International Immunopharmacology 67 (2019) 1–12

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

# Fraxin ameliorates lipopolysaccharide-induced acute lung injury in mice by inhibiting the NF-κB and NLRP3 signalling pathways

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ARTICLE INFO

*Keywords:*
Fraxin
Acute lung injury
NF-κB pathway
NLRP3 inflammasome

ABSTRACT

Fraxin, the effective component of the Chinese traditional medicine Cortex Fraxini, is reported to have anti-inflammatory effects. This study assessed the anti-inflammatory effect of fraxin on the lipopolysaccharide (LPS)-induced inflammatory response in A549 cells and the protective efficacy on LPS-induced acute lung injury (ALI) in mice. Fraxin reduced LPS-induced TNF-α, IL-6 and IL-1β production in A549 cells and alleviated the LPS-induced wet/dry (W/D) weight ratio and the effects observed via histopathological examination of the lung in vivo. Furthermore, fraxin reduced the protein concentrations in the broncho-alveolar lavage (BAL) fluid and cytokine production in the sera. Fraxin also clearly attenuated the oxidation index, including the activity of myeloperoxidase (MPO), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH). Immunohistochemistry analysis showed that fraxin suppressed LPS-induced inflammatory damage. The expression of proteins involved in the NF-κB and NLRP3 inflammatory corpuscle signalling pathways was consistent between the lung tissues and cell samples. Overall, fraxin played a protective role in LPS-induced lung injury by inhibiting the NF-κB and NLRP3 signalling pathways.

## 1. Introduction

Experimental models of acute lung injury (ALI) in animals that clinically resemble acute respiratory distress syndrome (ARDS) will improve our understanding of its pathogenesis and provide a means of exploring new therapies [1–3]. Lipopolysaccharide (LPS) is the major component of endotoxin on the membrane of gram-negative bacteria and is commonly recognized to induce an ALI model [4–6].

Lipopolysaccharide causes an imbalance between the inflammatory response and the redox process. Upon LPS stimulation, key inflammatory cytokines are activated, such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) [7]. The increase in inflammatory cytokines is associated with ALI pathogenesis and may impair epithelial cell permeability and cause pulmonary oedema [8,9]. Previous studies have demonstrated that inflammatory cytokines can mediate toxicity and damage in the lung [10]. In addition, LPS causes oxidative stress, which leads to excessive production of myeloperoxidase (MPO) and malondialdehyde (MDA). Conversely, LPS reduces antioxidant enzymes, such as superoxide dismutase (SOD), and glutathione (GSH), which protect the lung tissue from oxidative damage [11].

A number of studies have indicated that LPS can activate NF-κB [12], which is the upstream regulatory protein for a variety of effector factors, including inflammatory cytokines, chemokines, adhesion molecules, and surface receptors [13]. The inflammatory corpuscle NLRP3 is a macromolecular multiprotein complex [14]. Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are recognized by NLRP3 receptors [15,16]. After signal stimulation, a series of cascade reactions is triggered and promotes caspase-1 production. Then, caspase-1 cleaves the inflammatory factor precursors of IL-1β and IL-18 to accelerate their maturation and release [17]. Mature IL-1β and IL-18 induce the release of other inflammatory factors, leading to further inflammatory reactions [18].

Fraxin (8-(beta-D-(beta-glucooxygenyl)-7-hydroxyl-6-methoxy coumarin) (Fig. 1) is the main active ingredient in the Chinese traditional medicine Cortex Fraxini [19]. A previous study showed that fraxin possessed a variety of bioactivities, including anti-inflammatory, antioxidant, analgesic, antibacterial, antiviral, immune regulation, antihyperuricaemia and diuresis activity [20]. However, whether it has a direct inhibitory effect on LPS-induced lung injury is unclear. Hence, the aim of this work was to explore the potential effect of fraxin on LPS-induced lung injury and the underlying mechanisms in mice.

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https://doi.org/10.1016/j.intimp.2018.12.003
Received 28 September 2018; Received in revised form 29 November 2018; Accepted 2 December 2018
Available online 06 December 2018
1567-5769/ 

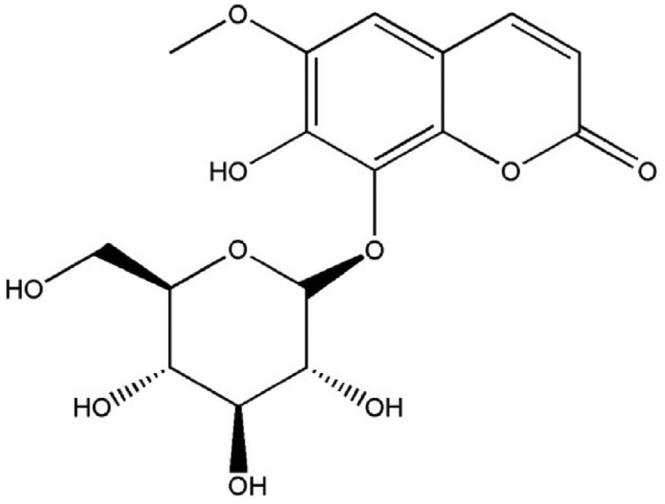


**Fig. 1.** The chemical structure of fraxin.

## 2. Materials and methods

### 2.1. Animals

Healthy 8-week-old Kunming male mice (weighing 18–22 g) were supplied by the Experimental Animal Centre of Xi'an Jiaotong University (Xi'an, China). All mice were kept under standard experimental conditions (temperature: 24 ± 1 °C, relative humidity: 40%–80%). The mice were fed standard rodent feed and had free access to water. The experimental procedures were performed according to the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Xi'an Jiaotong University.

### 2.2. Reagents and antibodies

Fraxin was purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). Dexamethasone (DEX) was purchased from Zhejiang Xianju Pharmaceutical Company (Zhejiang, China). Lipopolysaccharide (LPS, from *Escherichia coli* 055:B5) was purchased from Sigma (St. Louis, MO, USA). The enzyme-linked immunosorbent assay (ELISA) kits for mouse TNF-α, IL-6, and IL-1β were purchased from Shanghai Xitang Biotechnology Co., Ltd. (Shanghai, China). The GSH-Px, MDA, MPO, SOD and BCA detection kits were provided by the Jiancheng Bioengineering Institute of Nanjing (Nanjing, Jiangsu, China). The primary antibodies for p-p65NF-κB, p-IκBα, NLRP3, caspase-1, ASC, IL-1β and β-actin were from ABclonal (Boston, MA, USA). The other reagents were analytical grade or better.

### 2.3. Cell viability assay

A549 cells were incubated in RPMI-1640 medium with 10% foetal bovine serum (FBS) and cultured in an incubator (37 °C, 5% $CO_2$). To select the appropriate dose, cell viability was examined with different fraxin doses. The cells were seeded into 96-well plates at a density of $1 \times 10^4$ cells/well with 180 μL of medium at the exponential growth phase and incubated for 24 h. Then, the cells were treated with fraxin in a volume of 20 μL/well (0, $1 \times 10^{-3}$, $1 \times 10^{-2}$, $1 \times 10^{-1}$ and 1 mg/mL) and cultured for 24 h. The supernatant was removed, and 200 μL of MTT (0.5 mg/mL) was added to each well and incubated for 4–6 h. The formazan crystals were dissolved in 150 μL of DMSO/well. The optical density (OD) was measured at 490 nm on a microplate reader (BioTek ELx800, USA).

### 2.4. LPS-induced lung injury model

After 5 days of adaptation, twenty-five mice were divided into five groups: control group and LPS (1, 2, 4 and 6 h) groups. After LPS (30 mg/kg) challenge for different time, the lung tissues were collected to measure the W/D ratio. Then, the appropriate time of LPS-administration was selected.

The other mice were randomly separated into six groups (10 mice/group) as follows: control group; LPS (30 mg/kg) group; fraxin (10, 20, and 40 mg/kg) plus LPS groups and DEX (5 mg/kg) plus LPS group. Before LPS administration, the mice were gavaged with Fraxin or DEX (in the Fraxin + LPS and DEX + LPS groups) or normal saline alone (in the control and LPS groups) once daily for 7 days. Then, the mice (except for the control group) received an intraperitoneal injection of LPS (30 mg/kg). Blood serum samples were collected and stored at −20 °C for cytokine measurement. The lung tissues were divided into two parts: the first part was used to estimate the biochemical parameters and measure the lung W/D ratio, and the second part was kept in 4% formalin for H&E staining and immunohistochemistry. All tissues were kept at −80 °C.

### 2.5. Lung W/D ratio

The left upper lobe lung was taken and weighed immediately; this weight was recorded as the wet weight. Then, the lung tissues from

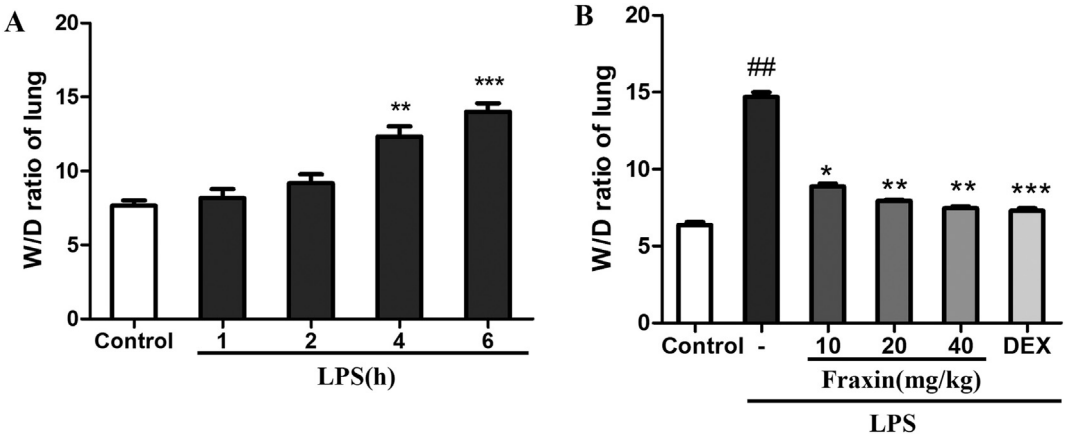


**Fig. 2.** Effect of fraxin on the lung wet-to-dry ratio in an LPS-induced ALI model. (A) Mice were stimulated with LPS (1, 2, 4 and 6 h) for different time. (B) Before administrating with LPS for 4 h, Fraxin (10, 20 or 40 mg/kg) and DEX (5 mg/kg) were administered to the mice. After LPS (30 mg/kg) challenge, the lungs were harvested from the mice. All data are presented as the mean ± SEM. ## $P < 0.01$ vs. the control group; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. the model group.

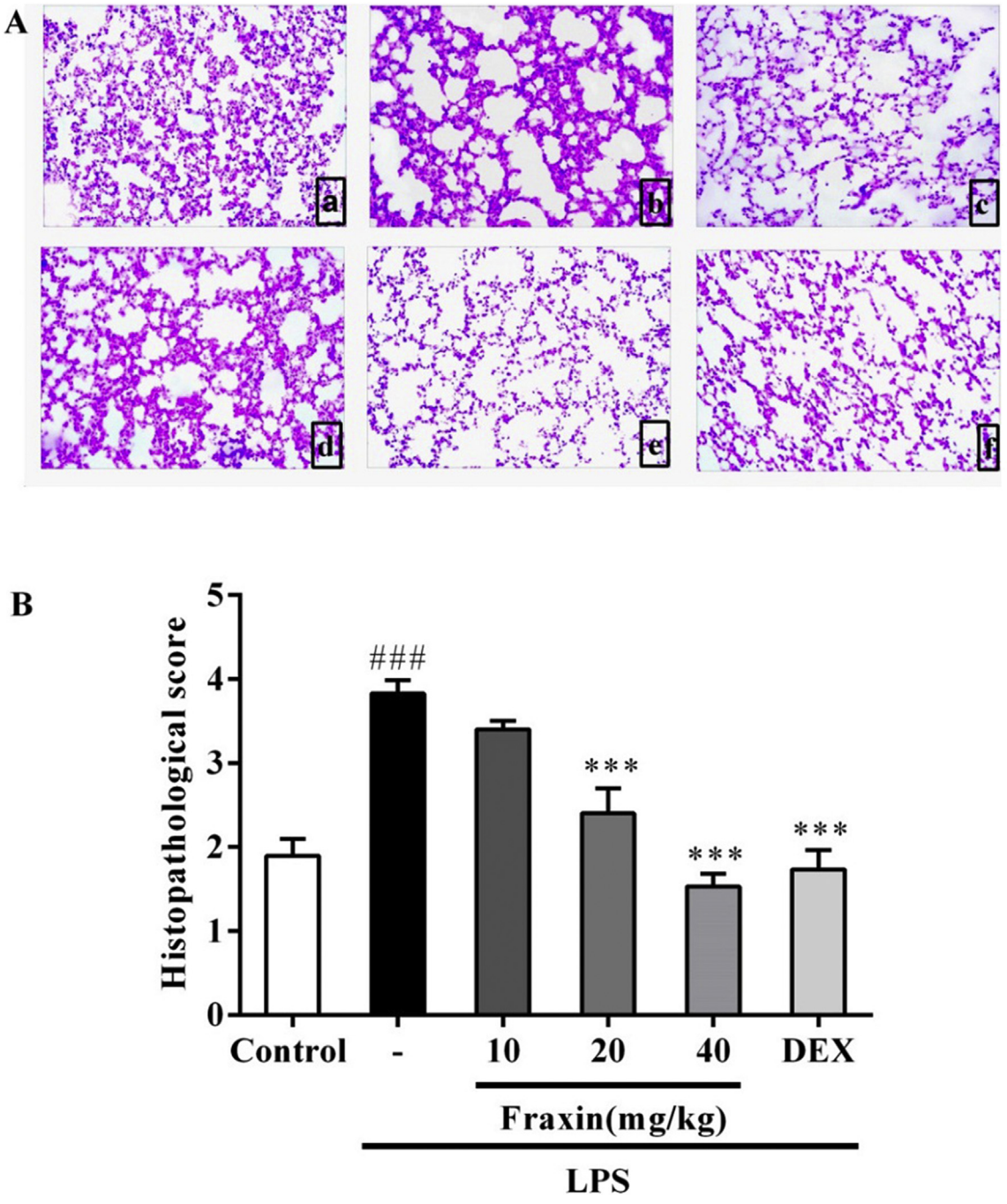


**Fig. 3.** Effect of fraxin on the LPS-induced histopathological changes in the lung. Fraxin (10, 20 or 40 mg/kg) and DEX (5 mg/kg) were administered to the mice 1 h prior to LPS administration. After LPS (30 mg/kg) challenge for 4 h, the lungs of the mice were harvested. (A) Histopathological evaluation of the (a) control, (b) LPS, (c) LPS + fraxin (10 mg/kg), (d) LPS + fraxin (20 mg/kg), (e) LPS + fraxin (40 mg/kg), and (f) LPS + DEX (5 mg/kg) groups. (B) The lung injury score was graded according to a five-point scale from 0 to 4 as follows: 0, l, 2, 3, and 4 represented no damage, mild damage, moderate damage, severe damage, and very severe damage, respectively. A representative histological section of the lungs was stained with haematoxylin and eosin (H&E staining, magnification ×100). All data are presented as the mean ± SEM. ### $P < 0.001$ vs. the control group; *** $P < 0.001$ vs. the model group.

each group were placed in an 80 °C oven for 48 h and dried to a constant weight, which was recorded as the dry weight. Finally, the mouse lung W/D ratio was calculated and used to evaluate the degree of pulmonary oedema.

### 2.6. BAL fluid collection and cell count

After LPS treatment, all mice were sacrificed, and the chest was opened. The BAL fluid was collected by three intratracheal injections of 1.5 mL of PBS. The collected fluid was centrifuged for 10 min at 1500 r/min at 4 °C. The cytokine levels in the supernatants were assessed using ELISA kits. The cell precipitate was resuspended in 100 μL of PBS, centrifuged onto slides and stained with Giemsa. The cell quantities were counted by a cell counting plate to obtain the total numbers of inflammatory cells, neutrophils and macrophages.

### 2.7. Cytokine assay

The TNF-α, IL-6, and IL-1β levels in the sera, BAL fluid and culture supernatant were detected by ELISA. According to the manufacturer's

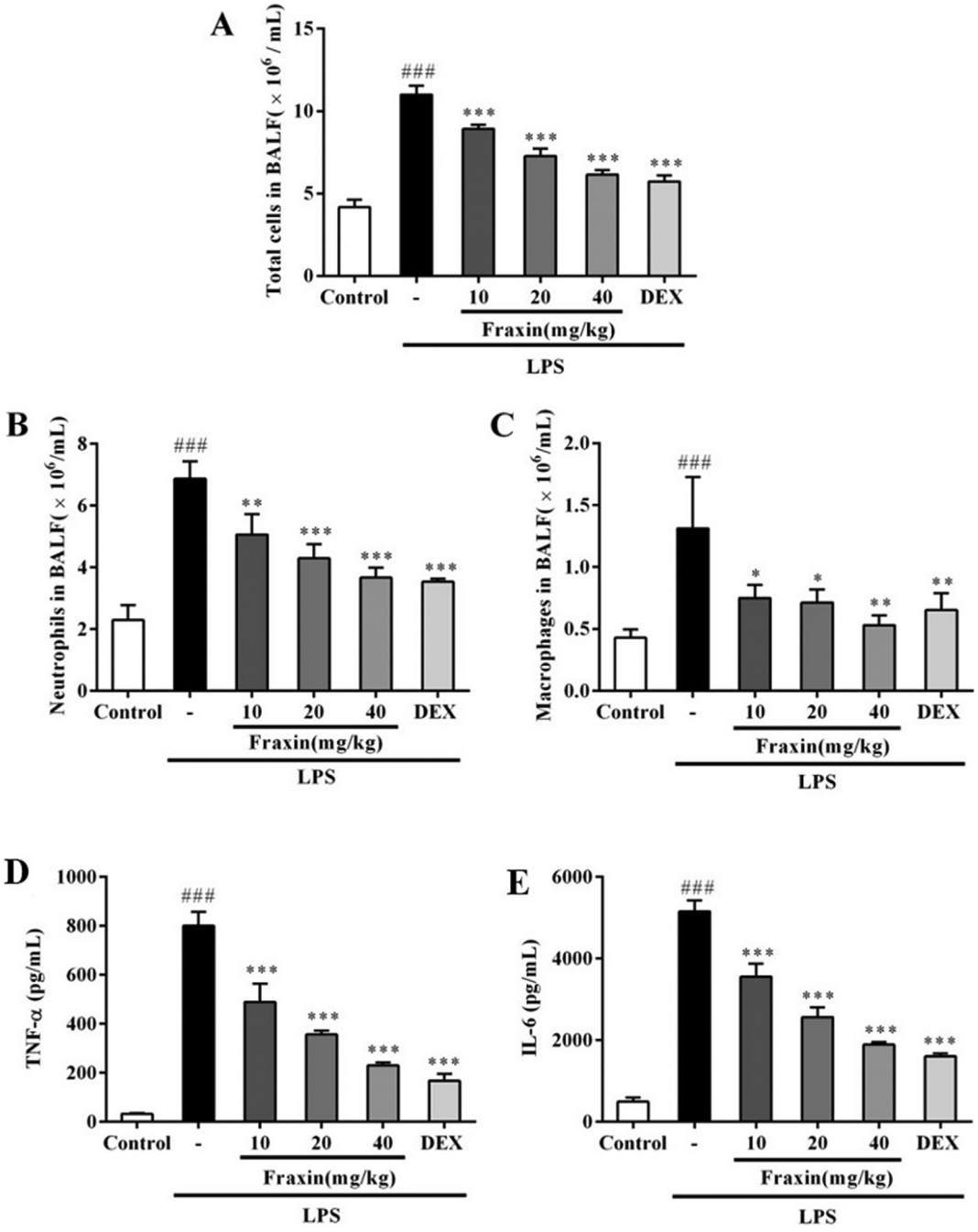


**Fig. 4.** Effect of fraxin on LPS-induced lung injury based on analysis of the BAL fluid. Fraxin (10, 20 or 40 mg/kg) and DEX (5 mg/kg) were administered to the mice 1 h prior to LPS administration. After LPS (30 mg/kg) challenge for 4 h, the BAL fluid was harvested from the mice. (A) Total cells. (B) Neutrophils. (C) Macrophages. (D) The TNF-α content. (E) The IL-6 content. All data are presented as the mean ± SEM. ### $P < 0.001$ vs. the control group; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. the model group.

protocols, the work reagents were added successively, and the optical density (OD) was measured at 450 nm in a microplate reader (Bio-Tek ELx800, USA).

### 2.8. Oxidative stress in the lung

MDA, MPO, SOD and GSH were used to evaluate the organizational damage to the lung. According to the instructions of the experimental manual, 100 mg of lung tissue was treated with different concentrations of tissue homogenate. Then, different working fluids were added and incubated for appropriate times at a suitable temperature prior to measurement of the OD value at the corresponding wavelength.

### 2.9. Histopathological examination

The collected lung tissues were fixed with a 4% buffered paraformaldehyde phosphate solution for 48 h. Then, the samples were embedded in paraffin and sliced into 5 μm thick sections and stained with haematoxylin and eosin (H&E). The pathological changes were observed under a light microscope (Olympus Optical, Tokyo, Japan).

### 2.10. Immunohistochemistry

The lung tissue sections were incubated with NLRP3, Nfr2 and p65 antibodies overnight at 4 °C and reacted with alkaline phosphatase

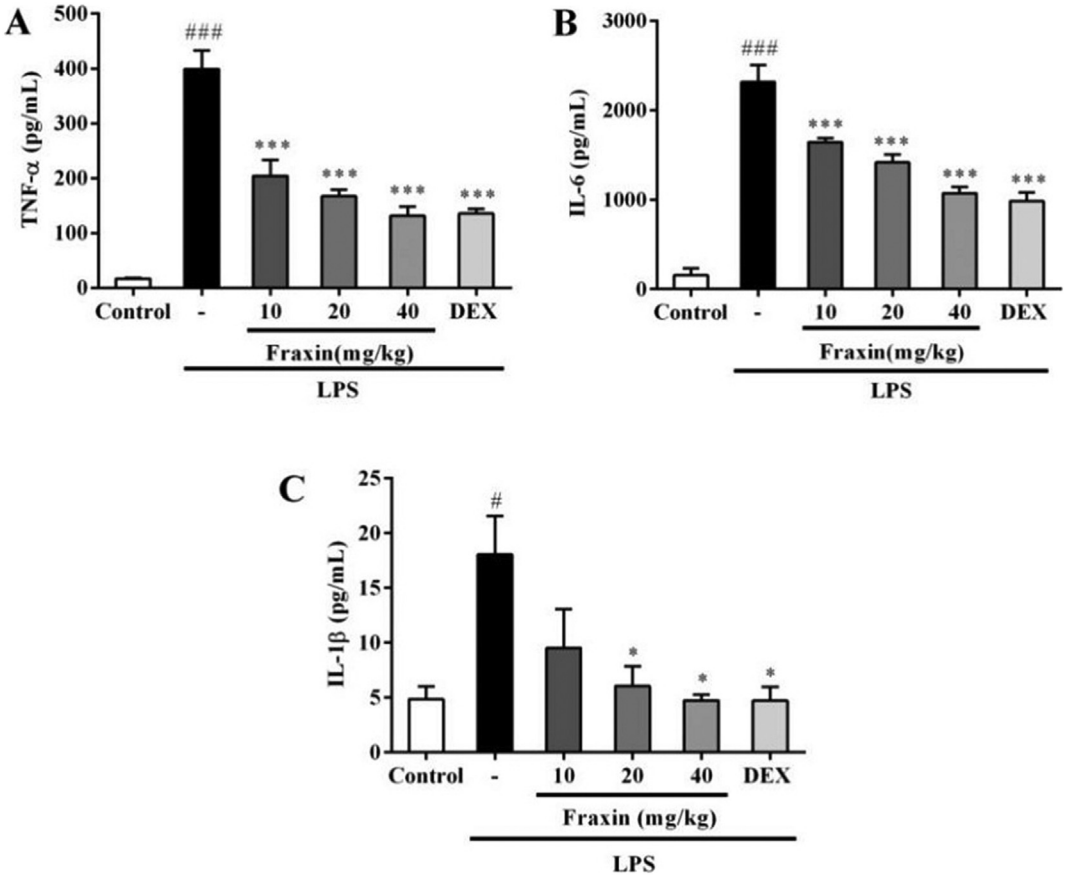


**Fig. 5.** Effect of fraxin on the serum TNF-α, IL-6 and IL-1β contents. The mice were administered fraxin (10, 20 or 40 mg/kg) and DEX (5 mg/kg) once daily for 1 week prior to LPS exposure. After LPS (30 mg/kg) challenge for 4 h, the serum was gathered. The TNF-α (A), IL-6 (B), and IL-1β (C) levels were measured. All data are presented as the mean ± SEM. ## $P < 0.01$ and ### $P < 0.001$ vs. the control group; * $P < 0.05$ and *** $P < 0.001$ vs. the model group.

(AP)-conjugated secondary antibodies for 1 h at room temperature under alkaline conditions. After staining with 3,3′-diaminobenzidine (DAB, Sigma, St Louis, MO, USA) and counterstaining with haematoxylin, the slices were visualized under an Olympus DP-71 microscope equipped with a digital camera. The buffy or brown diaminobenzidine precipitates were considered positive. Finally, the results were evaluated semi-quantitatively according to the percentages of positive cells in ten random images viewed at x100 magnification.

### 2.11. Western blotting

The lung tissues or cell samples were lysed in lysis buffer, and the total proteins were quantified using a BCA kit. Equal amounts of protein were transferred onto a PVDF membrane after separation on a 10% SDS-PAGE gel. The membranes were probed with primary antibodies (4 °C, overnight) and secondary antibodies (room temperature, 1 h) after blocking with 5% skim milk in TBST buffer (20 mM Tris, 500 mM NaCl, pH 7.5, and 0.1% Tween 20). The total density of the protein bands was detected with an enhanced chemiluminescence system.

### 2.12. Statistical analyses

All data are presented as the mean ± S.E.M. The statistical analyses were performed with GraphPad Prism 5.0. All statistical tests were analysed by one-way ANOVA. Significant differences were considered if $P < 0.05$.

## 3. Results

### 3.1. Effect of fraxin on the LPS-induced lung W/D ratio

In this study, we used the lung W/D ratio to determine the effect of LPS and fraxin on pulmonary oedema. As shown in Fig. 2A, after stimulating mice with LPS for 4 h and 6 h, the W/D ratio was obviously higher than that in the control group ($P < 0.01$). Then, LPS stimulation for 4 h was chosen to following experiment. In Fig. 2B, LPS significantly increased the W/D ratio compared with that of the control group ($P < 0.01$). The ratio in the Fraxin group decreased by > 40% compared to that in the LPS group ($P < 0.05$). The lung W/D ratio in the DEX control group was also lower than that in the LPS group ($P < 0.001$). These data indicated that fraxin markedly reduced LPS-induced pulmonary oedema (Fig. 2).

### 3.2. Effect of fraxin on LPS-induced histopathological changes in the lung

As shown in Fig. 3, we performed a histopathological evaluation (A) of the lung tissues and determined the histological scores (B). The lung tissues from the control group were normal, with intact alveoli. At the same time, occasional inflammatory cell infiltration was observed around the bronchus. The lung tissue sections from the LPS group showed obvious pathological changes, including serious pulmonary oedema, haemorrhage, alveolar disarray and inflammatory cell infiltration in the alveolar cavity. After administration of fraxin and DEX, the pathological damage in the lung tissues was alleviated. These changes were assessed using the lung injury score. The results demonstrated that fraxin effectively alleviated LPS-induced pathological damage in the lung tissue (Fig. 3).

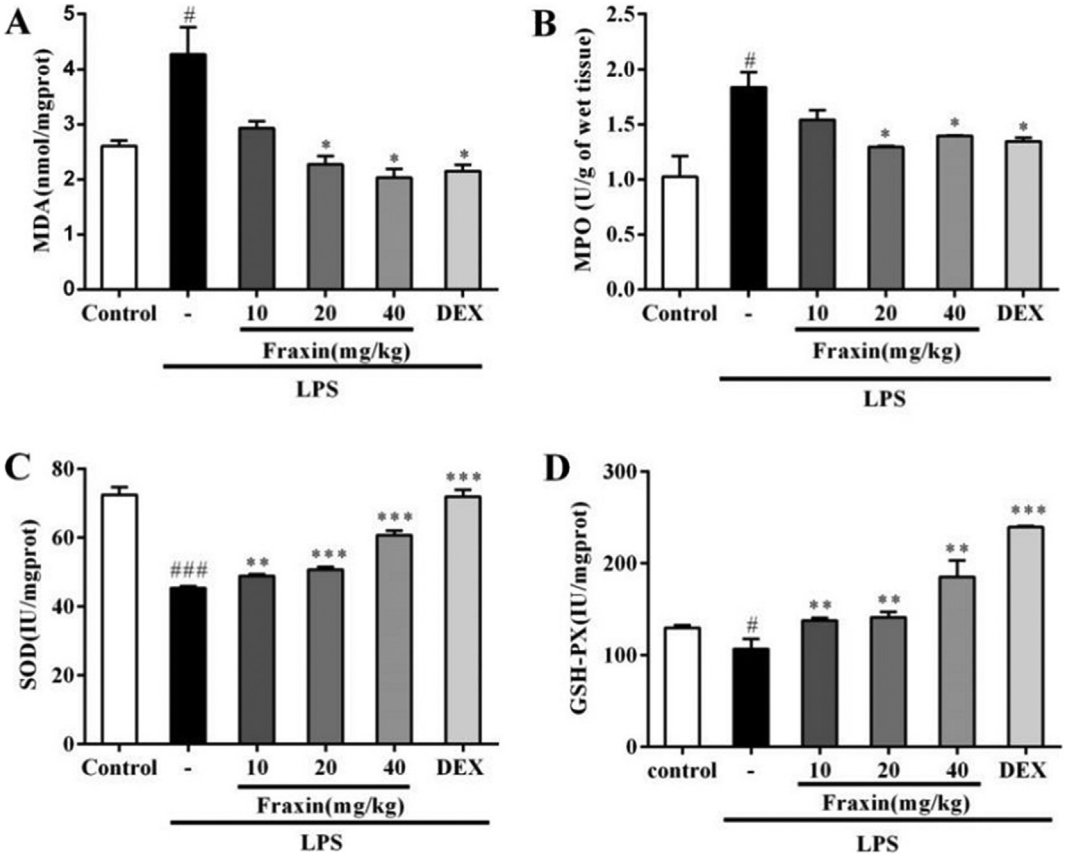


**Fig. 6.** Effect of fraxin on LPS-induced activity of MDA, MPO, SOD and GSH-Px in the lung tissues. The mice were administered fraxin (10, 20 or 40 mg/kg) and DEX (5 mg/kg) once daily for 1 week prior to LPS exposure. After LPS (30 mg/kg) challenge for 4 h, the lungs were harvested. The lung homogenates were prepared for MDA (A), MPO (B), SOD (C) and GSH-Px (D) measurement. All data are presented as the mean ± SEM. # $P < 0.05$ and ### $P < 0.001$ vs. the control group; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. the model group.

### 3.3. Effect of fraxin on LPS-induced lung injury in the BAL fluid

The numbers of inflammatory cells, neutrophils, and macrophages in the BAL fluid increased after 4 h of LPS stimulation ($P < 0.001$). Compared with that of the LPS group, the fraxin group revealed significant inhibition of the total numbers of inflammatory cells, neutrophils and macrophages in the BAL fluid ($P < 0.05$). The TNF-α and IL-6 levels in the BAL fluid were obviously increased in the LPS group ($P < 0.001$). In contrast, fraxin and DEX remarkably reduced the TNF-α and IL-6 levels ($P < 0.001$). The results showed that fraxin effectively suppressed the total numbers of macrophages, neutrophils and inflammatory cells and reduced the TNF-α and IL-6 levels in the BAL fluid (Fig. 4).

### 3.4. Effect of fraxin on LPS-induced cytokine production in the sera

The effect of fraxin on the TNF-α, IL-6 and IL-1β levels in mouse sera was determined. After treating the mice with LPS, the levels of these cytokines in the LPS group were different from those in the control group. Fraxin alleviated the TNF-α, IL-6 and IL-1β levels by > 50%, 30% and 41%, respectively, compared with those of the LPS group. These results confirmed that fraxin obviously reduced LPS-induced cytokine production in the sera (Fig. 5).

### 3.5. Effect of fraxin on the LPS-induced MDA, MPO, SOD and GSH-Px levels in the lung tissues

The MDA and MPO concentrations in the lung tissues of each group are shown in Fig. 6A and B. The MDA and MPO levels in the LPS group were significantly higher than those in the control group ($P < 0.05$). Fraxin and DEX both reduced MDA and MPO ($P < 0.05$). The SOD and GSH-Px levels in the lung tissues from the LPS group were clearly decreased (Fig. 6C and D) and were significantly different from those of the control group ($P < 0.01$). Fraxin and DEX administration effectively increased the SOD and GSH-Px concentrations ($P < 0.05$) in a dose-dependent manner. The results showed that fraxin increased the activity of antioxidant enzymes, suppressed lipid peroxidation, and alleviated LPS-induced lung injury in the mice (Fig. 6).

### 3.6. Effect of fraxin on LPS-induced NLRP3, Nrf2 and p65 expression in the lung tissues

NLRP3, Nrf2 and p65 expression in the lung tissue was analysed by immunohistochemistry. As shown in Fig. 7A and C, NLRP3 and p65 were expressed at low levels in the control group, whereas their expression levels in the LPS group were markedly increased ($P < 0.001$). Compared with the LPS group, the Fraxin and DEX group showed reduced NLRP3 and p65 expression ($P < 0.001$). Fig. 7B shows that Nrf2 protein expression was obviously lower in the LPS group than in the control group ($P < 0.01$). With fraxin administration (20 and 40 mg/kg), Nrf2 protein expression increased by > 180%. Thus, fraxin significantly reduced LPS-induced NLRP3 and p65 protein expression and elevated Nrf2 protein expression in the lung tissue (Fig. 7).

### 3.7. Effect of fraxin on LPS-induced NF-κB and NLRP3 inflammasome expression in the lung tissue

The p-p65 and p-IκBα expression levels were significantly increased

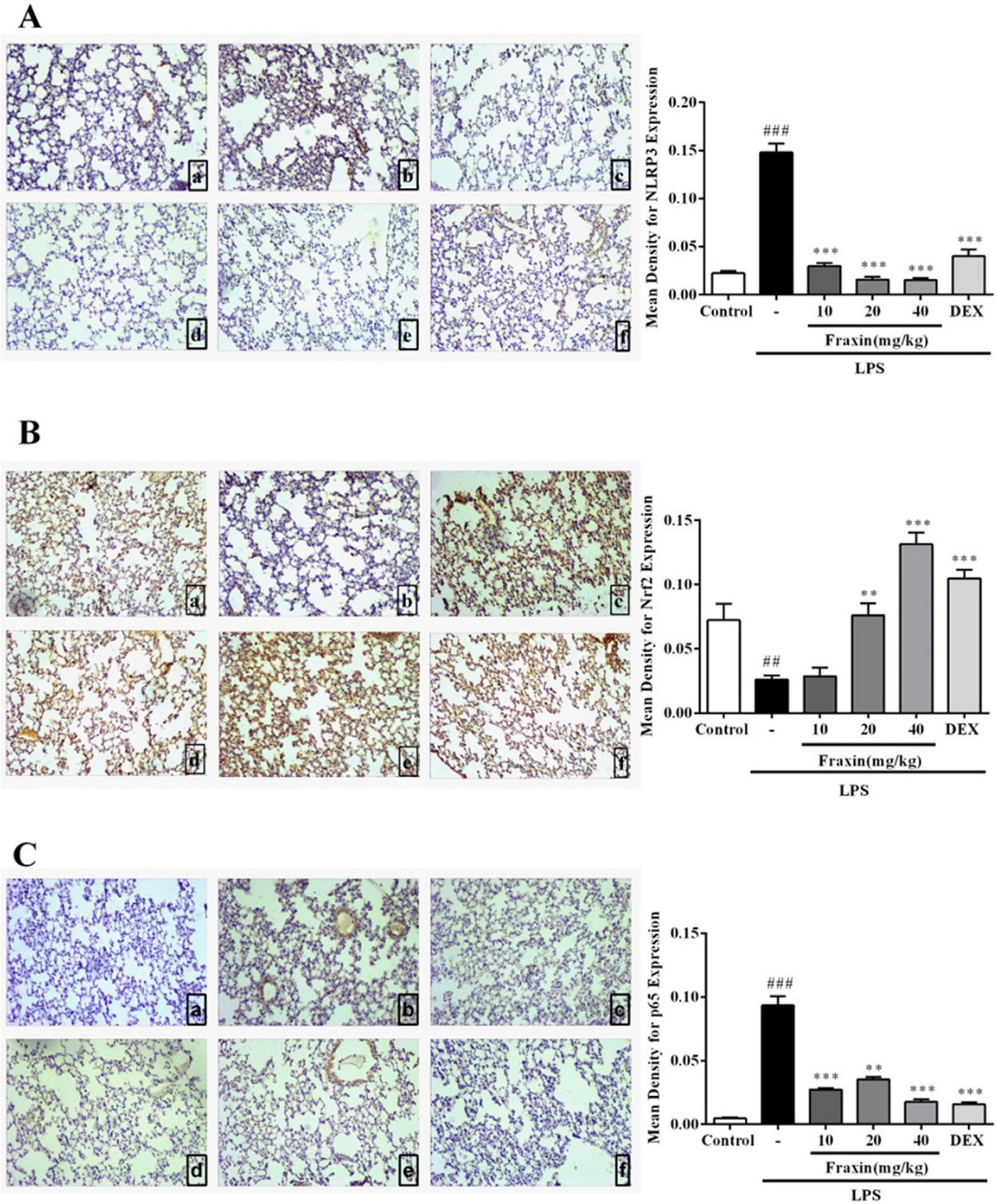


**Fig. 7.** Effect of fraxin on NLRP3, Nrf2 and p65 protein expression in the lung tissue. Fraxin (10, 20 or 40 mg/kg) and DEX (5 mg/kg) were administered intragastrically to the mice 1 h prior to LPS treatment. All animals were sacrificed 4 h after LPS (30 mg/kg) stimulation. The lungs were collected from the mice, and the NLRP3 (A), Nrf2 (B) and p65 (C) concentrations were measured. All data are presented as the mean ± SEM. ### $P < 0.001$ vs. the control group; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. the model group.

in the LPS group. In contrast, fraxin administration reduced the p-p65 ($P < 0.001$) and p-IκBα ($P < 0.05$) expression levels. The results indicated that fraxin effectively alleviated NF-κB protein expression in the lung tissue. After LPS stimulation, NLRP3, IL-1β, caspase-1 and ASC protein expression was obviously elevated in the lung tissues compared with that in the control group ($P < 0.001$). Fraxin and DEX inhibited the protein expression levels in the lung tissues ($P < 0.05$). The results showed that fraxin effectively reduced the expression of the inflammatory corpuscle protein NLRP3 in the lung tissue (Fig. 8).

### 3.8. Effect of fraxin on A549 toxicity

No significant effect on cell viability was observed following pretreatment with fraxin when the fraxin concentration ranged from 10 to 320 μM compared with that of the control group. When the fraxin concentration was > 320 μM, cell survival was significantly inhibited

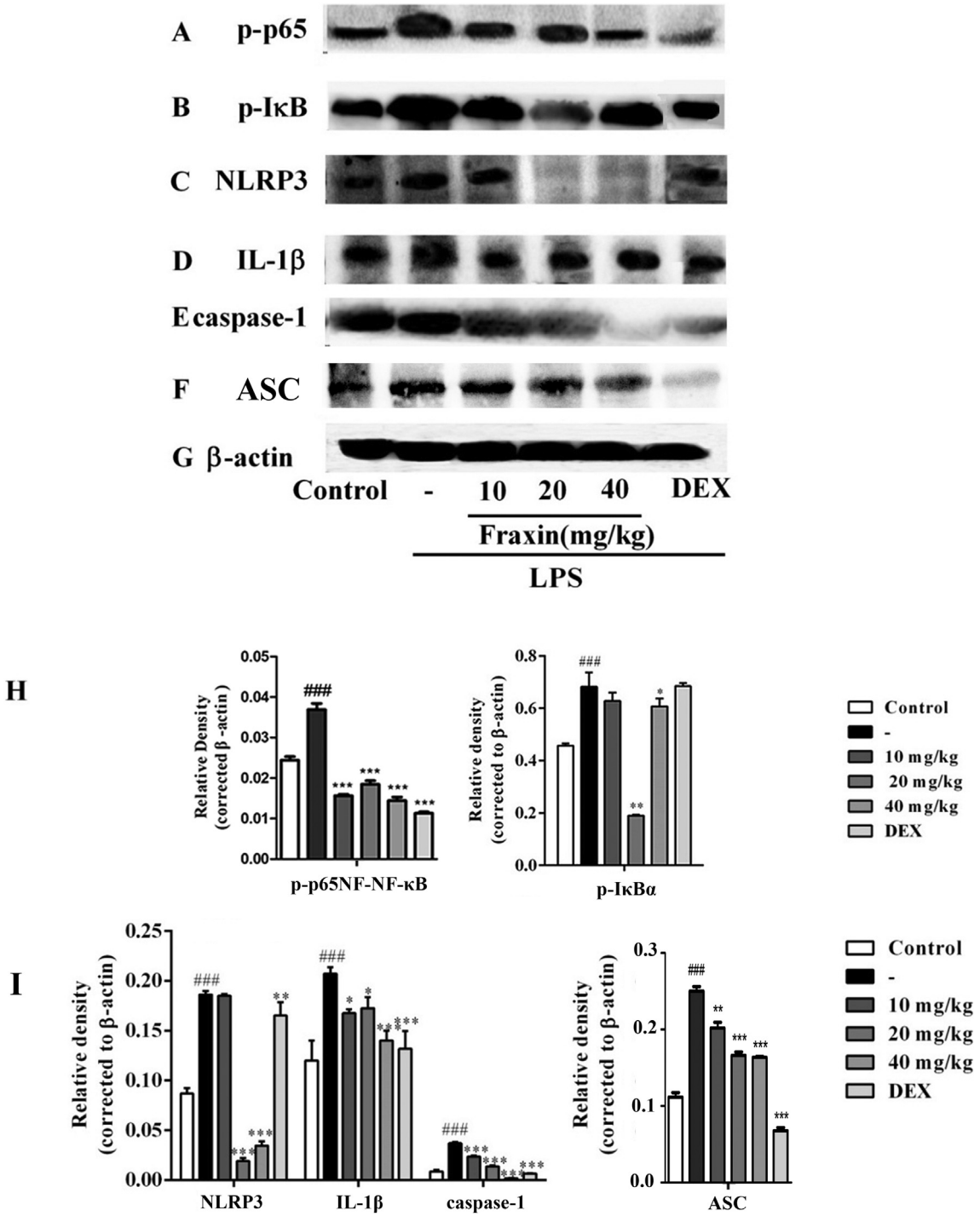


**Fig. 8.** Effect of fraxin on NF-κB and NLRP3 inflammasome protein expression in the lung tissues was assessed by Western blotting. Fraxin (10, 20 or 40 mg/kg) and DEX (5 mg/kg) were administered to the mice 1 h prior to LPS treatment. After LPS (30 mg/kg) challenge for 4 h, the lungs of the mice were harvested. Protein samples were extracted from the lungs and analysed by Western blotting. The relative expression of p-p65 (A), p-IκBα (B), NLRP3 (C), IL-1β (D), caspase-1 (E), ASC (F) and β-actin (G) was quantified by densitometric analysis. All data are presented as the mean ± SEM. ### $P < 0.001$ vs. the control group; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. the model group.

($P < 0.05$). The cell viability results provided safe concentrations for the subsequent experiments. Therefore, in the subsequent experiments, the selected fraxin concentrations used to study the protective effects of fraxin on LPS-stimulated A549 cells were 10, 20 and 40 μM (Fig. 9).

### 3.9. Effect of fraxin on LPS-induced cytokine production in A549 cells

The effect of fraxin on the inflammatory factor TNF-α, IL-6 and IL-1β levels in A549 cells is shown in Fig. 10. After stimulating the A549 cells with LPS, the TNF-α content in the LPS group was significantly increased compared with that of the control group ($P < 0.05$) but decreased after administration of 40 μM fraxin ($P < 0.05$). IL-6 and IL-1β in the LPS group were 2.7 times and 1.5 times greater than the levels in the control group, respectively. Fraxin clearly inhibited the LPS-induced release of the inflammatory factors TNF-α, IL-6 and IL-1β in A549 cells (Fig. 10).

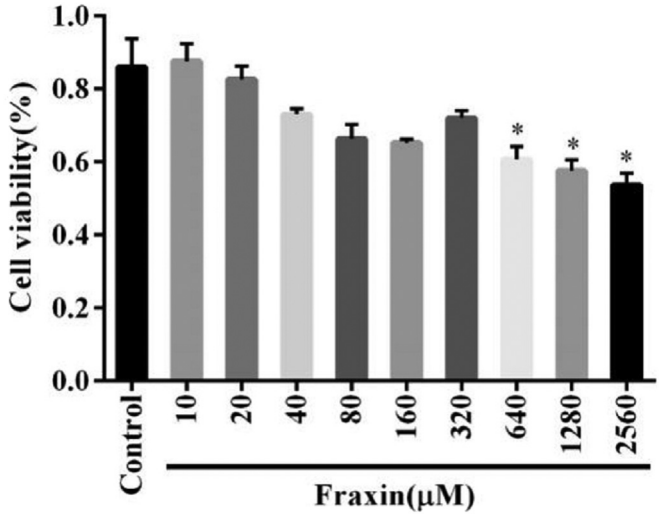


**Fig. 9.** Effect of fraxin on A549 cell viability. A549 cells were subjected to different fraxin concentrations for 24 h, and cell viability was measured by the MTT assay. All data are presented as the mean ± SEM. * $P < 0.05$ vs. the control group.

### *3.10. Effect of fraxin on LPS-induced NF-κB and NLRP3 inflammasome expression in A549 cells*

The results showed that p-p65 and p-IκBα expression in the LPS group increased by > 80% and 110% compared with that in the control group, respectively, and that the protein concentrations were reduced after injection of fraxin ($P < 0.05$). The results indicated that fraxin reduced NF-κB protein expression in the LPS-stimulated cells. Western blotting was also performed to detect the expression of key proteins related to the NLRP3 inflammatory corpuscle pathway. After LPS stimulation, NLRP3, IL-1β, caspase-1 and ASC were elevated in the LPS group ($P < 0.05$) and were significantly inhibited by fraxin treatment ($P < 0.05$). The results demonstrated that fraxin effectively reduced the expression of the inflammatory corpuscle protein NLRP3 in the A549 cells (Fig. 11).

## 4. Discussion

ARDS is caused by a variety of factors, including sepsis, systemic inflammation and pneumonia [3], and is a common emergency and severe disease. LPS is widely applied to establish an inflammatory response model [21]. In this study, an LPS-induced ALI mouse model and an A549 cell-injury model were used to examine the protective effects and mechanism of fraxin.

After LPS stimulation, the alveolar capillary barrier was destroyed, permeability increased, and inflammatory leachate infiltrated the lungs, which resulted in pulmonary oedema [22]. The results showed that lung swelling in the LPS group was significantly more serious than that in the control group. Preadministration of fraxin inhibited penetration of the sera into the lung tissue and alleviated the LPS-induced pulmonary oedema. The results were consistent with changes in the number of inflammatory cells in the BAL fluid of each group. Increased inflammatory cells in the BAL fluid are regarded as one feature of ALI [23]. These findings indicated that fraxin effectively mitigated the pulmonary oedema induced by LPS and reduced the number of inflammatory cells in the BAL fluid.

The current study shows that oxidative stress is involved in the pathogenesis of acute lung injury [25]. In this study, we measured four

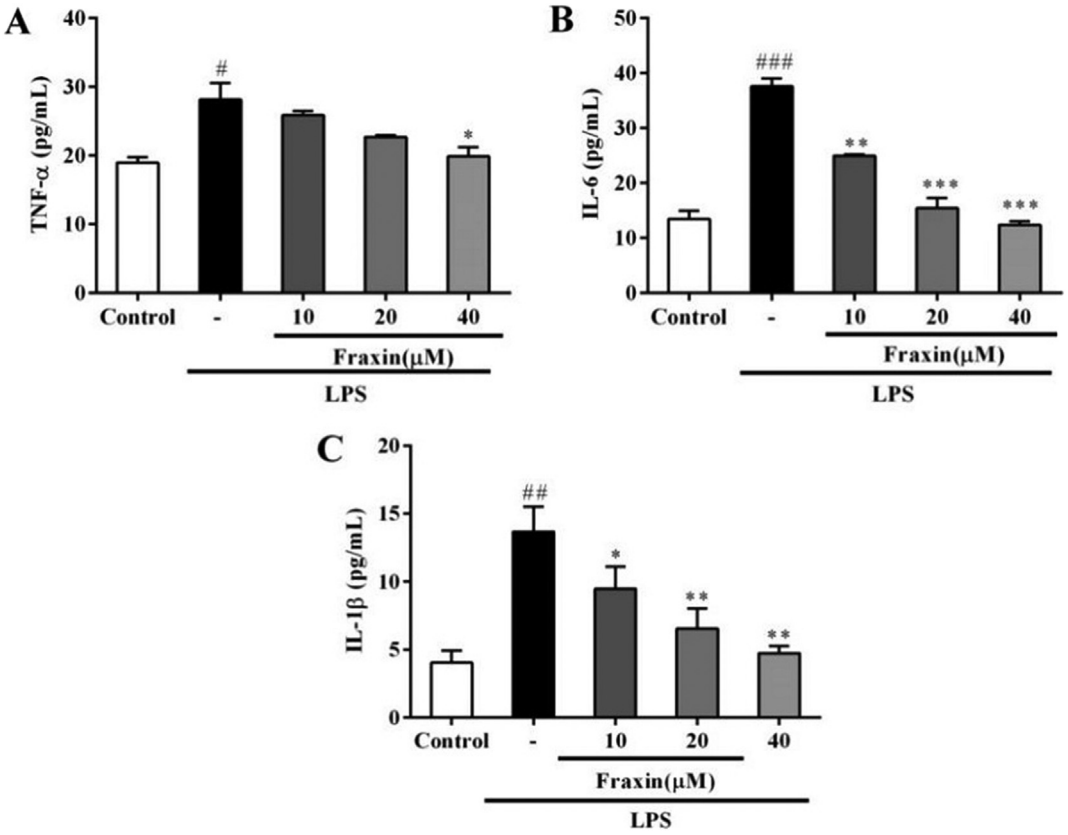


**Fig. 10.** Effect of fraxin on the TNF-α, IL-6 and IL-1β contents in the A549 cells. The cells were subjected to fraxin (10, 20 or 40 μM) for 24 h and then exposed to LPS (10 μg/mL) for 12 h. The TNF-α (A), IL-6 (B) and IL-1β (C) levels in the culture supernatants were measured by ELISA. All data are presented as the mean ± SEM. $^{\#}P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. the control group; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. the model group.

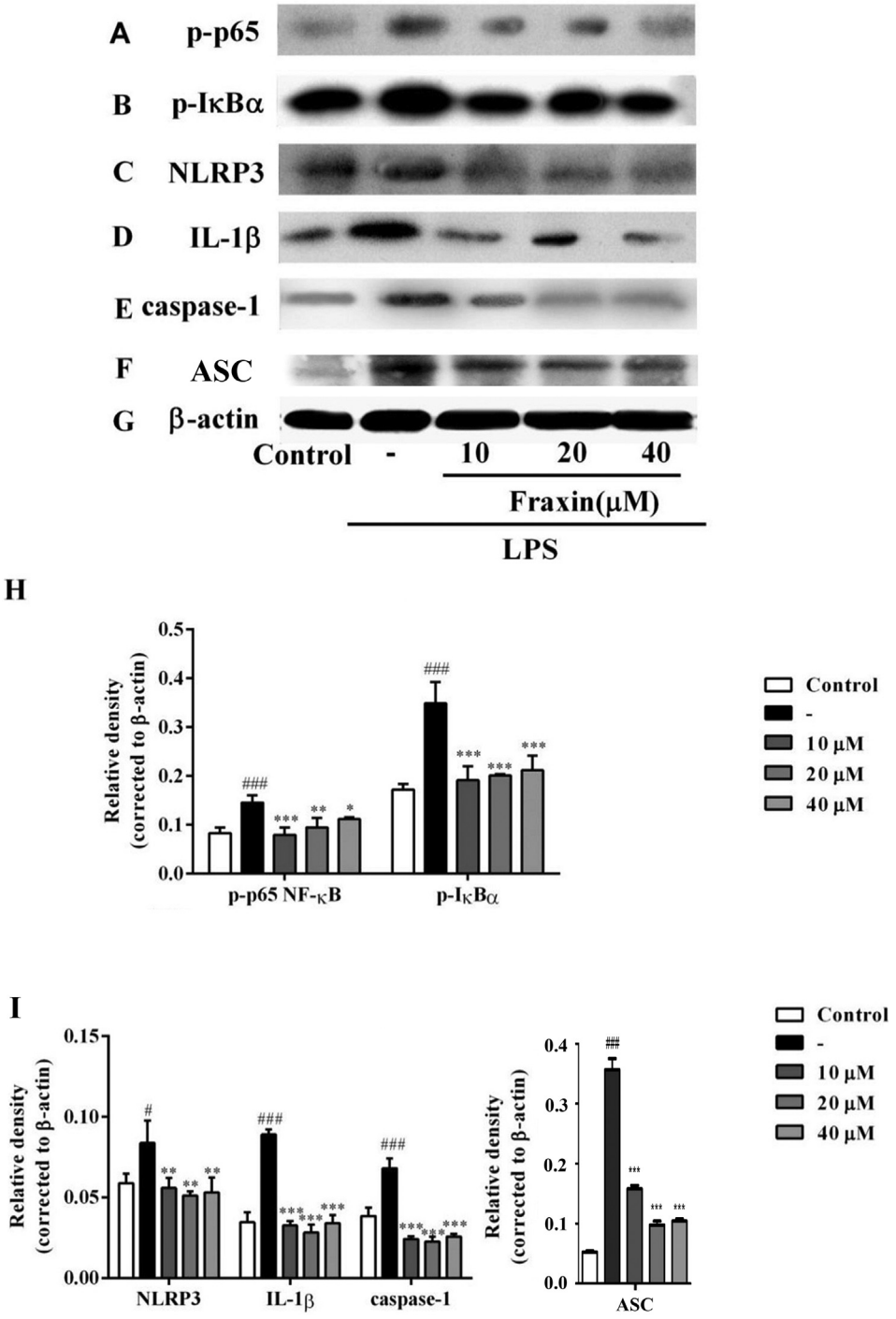


**Fig. 11.** Effect of fraxin on NF-κB and NLRP3 expression in LPS-induced A549 cells. The cells were subjected to fraxin (10, 20 or 40 μM) for 24 h and then exposed to LPS (10 μg/mL) for 12 h. Protein expression was analysed by Western blotting. The relative expression of p-p65 (A), p-IκBα (B), NLRP3 (C), IL-1β (D), caspase-1 (E), ASC (F) and β-actin (G) was quantified by densitometric analysis. All data are presented as the mean ± SEM. # $P < 0.05$, ### $P < 0.001$ vs. the control group; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. the model group.

important indexes of oxidative stress. MPO activity is a sign of neutrophil infiltration in the lung tissue [24,25]. With development of ALI, numerous neutrophils infiltrate the alveolar spaces, and MPO activity increases [26]. Studies have shown that fraxin is effective in reducing LPS-induced neutrophil accumulation. SOD is an antioxidant enzyme in the body. When the lung tissue is damaged, scavenging oxygen free radicals are consumed in large amounts, and SOD activity decreases [27]. MDA is a lipid peroxidation product that increases in proportion to the oxygen free radical content when lung tissue is damaged [28]. GSH-Px is a peroxide-degrading enzyme that protects the cell membrane integrity and function [29,30]. The results demonstrated that LPS increased the MDA content in the lung tissue and inhibited the activity of SOD and GSH-Px. The MDA level in the fraxin group decreased, whereas the activity of SOD and GSH-Px increased, indicating that fraxin alleviated the oxidative stress induced by LPS and protected the lung tissue.

In addition to oxidative stress, inflammation also plays a role in the pathogenesis of ALI. The inflammatory factors TNF-α, IL-6 and IL-1β regulate signals during the inflammatory process [31]. TNF-α has a strong inflammatory damage ability and is one of the strongest

inflammatory mediators in the body [32]. IL-6 is an important indicator that characterizes the severity of inflammatory diseases [33]. IL-1β is a central mediator of inflammation that damages vascular endothelial and lung epithelial cells [34]. These cytokines exacerbate tissue damage. The results indicated that LPS elevated the release of TNF-α, IL-6 and IL-1β, whereas fraxin inhibited the upregulation of these inflammatory mediators induced by LPS. These changes were supported by the results of the histopathological examination, which showed that pretreatment with fraxin dramatically protected the lung from LPS-induced injury.

NF-κB is an important nuclear transcription factor in cells and an upstream regulatory protein of various inflammatory factors [35], such as adhesion and growth factors. Therefore, NF-κB plays an effective role in immune regulation, inflammation, infection and cell apoptosis [36]. NLRP3 inflammatory bodies activate caspase-1 to cause maturation and secretion of downstream cytokines, such as IL-1β and IL-18, which mediate and expand the inflammatory cascade [17,18]. Research has shown that activation of NLRP3 inflammatory bodies is regulated by NF-κB. NF-κB and NLRP3 inflammatory bodies jointly regulate the occurrence and development of inflammatory responses. The NLRP3 inflammatory corpuscle increases the permeability of alveolar epithelial cells and induces pro-inflammatory cytokine production [37,38]. In different ALI mouse models, different stimulatory factors activate NLRP3 inflammatory bodies, which release large amounts of mature IL-1β and IL-18 [17]. Then, the inflammatory response cascade amplifies, leading to increased permeability of alveolar epithelial cells and alveolar barrier dysfunction, which indicates that the NLRP3 inflammatory body plays an important role in the pathological process of ALI and has an extremely important relationship with ALI. Western blotting and immunohistochemistry showed that fraxin obviously suppressed NF-κB protein expression and NLRP3 inflammatory bodies in the lung tissue. Fraxin has protective effects in ALI mice, which may be related to inhibition of NF-κB and NLRP3 inflammatory signalling pathway activation.

We also studied the anti-inflammatory activity of fraxin in A549 cells. A549 cells are widely used in protection mechanistic studies of alveolar epithelial cells. LPS stimulated A549 cells to release inflammatory mediators, which led to structural damage, apoptosis and necrosis [39,40]. The findings demonstrated that fraxin markedly alleviated the IL-6, TNF-α and IL-1β levels in the A549 cell supernatant and reduced NF-κB and NLRP3 inflammatory pathway activation.

In conclusion, fraxin confers protection against LPS-induced lung injury and the inflammatory response in A549 cells, and its effect may be achieved by inhibiting the activity of the NF-κB and NLRP3 inflammatory body pathways, downregulating the expression of inflammatory mediators and reducing oxidative stress.

## Conflict of interests

The authors declare that there are no conflicts of interest.

## Acknowledgements

This research was financially supported, in part, by the Shanxi National Science Foundation of International Cooperation projects (No. 2013KW26-02) to W. F. Li.

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