



Hydrogen gas reduces HMGB1 release in lung tissues of septic mice in an Nrf2/HO-1-dependent pathway



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ABSTRACT

Background: Lung injury is a vital contributor of mortality in septic patients. Our previous studies have found that molecular hydrogen (H₂), which has anti-oxidant, anti-inflammatory, and anti-apoptosis effects, had a therapeutic effect on a septic animal model through increasing expression of nuclear factor-erythroid 2-related factor 2 (Nrf2). The aim of this research was to investigate the effects of 2% H₂ gas inhalation on sepsis-induced lung injury and its underlying mechanisms.

Methods: Male wild-type (WT) and Nrf2-knockout (Nrf2-KO) ICR mice underwent sham or cecal ligation and puncture (CLP) operation. Two percent of H₂ gas was inhaled for 60 min beginning at both 1 h and 6 h after sham or CLP surgery. To assess the severity of septic lung injury, the 7-day survival rate, wet/dry (W/D) weight ratio of lung tissue, lung histopathologic score, pro-inflammatory cytokines (tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), high-mobility group box 1 (HMGB1)), anti-inflammatory cytokine (interleukin 10 (IL-10)), antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and heme oxygenase 1 (HO-1)), and an oxidative product (malondialdehyde (MDA)) were detected after sham or CLP operation. The histopathologic changes were observed in lung tissues by hematoxylin and eosin (HE) staining, and pro-inflammatory cytokines (TNF-α and IL-6), anti-inflammatory cytokine (IL-10), antioxidant enzymes (SOD and CAT), and MDA were detected in lung tissues by an enzyme-linked immunosorbent assay (ELISA).

Results: The results indicated that 2% H₂ gas treatment increased the survival rates, decreased the W/D weight ratio and the lung injury score, alleviated the injuries caused by oxidative stress and inflammation, and induced HO-1 level but reduced HMGB1 level in WT but not Nrf2-KO mice. These data reveal that H₂ gas could suppress lung injury in septic mice through regulation of HO-1 and HMGB1 expression and that Nrf2 plays a main role in the protective effects of H₂ gas on lung damage caused by sepsis.

1. Introduction

As a severe systemic inflammatory response syndrome, sepsis is a primary cause of death in critical patients due to its high incidence, morbidity, and mortality. Major mechanisms of sepsis include unbalanced overproduction of inflammatory molecules, increased venular leukocyte aggregation, microthrombosis, and microvascular vasoconstriction. The overwhelming inflammatory responses eventually lead to lethal multiple-organ failure [1]. The lung is the first organ to fail in a sepsis-induced multiple organ dysfunction syndrome [2]. Pulmonary inflammatory response caused by upregulation of various pro-inflammatory factors (such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and high-mobility group box 1 (HMGB1)) and an infiltration of many inflammatory cells obviously contributes to high

mortality in sepsis [3]. Therefore, alleviating excessive pulmonary inflammation during sepsis reduces the mortality rate of septic patients.

Molecular hydrogen (H₂) is a kind of selective antioxidant which can be effectively used to treat > 70 kinds of diseases so far [4]. Our previous researches have already reported that H₂ gas or H₂-rich saline exerted protective effects on many diseases, such as sepsis, stroke, traumatic brain injury, ischemia-reperfusion injury and neurodegenerative diseases through regulating of inflammatory response, oxidative stress, and neuronal apoptosis [5–10]. We have also reported that 2% H₂ gas inhalation may be useful for alleviating lung injuries [11]. However, its specific mechanisms have not yet been found.

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is one of the major factors that may regulate a proper innate immune response, which determines the survival rates during sepsis [7]. Heme oxygenase-1 (HO-

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1), as one of the main downstream molecules of Nrf2, has been reported to regulate oxidative stress and inflammatory responses in multiple diseases and pathological conditions [12–14]. Under the conditions of sepsis, Nrf2 moves into the nucleus to attach to the cis-acting enhancer antioxidant response element (ARE) sequence and then activates its downstream molecule HO-1. However, the increasing activation of HO-1 depressed the expression of HMGB1, which is one of the potential “late” pro-inflammatory mediators and may serve as a diagnostic biomarker of sepsis [6,15].

Based on our previous studies, this research aimed to investigate the key role of Nrf2 in the protective effects of H₂ gas on lung injury induced by severe sepsis through constructing cecal ligation and puncture (CLP) models both in wild-type (WT) and Nrf2 knockout (Nrf2-KO) mice.

2. Materials and methods

2.1. Experimental animals

Male WT and Nrf2-KO ICR mice (both 6 to 8 week-old and weighing 20 to 25 g) were obtained from the Better Biotechnology Company (Nanjing, China). Mice (5 mice per cage) were housed in a controlled 12-h light-dark environment at 22 to 25 °C with enough access to water and food. All experiments were approved by the Animal Experimental Ethics Committee of Tianjin Medical University General Hospital, Tianjin, China.

2.2. Reagents

Superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) testing kits (Nanjing Jiancheng Bioengineering Institution, China), enzyme-linked immunosorbent assay (ELISA) kits for TNF- α , IL-6, IL-10 (Solarbio, China), polyclonal anti-rabbit HMGB1 (Proteintech, China), polyclonal anti-mouse HO-1 (Abcam, USA), anti-rabbit β -actin (Abcam, USA), horseradish peroxidase-conjugated secondary antibody (Zhongshan Jinqiao Biological Technology Co., Ltd. China), and anti-mouse FITC fluorescent-labeled secondary antibody were used for this study (or the present experiments).

2.3. Protocols

Male WT and Nrf2-KO ICR mice were randomly divided into 4 \times 2 groups (4 groups for WT mice and 4 groups for Nrf2-KO mice, $n = 48$ /group, $N = 384$): Sham group, Sham + H₂ group, CLP group, and CLP + H₂ group. The sepsis model was produced by proceeding CLP operation in CLP and CLP + H₂ groups. The Sham group underwent the same process except for cecal ligation and puncture. H₂ gas (2%) was inhaled for 60 min by Sham + H₂ and CLP + H₂ groups at 1 h and 6 h after the operation, while Sham and CLP groups treated with air inhalation only. At 24 h after sham or CLP operation, 20 mice of each group (160 in total) were randomly separated to record survival rates. Other mice were sacrificed and obtained the lung tissues in different groups. Lungs from 6 mice of each group were used for measurement of lung wet/dry (W/D) weight ratio (48 in total) and lung tissue slices were prepared from 4 mice for histomorphological procedure, immunohistochemistry (HMGB1), and immunofluorescence (HO-1) (32 in total). Six mice from each group were used for each of ELISA test of TNF- α , IL-6, and IL-10 (48 in total), detection of the SOD and CAT activity, and MDA levels (48 in total), and western blotting (48 in total) (Fig. 1).

2.4. CLP model

CLP models were established under the instruction of our previous study [7]. In brief, mice were anesthetized and disinfected routinely. Abdominal skin was cut through the midline. The cecum was found and

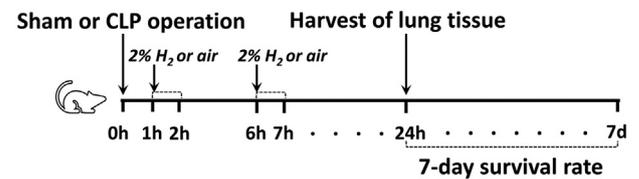


Fig. 1. Experimental design.

Male WT and Nrf2-KO ICR mice (aged 6–8 weeks, weighing between 20 and 25 g) received the sham or CLP operation. Two percent hydrogen gas (H₂) or fresh air was inhaled for 60 min beginning at both 1 h and 6 h after sham or CLP operation. Mice in different groups were randomly separated for survival rate recording from day 1 to day 7. The lung tissues in different groups were harvested at 24 h after sham or CLP operation. Different groups of lung tissues were used for all the tests, except for 7-day survival rates, as described in [Materials and methods](#).

ligated at approximately 3/4 of it at the back of the ileocecal valve. Intestinal content was squeezed out through a puncture in the cecum. The peritoneum and skin was sutured after all the procedures were completed. Saline (1 ml) was injected subcutaneously into the back of each mouse in all the groups immediately after operation as liquid resuscitation.

2.5. H₂ gas inhalation

We used a CGH-300 high-purity H₂ generator (Tianjin TongPu Analysis Instrument Technology Co., Ltd.) to produce H₂ gas as described previously [6]. Mice were placed into a sealed resin box with air inlet and outlet. A mixture of H₂ gas and air was transported into the box by the generator. Concentration of H₂ gas was analyzed and maintained at 2% by a PG210 H₂ detector (Henan Inte Electrical Equipment Co., Ltd.).

2.6. Survival rates

The survival rates of mice (20 mice in each group) were recorded for 7 days after operation procedures. All the mice were bred under the same controlled conditions with enough access to food and water.

2.7. Preparation of lung tissue slices

At 24 h after sham or CLP operation, mice were killed and perfused with 4% paraformaldehyde, and all the lung samples were obtained. Samples were fixed in 10% buffered formalin for 24 h at room temperature, then dehydrated with ethyl alcohol, embedded in paraffin blocks, and sectioned into coronal sections with a thickness of 5 μ m.

2.8. Histomorphological procedure

Lung sections of different groups were stained after deparaffinization and rehydration for the hematoxylin and eosin (HE) staining. The morphological structure was observed and compared by light microscopy at a magnification of 400 times (X40, Leica, Germany). Lung injury was evaluated by observing edema, congestion, neutrophil accumulation, endobronchial hemorrhage, and cell proliferation. The score of each item was divided by the order of severity into 0, 1, 2, and 3. The lung injury degree was evaluated by the sum points of all 5 items.

2.9. Lung wet/dry (W/D) weight ratio measurement

As soon as lung tissues were obtained, washed thoroughly with saline, and excess moisture was absorbed by a filter paper, the wet weight (W) was obtained using an electronic balance, then the dry weight (D) was measured after the samples were dried at 80 °C by an

oven for 24 h. Lung W/D weight ratio was calculated as follows: $W/D = [\text{wet weight} / \text{dry weight}] \times 100\%$.

2.10. Immunohistochemistry

After slices were deparaffinized and antigen retrieval was performed by sodium citrate, slices were blocked with H_2O_2 for 10 min, rinsed in PBS 3 times for 5 min each, and incubated in polyclonal rabbit anti-mouse HMGB1 primary antibody (1:100) in 4°C for overnight. Slices were rinsed for 3 times every other day and incubated with goat anti-rabbit secondary antibody at 37°C for 1 h. Slices were developed using the 3,3'-diaminobenzidine (DAB) method and then redyed with hematoxylin. The expression of HMGB1 was observed by calculating the percentage of the brown particles (HMGB1 positive cells) to all the cells under a light microscope.

2.11. Immunofluorescence

Slices were fixed with 4% paraformaldehyde for 20 min and rinsed for 3 times in 1xPBS. After permeabilized with 0.3% Triton X-100 for 20 min and rinsed for 3 times, The slices were blocked with 10% FBS for 2 h. Diluted anti-mouse HO-1 (1:100, Abcam) antibody in 1xPBS was applied to slices and the slices were incubated overnight at 4°C . Incubating buffer was removed and the slices were rinsed 3 times, then incubated with diluted corresponding fluorescent-labeled secondary antibodies (anti-mouse fluorescein isothiocyanate (FITC), 1:100) in the dark for 1 h. The slices were rinsed 3 times with the buffer and incubated in 4,6-diamidino-2-phenylindole (DAPI) for 5 min, and then we chose 3 random fields under a fluorescence microscope and observed the fluorescence field.

2.12. Elisa

Animals were sacrificed and perfused using saline at 24 h after modeling. Lungs were harvested, grinded, and centrifuged. TNF- α , IL-6, and IL-10 levels in the supernatant were tested using an ELISA kit. Experiments were carried out following the kit instructions.

2.13. Detection of the antioxidant enzymes SOD and CAT activity and the oxidative product MDA production

The lung tissues were obtained to analyze the activities of SOD, CAT, and level of oxidative product MDA at 24 h after operation by using the SOD, CAT testing kits (Nanjing Jiancheng Bioengineering Institution, China), and MDA testing kits (Nanjing Jiancheng Bioengineering Institution, China). The experiments were performed according to the kit instructions.

2.14. Western blotting

After perfusion with saline, lung tissues obtained were weighted and homogenized on ice with phenylmethylsulfonyl fluoride (PMSF) for radioimmunoprecipitation assay (RIPA), followed by centrifugation at 10,000 R/min at 4°C for 10 min. Samples were set still on the ice for 30 min, then centrifuged at 15,000 R/min at 4°C for 10 min, and the supernatant was collected. Total protein content was detected by a Bradford method.

The proteins in different groups were size-separated in 10% sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 90 min and transferred to polyvinylidene difluoride (PVDF) membrane at 100 V for 1 h. Then, the membranes were blocked with 5% nonfat milk for 2 h. The membranes were incubated with polyclonal anti-rabbit HMGB1 (1:6000, Proteintech), polyclonal anti-mouse HO-1 (1:6000, Abcam), and anti-rabbit β -actin (1:8000, Abcam) diluted in tris-buffered saline with Tween (TBST) buffer overnight at 4°C . All membranes were rinsed for 5 times with $1 \times$ TBST buffer, then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Protein bands were scanned, and integrated density values (IDVs) were calculated with the GelPro program and normalized with that of β -actin.

2.15. Statistics

Survival rates in different groups are reported as percentages (%) and the other data are expressed as means \pm standard deviation (SD). Long-rank (Mantel-Cox) test was used to analyze the difference of survival rates among groups, and unpaired *t*-test (if the values were Gaussian distribution) or Mann-Whitney test (if values were not Gaussian distribution) was used to analyze the difference between sham and CLP or CLP + H_2 with CLP groups. One-way ANOVA was used to analyze the interaction among all groups. $P < 0.05$ was considered to be statistically significant, and the significance testing was two-tailed. Statistical analysis was conducted by a GraphPad Prism software (version 5.0) and SPSS statistic software (version 16.0).

3. Results

3.1. Survival rates of WT septic mice but not Nrf2-KO septic mice were enhanced by 2% H_2 gas treatment

When compared to the Sham and Sham + H_2 groups, the 7-day survival rates of both the WT CLP and Nrf2-KO CLP groups decreased significantly ($P < 0.05$). After H_2 treatment, survival rates of WT mice in the CLP + H_2 group rose up (vs. CLP in WT mice: $P < 0.05$). However, the survival rate of Nrf2-KO mice in the CLP + H_2 group showed no difference compared to its CLP group ($P > 0.05$) (Fig. 2).

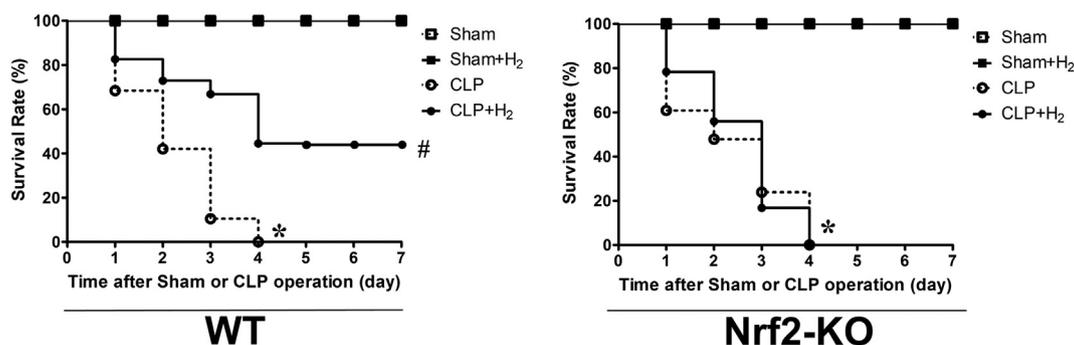


Fig. 2. Effects of H_2 on survival rates in WT and Nrf2-KO mice.

The survival rates were monitored for 7 days in both WT and Nrf2-KO mice. Values are shown as survival percentage ($n = 20$ per group). * $P < 0.05$ vs. the sham group; # $P < 0.05$ vs. the CLP group.

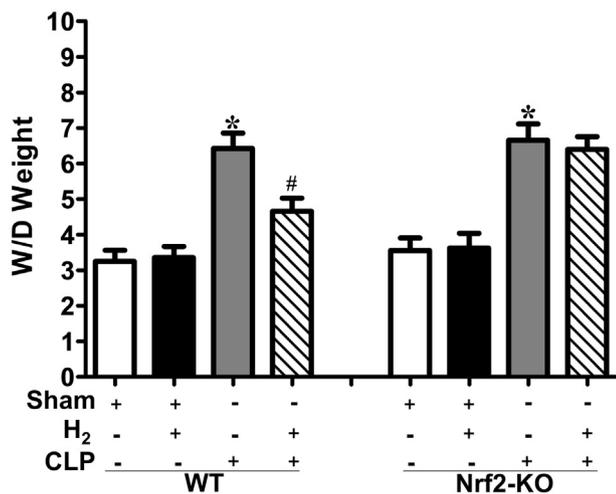


Fig. 3. Effects of H₂ on lung wet/dry weight ratio in WT and Nrf2-KO mice. Mice were sacrificed at 24 h after CLP or sham operation, and wet weight was obtained as soon as lungs were harvested. The dry weight was measured after the samples were dried at 80 °C by an oven for 24 h (n = 6 per group). Lung wet/dry weight ratio was calculated as follows: W/D = [wet weight/dry weight] × 100%. *P < 0.05 vs. the sham group; #P < 0.05 vs. the CLP group.

3.2. Lung W/D weight ratio of WT septic mice but not Nrf2-KO septic mice decreased after 2% H₂ treatment

Lung W/D weight ratios were higher in CLP groups both in WT and Nrf2-KO mice compared to their Sham groups separately (P < 0.05). After H₂ treatment, lung W/D weight ratios in CLP + H₂ group decreased significantly in WT mice (vs. CLP in WT mice: P < 0.05), while there was no significant difference between the CLP + H₂ and CLP groups in Nrf2-KO mice (vs. CLP in Nrf2-KO mice: P > 0.05). (Fig. 3).

3.3. Lung injury and inflammation were alleviated by 2% H₂ gas treatment in WT septic mice but not in Nrf2-KO septic mice

HE staining showed that in CLP groups in both WT and Nrf2-KO mice, lung tissue fractured, alveolar collapsed with obvious

inflammatory cell infiltration, and lung injury scores increased compared with their Sham groups (P < 0.05) (Fig. 4A). After H₂ inhalation, lungs in WT mice of the CLP + H₂ group showed a more complete lung tissue structure with fewer inflammatory cells, and lung injury score was also decreased (vs. CLP in WT mice: P < 0.05). However, the CLP + H₂ group in Nrf2-KO mice showed no obvious difference compared with its CLP group (P > 0.05) (Fig. 4B).

3.4. Two percent H₂ gas treatment decreased the expression of TNF-α and IL-6 but increased IL-10 in WT but not Nrf2-KO mice with severe sepsis

Compared to their Sham and Sham + H₂ groups, the expression of TNF-α, IL-6, and IL-10 increased obviously in CLP groups both in WT and Nrf2-KO mice (P < 0.05). In WT mice, after inhalation of H₂ gas, the levels of pro-inflammatory cytokines, such as TNF-α and IL-6, decreased markedly in the CLP + H₂ group compared with those of the CLP group, but the level of anti-inflammatory cytokine IL-10 increased compared to CLP injury alone (P < 0.05). However, Nrf2-KO mice still showed no significant differences between the CLP + H₂ and CLP groups (P > 0.05) (Fig. 5).

3.5. Two percent H₂ gas inhalation reversed the imbalance of the oxidative stress status in WT but not Nrf2-KO septic mice

The activity of antioxidant enzymes including SOD and CAT decreased significantly, but the oxidative product MDA increased markedly after CLP procedure in WT mice (vs. Sham in WT mice, P < 0.05). H₂ gas inhalation could increase the activity of SOD and CAT and decrease the level of MDA in WT septic mice (P < 0.05), but not in Nrf2-KO septic mice (CLP + H₂ vs. CLP in Nrf2-KO mice, P > 0.05) (Fig. 6).

3.6. Expressions of HO-1 and HMGB-1 changed differently in WT and Nrf2-KO septic mice after H₂ gas treatment

The HO-1 expression was observed using immunofluorescence. Fluorescence was stronger in the CLP group compared to the Sham group in WT mice. Moreover, H₂ gas treatment was found to increase the HO-1 level in the CLP + H₂ group in comparison with that of the CLP group in WT mice (vs. CLP in WT mice, P < 0.05). However, the CLP and CLP + H₂ groups in Nrf2-KO mice showed no statistically significant difference (CLP vs. CLP + H₂ in Nrf2-KO mice, P > 0.05)

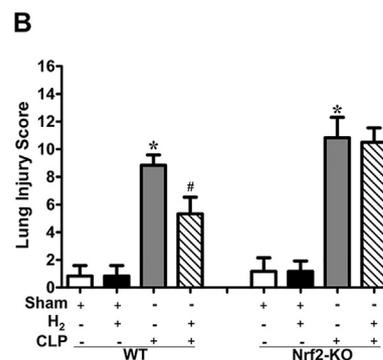
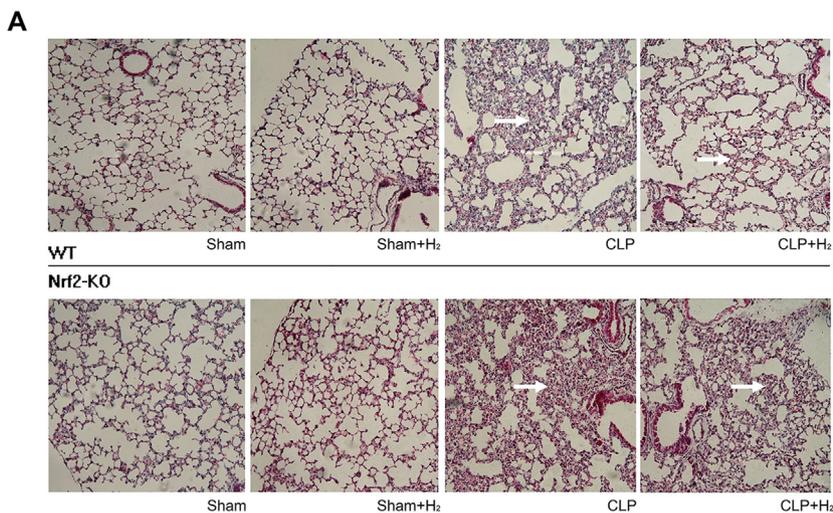


Fig. 4. Effects of H₂ on lung injury in WT and Nrf2-KO mice. Mice were sacrificed at 24 h after CLP or sham operation, lung tissue sections were made and stained with hematoxylin and eosin, morphological structure (A) was observed, and the inflammatory foci are indicated by horizontal arrows (original magnification ×200). Lung injury was scored by observing the morphological structure (B). Data are shown as mean ± SD (n = 4 per group). *P < 0.05 vs. the sham group; #P < 0.05 vs. the CLP group.

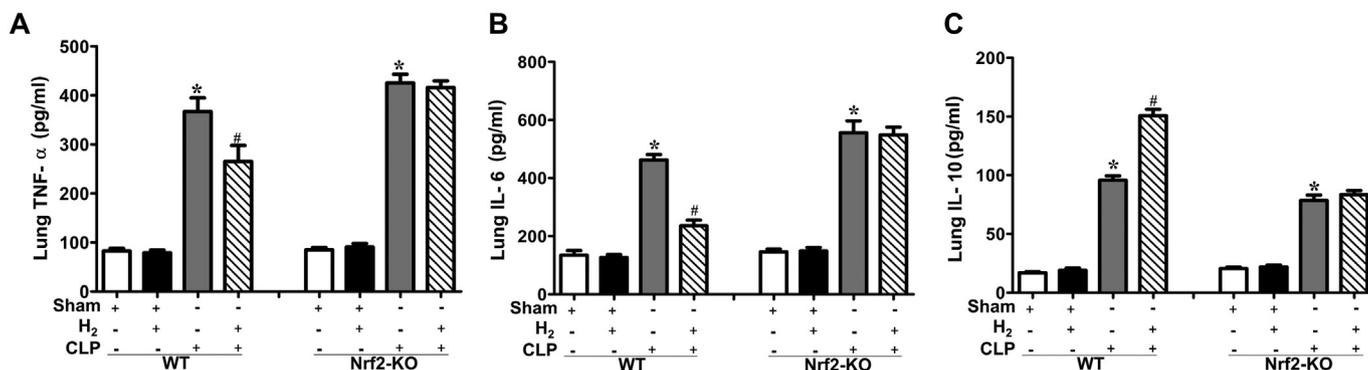


Fig. 5. Effects of H₂ on pro-inflammatory and anti-inflammatory cytokines in lung tissue of WT and Nrf2-KO mice. Mice were sacrificed at 24 h after sham or CLP operation, and the lung tissue in different groups was harvested for testing (A) TNF-α levels, (B) IL-6 levels, and (C) IL-10 levels. Data are shown as mean ± SD (n = 6 per group).

*P < 0.05 vs. the sham group; #P < 0.05 vs. the CLP group.

(Fig. 7A, B).

Then, expression of HMGB1 was tested with immunohistochemistry. In WT mice, compared to Sham, brown staining (positive cells) increased in the CLP group. However, in the CLP + H₂ group, the expression of HMGB1 was alleviated (vs. CLP in WT mice, P < 0.05). In addition, the CLP and CLP + H₂ groups in Nrf2-KO mice showed no statistically significant difference (CLP vs. CLP + H₂ in Nrf2-KO mice, P > 0.05) (Fig. 8A, B).

To further assure the expression of these 2 proteins, western blot was used. As the western blot results between CLP and Sham groups in pathologic conditions such as sepsis showed that expressions of HO-1 and HMBG-1 were increased in both WT and Nrf2-KO mice (P < 0.05). H₂ treatment increased HO-1 but decreased HMGB-1 levels in the CLP + H₂ group in WT mice (vs. CLP in WT mice, P < 0.05). However, H₂ showed anti-inflammatory effects in WT mice, which were reversed in Nrf2-KO mice, and there was no significant difference between the CLP and CLP + H₂ groups in Nrf2-KO mice (CLP vs. CLP + H₂ in Nrf2-KO mice, P > 0.05) (Figs. 7C, D, 8C, D).

4. Discussion

Multi-organ dysfunction syndrome (MODS) is considered to be one of the deadly complications of sepsis. Since the lungs are commonly affected by MODS, lung injury is an important cause of mortality in sepsis, which occurs in approximately 30% of septic patients [16]. Unfortunately, other than protective ventilator strategies, there have been no specific treatments available until now [17]. The damage of alveolar endothelial cells is an important mechanism of sepsis-induced lung injury. Damaged endothelial cells can lead to fluid transport

disorder and endothelial cell clearance, which causes inflammatory cell infiltration, then resulting in pulmonary edema and pulmonary parenchymal injury with pulmonary dysfunction [5,18]. Other mechanisms include oxidative stress and apoptosis.

H₂ is an odorless, colorless reducing agent. H₂ cannot be absorbed easily by the human body for its low solubility. Hence, its biological ability in medicine has been neglected [19]. However, a study by Oh-sawa et al. [20] demonstrated in 2007 that H₂ showed a protective effect against oxidative damage. Following studies revealed that H₂ has anti-inflammatory, anti-oxidative, and anti-apoptotic effects [5,7]. Recent researches have reported that H₂ gas plays a protective role against sepsis in vital organs, such as brain, intestines, and kidneys, in septic models [15,21,22]. Our previous studies [6,7,23] showed that H₂ could reduce acute lung injury caused by sepsis in CLP-induced septic mice by alleviating inflammatory response and releasing oxidative stress; however, the underlying mechanism is still ambiguous.

To explore the mechanism, Nrf2 knock-out mice were used to make a contrast. As one of the CNC family members, Nrf2 is a crucial transcription factor that regulates immune stress, antioxidant and anti-inflammatory responses in pathological situations, including sepsis and ischemic stroke [24,25]. In order to evaluate the effect of H₂ gas treatment in septic mice in general, the survival rates of mice were recorded for 7 days. H₂ gas inhalation significantly improved the survival rates in WT septic mice, while it failed to protect Nrf2-KO mice. Moreover, for confirming the damage caused by sepsis, lung injury score and lung water content were calculated in lung tissue as indexes for evaluating lung injury. Inflammatory status causes the disruption of the pulmonary endothelial barrier, increases the permeability of alveolar epithelial cells, damages liquid transferring ability of lung cells,

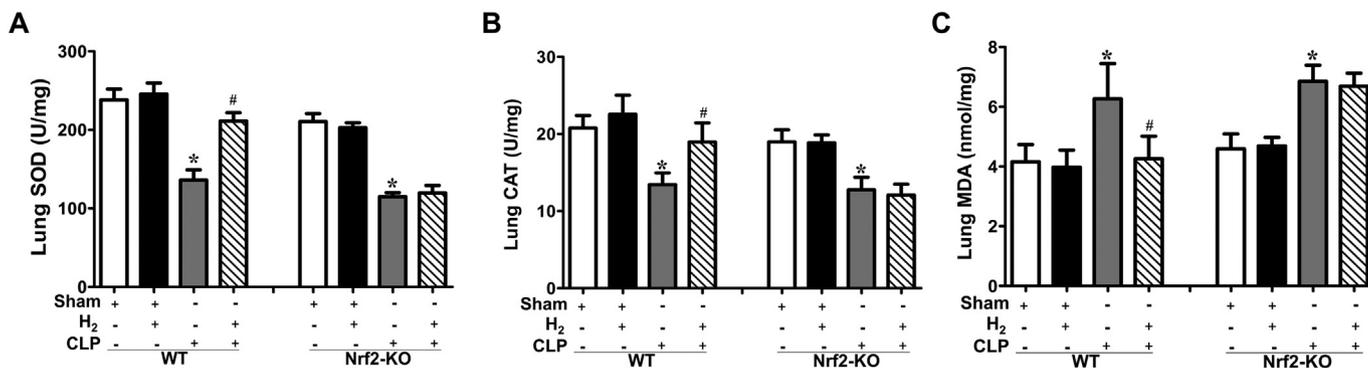


Fig. 6. Effects of H₂ on antioxidant enzymes and oxidative products in lung tissue of WT and Nrf2-KO mice. At 24 h after sham or CLP operation, lung tissue was harvested to determine the levels of (A) SOD, (B) CAT, and (C) MDA. Values are expressed as mean ± SD (n = 6 per group).

*P < 0.05 vs. the sham group; #P < 0.05 vs. the CLP group.

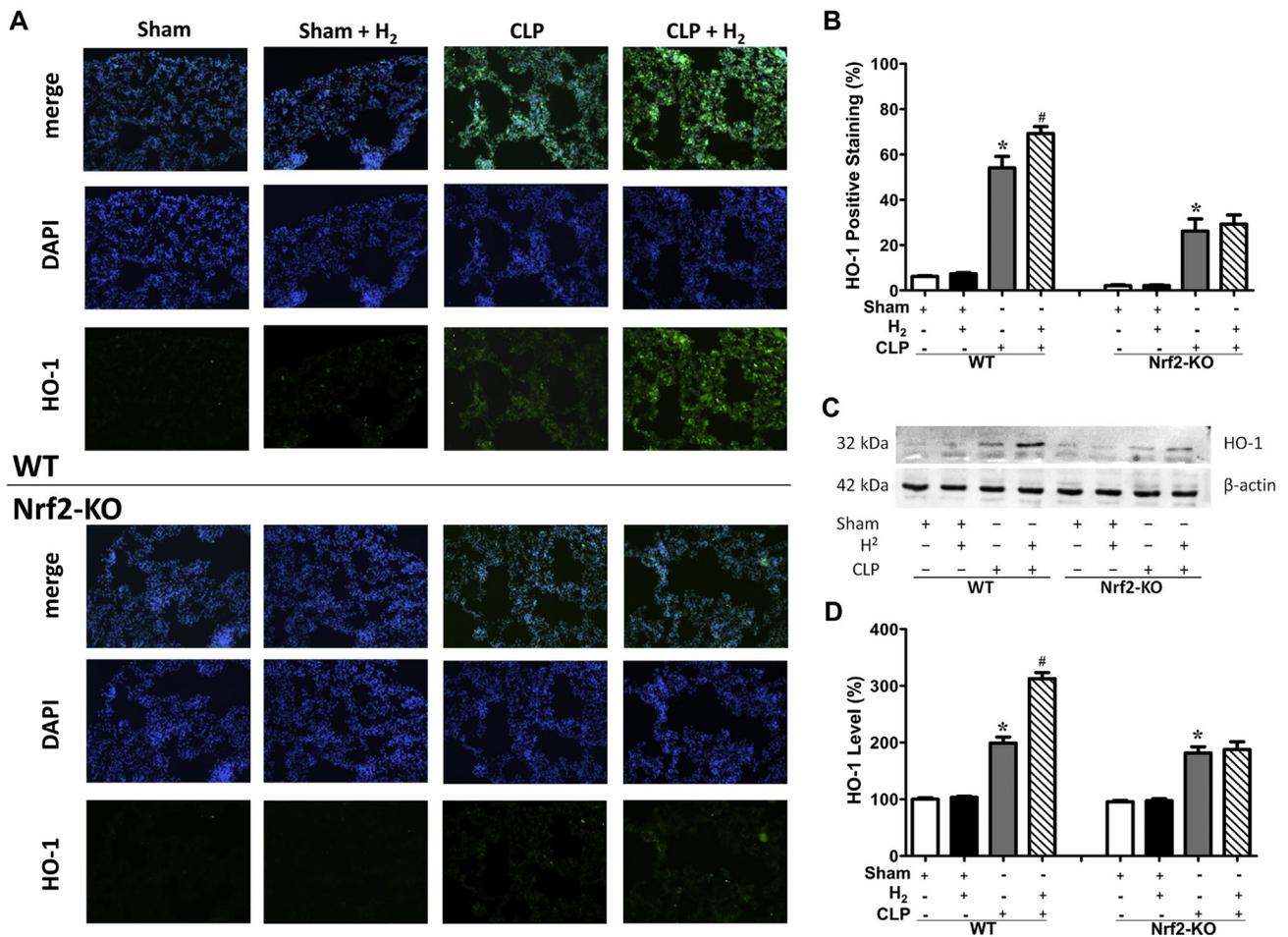


Fig. 7. Effects of H₂ on HO-1 expression in WT and Nrf2-KO mice. At 24 h after sham or CLP operation, (A) immunofluorescence (original magnification, 200×; exposure time was 0.5 s for HO-1 and 0.1 s for DAPI) and (C) western blotting were used to detect HO-1 expression. Quantitative analyses of HO-1 expression observed with (B) immunofluorescence staining and (D) are represented as percentage of positive staining and percentage change of the protein level compared to sham levels, respectively. Values are shown as mean ± SD (n = 4 per group in immunofluorescence and n = 6 per group in western blotting test). *P < 0.05 vs. the sham group; #P < 0.05 vs. the CLP group.

and eventually causes lung edema [26,27]. Lung edema is positively correlated with lung inflammatory status, as lung W/D weight ratios of CLP groups were higher in both WT and Nrf2-KO mice, corresponding to the results of lung injury scores. H₂ gas treatment alleviated edema, inflammatory infiltrate, and associated lung injury in WT but not Nrf2-KO mice.

Oxidative stress is shown as a result of the insufficient activity of endogenous antioxidant defense system against reactive oxygen species (ROS) [28]. For one thing, excessive ROS can lead to oxidative damage of organisms, which produces relatively stable oxidized biomolecule products, including MDA and 8-hydroxy-2-deoxyguanosine (8-OHdG). For another, the antioxidant levels and antioxidant enzyme activity (SOD, CAT, and HO-1) obviously reduced in the oxidative stress-related diseases [29]. Therefore, either impaired antioxidant systems or enhanced ROS production will result in the change of cellular redox balance to oxidative imbalance and leading to the over-production of ROS [30]. During sepsis caused by CLP, oxidant production MDA increased dramatically, whereas the activity of SOD, CAT, and HO-1 was suppressed. After H₂ gas treatment, the level of MDA dropped, and the activity of SOD, CAT, and HO-1 were enhanced significantly in WT but not Nrf2-KO mice.

The suppression of inflammatory status is considered to be crucial importance in treating septic lung injury [31]. Potent pro-inflammatory cytokines, including TNF-α, IL-6, HMGB1, and anti-inflammatory

cytokines, such as IL-10, are vital in inflammatory responses during the onset and progression of lung injury [32]. When the tissues or organs are injured by sepsis, the mononuclear phagocyte system in the human body will release various types of “early” pro-inflammatory cytokines that have thus far been identified as TNF-α and IL-6, and a type of “late” pro-inflammatory cytokine named HMGB1. HMGB1, which appears first in the extracellular matrix at 8–12 h after the initial reaction in which macrophages respond to proinflammatory stimulation, is a widely-expressed and highly abundant protein that acts as an extracellular signal upon active secretion by immune cells or passive release by dead, dying, and injured cells [33]. At the same time, our mononuclear phagocyte system also releases IL-10, an anti-inflammatory factor, which plays a crucial role in reducing inflammatory response and antagonizing inflammatory mediators under the condition of sepsis. In our present study, there were TNF-α, IL-6, HMGB1, and IL-10 expressed in the CLP group more than those in the Sham group both in WT and Nrf2-KO mice. However, 2% H₂ gas inhalation reversed the imbalance of inflammatory status by decreasing the production of pro-inflammatory cytokines (TNF-α, IL-6, and HMGB1) and increasing the production of anti-inflammatory cytokines (IL-10), thus improving the recovery of pulmonary injury in the CLP-induced lung injured WT mice model, but not the Nrf2-KO mice model.

In our study, 2% H₂ gas inhalation successfully alleviated morphological injury of lung tissue, reduced inflammatory cell infiltration, and

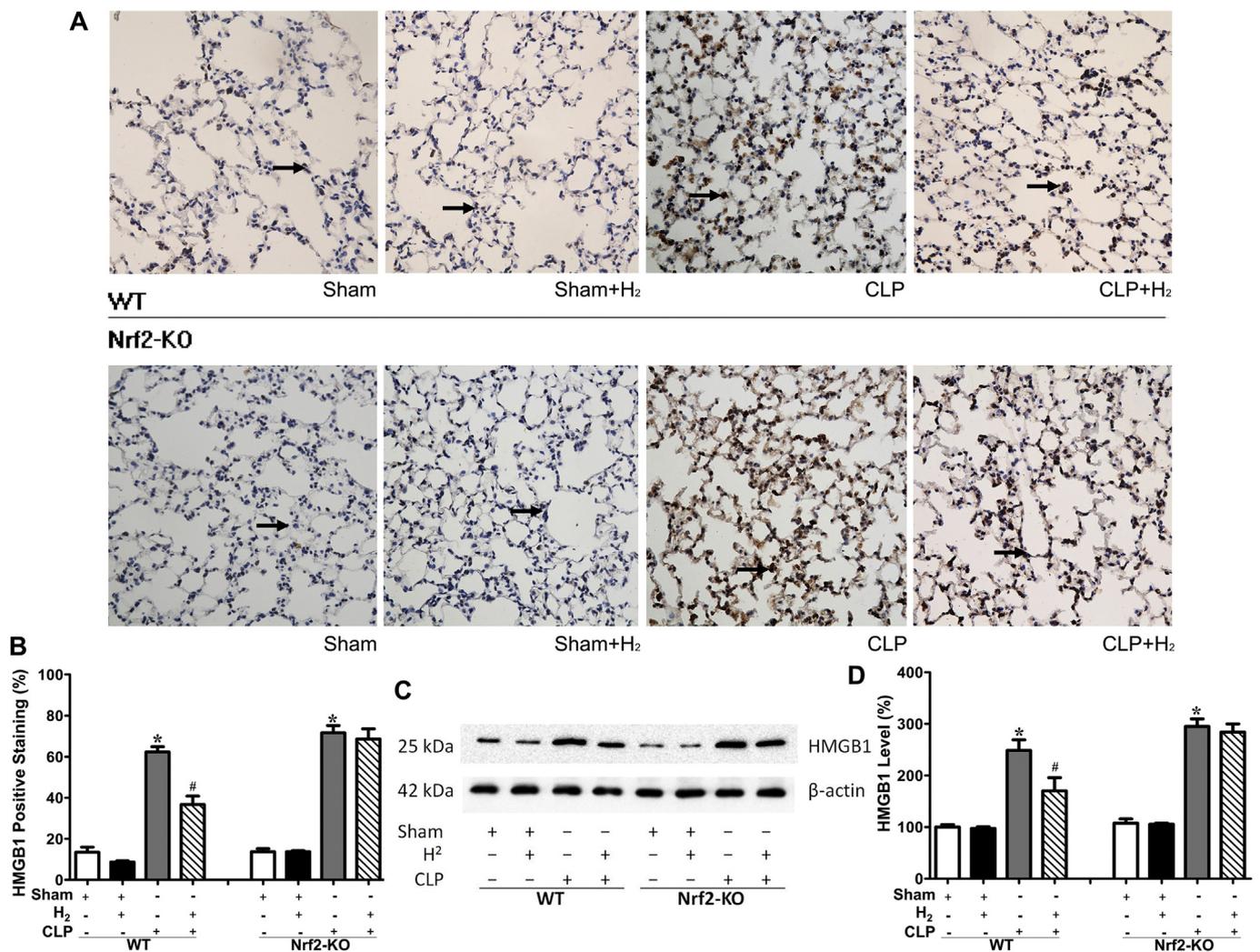


Fig. 8. Effects of H₂ on HMGB1 expression in WT and Nrf2-KO mice. At 24 h after sham or CLP operation, (A) immunohistochemical staining (original magnification, 200×) and (C) western blotting were used to detect HMGB1 expression. Quantitative analyses of HMGB1 expression observed with (B) immunohistochemical staining and (D) are represented as percentage of positive staining (black arrows) and percentage change of the protein level compared to sham levels, respectively. All data in this part are expressed as mean ± SD (n = 4 per group in immunohistochemical staining and n = 6 per group in western blotting test). *P < 0.05 vs. the sham group; #P < 0.05 vs. the CLP group.

enhanced the expression of protective factor HO-1, but reduced the transfer of HMGB1. However, this protective effect was regulated by Nrf2. H₂ showed no protective effect in all Nrf2-KO mice, indicating that Nrf2 regulates the expression of HMGB1 and HO-1, therefore releases the damage of oxidative stress and sepsis-induced lung injury. Together, these data indicated that H₂ gas plays a key role in protecting against inflammation and oxidative stress through stimulating HO-1 and suppressing HMGB1 expression. However, H₂ gas had no therapeutic effects on septic mice without the expression of Nrf2.

5. Conclusion

Collectively, H₂ gas could suppress lung injury in septic mice via regulating of HO-1 and HMGB1 expression, and Nrf2 plays a main role in the protective effects of H₂ against lung injury caused by sepsis.

Conflict of interest statement

The authors declare no conflicts of interest, financial or otherwise.

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