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Helix B surface peptide protects against acute lung injury through reducing oxidative stress and endoplasmic reticulum stress *via* activation of Nrf2/HO-1 signaling pathway

X.-G. BI, M.-L. LI, W. XU, J.-Y. YOU, D. XIE, X.-F. YUAN, Y. XIANG

Department of General ICU, The Third Affiliated Hospital of Sun Yat-sen University-Lingnan Hospital, Guangzhou, China

Abstract. – OBJECTIVE: Acute lung injury (ALI) is a clinical problem with poor prognosis and high mortality. The purpose of this study was to explore the effects of helix B position peptide (HBSP) on ALI and its mechanism.

MATERIALS AND METHODS: C57/BL6 male mice were used to construct ALI models by LPS tracheal injection and detect the effect of HBSP on mouse ALI by subcutaneously injecting HBSP. In addition, normal human lung epithelial cell line (BEAS-2B) were cultured and stimulated with HBSP. Then, the effects of HBSP on oxidative stress and endoplasmic reticulum stress (ERS) in BEAS-2B cells were examined. Finally, the effect of HBSP on the Nrf2/HO-1 signaling pathway was examined, and the mechanism of action of HBSP was verified using the Nrf2/HO-1 signaling pathway inhibitor ML385.

RESULTS: *In vitro*, HBSP increased the expression of SOD1/2 and decreased the expression of ERS-related molecules such as CHOP, GRP-78, and caspase-12, indicating that HBSP effectively reduces the level of oxidative stress and ERS in BEAS-2B cells. In addition, HBSP also increased the activity of the Nrf2/HO-1 signaling pathway and ML385 reduced the protective effect of HBSP on BEAS-2B cells. *In vivo*, HBSP significantly reduced LPS-induced mouse ALI. W/D and inflammatory factors in the BALF of the mouse lung were significantly reduced and the level of oxidative stress was also reduced.

CONCLUSIONS: HBSP plays an important role in relieving ALI by activating Nrf2/HO-1 signaling pathway, which reduces the level of inflammation in lung tissue and oxidative stress and ERS in lung epithelial cells.

Key Words:

Helix B surface peptide, Acute lung injury, Oxidative stress, Endoplasmic reticulum stress, Nrf2/HO-1 signaling pathway.

List of Abbreviations

Acute lung injury (ALI); helix B position peptide (HBSP); endoplasmic reticulum stress (ERS); reactive oxygen species (ROS); superoxide dismutase (SOD); erythropoietin (EPO); EPO receptors (EPOR); β-common receptors (βcR); lipopolysaccharide (LPS); Wet/dry Weight Ratio (W/D); Malondialdehyde (MDA); glutathione Peroxidase (GSH-Px); phosphate-buffered saline (PBS); Bronchoalveolar Lavage Fluid (BALF); enzyme-linked immunosorbent assay (ELISA); Hematoxylin-Eosin (HE); American Type Culture Collection (ATCC); Dulbecco's Modified Eagle's Medium (DMEM); fetal bovine serum (FBS); bicinchoninic acid (BCA); sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); polyvinylidene difluoride (PVDF); phosphate-buffered saline and tween-20 (PBST); quantitative real time-polymerase chain reaction (qRT-PCR); complementary deoxyribose nucleic acid (cDNA); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Cell Counting Kit-8 (CCK-8); immunocytofluorescence (IF); 4',6-diamidino-2-phenylindole (DAPI); Statistical Product and Service Solutions (SPSS); analysis of variance (ANOVA).

Introduction

Acute lung injury (ALI) is one of the most common refractory complications in the clinic, with a high mortality rate. It is a diffuse injury of lung parenchyma caused by various infections such as severe infection, various shocks, trauma, and surgery[1]. There are many pathogenic factors in ALI and the common pathophysiological changes are acute diffuse lesions of alveolar capillary endothelial cells, alveolar epithelial cells, and interstitial lung. The pathogenesis of ALI is also complex and has not yet been fully understood[2]. Despite this, there is increasing evidence that large amounts of reactive oxygen species (ROS) and oxidative stress are involved in ALI

Corresponding Author: Xiaogang Bi, BM; e-mail: bxg655@sina.com

and acute respiratory distress syndrome characterized by pulmonary vascular endothelial cell injury[3]. First, H_2O_2 can be detected in exhaled gases from patients with respiratory distress syndrome, and superoxide dismutase (SOD) and oxidized trypsin can also be detected in alveolar lavage fluid. Moreover, lipid peroxidase in the plasma of severely ill patients and sepsis patients prone to acute respiratory distress syndrome is also significantly elevated. Oxidative stress is an important factor in the development and progression of ALI. Excessive production of ROS can cause oxidative stress in cells, which can lead to cell damage and even apoptosis. The inflammatory response of the respiratory tract can lead to an increase in the production of ROS, disrupting the balance of redox in the lungs. Bender et al[4] have shown that removal of excess ROS can reduce lung damage caused by endotoxin.

Erythropoietin (EPO) is a hormone-like substance secreted by the interstitial cells of the renal cortex and liver, which promotes erythropoiesis and is mainly used for the treatment of renal anemia. In recent years, Edwards[5] found that EPO and its receptors with biological functions and pleiotropic effects are widely distributed in non-hematopoietic tissues and cells and exert various non-hematopoietic functions such as inhibiting apoptosis, antagonizing oxidative stress, and inhibiting excessive inflammatory reaction. EPO receptors (EPOR) include two classical EPORs and two β-common receptors (βcR)[6]. EPO combined with the classic EPOR can stimulate the proliferation and activation of red blood cells, reduce the apoptosis of red blood cells, and improve various types of anemia. EPO binding to heterodimerization of EPOR and βcR subunits has a variety of cytoprotective effects. However, the binding ability of EPO to traditional EPO homodimers is stronger than that of EPOR-βcR to protective subunits, so it is necessary to give very high doses of EPO to initiate tissue protection, while high-dose EPO inevitably causes adverse reactions such as hypertension and thrombosis, making it impossible to use in clinical practice[6].

The conformation of EPO consists of 4 hydrophobic Helixes (Helix A, B, C, and D)[7]. Brines et al[7] found that when EPO combined with EPOR, Helix B's hydrophilic surface does not contact it. Imamura et al[8] showed that after modification by carbamylation, EPO can still exert tissue protection but not promote erythropoiesis, indicating that the structure that cannot be modified by carbamylation in EPO contains components that play a tissue protection role. Considering that only Helix B in the molecular conformation of EPO does not contain lysine and cannot be modified by carbamylation, it is believed that Helix B may have a component that can protect tissues. Brines et al[7] discovered that when EPO binds to EPOR, the amino acid sequence of the hydrophilic group of the Helix B segment is QEQLERAL, and its activity is controlled by three amino acid residues on the proximal BC loop, so he synthesized Helix B surface peptide (HBSP) containing 11 amino acid residues: QEQLERALNSS. HBSP cannot bind to EPOR, so it has no effect of erythropoiesis, but it can play a universal tissue protection role through EPOR-βcR heterodimer receptor. HBSP has also been shown to exert tissue protective activity through anti-apoptosis, anti-oxidative stress, inhibition of excessive inflammatory response, and angiogenesis[8]. However, it is unclear whether HBSP can play a protective role in ALI.

Therefore, C57/BL6 male mice were used to make ALI models and BEAS-2B cells were cultured to study the effects of HBSP on the lungs. This study is expected to provide a new direction for the clinical treatment of ALI.

Materials and Methods

Animals and Grouping

Male C57/BL6 mice were purchased from Beijing Charles River Experimental Animal Technology Co., Ltd. (Beijing, China) and raised in a barrier facility. Five mice were housed in a squirrel cage. Mouse chow and clean drinking water for SPF grade experiments were used to feed mice. The mice in this study were divided into three groups, namely, control group, ALI group, and ALI+HBSP group. Mice in the ALI and ALI+HBSP groups were modeled in ALI, and those in ALI+HBSP were given subcutaneous injections of HBSP (Ketai, Shanghai, China) 60 μg/kg daily for the week prior to modeling. This investigation was approved by the Animal Ethics Committee of Sun Yat-sen University Animal Center.

Operative Procedure of Mice ALI

After anesthetizing the mice with 5% chloral hydrate (10 μL/g), sterile scissors were used to remove the fur from the neck of the mice. Then, scissors were used to cut the neck skin and separate the tissue until the mouse's trachea is exposed, and lipopolysaccharide (LPS) dilution (5 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) were intra-

tracheally injected into mice using a microsyringe. Immediately after the injection, the mice were placed in a straight position and the mice were gently shaken left and right so that the LPS was evenly distributed in the mouse trachea. Then, the surgical incision was sutured in the neck of the mouse and the mouse was returned to the squirrel cage. At 24 h, the mice were killed by breaking the neck.

Wet/dry Weight Ratio (W/D) of Lung Tissue

After the mice were sacrificed, the lung tissue of the mice was removed, and a filter paper was used to dry the blood on the surface of the lungs. Then, the lungs were weighed at this time, which was marked as wet weight. The lungs were then placed in an oven at 60°C for 48 h. At the end of the baking, the lungs were taken out and weighed again, and the result was marked as dry weight.

Malondialdehyde (MDA), SOD and Glutathione Peroxidase (GSH-Px) Activity Assay

The lung tissue was ground at low temperatures and dissolved in phosphate-buffered saline (PBS). Then MDA kit, SOD kit, and GSH-Px kit were used to detect the content of MDA, SOD, and GSH-Px in lung tissue according to the manufacturer's instructions.

Preparation of Bronchoalveolar Lavage Fluid (BALF)

The lungs of the mice were removed and rinsed from the main bronchus with PBS, and then collected the washed PBS. After that, the number of neutrophils was observed and counted under the microscope, and some of the BALF was retained for the detection of inflammation markers.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA kits were used to detect the levels of IL-6, IL-8, and TNF-α in mouse BALF. BALF were collected and the impurities were removed by centrifugation. Then, the IL-6 ELISA kit (Lianke, Hangzhou, China), IL-8 ELISA kit (Lianke, Hangzhou, China), and TNF-α ELISA kit (Lianke, Hangzhou, China) were used to determine the level of inflammatory factors in BALF according to the manufacturer's instructions.

Hematoxylin-Eosin (HE) Staining

The mouse lungs were soaked in 4% paraformaldehyde. After 24 h, the lungs were washed with PBS and embedded in paraffin after dehydration. Then, a slicer as used to make paraffin sections, which were dewaxed and hydrated and soaked in hematoxylin for 10 min. After washing, the paraffin sections were soaked in 1% hydrochloric acid for 30 s and then quickly rinsed into running water for 3 min. After the rinsing, the paraffin sections were placed in a 0.5% eucalyptus solution for 3 min. After the dyeing, they were dehydrated and sealed.

Cell Culture and Treatment

BEAS-2B is normal human lung epithelial cells purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin plus streptomycin (Gibco, Rockville, MD, USA) was used to culture BEAS-2B cells. The cells in the culture dish were cultured in an incubator at 37°C and 5% CO_2. After the cell growth density reached 60-70%, the cells were treated with drugs. ML385 (10 μL, Selleck, Houston, TX, USA), an inhibitor of the Nrf2/HO-1 signaling pathway, was used to inhibit the Nrf2/HO-1 signaling pathway in BEAS-2B cells. Finally, LPS (500 ng/mL) was used to construct a model of ALI at the cellular level.

Western Blot

After the BEAS-2B cell growth density reached 60%, the cells were stimulated with LPS or HBSP. After 24 h, the culture dish was taken out and the medium was discarded. After washing the cells with PBS, the total protein was extracted in the cells using protein lysate and detected the protein concentration using the bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). Thereafter, 30 μg of protein was added to each well of the electrophoresis gel for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was then transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and the PVDF membranes were then immersed in 5% skim milk for 1 h. After washing with phosphate-buffered saline and tween-20 (PBST), the PVDF membranes were incubated at 4°C overnight with primary antibody dilution (SOD1 1:3000, Rabbit, Abcam, Cambridge, MA, USA, SOD2, 1:3000, Rabbit, Abcam, Cambridge, MA, USA, CHOP, 1:2000, Rabbit, Abcam, Cambridge, MA, USA, GRP-78, 1:1000, Rabbit, Abcam, Cambridge, MA, USA,

caspase-12, 1:3000, Rabbit, Abcam, Cambridge, MA, USA, Nrf2, 1:2000, Rabbit, Abcam, Cambridge, MA, USA, HO-1, 1:1000, Rabbit, Abcam, Cambridge, MA, USA, β-actin, 1:3000, Rabbit, Abcam, Cambridge, MA, USA). After washing the PVDF membrane the next day, secondary antibody dilution (Goat anti-rabbit, 1:3000, Abcam, Cambridge, MA, USA) was used to incubate the PVDF membranes for 2 h, followed by washing. Finally, enhanced chemiluminescence (ECL) was used to detect protein expression.

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

After the BEAS-2B cell growth density reached 60%, the cells were stimulated with LPS or HBSP. At 24 h, the culture dish was taken out and the medium was discarded. After washing the cells with PBS, the RNA in the cells was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). A spectrophotometer was used to detect the RNA concentration. Next, the mRNA was reversed to complementary deoxyribose nucleic acid (cDNA) using the reverse transcription kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then cDNA was amplified using the SYBR Green kit (Invitrogen, Carlsbad, CA, USA). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as endogenous controls. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression level. The primer sequences of mRNA are shown in Table I.

Cell Counting Kit-8 (CCK-8) Assay

The optimal concentration of HBSP on BEAS-2B cells was determined by the CCK-8 method. 10, 20, 30, 50, 100 ng/mL of HBSP stimulated BEAS-2B cells, respectively. After 24 h, 10 μL of CCK-8 reagent (Yifeixue, Shanghai, China) was added to each well and cell incubation was continued in the incubator for 2 h. Finally, a microplate reader was used to measure the absorbance of each well.

Immunocytofluorescence (IF) Staining

BEAS-2B cells were passaged into 24-well plates. After treating the cells, the culture dishes were taken out and the medium was discarded. The cells were sequentially soaked in 4% paraformaldehyde and 0.2% Triton-PBS for 15 min each. Then, 10% goat serum was used to incubate the cells for 30 min at room temperature. In addition, primary antibody dilution (SOD1, 1:500, rabbit, Abcam, Cambridge, MA, USA, CHOP, 1:500, rabbit, Abcam, Cambridge, MA, USA, Nrf2, 1:500, rabbit, Abcam, Cambridge, MA, USA, IL-1β, 1:500, rabbit, Abcam, Cambridge, MA, USA) was used to incubate the cells overnight at 4°C. After washing the next day, the cells were incubated with fluorescent secondary antibody dilution (Goat anti-rab-

Table I. RT-PCR primer sequences.

Name	sense/anti-sense	Sequence (5'-3')
SOD1	sense	GGTGAACCAGTTGTGTTGTC
	anti-sense	CCGTCCTTTCCAGCAGTC
SOD2	sense	CAGACCTGCCTTACGACTATGG
	anti-sense	CTCGGTGGCGTTGAGATTGTT
GPX1	sense	ATCATATGTGTGCTGCTCGGCTAGC
	anti-sense	TACTCGAGGGCACAGCTGGGCCCTTGAG
GPX3	sense	AGAGCCGGGGACAAGAGAA
	anti-sense	ATTTGCCAGCATACTGCTTGA
CHOP	sense	CAGCGACAGAGCCAGAATAAC
	anti-sense	ACCGTCTCCAAGGTGAAAGG
GRP-78	sense	TACCCCAGATTGAAGTCACCT
	anti-sense	TTCTCGGCGTCATTGACCA
caspase-12	sense	TTTACGGACTTGCATGCACACGT
	anti-sense	TTGCAGCACTACGAGTAATCG
Nrf2	sense	TATGTCGACTGACTGACGTAC
	anti-sense	GACGGACCTATATGCGATTT
HO-1	sense	GGCTATATCGGCGATTTGCGC
	anti-sense	ACGGTTATTGGGATTACGACA
GAPDH	sense	ACAACTTTGGTATCGTGGAAGG
	anti-sense	GCCATCACGCCACAGTTTC

bit-FITC, 1:500, Abcam, Cambridge, MA, USA) for 1 hour. Finally, 4',6-diamidino-2-phenylindole (DAPI)-containing closure was used to seal the cells onto a glass slide.

ROS Level Detection

ROS levels in BEAS-2B cells was measured by flow cytometry. After the cells were passaged to 6-well plates, drugs were used to stimulate the cells, and the DCFH-DA kit (Keygen, Nanjing, China) was used to detect ROS levels in BEAS-2B cells according to the manufacturer's instructions.

Statistical Analysis

All experiments were repeated more than 3 times. Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) and GraphPad prism 7.0 (La Jolla, CA, USA) were used to process experimental data for this study. Differences between two groups were analyzed using the Student's *t*-test. Comparison between multiple groups was done using One-way analysis of variance (ANOVA) test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered to be statistically significant.

Results

HBSP Reduces Oxidative Stress in BEAS-2B Cells Induced by LPS

The effects of different concentrations (10, 20, 30, 50, 100 ng/mL) of HBSP on the activity of BEAS-2B cells were by CCK-8 assay (Figure 1A). The results showed that 50 ng/mL HBSP was the optimal concentration. The results of Western blot (Figure 1B) and RT-PCR (Figure 1C-1F) showed that LPS significantly decreased the expressions of SOD1, SOD2, GPX1, and GPX3 in cells, and HBSP effectively increased their expressions. In addition, HBSP reduced ROS levels in BEAS-2B cells (Figure 1G). The results of IF staining (Figure 1H) also confirmed the promotion of SOD1 by HBSP. These results indicate that HBSP reduces oxidative stress in BEAS-2B cells.

HBSP Reduces Endoplasmic Reticulum Stress (ERS) Induced by LPS in BEAS-2B Cells

Differences in expression of ERS-related markers in BEAS-2B cells were examined. The results of Western blot (Figure 2A) and RT-PCR (Figure 2B-2D) showed that the expressions of CHOP, GRF-78, and caspase-12 in LPS group were significantly increased, indicating that LPS increases ERS in BEAS-2B cells. The expression of ERS-related molecules in the LPS+HBSP group was significantly lower than that in the LPS group, indicating that HBSP significantly reduces ERS. The results of IF staining (Figure 2E) also indicated that HBSP reduced the expression of CHOP.

HBSP Increases the Activity of Nrf2/HO-1 Signaling Pathway in BEAS-2B Cells

The Nrf2/HO-1 signaling pathway is a classical antioxidant stress signaling pathway. The results of Western blot (Figure 3A) and RT-PCR (Figure 3B, 3C) showed that the activity of Nrf2/HO-1 signaling pathway was significantly decreased in BEAS-2B cells of LPS group and HBSP could promote the expression of Nrf2 and HO-1. The results of IF staining (Figure 3D) also indicated that HBSP increased the expression of Nrf2.

Inhibition of Nrf2/HO-1 Signaling Pathway Attenuates the Protective Effect of HBSP on BEAS-2B Cells

ML385 is an inhibitor of the Nrf2/HO-1 signaling pathway. The results of Western blot (Figure 4A) and RT-PCR (Figure 4B-4F) showed that after stimulating BEAS-2B cells with ML285, the protective effect of HBSP on cells was significantly decreased and showed a decrease in SOD1 and SOD2, as well as an increase in CHOP, GRF-78, and caspase-12. The results of IF staining (Figure 4G) also indicated that ML385 reduced the anti-inflammatory effect of HBSP on BEAS-2B cells and increased the expression of TNF-α. This suggested that HBSP may attenuate ALI by activating Nrf2/HO-1.

Exogenous HBSP Attenuates LPS-Induced Mouse ALI

To determine the effect of HBSP on mouse ALI, LPS was used to induce mouse ALI and subcutaneous injection of HBSP was administered. The results of HE (Figure 5A) showed that there was significant edema in the pulmonary interstitium of LPS-induced mice, and HBSP significantly improved pulmonary interstitial edema and improved lung tissue morphology. The W/D of lung tissue in the ALI group was also significantly higher than that in the control group, while HBSP significantly reduced W/D (Figure 5B).

In addition, HBSP significantly reduced neutrophils (Figure 5C) and inflammatory factors (IL-6, IL-8, and TNF-α) (Figure 5D-5F)

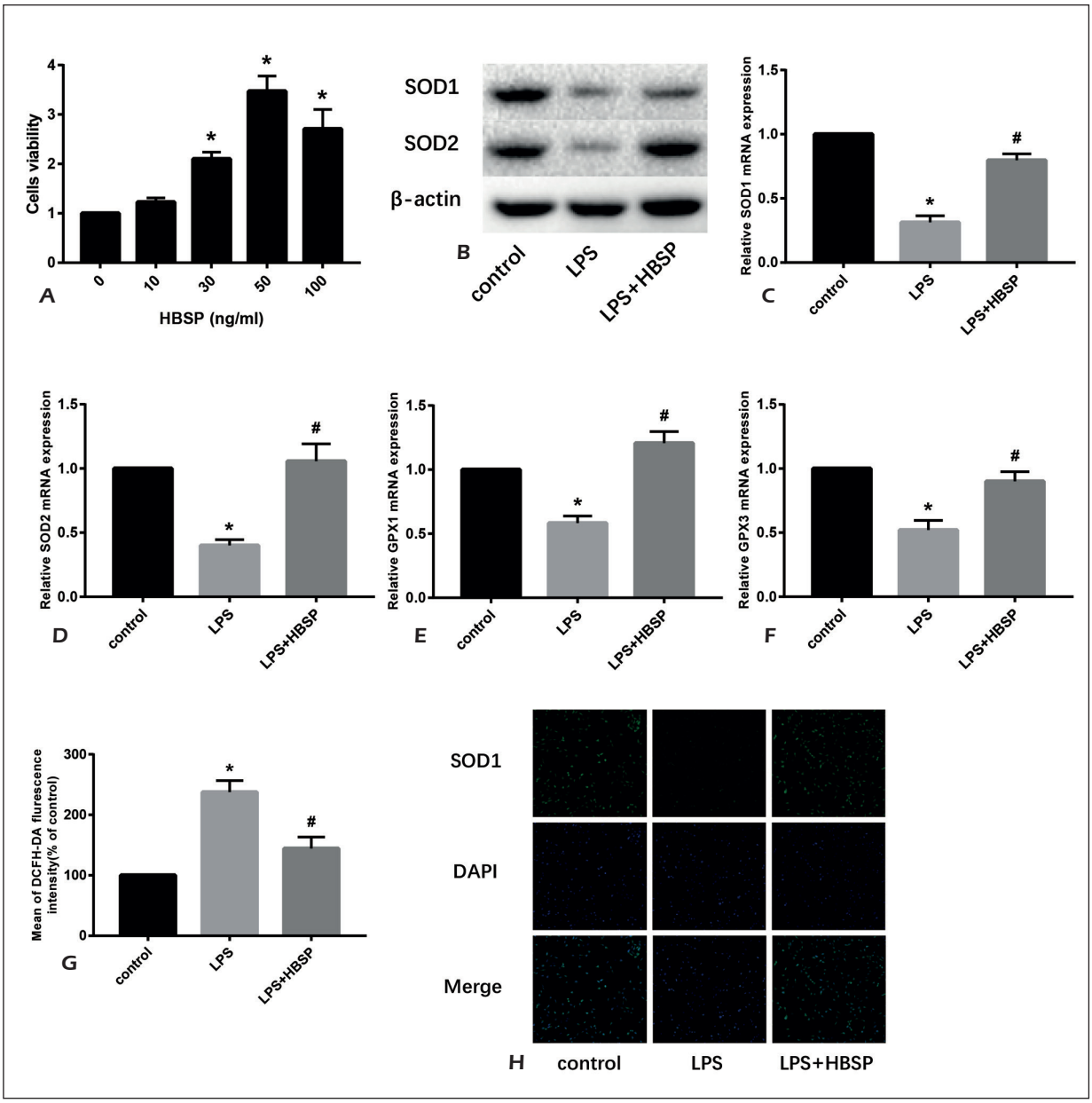

Figure 1. HBSP reduces oxidative stress in BEAS-2B cells induced by LPS. CCK-8 reveals that 50 ng/ml is optimum concentration (**A**). Expressions of SOD1, SOD2, GPX1, and GPX3 in three groups are determined by Western blot (**B**) and RT-PCR (**C-F**). ROS levels in three groups are detected by flow cytometry (**G**). IF staining detects the expression of SOD1 (**H**) (magnification 200×; "*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the LPS group).

in BALF. MDA, SOD, and GSH-Px are classical indicators of oxidative stress. The results showed that HBSP effectively reduced the expression of MDA and increases the expression of SOD and GSH-Px, indicating that HBSP inhibits the level of oxidative stress in mouse lung tissue (Figure 5G-5I).

Discussion

ALI is a critical respiratory disease caused by severe infection, massive blood transfusion, and trauma, which seriously affects the life and health of patients[9]. Currently, there is no effective treatment for LPS-induced ALI, so it is important to

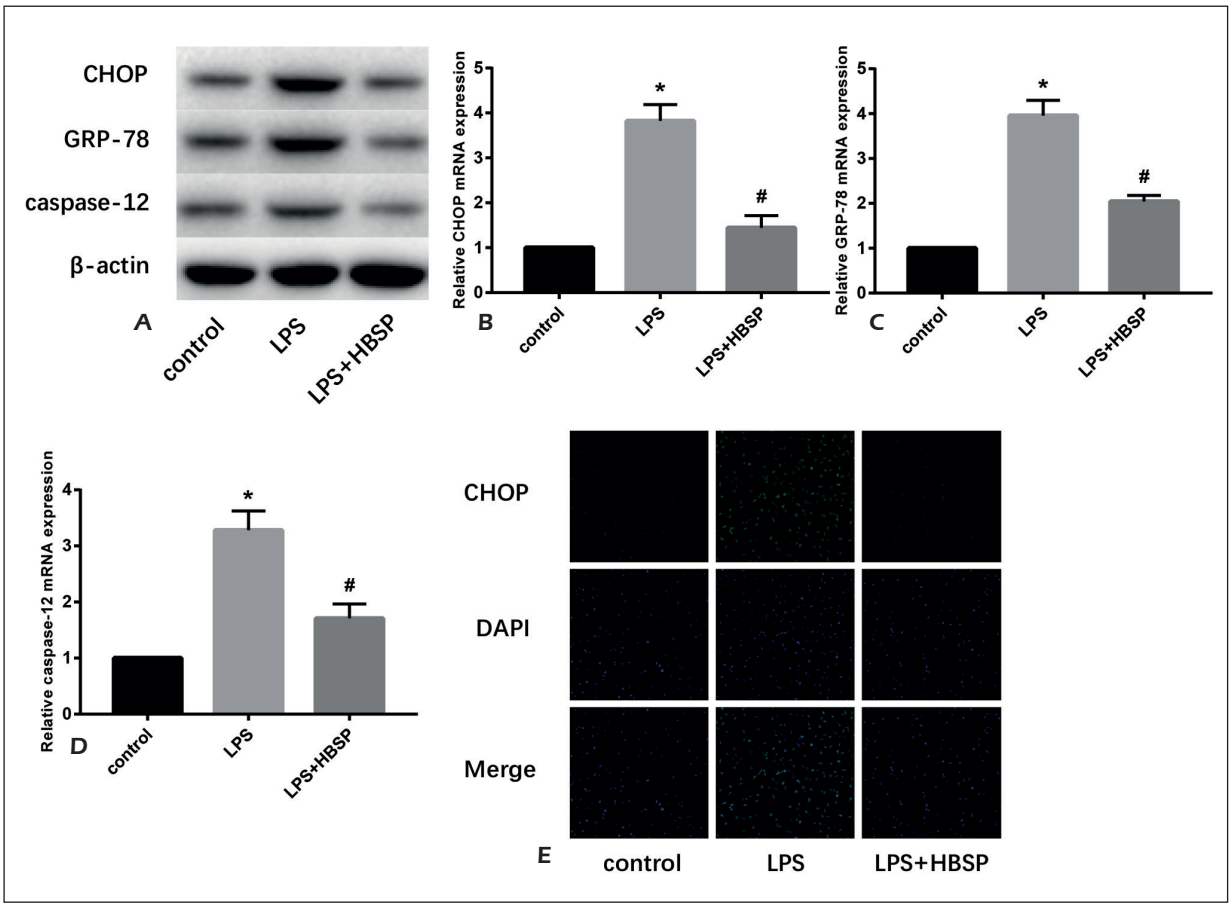

Figure 2. HBSP reduces ERS induced by LPS in BEAS-2B cells. Expressions of CHOP, GRP-78, and caspase-12 in three groups are determined by Western blot (**A**) and RT-PCR (**B**-**D**). IF staining (**E**) detects the expression of CHOP (magnification 200×; "*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the LPS group).

find effective treatments for ALI[9]. As a synthetic peptide, HBSP has been found to have a variety of tissue protection effects. Ueba et al[10] found that HBSP can inhibit TNF-α-induced cardiomyocyte apoptosis by activating intracellular signaling pathways such as Akt, ERK1/2, and STAT3 through cardiomyocytes cultured *in vitro*, and exert similar cardiomyocyte protection effects as EPO. Patel et al[11] constructed the acute renal ischemia-reperfusion model of the rat by temporarily clamping the renal pedicles on both sides of the rat and found that HBSP can reduce glomerular and renal tubule dysfunction caused by ischemia-reperfusion. Robertson et al[12] induced the model of mild cerebral cortex injury through hemorrhagic shock in rats and found that HBSP is similar to EPO, which can accelerate the recovery of cerebral blood flow after brain injury and improve the time of persistence on the balance beam and the speed of crossing the beam after brain injury in rats. As a derivative of EPO, HBSP can act on the cardiovascular system, kidney, nervous system, etc., and exert similar tissue protection effects as EPO, but it does not stimulate the hematopoietic system of the bone marrow, avoiding polycythemia, elevated blood pressure, and thrombosis[13]. Therefore, HBSP has a good prospect of clinical application. However, there is no relevant research on the effect of HBSP on ALI. Therefore, a mouse ALI model was made to study the effect of HBSP on mouse ALI and hope to provide a theoretical basis for clinical application.

Most of ALI is characterized by polymorphonuclear neutrophils (PMN) dependence, and activation of PMN is the initiator of its excessive inflammatory response[14]. On the one hand, PMN is necessary for the elimination of pathogens, on the other hand it also has a pro-inflammatory effect. PMN generally only plays a role in infection or trauma, but under the pathological conditions

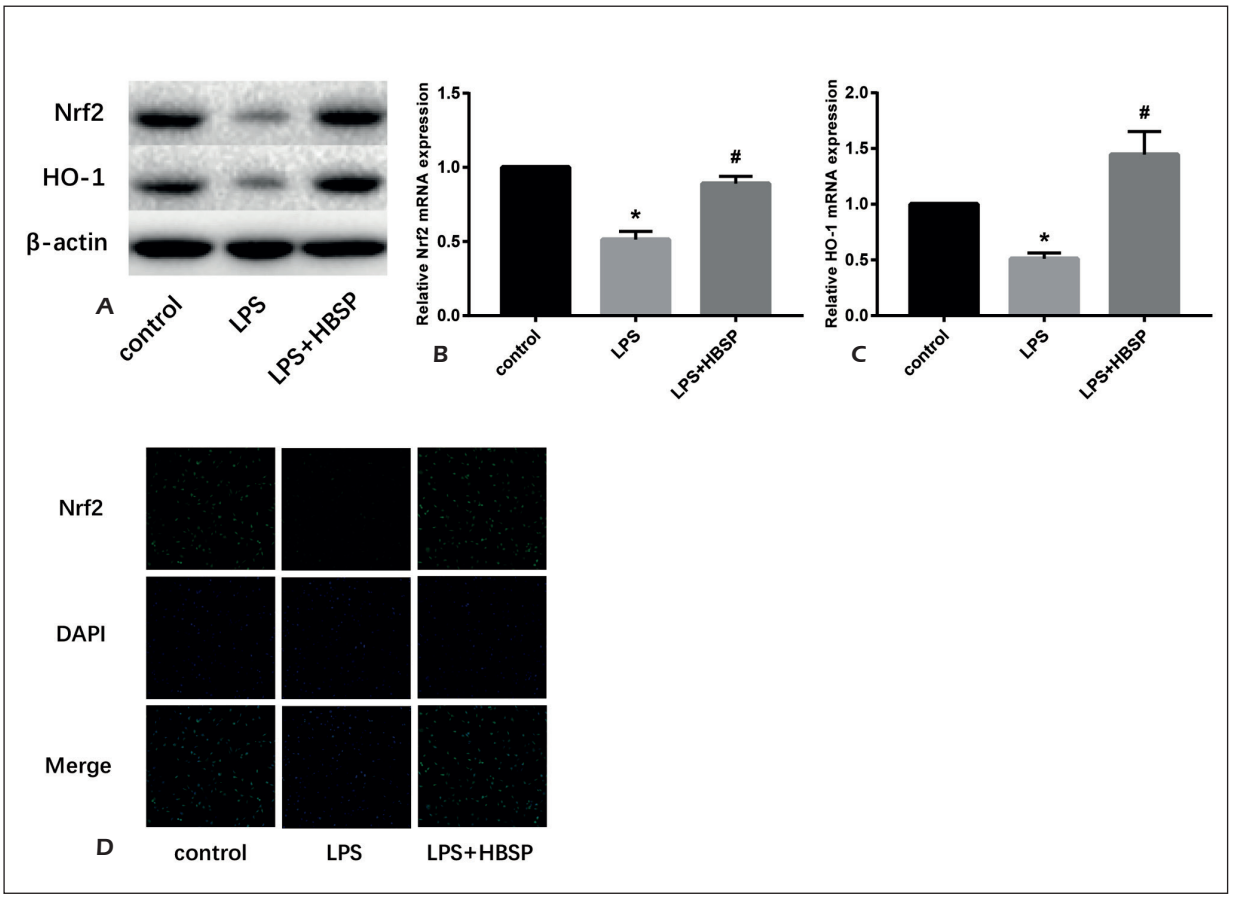

Figure 3. HBSP increases the activity of Nrf2/HO-1 signaling pathway in BEAS-2B cells. Expressions of Nrf2 and HO-1 in mice are determined by Western blot (**A**) and RT-PCR (**B**-**C**). IF staining (**D**) detects the expression of Nrf2 (magnification 200×; "*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the LPS group).

such as sepsis and severe trauma, PMN changes in cell biomechanics and cell dynamics and its deformation ability decreases. PMN adheres to endothelial cells under the action of adhesion molecules such as P-selectin, β2 integrin, and ICAM-1, and remains in the lung tissue. PMN is activated under the action of inflammatory factors such as TNF-α, IL-1, IL-6, and integrin receptor ICAM-1, etc., and then, mediated by IL-8, CD11b/CD18, etc., passes through the endothelium into the pulmonary interstitium and alveolar cavity[15]. Finally, respiratory burst degranulation results in oxidative stress state, producing and releasing large amounts of oxygen free radicals, proteases, and lipid mediators. These substances, which are mainly released by activated effector cells such as alveolar macrophage (AM) and PMN, cannot only directly damage lung tissue, but also promote the activation of AM and PMN in the inflammatory region, further releasing more oxygen free radicals and lysosomal enzymes form a vicious circle. In addition, oxygen free radicals can also induce the release of IL-1, TNF-α, and IL-8 by activating NF-κB, which induces the inflammatory reaction cascade effect and promotes the development of ALI[16]. In this study, several inflammatory factors (IL-6, IL-8, and TNF-α) were also detected in BALF of mice induced by LPS, and W/D and pathological staining of mouse lung tissues revealed that there was significant edema in the interstitial lung, indicating that LPS successfully induced ALI in mice. After intraperitoneal injection of HBSP into mice, the number of neutrophils and inflammatory factors in the BALF of the mice decreased significantly, and HE showed that the pulmonary interstitial edema of the mice was also significantly improved. In HBSP-treated BEAS-2B cells, the expression of SOD1/2 was significantly increased and the ROS content in the cells was decreased, indicating that HBSP can

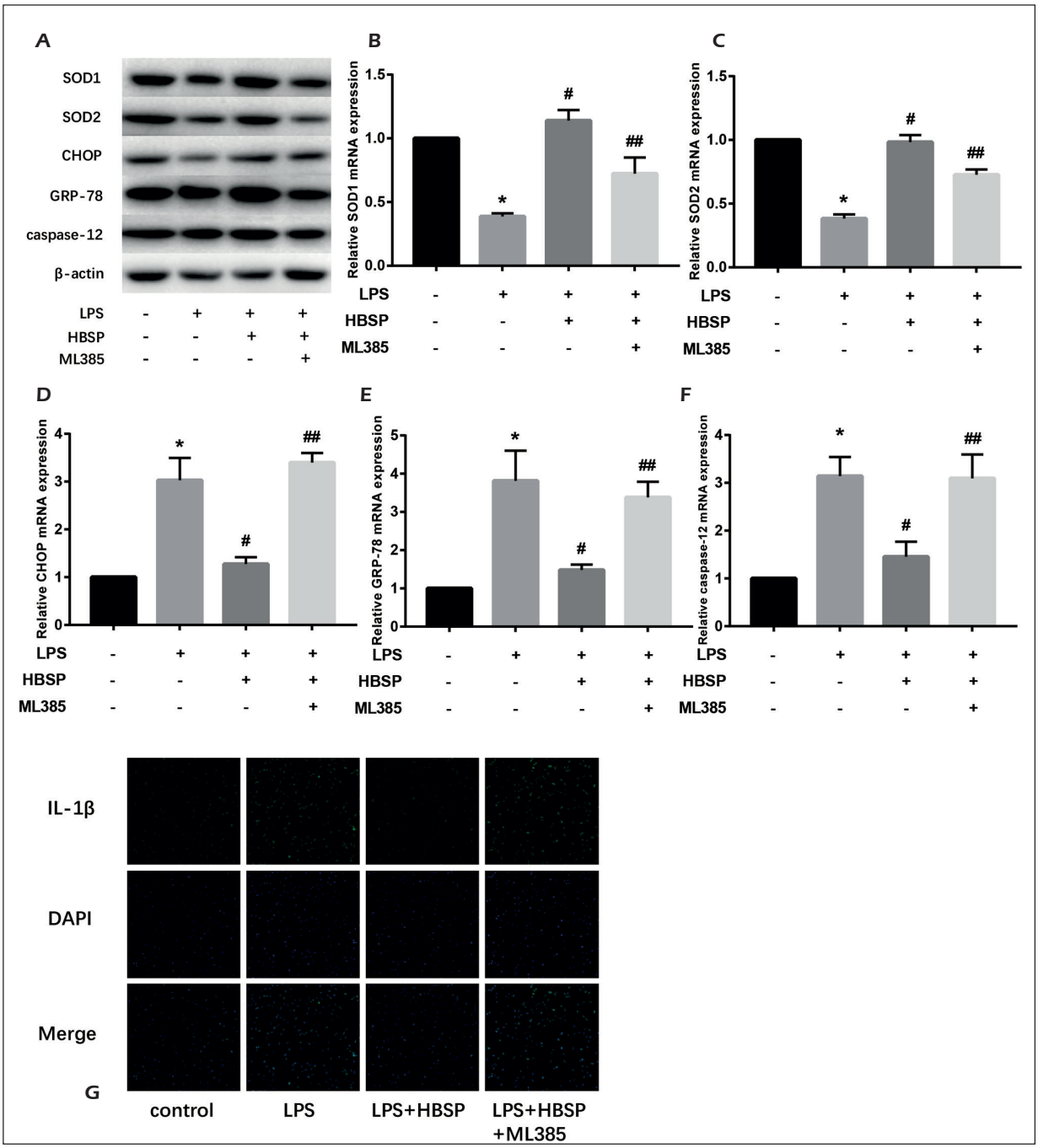

Figure 4. Inhibition of Nrf2/HO-1 signaling pathway attenuates the protective effect of HBSP on BEAS-2B cells. Expressions of SOD1, SOD2, CHOP, GRP-78, and caspase-12 in four groups are determined by Western blot (**A**) and RT-PCR (**B**-**F**). IF staining (**G**) detects the expression of IL-1β (magnification 200×; "*" means there is a statistical difference with the control group, "#" means there is a statistical difference with the LPS group and "##" means there is a statistical difference with the LPS+HBSP group).

significantly reduce the oxidative stress level of BEAS-2B cells and improve the ability to scavenge oxygen free radicals. Therefore, HBSP has significant anti-inflammatory and anti-oxidative effects on the lungs, which is very beneficial for the treatment of ALI.

Under physiological conditions, three types of transmembrane proteins on the endoplasmic re-

ticulum membrane, namely protein kinase-like endoplasmic reticulum kinase (PERK), endoribonuclease inositol-requiring enzyme 1 (IRE1), and activated transcription factor 6 (ATF6), bind to intracellular GRP78/BiP to form a stable complex and the downstream associated signaling pathway is inactivated[17]. When unfolded proteins or misfolded proteins aggregate in the endoplasmic reticulum, GRP78/BiP receives signals and dissociates with the transmembrane proteins PERK, IRE1, and ATF6, and simultaneously binds to unfolded proteins to activate the three classical signal transduction pathways of PERK, IRE1, and ATF6. The signal transduction pathways promote the correct folding of proteins, inhibit the production of proteins, accelerate the degradation of non-functional proteins, and strengthen the self-repairing ability of the endoplasmic reticulum[18]. Various self-factors and external environment, such as lack of oxygen in cells, oxidative stress in cells, nutritional deficiencies, chemical drug stimulation, and loss of calcium homeostasis, can trigger abnormal protein accumulation in the endoplasmic reticulum, which will induce ERS. There are three ways to activate apoptosis through endoplasmic reticulum stress: (1) The PERK signaling pathway activates the transcription of the CHOP gene, which is conducive to the downregulation of anti-apoptotic genes Bcl-2, thereby promoting apoptosis. (2) The IRE1 signaling pathway can also down-regulate Bcl-2 expression and up-regulate the apoptosis-promoting gene BIM by activating the JNK signaling pathway. (3) The classical apoptotic pathway involving the Caspase family, which is unique to ERS, is mainly due to the activation of caspase-12, which leads to the involvement of Caspase family members and

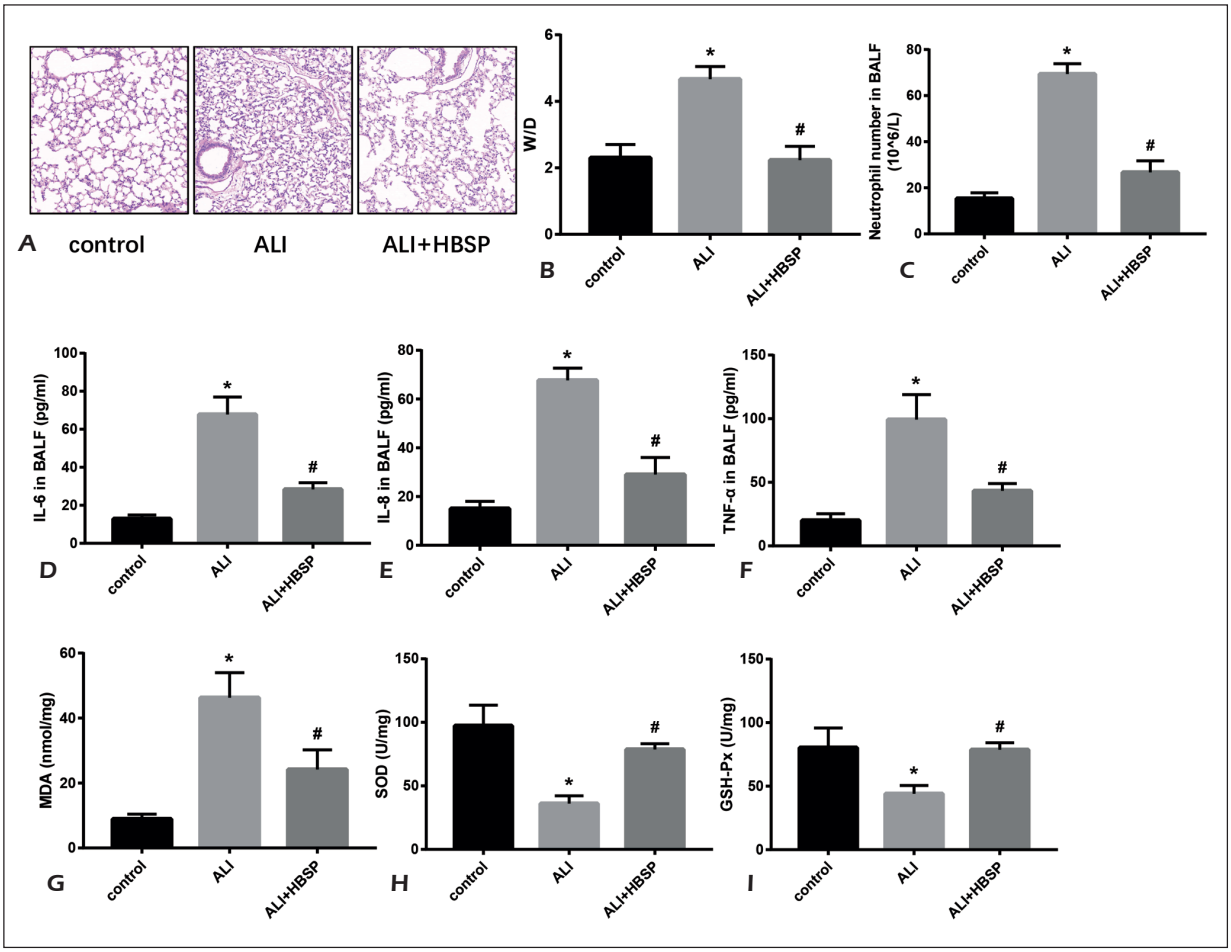

Figure 5. Exogenous HBSP attenuates LPS-induced mouse ALI. **A**, HE staining of mice lung tissue (magnification 200×). B, W/D in three groups is detected. Nneutrophil number (**C**) in BALF were detected. Expressions of IL-6 (**D**), IL-8 (**E**), and TNF-α (**F**) in BALF are determined by ELISA. MDA (**G**), SOD (**H**) and GSH-Px (**I**) activity in three groups ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the ALI group).

ultimately leads to cell apoptosis[19]. LPS is a component of the cell wall of Gram-negative bacteria and is the main active component of bacterial endotoxin, which can cause fever, microcirculatory disturbance, endotoxin shock, and disseminated intravascular coagulation, and can induce excessive inflammation of the body[20]. The reaction is a key pathogenic factor in the pathological process of endotoxemia, ALI and even sepsis. Guo et al[21] established a model of ALI by injecting LPS into the tail vein of rats, and observed the expression of GRP-78, CHOP, and caspase-12 in the lung tissue of rats at 1, 6, and 12 h after the establishment. The results showed that the expression of GRP-78 was up-regulated and the protein level was increased in the LPS group at early stage, suggesting that ERS has occurred in the lung tissue cells at the time of ALI. The expression of CHOP was observed at 6 and 12 h after ALI modeling, and the change at 1 h is not evident. The expression of caspase-12 in lung tissue of ALI rats gradually increased with time, which was consistent with the apoptosis of lung epithelial cells and pulmonary vascular endothelial cells. Similarly, activation of caspase-12 is a key downstream executive molecule of ERS-mediated apoptosis[22]. Animal experiments show that caspase-12-deficient mice have significantly higher anti-pulmonary infections than wild type mice. This result indicates that ERS is involved in the pathophysiological process of ALI and is one of the important mechanisms of endotoxin-induced lung injury[23]. Similar results were found when BEAS-2B cells were stimulated with LPS. However, CHOP, GRP-78, and caspase-12 were significantly reduced after stimulation with HBSP, indicating that HBSP reduced ERS levels in BEAS-2B cells.

HBSP has biological activity and neuroprotective effects. Liu et al[24] established a myocardial ischemia/reperfusion (I/R) model in mice. The study found that the I/R+HBSP group had significantly improved cardiac systolic function and reduced myocardial infarct size. The mechanism was that HBSP could activate PI3K/Akt/Mtor transduction pathway and inhibits autophagy to reduce myocardial injury in mice. HBSP has significant neuroprotective effects and can significantly improve cerebral blood flow recovery after injury. HBSP can also significantly reduce diabetes-related glial cell dysfunction and neuronal damage, inhibit the release of local pro-inflammatory factors in the retina, and effectively improve diabetic retinopathy. HBSP also has a protective effect on acute kidney injury. Wu et al[25] have found that HBSP can protect kidney I/R in mice by enhancing cell survival rate and inhibiting apoptosis. In this study, it was found that HBSP had significant anti-inflammatory and anti-oxidative effects on alveolar epithelial cells. In addition, HBSP increased the activity of the Nrf2/HO-1 signaling pathway in BEAS-2B cells. The Nrf2/HO-1 signaling pathway is a classical oxidative stress-related pathway. Activation of the Nrf2/HO-1 signaling pathway reduces intracellular oxidative stress levels and increases the cell's ability to resist oxidative stress. The activation of Nrf2/HO-1 signaling pathway by HBSP may be the mechanism by which it improves ALI. We believe that through the study of HBSP, we can provide new targets and directions for the clinical treatment of ALI.

Conclusions

These data showed that HBSP significantly reduces LSP-induced mouse ALI, as well as its levels of inflammation and oxidative stress. *In vitro*, HBSP also significantly reduces oxidative stress and ERS in BEAS-2B cells. In addition, HBSP can activate the Nrf2/HO-1 signaling pathway associated with anti-oxidative stress, which may be the mechanism by which HBSP exerts anti-oxidative stress.

Conflict of Interests

The authors declare that they have no conflict of interests.

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