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# ZJ01, a Small Molecule Inhibitor of the Kelch-Like ECH-Associated Protein 1-Nuclear Factor Erythroid 2-Related Factor 2 (Keap1-Nrf2) Protein-Protein Interaction, Reduces Hyperoxic Acute Lung Injury in a Mouse Model

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Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
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**Background:** Hyperoxic acute lung injury (ALI) is a complication of ventilation in patients with respiratory failure. Nuclear factor erythroid-2-related factor 2 (Nrf2) has an important role in ALI. Kelch-like ECH-associated protein 1 (Keap1) binds to Nrf2. ZJ01 is a small molecule inhibitor of Keap1-Nrf2 protein-protein interaction (PPI) that can reduce Keap1-induced inhibition of Nrf2. This study aimed to investigate the effects of ZJ01 and the heme oxygenase-1 (HO-1) inhibitor, zinc protoporphyrin IX (ZnPP IX), in a mouse model of hyperoxic ALI.





**Material/Methods:** C57BL/6J mice included five study groups: the room air+vehicle-treated group; the room air+ZJ01 group; the hyperoxia+vehicle-treated group; the hyperoxia+ZJ01 group; and the hyperoxia+ZJ01+ZnPP IX group. ZJ01, ZnPP IX, or vehicle were given 1 h after the hyperoxia challenge. The lungs from the mice were harvested at 72 h following the hyperoxia challenge.

**Results:** Hyperoxia exposure for 72 h increased the activity of myeloperoxidase, the lung water content, the levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and matrix metalloproteinase-9 (MMP-9) in the vehicle-treated mice. ZJ01 treatment reduced hyperoxia-induced inflammation and increased the activation of Nrf2 and HO-1 compared with the vehicle-treated mice. Histology of the lungs showed that ZJ01 treatment reduced the changes of hyperoxia-induced ALI. Pretreatment with ZnPP IX reversed the beneficial effect of ZJ01.

**Conclusions:** ZJ01, a Keap1-Nrf2 PPI inhibitor, reduced hyperoxic ALI in a mouse model through the Nrf2/HO-1 pathway.

**MeSH Keywords:** **Acute Lung Injury • Inflammation • NF-E2-Related Factor 1 • Oxidative Stress**

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## Background

Hyperoxic acute lung injury (ALI) is a complication of ventilation in patients with respiratory failure. During oxygen therapy, the generation of oxidants, including reactive oxygen species (ROS), trigger ALI and acute respiratory distress syndrome (ARDS) [1–5]. Nuclear factor erythroid 2-related factor 2 (Nrf2) has an important role in maintaining redox homeostasis by regulating the expression of antioxidants [6,7]. In a previous study, using a mouse model of ARDS, increased mortality in Nrf2-deficient mice occurred following hyperoxia [8]. The induction of Nrf2 expression has been reported to reduce the severity of ALI in studies using preclinical models and has been proposed as a potential strategy for the treatment of ALI and ARDS [6,9].

Normally, Nrf2 is inhibited by kelch-like ECH-associated protein 1 (Keap1) to control the activation of Nrf2. Keap1 inhibits Nrf2 activation through the protein-protein interaction (PPI), which results in Nrf2 ubiquitination and degradation. Therefore, disrupting the PPI of Keap1 and Nrf2 is a potential strategy to enhance Nrf2 activation and rebalance oxidants and antioxidants during ALI. Inhibitors of Keap1-Nrf2 PPI have been shown to induce Nrf2 and reduce inflammation and oxidative stress in cell and animal studies [10,11]. Recently, Lu et al. studied *in vitro* cell models and showed that acetaminophen-induced liver injury was reduced using an inhibitor of Keap1-Nrf2 PPI [10]. The same research group also recently investigated a mouse model and showed that the Keap1-Nrf2 PPI inhibitor, CPUY192018, reduced chronic renal inflammation [11].

ZJ01 is a novel small molecule inhibitor of Keap1-Nrf2 PPI, which has recently been shown to trigger Nrf2 nuclear translocation in H9c2 cardiac cells *in vitro*, resulting in increased mRNA expression levels of Nrf2 target genes, including the heme oxygenase-1 (HO-1) inhibitor gene [12]. Also, reactive oxygen species (ROS) levels were reduced by ZJ01 *in vitro* [12]. Therefore, this study aimed to investigate the effects of ZJ01 and the HO-1 inhibitor, zinc protoporphyrin IX (ZnPP IX), in a mouse model of hyperoxia-induced ALI.

## Material and Methods

### Animals and the study groups

The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Nanfang Hospital. The animal studies were performed according to the recommendations of the Helsinki Convention for the Care and Use of Experimental Animals.

Wild-type C57BL/6J mice, weighing between 18–22 gm, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed in cages at a temperature of between 19–25°C and a 12-hourly light and dark cycle. Food and water were provided *ad libitum*. The mice were acclimatized for at least one week before the study began, and efforts were made to ensure that the experimental animals did not suffer from distress.

The treatments used in this study included room air inhalation; intraperitoneal injection with dimethyl sulfoxide (DMSO), which was used as a solvent and as the vehicle; ZJ01, the small molecule inhibitor of Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid-2-related factor 2 (Nrf2) protein-protein interaction (PPI); and the heme oxygenase-1 (HO-1) inhibitor, zinc protoporphyrin IX (ZnPP IX). The C57BL/6J mice were assigned to five study groups: the room air+vehicle-treated group; the room air+ZJ01 group; the hyperoxia+vehicle-treated group; the hyperoxia+ZJ01 group; and the hyperoxia+ZJ01+ZnPP IX group. ZJ01, ZnPP IX, or vehicle were given 1 h after the hyperoxia challenge. During the study, the mice were maintained in an air-tight cage and exposed to hyperoxia for 72h to induce hyperoxic acute lung injury (ALI), with the oxygen concentration maintained at 100%. Room air exposure was given to the control animals.

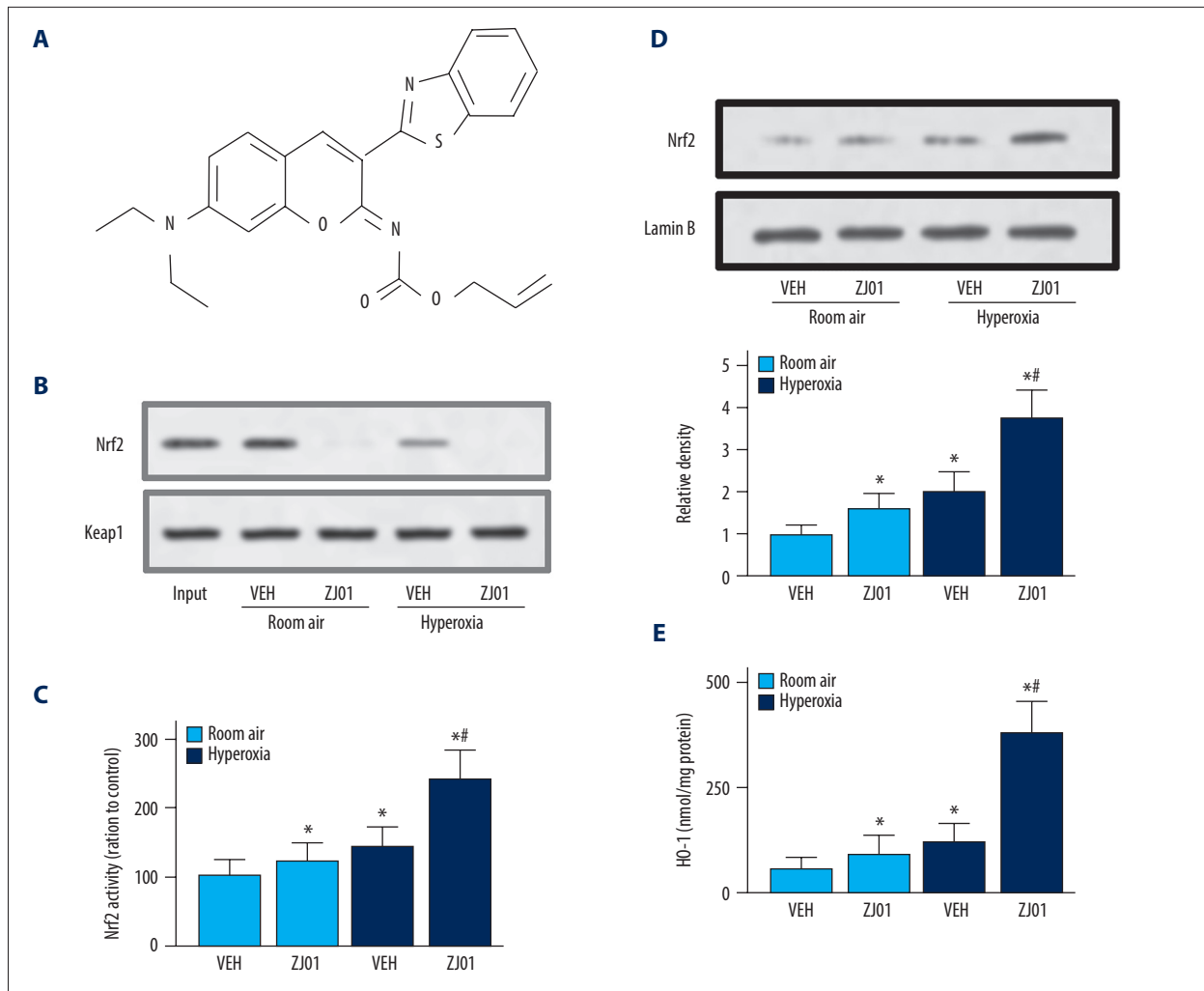
### Synthesis and use of the Keap1-Nrf2 PPI inhibitor, ZJ01

Figure 1A shows the structure of ZJ01, which was synthesized as previously reported [12]. ZJ01 (10mg/kg) or vehicle was administered intraperitoneally at 1 h after the hyperoxia challenge, and an additional dose of ZJ01 or vehicle was administered every 24 h. On review of the protocols from a previous study [12], a preliminary study was performed to determine the dose of ZJ01 used in this study (data not shown).

ZJ01 was freshly dissolved in the vehicle (DMSO: normal saline (v/v)=5: 95). Thirty minutes before ZJ01 treatment, the HO-1 inhibitor, ZnPP IX (50 mg/kg<sup>-1</sup>), was given by intraperitoneal injection (Sigma Chemical Co. St. Louis, MO, USA.) [13]. An additional dose of ZnPP IX (50 mg/kg) was administered every 24 h. The animals were then euthanized under anesthesia by 50 mg/kg<sup>-1</sup> of pentobarbitone by intraperitoneal injection at 72 h after room air or hyperoxia stimulation. The left lung was removed for the measurement of lung edema. The right lung was harvested and immediately frozen in liquid nitrogen for further study.

### Immunoprecipitation

Cytosolic protein was extracted by using a nuclear/cytosol fractionation kit (CellBiologics, Chicago, IL, USA). Dynabeads® Protein G (Thermo Fisher Scientific, Shanghai, China) was



**Figure 1.** C57BL/6J mice were exposed to room air or hyperoxia and treated with and without ZJ01, an inhibitor of Kelch-like ECH-associated protein 1 (Keap1) protein-protein interaction (PPI). **(A)** The structure of the small molecule, ZJ01. **(B)** Kelch-like ECH-associated protein 1 (Keap1) and nuclear factor erythroid-2-related factor 2 (Nrf2) protein-protein interaction (PPI) were detected by co-immunoprecipitation using antibodies to Keap1 in hyperoxic mice and room air-stimulated mice. Western blot was performed using antibodies to Keap1 and Nrf2. **(C)** Nrf2 activity. **(D)** Nrf2 protein levels. **(E)** Heme oxygenase-1 (HO-1) activity. VEH, vehicle. \*  $P < 0.05$  vs. the room air+VEH group; #  $P < 0.05$  vs. the hyperoxia+VEH group.

incubated with anti-Keap1 antibody (Thermo Fisher Scientific, Shanghai, China) for 10 minutes at room temperature with rotation. Immunoprecipitation was performed by incubating extracted cytosolic protein with the Dynabeads® Protein G-antibody complex in a rotating incubator at 4°C for 4 h. The complex was washed, and the target antigen was eluted. Immunoblot analysis was performed to investigate the target antigen.

### Measurement of Nrf2 activity

The activity of Nrf2 was measured using a commercial TransAM™ Nrf2 kit (Active Motif, Carlsbad, CA, USA), according to the manufacturer's instructions.

### Western blot

Protein concentration was measured by a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Asheville, NC, USA), according to the manufacturer's instructions. Total protein (50 µg) underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was transferred to nitrocellulose membranes. Before incubation with specific antibodies, nonspecific binding was blocked by incubation with 5% dried skimmed milk powder. Primary antibodies used were to Nrf2, lamin B, HO-1, and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) and Keap1 (Thermo Fisher Scientific, Shanghai, China) and were incubated with the membranes at 4°C overnight. The blots were incubated with secondary horseradish

peroxidase (HRP)-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at room temperature. The bands were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### Measurement of HO-1

The activity of HO-1 in lung tissue samples was measured by detecting the level of bilirubin, a product of HO-1 [14]. Briefly, lung tissue supernatant was mixed with heme, NADPH, and  $MgCl_2$  in phosphate-buffered saline (PBS) (pH 7.4) in an incubator at 37°C for 1 h. The reaction in the mixture was stopped by placing the mixture on ice. The levels of bilirubin generated in the mixture were detected using a Multiskan Sky Microplate Spectrophotometer at 530 nm (Thermo Scientific, Asheville, NC, USA).

### Analysis of glutathione peroxidase (GPx) and glutathione (GSH) levels

The levels of GSH were measured using a kit (Cayman Chemical, Ann Arbor, USA) and the measurement of GPx activity was measured using a kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions

### Analysis of lipid hydroperoxide levels

Levels of lipid hydroperoxide were measured using a commercial kit (Cayman Chemical, Ann Arbor, USA), according to the manufacturer's instructions.

### Measurement of myeloperoxidase (MPO)

The activity of MPO in the lung supernatant was measured using a commercial kit (Cayman Chemical, Ann Arbor, USA), according to the manufacturer's instructions. Briefly, samples were mixed with  $H_2O_2$ , o-dianisidine hydrochloride, and PBS (pH 7.4). MPO activity was detected using a Multiskan Sky Microplate Spectrophotometer at 530 nm (Thermo Scientific, Asheville, NC, USA).

### The lung wet to dry weight (W/D) ratio

The lung water content was detected at 72 h after hyperoxia or room air stimulation. Dried lung weight was detected at 48 h after placing the lung in an oven at 70°C.

### Measurement of tumor necrosis factor- $\alpha$ (TNF- $\alpha$ ) and matrix metalloproteinase-9 (MMP-9)

TNF- $\alpha$  and MMP-9 levels were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc, Minneapolis, MN, USA), according to the manufacturer's instructions.

### Lung histology

Two histopathologists, who were unaware of the study groups, conducted the histological evaluation of the mouse lung tissue sections, using a scoring system for ALI, according to the American Thoracic Society [15]. In the scoring system, infiltration of neutrophils and proteinaceous debris, hyaline membranes, and septal thickening in the lung were evaluated.

### Statistical analysis

Data were analyzed using SPSS version 20.0 software (IBM, Armonk, NY, USA). Values were expressed as the mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare differences between the study groups. A P-value <0.05 was considered to be statistically significant.

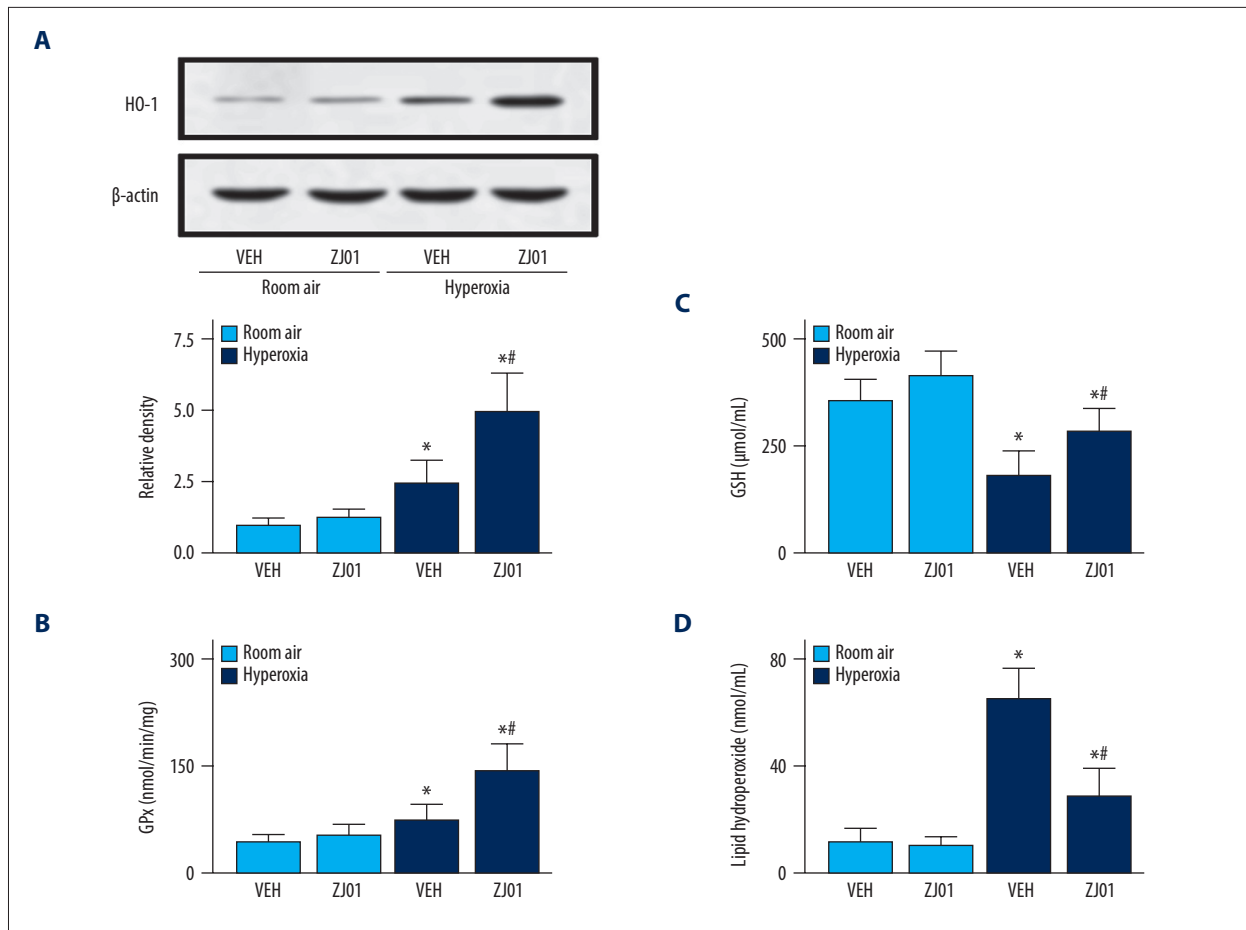
## Results

### ZJ01 increased the activation of nuclear factor erythroid-2-related factor 2 (Nrf2) in the mouse model of hyperoxic acute lung injury (ALI)

Activation of Nrf2 has previously been shown to reduce the effects of ALI [6,9]. In this study, a co-immunoprecipitation assay was performed to investigate the inhibitory effect of ZJ01, a small molecule inhibitor of Keap1-Nrf2 protein-protein interaction (PPI) that can reduce Keap1-induced inhibition of Nrf2. The results showed that ZJ01 reduced the PPI of Keap1 and Nrf2 both in lung tissues of the control and hyperoxia-stimulated mice (Figure 1B). As shown in Figure 1C and 1D, ZJ01 treatment activated Nrf2. When mice were subjected to hyperoxia, ZJ01 significantly increased the activation of Nrf2 compared with the dimethyl sulfoxide (DMSO) vehicle. In the hyperoxia-stimulated mice, ZJ01 increased heme oxygenase-1 (HO-1) activity by 3.1-fold compared with the vehicle (Figure 1E). The levels of HO-1 protein were increased by ZJ01 (Figure 2A). ZJ01 increased the activity of glutathione peroxidase (GPx) by 57% when compared with the dimethyl sulfoxide (DMSO) vehicle (Figure 2B).

### The effects of ZJ01 on glutathione (GSH) and lipid hydroperoxide

GSH is an antioxidant. As shown in Figure 2C, the GSH level was significantly increased in the lung tissue from ZJ01-treated mice compared with mice treated with the DMSO vehicle. Oxidants cause cell damage in ALI [6,9]. The results showed that the vehicle did not inhibit the hyperoxia-induced increase in lipid hydroperoxide (Figure 2D). However, the level of lipid hydroperoxide was reduced in ZJ01-treated mice (Figure 2D).



**Figure 2.** C57BL/6 mice were exposed to room air or hyperoxia and treated with and without ZJ01, an inhibitor of Kelch-like ECH-associated protein 1 (Keap1) protein-protein interaction (PPI). **(A)** Heme oxygenase-1 (HO-1). **(B)** Glutathione peroxidase (GPx) activity. **(C)** Glutathione (GSH) levels. **(D)** Lipid hydroperoxide levels. VEH, vehicle. \*  $P < 0.05$  vs. the room air+VEH group; #  $P < 0.05$  vs. the hyperoxia+VEH group.

### The effects of ZJ01 on hyperoxia-induced ALI and myeloperoxidase (MPO) in the mouse model

As shown in Figure 3A, the MPO level of lung tissues was increased significantly in vehicle-treated mice exposed to hyperoxia. In contrast, the hyperoxia-induced increase in the level of MPO was inhibited by ZJ01 treatment (Figure 3A). ZJ01 also reduced hyperoxia-induced pulmonary edema (Figure 3B). As shown in Figure 3C, hyperoxia exposure significantly increased the levels of TNF- $\alpha$ . The levels of matrix metalloproteinase-9 (MMP-9) were increased by hyperoxia (Figure 3D). As shown in Figure 3C and 3D, these hyperoxia-induced changes were reduced by ZJ01 treatment.

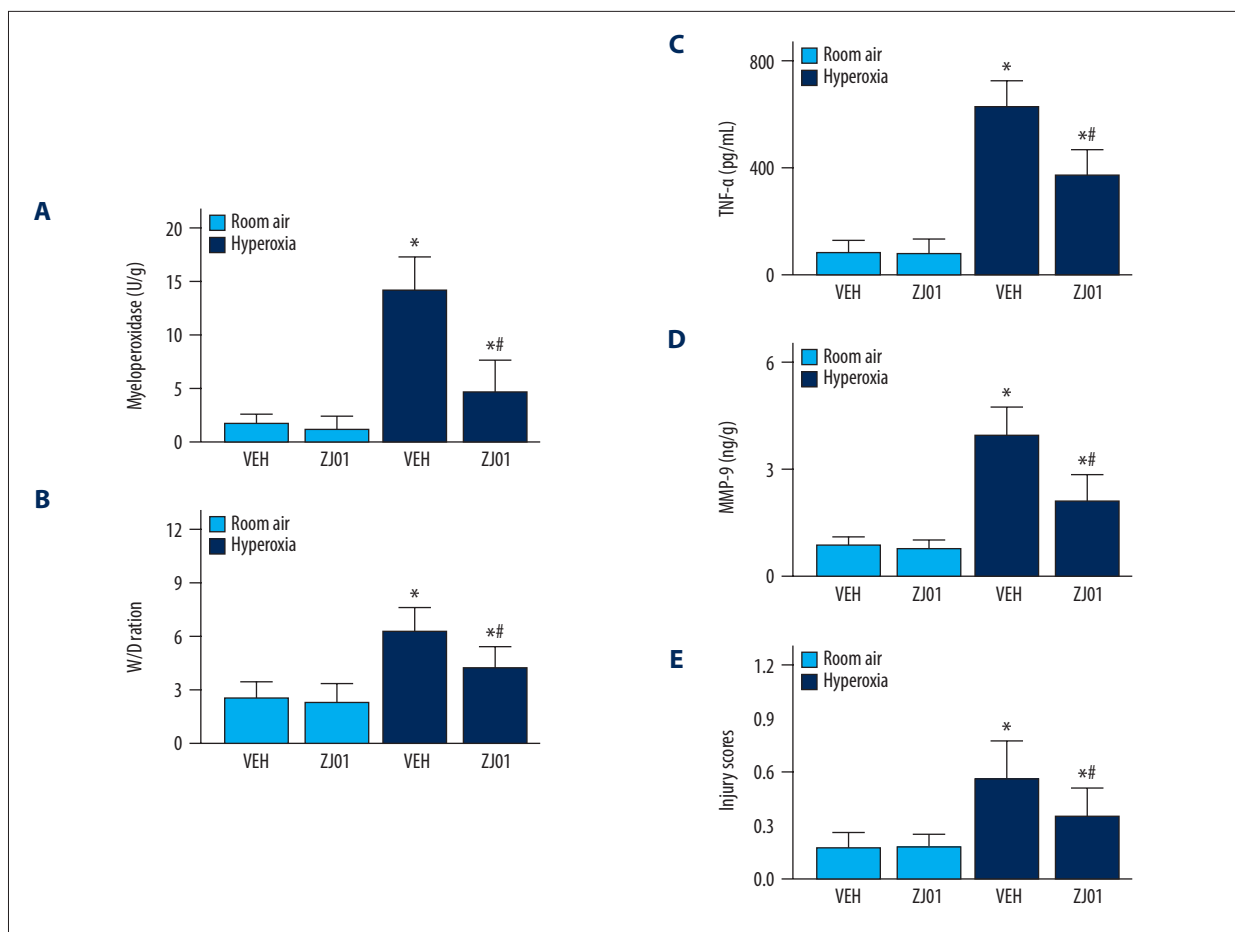
### The effects of ZJ01 on lung histology

Histology of the lung tissue in the mouse study groups was performed (Figures 3E, 4). Neutrophil infiltration and lung edema were found in mice that were exposed to

hyperoxia for 72 h (Figure 4C). ZJ01 treatment reduced the degree of hyperoxia-induced pulmonary edema and inflammation (Figures 3E, 4D).

### The effects of ZJ01 on heme oxygenase-1 (HO-1)

HO-1 is an important downstream effector of Nrf2. In this study, ZJ01 significantly increased the activity of HO-1. The mice were pretreated with the heme oxygenase-1 (HO-1) inhibitor, zinc protoporphyrin IX (ZnPP IX). As shown in Figure 5A, the ZJ01-triggered increase of HO-1 activity was significantly inhibited by ZnPP IX. Also, ZJ01-triggered reduction in the markers of inflammation and edema, including lipid hydroperoxide, MPO, the W/D ratio, and histological changes in the lung tissues, which were reversed by inhibition of HO-1 activity (Figures 4E, 5B–5D).



**Figure 3.** C57BL/6 mice were exposed to room air or hyperoxia and treated with and without ZJ01, an inhibitor of Kelch-like ECH-associated protein 1 (Keap1) protein-protein interaction (PPI). **(A)** Myeloperoxidase activity. **(B)** The ratio of wet to dry weight (W/D) of the lungs. **(C)** Levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). **(D)** levels of matrix metalloproteinase-9 (MMP-9). **(E)** Histology of the lungs. VEH, vehicle. \*  $P < 0.05$  vs. the room air+VEH group; #  $P < 0.05$  vs. the hyperoxia+VEH group.

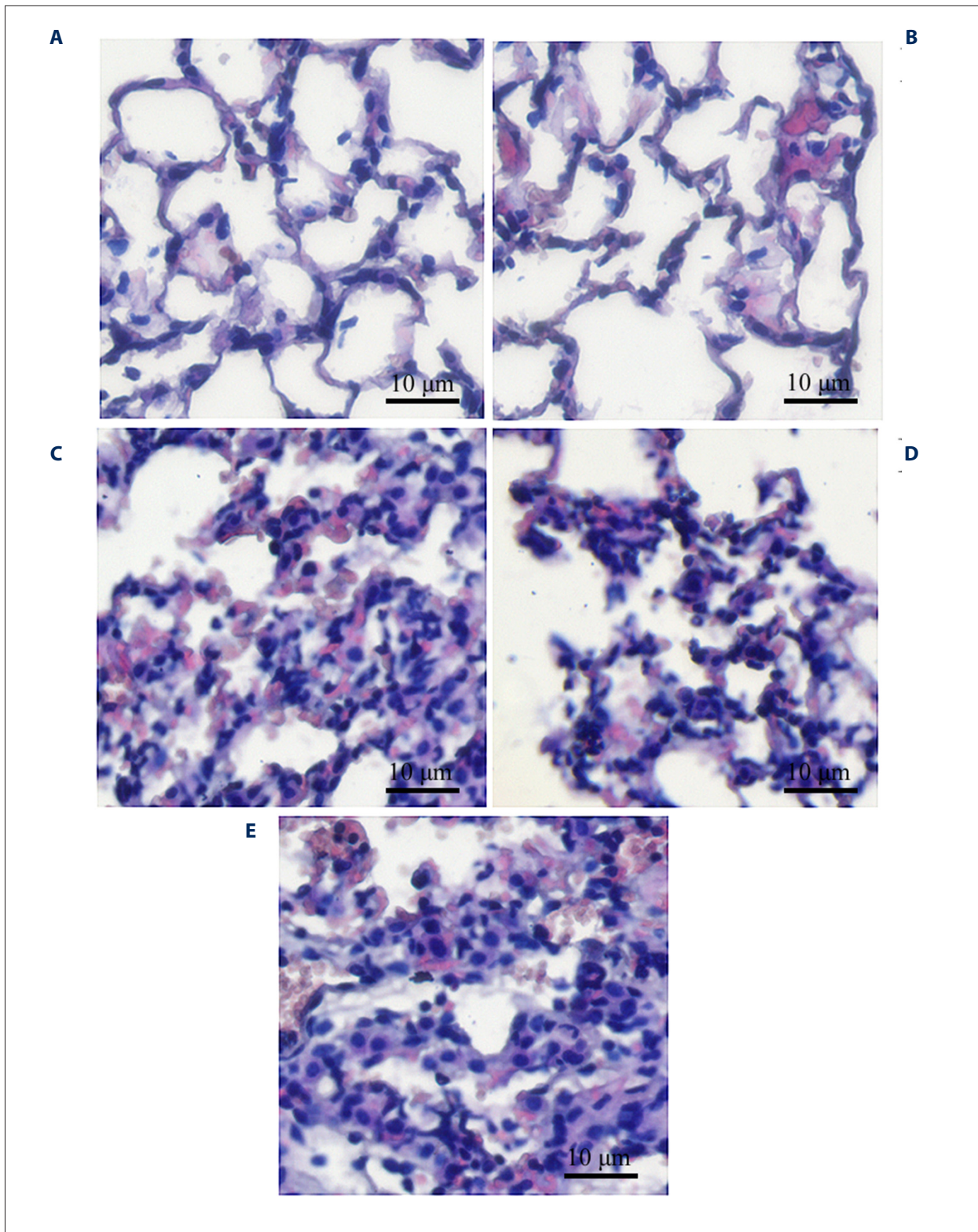
## Discussion

The aims of this study were to investigate the effects of ZJ01 and the heme oxygenase-1 (HO-1) inhibitor, zinc protoporphyrin IX (ZnPP IX), in a mouse model of hyperoxic acute lung injury (ALI). ZJ01 is a small molecule inhibitor of kelch-like ECH-associated protein 1 (Keap1) and nuclear factor erythroid-2-related factor 2 (Nrf2) protein-protein interaction (PPI) that can reduce Keap1-induced inhibition of Nrf2. Using a mouse model of ALI, the findings showed that ZJ01 reduced hyperoxia-induced histological lung changes, including neutrophil infiltration and lung edema. Also, the study findings showed that HO-1 was an effector in the effects of ZJ01 on the reduction of ALI in the mouse model.

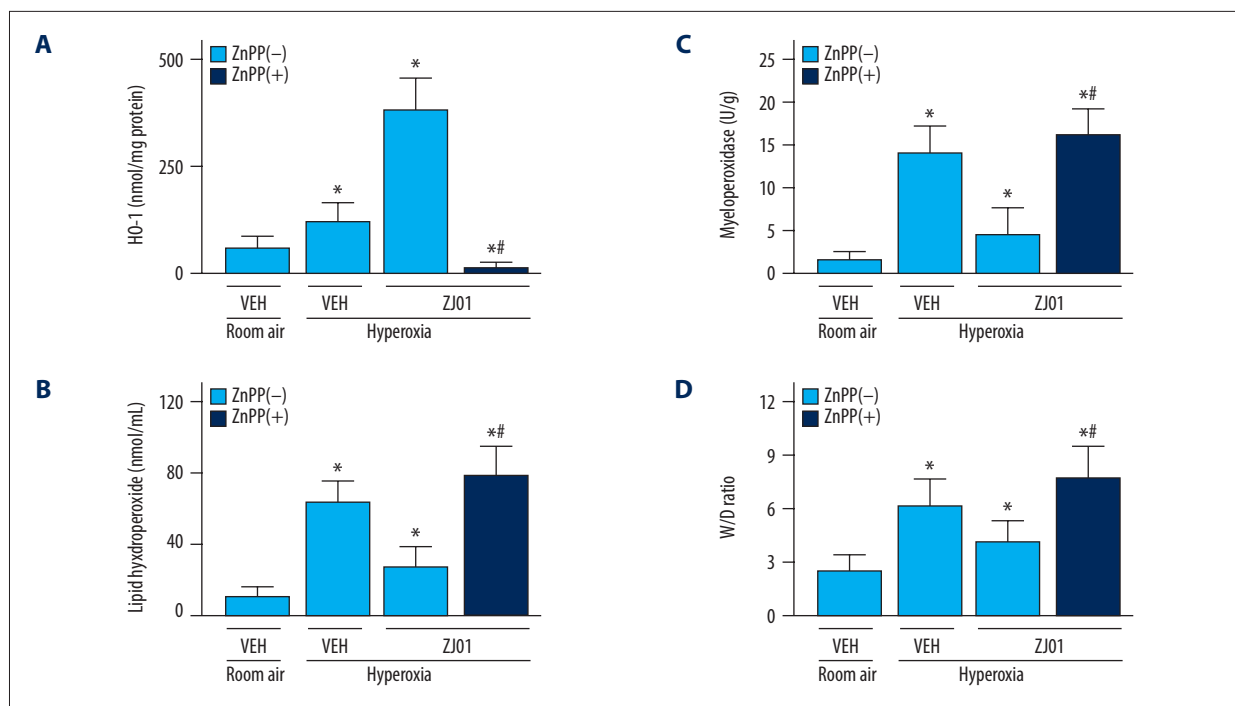
Acute respiratory distress syndrome (ARDS) is an inflammatory disorder [1]. Activated neutrophils infiltrate the lung and the release of pro-inflammatory mediators that have a central role in ARDS [1]. The activity of myeloperoxidase (MPO)

is a useful marker for the detection of neutrophil infiltration. The findings from the present study showed that the activity of MPO was inhibited by ZJ01 treatment. Also, the level of matrix metalloproteinase-9 (MMP-9) in the lung was reduced in ZJ01-treated mice. MMP-9 contributes to lung injury by degrading the extracellular matrix (ECM) during the progression of ALI [16]. Activated neutrophils are a major source of MMP-9 [17]. Inhibition of neutrophil infiltration in the lung by ZJ01 may contribute to the reduction of MMP-9 to reduce tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) levels in the lung. A previous study has shown that ZJ01 inhibited TNF- $\alpha$  in lipopolysaccharide (LPS)-stimulated H9c2 cardiac cells [12]. Another inhibitor of the Keap1-Nrf2 PPI, CPUY192018, reduced pro-inflammatory cytokines, including TNF- $\alpha$ , in HK-2 cells and in the kidney in mice that were stimulated by LPS [11]. The reduction of levels of TNF- $\alpha$  has previously been reported to be associated with reduced ALI [18].





**Figure 4.** Histology of the lung tissue sections from C57BL/6J mice in the five study groups exposed to room air or hyperoxia and treated with and without ZJ01 and zinc protoporphyrin IX (ZnPP IX). (A) The room air+vehicle group. (B) The room air+ZJ01 group. (C) The hyperoxia+vehicle group. (D) The hyperoxia+ZJ01 group. (E) The hyperoxia+ZJ01+zinc protoporphyrin IX (ZnPP IX) group.



**Figure 5.** C57BL/6 mice were exposed to room air or hyperoxia and treated with and without ZJ01, an inhibitor of Kelch-like ECH-associated protein 1 (Keap1) protein-protein interaction (PPI). **(A)** Heme oxygenase-1 (HO-1) activity. **(B)** The levels of lipid hydroperoxide. **(C)** The activity of myeloperoxidase. **(D)** The ratio of wet to dry weight (W/D) of the lungs. VEH, vehicle; ZnPP IX, zinc protoporphyrin IX. \*  $P < 0.05$  vs. the mice with room air+VEH treatment; #  $P < 0.05$  vs. the mice with hyperoxia+ZJ01 treatment.

ZJ01 has been previously reported to have antioxidant effects [12]. However, the effect of ZJ01 on ALI and the potential mechanisms involved have not previously been reported. Endogenous antioxidants are fundamental in host antioxidant defense mechanisms. The antioxidant effect of glutathione (GSH) has previously been reported [19]. An inhibitor of the Keap1-Nrf2 PPI has previously been shown to increase the levels of GSH levels in lipopolysaccharide (LPS)-stimulated HK-2 cells and in the kidney tissue in mice [7]. The findings from the present study showed that hyperoxia-induced reduction of GSH levels was reversed in the ZJ01-treated mouse model group.

Glutathione peroxidase (GPx) is a member of the reactive oxygen species (ROS)-scavenging enzymes. The upregulation of GPx is involved in the endogenous antioxidant capacity in several inflammatory lung diseases [20]. The findings from the present study showed that ZJ01 increased the activity of GPx. Also, the inhibition of the Keap1-Nrf2 PPI-induced increase in GPx was shown in a previous study [7]. Heme oxygenase-1 (HO-1) is an enzyme that has protective effects on the lungs during ALI [21,22]. HO-1 in modified bone marrow-derived mesenchymal stem cells reduced LPS-induced oxidative stress in cultured human pulmonary microvascular endothelial cells [23]. This study showed that HO-1 was significantly induced by ZJ01, which indicated that ZJ01 could induce antioxidants in hyperoxic ALI.

Nuclear factor erythroid-2-related factor 2 (Nrf2) is a potential therapeutic target for oxidative stress, as it regulates the expression of endogenous antioxidants [6,9]. Previous studies have shown that HO-1 is induced by an inhibitor of thioredoxin reductase-1 via Nrf2-dependent mechanisms in the lung epithelium [24]. Treatment with an activator of Nrf2 has previously been shown to reduce mortality in haem-induced acute chest syndrome in a mouse model of sickle cell disease, which was reduced when HO-1 was inhibited [25]. Intestinal ischemic post-conditioning induced upregulation of Nrf2 and HO-1 expression and was protective for intestinal ischemia-reperfusion injury-induced ALI [26]. These results support the vital role that Nrf2 plays in antioxidant defense mechanisms.

In 2018, Meng et al. showed that an inhibitor of the Keap1-Nrf2 PPI activated Nrf2 in H9c2 cardiomyocytes in a lipopolysaccharide (LPS)-induced mouse myocarditis model [27]. The findings from the present study showed that ZJ01 increased Nrf2 expression in hyperoxic ALI. The changes associated with hyperoxia-induced ALI were reduced in the ZJ01-treated group. These findings support that ZJ01, a small molecule inhibitor of Keap1-Nrf2 PPI that can reduce Keap1-induced inhibition of Nrf2 and hyperoxic ALI by the induction of Nrf2. Nrf2 inhibited oxidative stress and inflammation by regulating its downstream effectors. HO-1 was an important effector in the host



antioxidant defense mechanism in hyperoxia-induced ALI in mice following treatment with ZJ01.

## Conclusions

This study aimed to investigate the effects of ZJ01, a small molecule inhibitor of Kelch-like ECH-associated protein 1 (Keap1) protein-protein interaction (PPI), and the heme oxygenase-1 (HO-1) inhibitor, zinc protoporphyrin IX (ZnPP IX), in a mouse model of hyperoxia-induced acute lung injury (ALI). ZJ01 reduced hyperoxic ALI in the mouse model through the Nrf2/HO-1 pathway.

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## Conflict of interest

None.