chinese medicine, *Salvia miltiorrhizae* has been employed in Asian countries to cure and prevent various types of disease with high efficacy (11). In recent years, two active components with the highest pharmaceutical value have been extracted, i.e., tanshinone IIA and salvianolic acid B (Fig. 1) (12). The latter has been reported to aid in neural functional recovery in rats with cerebral injury (12). Furthermore, it was shown to have neuroprotective effects in rats with

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focal cerebral ischemic reperfusion (13). It has been suggested that the neural functional recovery effect of salvianolic acid B is dependent on its specific chemical structure, which are accountable for its neuroprotective effects (12,14). The present study assessed whether salvianolic acid B exerts a protective effect on LPS-induced ALI.

Materials and methods

Animals, experimental groups and LPS-induced ALI model. Adult male Sprague-Dawley (SD) rats weighing 50 ± 2 g were obtained from the Animal Center of Anhui Medical University (Hefei, China), and were housed in a humidity-controlled environment ($22 \pm 2^\circ\text{C}$; $55 \pm 5\%$ humidity) with a 12-h light/dark cycle and allowed free access to water and standard rat chow. The rats were anesthetized with an intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg body weight; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Subsequently, 0.01% LPS (100 $\mu\text{g/kg}$ body weight; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was injected into the tail vein of SD rats through a 24-gauge catheter. All 26 adult male SD rats were randomly divided into 3 groups: The control group (sham, $n=6$), LPS-induced ALI model group (model, $n=10$), salvianolic acid B-treated group (treated, $n=10$). The rats from the salvianolic acid B-treated group were pretreated with salvianolic acid B (1 mg/ml; 20 ml/kg body weight) 1 h prior to LPS challenge, then 20 ml/kg of salvianolic acid B every 2 days for 4 weeks thereafter.

Lung wet/dry weight ratio. The right edematous lung tissue from every group was weighed to determine the tissue's wet weight. Subsequently, the right lung edema tissue was placed in an oven at 80°C for 24 h and weighed to determine the tissue dry weight. The tissue wet/dry weight ratio was then calculated.

Lung histopathology. The left edematous lung tissue from every group was fixed in 4% paraformaldehyde for 24 h and dehydrated by an ethanol gradient. The tissue was embedded in paraffin and sectioned into 4- μm slices. Sliced tissue samples were stained with hematoxylin and eosin.

Determination of oxidative stress and inflammation. Lung homogenate 10% (w/v) was prepared and centrifuged at $12,000 \times g$ for 10 min. The supernatant was collected and standard enzyme-linked immunosorbent assay kits were used to measure the content of caspase-3 (DYC835-2; R&D Systems, Inc., Minneapolis, MN, USA), and malondialdehyde (MDA; A003-1), superoxide dismutase (SOD; A001-1), catalase (CAT; A007-1), glutathione peroxidase (GPx; A005; all from Nanjing Jiancheng Bioengineering Institute, Nanjing, China), tumor necrosis factor (TNF)- α (ab46070) and interleukin (IL)-6 (ab100772; both from Abcam, Cambridge, UK), according to the manufacturer's instructions.

Western blot analysis. Lung homogenate 10% (w/vol) was prepared and centrifuged at $12,000 \times g$ for 10 min. The supernatant was collected and used to measure protein contents using a bicinchoninic acid assay (Beyotime Institute of Biotechnology, Haimen, China). In brief, total protein (50 $\mu\text{g/lane}$) was subjected to 10-12% SDS-PAGE and subsequently transferred

onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked with 5% skimmed milk in Tris-buffered saline with 0.1% Tween-20 (pH 7.4) and incubated with anti-transforming growth factor (TGF)- $\beta 1$ (1:2,000 dilution; 3709; Cell Signaling Technology, Inc., Danvers, MA, USA), anti- α -smooth muscle actin (SMA; 1:2,000 dilution; ab5694; Abcam) anti-collagen I (1:2,000 dilution; ab34710; Abcam) or β -actin (1:5,000 dilution; sc-7210; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C . The membrane was incubated with horseradish peroxidase-conjugated secondary antibody (sc-2357, 1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37°C for 2 h.

Statistical analysis. Values are expressed as the mean \pm standard deviation by SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance and pairwise comparison were performed. $P < 0.05$ was considered to indicate a statistically significant difference between values.

Results

Salvianolic acid B attenuates LPS-induced increases in the lung wet/dry weight ratio in rats. As shown in Fig. 2, the lung wet/dry weight ratio in the model group was higher than that in the sham group. The LPS-induced increase in the lung wet/dry weight ratio was markedly attenuated by salvianolic acid B pre-treatment in LPS-induced ALI rats (Fig. 2).

Salvianolic acid B attenuates LPS-induced lung tissue injury in rats. As shown in Fig. 3, sham rats had a normal lung morphology, while LPS-induced ALI rats exhibited extensive lung damage. Of note, pre-treatment with salvianolic acid B significantly attenuated LPS-induced lung tissue injury in rats (Fig. 3).

Salvianolic acid B attenuates LPS-induced increases in the content of caspase-3 in rats. As shown in Fig. 4, the lung tissue of LPS-induced ALI rats showed a significant increase in caspase-3 content compared with that in the sham group. However, treatment with salvianolic acid B significantly attenuated LPS-induced increases in the content of caspase-3 in the rat lungs (Fig. 4).

Salvianolic acid B attenuates LPS-induced oxidative stress in rats. When compared to that in the sham-operated rats, the MDA content was enhanced and the SOD, CAT and GPx content was attenuated in LPS-induced ALI rats (Fig. 5). Of note, salvianolic acid B treatment significantly attenuated these LPS-induced changes associated with oxidative stress in rats (Fig. 5).

Salvianolic acid B attenuates LPS-induced inflammation in rats. After LPS exposure, the levels of the TNF- α and IL-6 content in the rat lungs were significantly elevated compared to those in sham-operated animals (Fig. 6). Salvianolic acid B treatment significantly attenuated LPS-induced increases in the TNF- α and IL-6 content in rat lungs (Fig. 6).

Salvianolic acid B attenuates LPS-induced Col-I in rats. As shown in Fig. 6, the protein expression of Col-I in the rat lungs

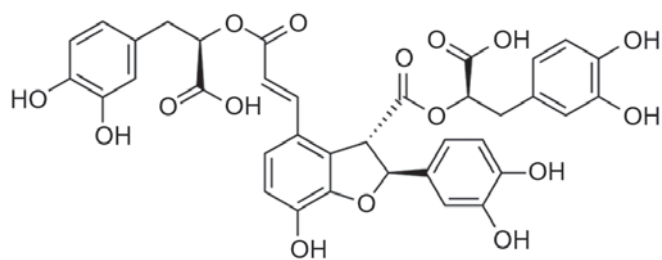


Figure 1. Chemical structure of salvianolic acid B.

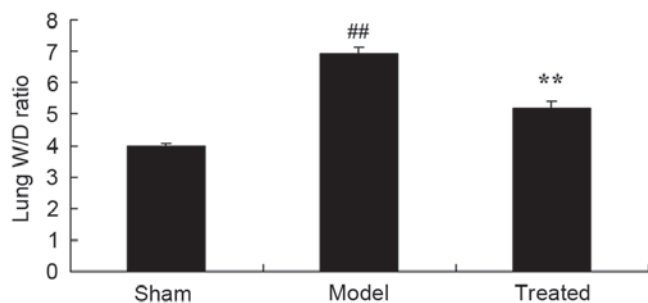


Figure 2. Salvianolic acid B attenuates LPS-induced increases in the lung W/D weight rate in rats. Groups: Sham, control group; model, LPS-induced acute lung injury model group; treated, salvianolic acid B-treated group (1 mg/ml; 20 ml/kg body weight). ^{##}P<0.01 vs. sham group, ^{**}P<0.01 vs. model group. W/D, wet/dry; LPS, lipopolysaccharide.

after LPS exposure was significantly higher than that in the sham rats (Fig. 7). The LPS-induced Col-I protein expression in the rat lungs was significantly attenuated by treatment with salvianolic acid B (Fig. 7).

Salvianolic acid B attenuates LPS-induced TGF- β 1 in rats. As shown in Fig. 8, TGF- β 1 protein expression was significantly activated in rats after LPS exposure compared with that in sham rats. Treatment with salvianolic acid B significantly attenuated LPS-induced TGF- β 1 protein expression in ALI rats (Fig. 8).

Salvianolic acid B attenuates LPS-induced α -SMA in rats. When compared to that in the sham control rats, α -SMA protein expression was observed to be increased in LPS-induced ALI rats (Fig. 9). Of note, salvianolic acid B treatment significantly attenuated LPS-induced α -SMA protein expression in rats (Fig. 9).

Discussion

ALI is a syndrome of pulmonary inflammation and permeability characterized by gas exchange dysfunction (15). If it aggravates, it is referred to as ARDS in the clinic. Its pathological features are pulmonary capillary endothelial cell damage, alveolar epithelial cell injury, extensive aqualung, tiny pulmonary atelectasis, microthrombi and microcirculation disturbance (16). Common incentives include severe infection, trauma, shock and intoxication, such as inhalation of toxic gases. While the morbidity and fatality rates of affected patients are high, the advance of clinical methods and comprehensive treatments have decreased its mortality rate, which, however, remains as high as 30-40% (3). The mortality rates is also increased in

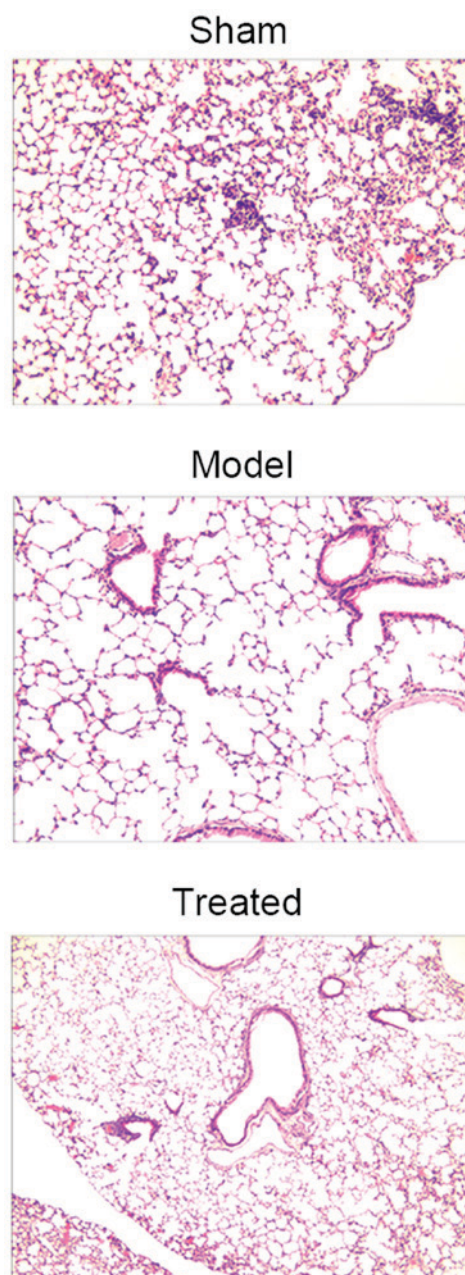


Figure 3. Salvianolic acid B attenuates LPS-induced lung tissue injury in rats. Representative histological images stained with hematoxylin and eosin are shown (magnification, x20). Groups: Sham, control group; model, LPS-induced acute lung injury model group; treated, salvianolic acid B-treated group (1 mg/ml; 20 ml/kg body weight). LPS, lipopolysaccharide.

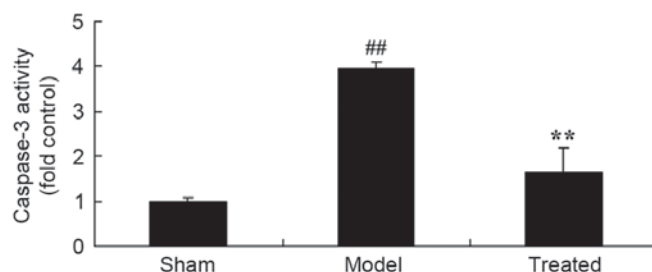


Figure 4. Salvianolic acid B attenuates LPS-induced increases of caspase-3 in the lung tissues of rats. Groups: Sham, control group; model, LPS-induced acute lung injury model group; treated, salvianolic acid B-treated group (1 mg/ml; 20 ml/kg body weight). ^{##}P<0.01 vs. sham group, ^{**}P<0.01 vs. model group. LPS, lipopolysaccharide.

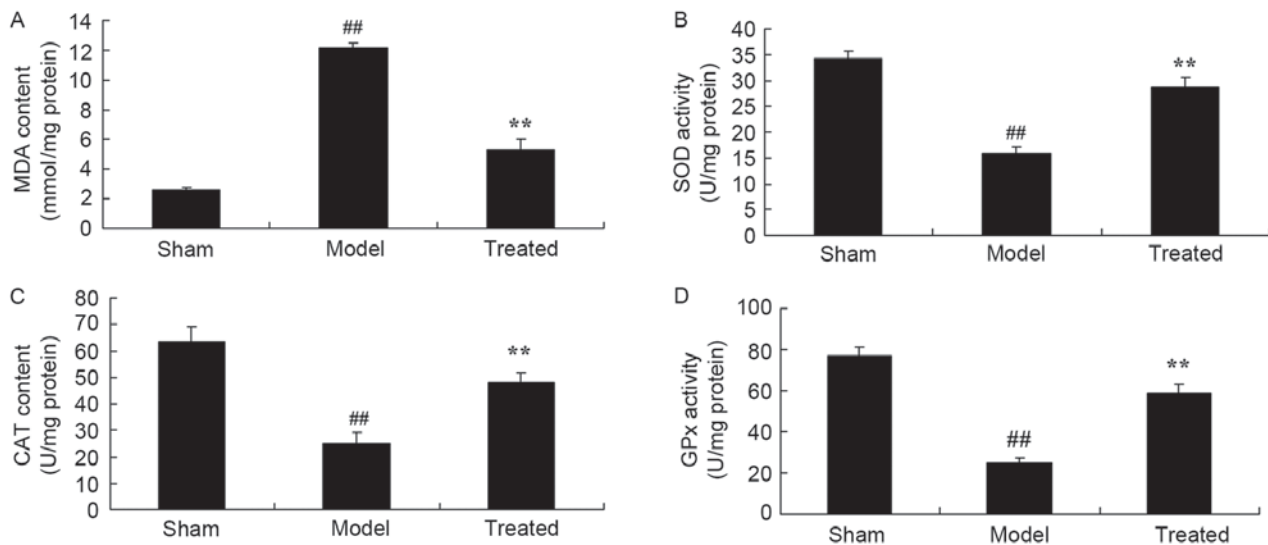


Figure 5. Salvianolic acid B attenuates LPS-induced oxidative stress in the lung tissues of rats. Treatment with salvianolic acid B inhibited LPS-induced (A) increases in MDA as well as decreases in the (B) SOD, (C) CAT and (D) GPx content in rats. Groups: Sham, control group; model, LPS-induced acute lung injury model group; treated, salvianolic acid B-treated group (1 mg/ml; 20 ml/kg body weight). ^{##}*P*<0.01 vs. sham group, ^{**}*P*<0.01 vs. model group. MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; LPS, lipopolysaccharide.

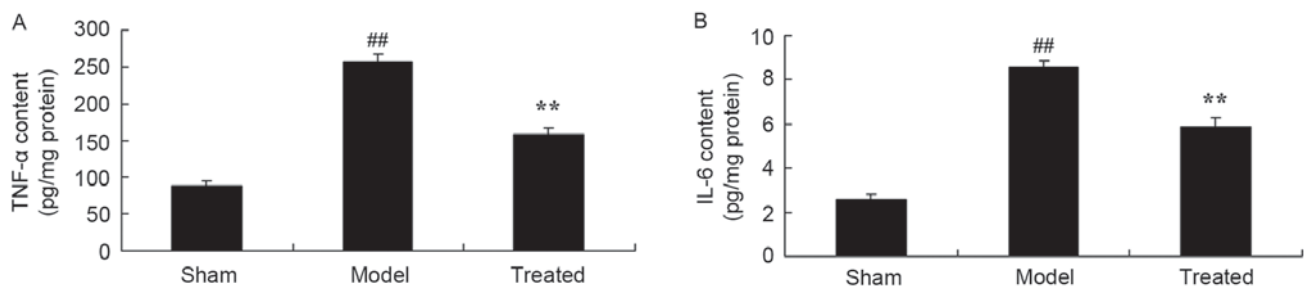


Figure 6. Salvianolic acid B attenuates LPS-induced inflammation in the lung tissues of rats. Treatment with salvianolic acid B attenuated LPS-induced increases in (A) TNF-α and (B) IL-6 contents in rats. Groups: Sham, control group; model, LPS-induced acute lung injury model group; treated, salvianolic acid B-treated group (1 mg/ml; 20 ml/kg body weight). ^{##}*P*<0.01 vs. sham group, ^{**}*P*<0.01 vs. model group. TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide.

patients with accompanying multiple organ dysfunction, with the mortality rate being 100% if more than four organ dysfunctions are present (17). In the present study, salvianolic acid B was shown to markedly attenuate the LPS-induced increase in the lung wet/dry weight ratio as well as lung tissue injury in ALI rats. Chen *et al* (12) reported that salvianolic acid B attenuates traumatic brain injury through exerting an anti-inflammatory effect in mice.

The pathogenesis of ALI is rather complex and it has remained to be fully clarified. However, oxidative stress is considered to be one of major pathogenic factors of ALI (18). Internally (hypoxia-ischemia or inflammation) and externally (pressure, empyrosis), organisms produce large amounts of reactive oxygen, which destroys the equilibrium state of the oxidation/antioxidant system (19). Cell or tissue damage may occur to cause associated diseases, such as tumors or diabetes (20). This process is referred to as oxidative stress. The results of the present study indicated that salvianolic acid B exerts protective effects against LPS-induced oxidative stress in ALI rats. Yang *et al* (14) suggested that salvianolic acid B protects against low-density lipoprotein oxidation and neointimal hyperplasia through inhibition of reactive oxygen

species production in endothelium-denuded hypercholesterolemia rabbits.

The current understanding of apoptotic processes has shifted from cell nucleus-centered to mitochondria-centered mechanisms (8). It is thought that mitochondria have a decisive role in apoptotic processes. As an endogenous apoptotic pathway, the mitochondrial apoptotic pathway is regarded as the key regulatory process (21). The primary functions of mitochondria in cell apoptosis include the release of activity factors of caspase, such as cytochrome *c*, the loss of the mitochondrial transmembrane potential and dysfunction of oxidative phosphorylation of mitochondria (22). During this process, cytochrome *c* and caspase-3 have an essential role. Consistent with this, the results of the present study demonstrated that treatment with salvianolic acid B significantly attenuated LPS-induced increases in the content of caspase-3 in the lungs of ALI rats. Tang *et al* (23) suggested that salvianolic acid B protects human endothelial progenitor cells through inhibiting oxidative stress and caspase-3 activation.

Cells as well as body fluids participate in the inflammatory response (24). Cells participating in inflammation include polymorphonuclear neutrophil (PMN), alveolar epithelial

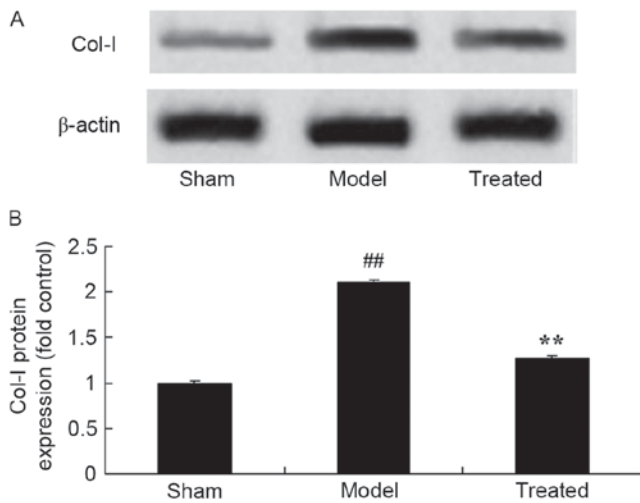


Figure 7. Salvianolic acid B attenuates LPS-induced Col-I in the lung tissues of rats. (A) Western blots showing that treatment with salvianolic acid B attenuated LPS-induced Col-I protein. (B) Quantified protein expression levels of Col-I in rats. Groups: Sham, control group; model, LPS-induced acute lung injury model group; treated, salvianolic acid B-treated group (1 mg/ml; 20 ml/kg body weight). ^{##}P<0.01 vs. sham group, ^{**}P<0.01 vs. model group. Col-I, collagen I; LPS, lipopolysaccharide.

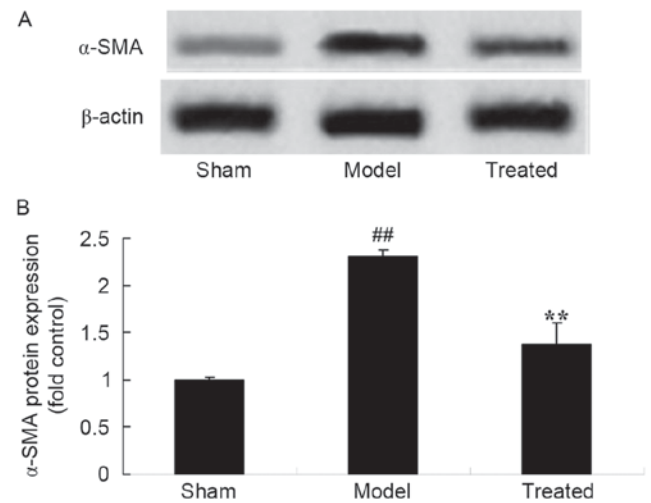


Figure 9. Salvianolic acid B attenuates LPS-induced α -SMA in the lung tissues of rats. (A) Western blot showing that treatment with salvianolic acid B reduced LPS-induced increases in α -SMA protein. (B) Quantified protein expression levels of α -SMA. Groups: Sham, control group; model, LPS-induced acute lung injury model group; treated, salvianolic acid B-treated group (1 mg/ml; 20 ml/kg body weight). ^{##}P<0.01 vs. sham group, ^{**}P<0.01 vs. model group. SMA, smooth muscle actin; LPS, lipopolysaccharide.

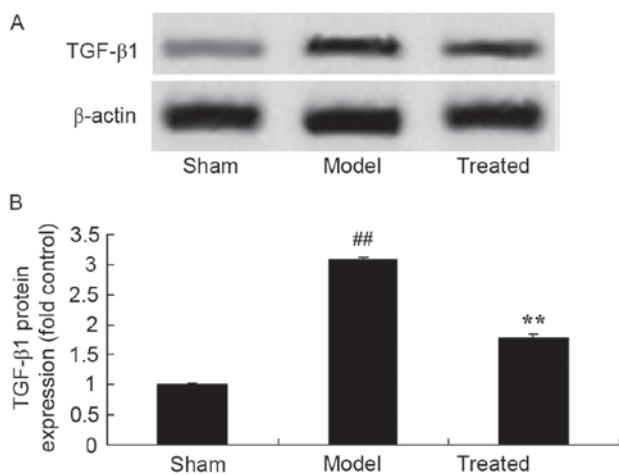


Figure 8. Salvianolic acid B attenuates LPS-induced TGF- β 1 in the lung tissues of rats. (A) Western blot showing that treatment with salvianolic acid B reduced LPS-induced increases in TGF- β 1 in rats. (B) Quantified protein expression levels of TGF- β 1. Groups: Sham, control group; model, LPS-induced acute lung injury model group; treated, salvianolic acid B-treated group (1 mg/ml; 20 ml/kg body weight). ^{##}P<0.01 vs. sham group, ^{**}P<0.01 vs. model group. TGF, transforming growth factor; LPS, lipopolysaccharide.

cells, vascular endothelial cells, pulmonary vascular endothelial cells, alveolar macrophages, pulmonary interstitial macrophages and pulmonary intravascular macrophages, among which PMN and pulmonary intravascular macrophage-shave a pivotal role in lung injury (25). Bodily fluids include cytokines, lipid mediators, oxygen radicals, proteases, alexin, cruor and the fibrinolytic system (7). Consistent with this, the present study found that salvianolic acid B treatment significantly attenuated LPS-induced increases in the TNF- α and IL-6 content in the lungs of ALI rats. Xu *et al* (11) suggested that salvianolic acid B attenuates platelet-mediated inflammatory responses in vascular endothelial cells.

Cytokines in the lung can be expressed in intrinsic pulmonary cells, such as epithelial cells, vascular endothelial cells and interstitial cells. Furthermore, they may be secreted by inflammatory cells activated during lung injury processes, including macrophages and lymphocytes, which may have biological effects in the lung (26). In cytokines participating in radiation-induced lung injury, TGF- β has a predominant role and is a major cytokine, which has been most thoroughly researched. It may induce inflammatory cells and fibroblasts to synthesize IL-1 and IL-6 (27). Furthermore, it may self-induce the production of more TGF- β and inhibit the degradation of extracellular matrix (ECM). It induces fibroblasts to interact with compound ECM and promotes the adhesion between ECM and cells. It stimulates fibroblasts to proliferate and induces the expression of collagen RNAI and III. The fastigium of TGF- β expression corresponds to the vigorous stage of fibroblast proliferation and collagen synthesis, suggesting that TGF- β and other cytokines mediate the early inflammatory response (27). α -SMA, which is the primary molecule of ALI, mainly exists in the cytoplasm. Studies found that TGF- β 1 induces the transformation of fibroblasts and pulmonary epithelial cells to generate ALI, which increases the expression of α -SMA (28). The present study found that salvianolic acid B attenuated LPS-induced increases in Col-I, TGF- β 1 and α -SMA protein expression in the lung tissues of ALI rats. Li *et al* (29) indicated that salvianolic acid B attenuated hepatic fibrosis through Col-I, α -SMA and TGF- β signaling in rats. Lv and Xu (30) reported that salvianolic acid B inhibits TGF- β 1 in the stimulated human hepatic stellate cell line LX-2.

In conclusion, the results of the present study indicated that salvianolic acid B markedly attenuated LPS-induced increases in the lung wet/dry weight ratio as well as lung tissue injury in ALI model rats via inhibition of apoptosis, oxidative stress and inflammation. These findings suggested that the clinical applicability of salvianolic acid B in the prevention and treatment of ALI warrants further exploration.

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