Protective Effects of Hydrogen Gas on Murine Polymicrobial Sepsis via Reducing Oxidative Stress and HMGB1 Release

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ABBREVIATIONS----ALI, acute lung injury; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BAL, bronchoalveolar lavage; BUN: blood urea nitrogen; CAT, catalase; CLP, cecal ligation and puncture; Cr: creatinine; DCS, decompression sickness; GSH-Px, glutathione peroxidase; H_2, hydrogen; H_2O_2, hydrogen peroxide; HMGB1, high-mobility group box 1; ICU, intensive care units; 8-iso-PGF2α, 8-iso-prostaglandin F2α; MPO, myeloperoxidase; •OH: hydroxyl radicals; ROS, reactive oxygen species; SOD, superoxide dismutase; W/D: wet-to-dry.

ABSTRACT----Despite recent advances in antibiotic therapy and intensive care, sepsis is still considered to be the most common cause of death in intensive care units (ICU). Excessive production of reactive oxygen species (ROS) plays an important role in the pathogenesis of sepsis. Recently, it has been suggested that molecular hydrogen (H_2) exerts a therapeutic antioxidant activity by selectively reducing hydroxyl radicals (•OH, the most cytotoxic ROS) and effectively protects against organ damage induced by ischemia/reperfusion. Therefore, we hypothesized that H_2 treatment had a beneficial effect on sepsis. In the present study, we found that H_2 inhalation starting at 1 and 6 hours after cecal ligation and puncture (CLP) or sham operation significantly improved the survival rate of septic mice with moderate or severe CLP in a concentration- and time-dependent manner. Furthermore, moderate or severe CLP mice showed significant multiple organ damage characterized by the increases of lung myeloperoxidase (MPO) activity, wet-to-dry (W/D) weight ratio, protein concentration in bronchoalveolar lavage (BAL), serum biochemical parameters, and organ histopathological scores at 24 hours after CLP operation, which was significantly attenuated by 2% H_2 treatment. In addition, we found that the beneficial effects of H_2 treatment on sepsis and sepsis-associated organ damage were associated with the decreased levels of oxidative product, increased activities of antioxidant enzymes and reduced levels of high-mobility group box 1 (HMGB1) in serum and tissue. Thus, H_2 inhalation may be an effective therapeutic strategy for septic patients.

KEY WORDS----sepsis; acute lung injury; organ damage; reactive oxygen species; high-mobility group box 1; antioxidant enzyme; hydrogen gas

INTRODUCTION

Despite recent advances in antibiotic therapy and intensive care, sepsis is still considered to be the most common cause of death in intensive care units (ICU), which is a complex, incompletely understood and often fatal disorder, typically accompanied by multiple organ dysfunction (1, 2). Over 750, 000 people become septic each year with a mortality rate of 30-40% and an approximate cost of $16.7 billion in the United States alone (1, 2). Because the factors responsible for the pathology and death associated with sepsis are not fully understood (3), it has been exceedingly difficult to develop measures that reduce so high mortality. Thus, there is considerable interest in identifying an effective novel therapy for this disorder.

A growing number of studies have found that excessive production of reactive oxygen species (ROS) and reduction of antioxidant defense systems play an important role in the pathogenesis of sepsis (4). Therefore, many researchers have focused on reducing the levels of ROS to treat sepsis (4). Hydrogen gas (H_2) has been used in medical applications to prevent decompression sickness (DCS) in deep divers for safety profiles (5). In 1997, Shirahata *et al*. (6) reported that electrolyzed-reduced water, which dissolved large amounts of H_2, had the ability to protect DNA from oxidative damage. Recently, it has been suggested that H_2 exerts a therapeutic antioxidant activity by selectively reducing hydroxyl radicals (•OH, the most cytotoxic ROS) and effectively protects against organ damage such as transient cerebral ischemia, neonatal cerebral hypoxia-ischemia, liver injury, lung injury and

myocardial injury induced by ischemia/reperfusion (7-13). These findings strongly indicate that H_2 treatment has antioxidant ability in vivo and may provide a beneficial effect on sepsis. However, no research about this has been reported.

It is well known that cecal ligation and puncture (CLP) causes lethal peritonitis and sepsis due to a polymicrobial infection that is accompanied by multiple organ dysfunction (14). Therefore, the present study was designed to investigate the possible therapeutic effects of H_2 on sepsis in a murine model of moderate or severe CLP. In addition, the roles of antioxidant enzymes and HMGB1, known as a key mediator in CLP-induced lethality, in the protective effects were studied.

MATERIALS AND METHODS

Animals

Adult male C57BL/6 mice weighing 20 to 25 g (specific pathogen free) were provided by the Laboratory Animal Center of Fourth Military Medical University. Animals were housed at 20 to 22 ℃ with a 12-h light/dark cycle. Standard animal chow and water were freely available. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Fourth Military Medical University, and performed in accordance with the National Institutes of Health (NIH, USA) guidelines for the use of experimental animals.

Cecal ligation and puncture (CLP) model

We performed CLP as described previously (15, 16). Briefly, we anesthetized mice deeply by intraperitoneal injection of 50 mg/kg pentobarbital sodium. We exposed the cecum by a 1 cm abdominal midline incision and subjected it to ligation below the ileocecal valve and a single 'through and through' perforation of the ligated segment. For severe CLP (100% lethality), we ligated the distal three-quarters of the cecum and made a single puncture with a 20-gauge needle; for moderate CLP (30-40% survival), we ligated the distal one-half of the cecum and made a single puncture with a 21-gauge needle. A small amount of stool was extruded through the puncture site. We then replaced the cecum into the abdomen and closed the incision using a sterile 6-0 silk suture. 1 ml of pre-warmed sterile saline (pyrogen-free 0.9%

NaCl, 37 ℃) was subcutaneously administered for fluid resuscitation. Animals with sham operation underwent the same procedure without CLP.

Hydrogen gas (H_2) treatment

The animals were put in a sealed plexiglas chamber with inflow and outflow outlets. H_2 was supplied through a gas flowmeter, TF-1 (YUTAKA Engineering Corp., Tokyo, Japan), and delivered by air into the chamber through a tube at a rate of 4 L/min. The concentration of oxygen in the chamber was maintained at 21% by using supplemental oxygen and continuously monitored with a gas analyzer (Medical Gas Analyzer LB-2, Model 40 M, Beckman, USA). The concentration of H_2 in the chamber was continuously monitored with a commercially available detector (Hy Alerta Handheld Detector Model 500, H_2 Scan, Valencia, CA, USA) and maintained at the predetermined level during the treatment. Carbon dioxide was removed from the chamber gases with baralyme. The animals without H_2 treatment were exposed to room air in the chamber. The room and chamber temperature was maintained at 20 to 22 ℃. Food and water were available *ad libitum* during the treatment.

Experimental design

Experiment One: Effects of H_2 treatment on the survival rate of septic mice with moderate or severe CLP

Effects of H_2 treatment on the survival rate of septic mice with moderate CLP.

120 animals were randomly divided into 4 groups (n = 30 per group): Sham,

Sham+2% H_2 for 60 min, Moderate CLP, and Moderate CLP+2% H_2 for 60 min groups. The animals in the Sham+2% H_2 for 60 min and Moderate CLP+2% H_2 for 60 min groups were exposed to 2% H_2 for 60 minutes starting at 1 and 6 hours after sham or moderate CLP operation, respectively. As a control, the animals from the Sham and Moderate CLP groups were given room air treatment at the same time points. The survival rate was observed on day 1, 2, 3, 5, 7 and 14 after CLP or sham operation. ***Effects of different concentrations of H_2 treatment on the survival rate of septic mice with moderate CLP.*** 120 animals were randomly divided into 4 groups (n = 30 per group): Moderate CLP, Moderate CLP+1% H_2 for 60 min, Moderate CLP+2% H_2 for 60 min, and Moderate CLP+4% H_2 for 60 min groups. The animals in all groups were subjected to moderate CLP operation. At 1 and 6 hours after CLP operation, the animals were exposed to different concentrations of H_2 (0%, 1%, 2% or 4%) for 60 minutes, respectively. The survival rate was observed on day 1, 2, 3, 5, 7 and 14 after CLP operation. ***Effects of H_2 treatment for different time on the survival rate of septic mice with moderate CLP.*** Based on the above experiments, 2% H_2 treatment was used in this experiment. 120 animals were randomly divided into 4 groups (n = 30 per group): Moderate CLP, Moderate CLP+2% H_2 for 30 min, Moderate CLP+2% H_2 for 60 min, and Moderate CLP+2% H_2 for 90 min groups. The animals in all groups were exposed to moderate CLP operation. At 1 and 6 hours after CLP operation, the animals were exposed to 2% H_2 for different time (0 min, 30 min, 60 min, or 90 min), respectively. The survival rate was observed on day 1, 2, 3, 5, 7 and 14 after CLP operation. ***Effects of H_2 treatment on the survival rate of septic mice***

with severe CLP. Based on the above experiments, 2% H_2 treatment for 60 min was used in this experiment. 60 animals were randomly divided into 2 groups (n = 30 per group): Severe CLP and Severe CLP+2% H_2 for 60 min groups. The animals in both groups were exposed to severe CLP operation. The animals in the Severe CLP+2% H_2 for 60 min group were exposed to 2% H_2 for 60 minutes at 1 and 6 hours after CLP operation, respectively. As a control, the animals from the Severe CLP group were given room air treatment at the same time points. The survival rate was observed on day 1, 2, 3, 5 and 7 after CLP operation.

Experiment Two: Effects of 2% H_2 treatment on sepsis-associated organ injury in mice with moderate or severe CLP

Based on the above experiments, 2% H_2 treatment for 60 min was used in this experiment. Additional 36 animals were used in this experiment and were assigned to 6 groups (n = 6 per group): Sham, Sham+2% H_2 for 60 min, Moderate CLP, Moderate CLP+2% H_2 for 60 min, Severe CLP, and Severe CLP+2% H_2 for 60 min groups. The detailed experimental protocols were the same as described above. Lung myeloperoxidase (MPO) activity, lung wet-to-dry (W/D) weight ratio, protein concentration in bronchoalveolar lavage (BAL) fluid and lung histopathology were observed at 24 hours after CLP or sham operation. In addition, we detected the serum biochemical parameters, as well as liver and kidney histopathology at 24 hours after CLP or sham operation.

Experiment Three: Effects of 2% H_2 treatment on cytokine as well as oxidant and antioxidant system in mice with moderate or severe CLP

Additional 36 animals were used in this experiment and were assigned to 6 groups (n = 6 per group). The grouping method and experimental protocols were the same as Experiment Two. At 24 hours after CLP or sham operation, the pro-inflammatory cytokine (high-mobility group box 1, HMGB1), antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT) and oxidative product (8-iso-prostaglandin F2α, 8-iso-PGF2α) in serum, lung, liver and kidney tissues were measured.

Lung MPO activity assay

At 24 hours after the CLP or sham operation, lungs were obtained and perfused with phosphate buffered saline (PBS) to remove all blood, then weighed and stored at -80 ℃ for no more than 1 week before the MPO assay was performed. The supernatant from lung homogenate was prepared for detecting the activity of MPO, an indicator of neutrophil infiltration in the lung tissue, which was measured as previously reported (17). MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per minute at 37 ℃ and was expressed in unit per gram weight of wet tissue. The change in absorbance was measured spectrophotometrically at 590 nm by spectrophotometer (DU 640B, Beckman, Fullerton, CA, USA).

Lung wet-to-dry (W/D) weight ratio

To quantify the magnitude of pulmonary edema, we evaluated lung W/D weight ratio. The harvested wet lung was weighed, and then placed in an oven for 24 hours at 80 ℃ and weighed when it was dried.

Bronchoalveolar lavage (BAL) and total protein assay

Animals were subjected to BAL for collecting BAL fluid (BALF) by the methods described previously (18). Animals were anesthetized, and the trachea was isolated by blunt dissection and a small-caliber tube was inserted into the airway and secured. Two volumes of 0.5 mL of PBS (pH 7.4) were instilled, gently aspirated, pooled and re-aspirated. Lavage samples were centrifuged at 1, 500 g for 10 minutes at 4 ℃. The supernatant was stored at -20 ℃. Total protein concentration in BAL was determined by using a standard commercial kit (Bio-Rad Laboratories, Hercules, CA, USA).

Organ histologic examination

Organ samples were taken at 24 hours after CLP or sham operation for observing morphologic alterations. The samples were fixed with 10% formalin for 6 hours at room temperature, embedded in paraffin, and sectioned at 5 μm thickness. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin. Organ histologic changes were evaluated by two pathologists who were blinded to the treatment regimen. A scoring system to grade the degree of lung injury was employed, based on the following histologic features: edema, hyperemia and congestion, neutrophil margination and tissue infiltration, intraalveolar hemorrhage

and debris, and cellular hyperplasia. Each feature was graded as absent, mild, moderate, or severe, with a score of 0-3. A total score was calculated for each animal (19). In addition, according to the scoring standard in our recently published papers (20, 21), the degree of liver and kidney injury was also graded.

Enzymatic activity assay

Blood and organ specimens (lung, liver and kidney) were collected at 24 hours after CLP or sham operation. The serum was separated by centrifugation at 3, 000 g for 15 minutes at 4 ℃, aliquoted, and stored at -80 ℃ until assayed. The tissue homogenates were prepared in chilled PBS (0.1 M, pH 7.4), and were centrifuged at 10, 000 g at 4 ℃ for 10 minutes. The supernatants were collected, aliquoted, stored at -80 ℃ until the following analysis.

The activities of SOD and CAT were measured using commercial kits purchased from Cayman Chemical Company (Ann Arbor, MI, USA). According to the manufacturer's instructions, total SOD activity was assayed by detecting superoxide radicals generated by xanthine oxidase and hypoxanthine. The reaction was monitored at 450 nm and one unit of SOD activity was defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical. The CAT activity was assayed by measuring the reduction of hydrogen peroxide at 540 nm and one unit was defined as the amount of enzyme that would cause the formation of 1.0 nmol of formaldehyde per minute at 25 ℃. All spectrophotometric readings were performed by using a spectrophotometer (DU 640B, Beckman, Fullerton, CA, USA). All assays were

conducted in triplicates. The tissue protein concentration was determined by using a standard commercial kit (Bio-Rad Laboratories, Hercules, CA, USA).

Detection of 8-iso-prostaglandin F2α (8-iso-PGF2α)

The serum and tissue homogenates (lung, liver and kidney) obtained above were also used for detecting the level of 8-iso-PGF2α. Measurement of 8-iso-PGF2α, free radical-catalysed products of arachidonic acid, can offer a reliable approach for quantitative measurement of oxidative stress status in vivo (22). The levels of serum and tissue 8-iso-PGF2α were detected by specific enzyme-linked immunosorbent assay (ELISA) kits (8-iso-PGF2α, Ann Arbor, MI, USA) using a microplate reader (CA 94089, Molecular Devices, Sunnyvale, Canada). All standards and samples were run in duplicate.

Detection of high-mobility group box 1 (HMGB1)

The serum and tissue homogenates (lung, liver and kidney) obtained above were also used for detecting the level of HMGB1. The levels of serum and tissue HMGB1 were detected by specific enzyme-linked immunosorbent assay (ELISA) kits (IBL, Hamburg, Germany) with a microplate reader (CA 94089, Molecular Devices, Sunnyvale, Canada). All standards and samples were run in duplicate.

Statistical analysis

The survival rates are expressed as percentage. The measurement data are

expressed as mean ± SEM. The analysis of survival rates was tested by Fisher's exact probability method. The inter-group differences of the rest data were tested by one-way ANOVA followed by LSD-*t* Test for multiple comparisons. The statistical analysis was performed with *SPSS* 16.0 software. In all tests, a *P* value of less than 0.05 was considered statistically significant.

RESULTS

H_2 inhalation at a 2% or 4% concentration had no significant effects on arterial pH, P_aO_2 and P_aCO_2 in mice with or without sepsis during the treatment

In the present study, we investigated the effects of H_2 inhalation on arterial pH, P_aO_2 and P_aCO_2 in mice with or without CLP operation during the treatment. The arterial blood gas was conducted at 0.5 hour after the onset of H_2 inhalation (1.5 hours after CLP or Sham operation) using a GEM Premier 3000 gas analyzer (Instrumentation Laboratory, Milan, Italy). There were no differences in the levels of arterial pH, P_aO_2 and P_aCO_2 among all groups (**Table 1**). The results demonstrate that H_2 inhalation at a 2% or 4% concentration has no significant effects on arterial pH, P_aO_2 and P_aCO_2 in mice with or without sepsis during the treatment.

H_2 treatment improved the survival rate of septic mice with moderate or severe CLP in a concentration- and time-dependent manner

In this study, we investigated the effects of H_2 treatment with different concentration or different therapeutic time on the survival rates of septic mice with moderate or severe CLP. The 14-day survival rate of moderate CLP mice was 30-40% ($P < 0.05$ vs. Sham group, n = 30 per group, **Fig. 1**). 2% H_2 inhalation for 60 minutes starting at 1 and 6 hours after CLP operation respectively improved the 14-day survival rate of moderate CLP mice to 80% ($P < 0.05$ vs. Moderate CLP group, n =

30 per group, **Fig. 1A**). **Fig. 1B** shows that the protective effects of H_2 treatment on septic mice are concentration-dependent. 1% H_2 treatment did not significantly increase the 14-day survival rate of moderate CLP mice ($P > 0.05$, n = 30 per group, **Fig. 1B**). However, 2% and 4% H_2 treatment increased the 14-day survival rate of moderate CLP mice to 80% and 90%, respectively ($P < 0.05$ vs. Moderate CLP group, n = 30 per group, **Fig. 1B**). **Fig. 1C** shows that the beneficial effects of H_2 treatment on septic mice are time-dependent. 2% H_2 inhalation for 30, 60 and 90 minutes starting at 1 and 6 hours after CLP operation respectively increased the 14-day survival rate of moderate CLP mice from 40% to 50%, 80% and 90%, respectively (**Fig. 1C**). In addition, 2% H_2 inhalation for 60 minutes starting at 1 and 6 hours after CLP operation respectively improved the 7-day survival rate of severe CLP mice from 0% to 60% ($P < 0.05$, n = 30 per group, **Fig. 1D**). The above data suggest that H_2 treatment can improve the survival rate of septic mice with moderate or severe CLP in a concentration- and time-dependent manner.

H_2 treatment attenuated acute organ injury in septic mice with moderate or severe CLP

As shown in **Fig. 2**, moderate and severe CLP mice appeared significant acute lung injury (ALI) at 24 hours after CLP operation, which was assessed by lung MPO activity, lung W/D ratio, protein concentration in BAL and lung histopathology. Moderate and severe CLP mice showed a significant increase in lung MPO activity,

lung W/D ratio, protein concentration in BAL and lung histologic scores ($P < 0.05$ vs. Sham group, n = 6 per group, **Fig. 2**). These abnormal changes were significantly attenuated by 2% H_2 treatment (**Fig. 2**).

With respect to histopathological changes, lung injury characterized by alveolar wall thickening, infiltration of neutrophils into the lung interstitium and alveolar space, consolidation and alveolar hemorrhage was present in mice with moderate or severe CLP. 2% H_2 treatment resulted in a reduction of infiltrated inflammatory cells and a marked improvement in lung architecture when compared with those in the Moderate CLP and Severe CLP groups (**Fig. 3**).

In addition, moderate and severe CLP mice appeared significant liver and kidney injury at 24 hours after CLP operation, which was assessed by serum biochemical parameters for liver and kidney (ALT, AST, Cr and BUN), and histopathology. Moderate and severe CLP mice showed a significant increase in the levels of serum ALT, AST, Cr and BUN, as well as liver and kidney histologic scores ($P < 0.05$ vs. Sham group, n = 6 per group, **Fig. 4**). These abnormal changes were significantly attenuated by 2% H_2 treatment (**Fig. 4**).

These data demonstrate that moderate or severe CLP mice appear significant organ damage at 24 hours after CLP operation, which is significantly attenuated by 2% H_2 treatment, suggesting that H_2 treatment has a beneficial effect on sepsis-induced multiple organ damage.

H_2 treatment prevented the abnormal changes of antioxidant enzymatic activities, oxidative product and inflammatory cytokine in septic mice with moderate or severe CLP

At 24 hours after CLP or sham operation, the activities of antioxidant enzymes SOD and CAT, the levels of oxidative product 8-iso-PGF2α and the levels of pro-inflammatory cytokine HMGB1 (a critical mediator of lethal sepsis) in serum and lung of all animals were observed. Our results showed that the decrease of SOD and CAT activities as well as the increase of 8-iso-PGF2α and HMGB1 levels in serum and lung occurred to mice with moderate or severe CLP ($P < 0.05$ vs. Sham group, n = 6 per group, **Fig. 5 and 6**). Treatment with 2% H_2 increased the SOD and CAT activities and decreased 8-iso-PGF2α and HMGB1 levels in serum and lung of septic mice with moderate or severe CLP ($P < 0.05$, n = 6 per group, **Fig. 5 and 6**). No statistically significant differences in the activities of SOD and CAT as well as the levels of 8-iso-PGF2α and HMGB1 were present between the Sham and Sham+2% H_2 groups ($P > 0.05$, n = 6 per group, **Fig. 5 and 6**).

In addition, we also detected the activities of SOD and CAT, the levels of 8-iso-PGF2α and the levels of HMGB1 in liver and kidney at 24 hours after CLP or sham operation. The results were similar with those in serum and lung, the detailed data were shown in **Fig. 7 and 8**.

These data suggest that H_2 treatment provides beneficial effects on sepsis and sepsis-associated organ damage, which are associated with the decreased levels of oxidative product, increased activities of antioxidant enzymes and reduced levels of

pro-inflammatory cytokine HMGB1 in serum and tissue.

DISCUSSION

In the present study, we found that 1) H_2 treatment starting at 1 and 6 hours after CLP or sham operation significantly improved the survival rate of septic mice with moderate or severe CLP in a concentration- and time-dependent manner. 2) Moderate or severe CLP mice showed significant organ injury characterized by the increase of lung MPO activity, lung W/D weight ratio, BAL total protein, serum biochemical parameters, and organ histopathological scores at 24 hours after CLP operation, which was significantly attenuated by 2% H_2 treatment. 3) The beneficial effects of H_2 treatment on sepsis and sepsis-associated organ injury were associated with the decreased levels of oxidative stress, increased activities of antioxidant enzymes and reduced levels of HMGB1 in serum and tissue.

Well-accepted and widely used CLP is considered to be a clinically relevant model for studying the pathogenesis and treatment of sepsis (14). CLP can cause lethal peritonitis and sepsis due to a polymicrobial infection that is accompanied by multiple organ damage. Therefore, the present study was designed to investigate the possible therapeutic effects of H_2 on sepsis in mice with moderate or severe CLP. In the present study, we successfully produced moderate or severe CLP model. Moderate CLP caused a 30-40% survival rate and moderate organ injury, while severe CLP caused 100% mortality and severe organ injury.

Sepsis, when accompanied by multiple organ injury, contributes to be the leading cause of death in ICU, with a mortality that has remained over 40% (23). In the present investigation, we also observed the increase of lung MPO activity, lung W/D weight ratio and protein concentration in BAL, as well as lung histopathological injury, indicating that CLP causes significant ALI. In addition, we also found that the increase of serum biochemical parameters and histopathological injury for liver and kidney occurred to mice with moderate or severe CLP, demonstrating that CLP also causes significant liver and kidney injury. Therefore, the development of novel strategies for treatment of organ injury is also critical for treatment of septic patient.

A growing number of studies have found that excessive production of ROS and reduction of antioxidant defense systems play an important role in the pathogenesis of sepsis (4). In excess, ROS and their by-products that are capable of causing oxidative damage may be detrimental to tissues and organs (24). It is reported that ROS include many types such as superoxide anion, hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2), and so on. One type of ROS can be converted into another type via antioxidant enzymes in vivo. For example, SOD converts superoxide anion radical into H_2O_2, which is detoxified into H_2O by either glutathione peroxidase (GSH-Px) or CAT (25). In addition, excess superoxide anion reduces transition metal ions such as Fe^{3+} and Cu^{2+}, the reduced forms of which in turn can react with H_2O_2 to produce •OH by the Fenton reaction (26). •OH is the strongest of the oxidant species and reacts indiscriminately with nucleic acids, lipids and proteins (27). There is no known detoxification system for •OH in vivo (27). Therefore, scavenging •OH is a critical

antioxidant process, which may be a good and critical measure for treating sepsis.

H_2 has been used in medical applications to prevent DCS in deep divers for safety profiles (5). In 1997, Shirahata *et al*. (6) reported that electrolyzed-reduced water, which dissolved large amounts of H_2, had the ability to protect DNA from oxidative damage. Recently, several studies demonstrate that H_2 exerts a therapeutic antioxidant activity by selectively reducing hydroxyl radicals (•OH, the most cytotoxic ROS) and effectively protected against tissue damage such as transient cerebral ischemia, neonatal cerebral hypoxia-ischemia, liver injury, lung injury and myocardial injury induced by ischemia/reperfusion, suggesting that H_2 has potential as an antioxidant for preventive and therapeutic applications (7-13). These findings strongly indicate that H_2 may provide a beneficial effect on sepsis. However, no research about this has been reported. In the present study, we found that H_2 treatment starting at 1 and 6 hours after CLP operation significantly improved the long-term survival rate of septic mice with moderate or severe CLP in a concentration- and time-dependent manner. Furthermore, we found that 2% H_2 treatment significantly attenuated sepsis-induced organ injury through observing the indicators including lung MPO activity, lung W/D weight ratio, BAL total protein, serum biochemical parameters, and organ histopathological scores at 24 hours after CLP operation. The above results demonstrate that H_2 treatment has a beneficial effect on sepsis and sepsis-induced organ injury in mice with moderate and severe CLP.

To further investigate the possible mechanism, we study the effects of H_2 treatment on oxidant and antioxidant system in moderate and severe CLP mice. In

rodent sepsis model induced by CLP, the activities of SOD, CAT and GSH-Px in tissue and serum were significantly decreased during the early and late phages, indicating that sepsis sets up an environment favorable for oxidative stress (28). The detection of products of lipid peroxidation has been widely used to estimate the overall status of oxidative stress. In the present study, we observed the decrease of SOD, CAT and the increase of oxidation product 8-iso-PGF2α in lung, liver, kidney and serum at 24 hours after moderate or severe CLP operation. We further showed that 2% H_2 treatment significantly improved the activities of CAT and SOD in these organs and serum, and decreased the levels of 8-iso-PGF2α in these organs and serum. These results suggest that the decrease of oxidative damage and the increase of endogenous antioxidant enzymatic activities may attribute to the protection of H_2 treatment.

Many researchers discovered that a ubiquitous protein, HMGB1, is released by activated macrophages/monocytes and so on, and functions as a late mediator of lethal endotoxemia and sepsis (29, 30). Recently, some studies have found that HMGB1 is a necessary and sufficient mediator of lethal organ damage in murine CLP sepsis (29, 30). Many animal and clinical experiments show that systemic HMGB1 level is significantly elevated in sepsis, while neutralizing antibodies directed against HMGB1 significantly reduce organ damage and improve survival even when the first doses are given 24 h after the onset of the disease (29, 30). Pharmacologic agents that reduce circulating HMGB1 levels, such as ethyl pyruvate, also provide significant protection against polymicrobial sepsis lethality (31). In addition, administration of

recombinant HMGB1 to mice recapitulates many clinical signs of sepsis, including fever, derangement of intestinal barrier function, and tissue injury (30). Here we found that 2% H_2 treatment significantly reduced serum and tissue HMGB1 levels in septic mice with moderate or severe CLP and thereby protected against the development of lethal organ damage.

In low concentration (< 4% in air), H_2 is neither explosive nor dangerous, which has been proved through 17-year long studies on cells, mice, monkeys and deep-sea divers (COMEX HYDRA program, Marseille). Inhaled H_2 at therapeutic dose has no adverse effects on the saturation level of arterial oxygen (S_pO_2) or hemodynamic parameters, and so on (13), which was also proved by the present study. H_2, as a potential antioxidant, has certain unique properties: unlike most known antioxidants, H_2 is permeable to cell membranes and can target organelles, including mitochondria and nuclei. Despite the moderate reduction activity of H_2, its rapid gaseous diffusion might make it highly effective for reducing cytotoxic radicals. H_2 specifically quenches exclusively detrimental ROS, such as •OH and peroxynitrite (ONOO-), while maintaining the metabolic oxidation-reduction reaction and other less potent ROS, such as superoxide anion and H_2O_2. It is likely that H_2 is mild enough not to disturb metabolic oxidation-reduction reactions or to disrupt ROS involved in cell signaling (unlike some antioxidant supplements with strong reductive reactivity, which increase mortality possibly by affecting essential defensive mechanisms). Ohsawa *et al*. (13) found that H_2 directly reacted with free radical species such as •OH although the kinetic favorability of this direct reaction may be uncertain. Further

studies will reveal the mechanisms by which H_2 protects cells and tissues against oxidative stress.

In summary, H_2 treatment starting at 1 and 6 hours after CLP operation is beneficial for sepsis and sepsis-associated organ injury in a concentration- and time-dependent manner, which is associated with the decrease of oxidative stress, improvement of endogenous antioxidant enzymatic activities, and reduction of late inflammatory cytokine HMGB1 in serum and tissue. The present study supports that H_2 inhalation may be a more effective therapeutic strategy for septic patients owing to its ability to rapidly diffuse across membranes.

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FIGURE LEGENDS

FIG. 1

H_2 treatment improved the survival rate of septic mice with moderate or severe CLP in a concentration- and time-dependent manner. The values are expressed as survival percentage (n = 30 per group). **(A)** Effects of 2% H_2 treatment for 60 minutes starting at 1 and 6 hours after CLP or sham operation respectively on the survival rate of septic mice with moderate CLP. $^{*}P < 0.05$ vs. Sham group; $^{\dagger}P < 0.05$ vs. Moderate CLP group. **(B)** Effects of different concentrations of H_2 treatment for 60 minutes starting at 1 and 6 hours after CLP operation respectively on the survival rate of septic mice with moderate CLP. $^{*}P < 0.05$ vs. Moderate CLP group; $^{\dagger}P < 0.05$ vs. Moderate CLP+1% H_2 for 60 min group. **(C)** Effects of 2% H_2 treatment for different time starting at 1 and 6 hours after CLP operation respectively on the survival rate of septic mice with moderate CLP. $^{*}P < 0.05$ vs. Moderate CLP group; $^{\dagger}P < 0.05$ vs. Moderate CLP+2% H_2 for 30 min group. **(D)** Effects of 2% H_2 treatment for 60 minutes starting at 1 and 6 hours after CLP operation respectively on the survival rate of septic mice with severe CLP. $^{*}P < 0.05$ vs. Sham group. CLP: cecal ligation and puncture; H_2: hydrogen.

FIG. 2

H_2 treatment attenuated acute lung injury in septic mice with moderate or severe CLP. **(A)** Lung MPO activity; **(B)** Lung BAL total protein; **(C)** Lung wet-to-dry

weight ratio; **(D)** Lung histologic scores. H_2 treatment was given by exposure to 2% H_2 for 60 minutes starting at 1 and 6 hours after CLP or sham operation respectively. These indicators were measured at 24 hours after CLP or sham operation. The values are expressed as means ± SEM (n = 6 per group). $^{*}P < 0.05$ vs. Sham group; $^{\dagger}P < 0.05$ vs. Moderate CLP group; $^{\ddagger}P < 0.05$ vs. Severe CLP group. MPO: myeloperoxidase; BAL: bronchoalveolar lavage; CLP: cecal ligation and puncture; H_2: hydrogen.

FIG. 3

H_2 treatment attenuated lung histopathological changes in septic mice with moderate or severe CLP. H_2 treatment was given by exposure to 2% H_2 for 60 minutes starting at 1 and 6 hours after CLP or sham operation respectively. The lungs were stained with hematoxylin-eosin at 24 hours after CLP or sham operation (original magnification: ×40). CLP: cecal ligation and puncture; H_2: hydrogen.

Fig. 4

H_2 treatment attenuated acute liver and kidney injury in septic mice with moderate or severe CLP. H_2 treatment was given by exposure to 2% H_2 for 60 minutes starting at 1 and 6 hours after CLP or sham operation respectively. These indicators were measured at 24 hours after CLP or sham operation. The values are expressed as means ± SEM (n = 6 per group). $^{*}P < 0.05$ vs. Sham group; $^{\dagger}P < 0.05$ vs.

Moderate CLP group; ‡ $P < 0.05$ vs. Severe CLP group. ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; Cr: creatinine; UI/L: International Unit per liter; CLP: cecal ligation and puncture; H_2: hydrogen.

FIG. 5

H_2 treatment up-regulated the activities of serum antioxidant enzymes, and reduced the levels of serum oxidative product and inflammatory cytokine in septic mice with moderate or severe CLP. (A): Serum SOD activity; **(B):** Serum CAT activity; **(C):** Serum 8-iso-PGF2α level; **(D):** Serum HMGB1 level. H_2 treatment was given by exposure to 2% H_2 for 60 minutes starting at 1 and 6 hours after CLP or sham operation respectively. The serum was harvested for measuring these indicators at 24 hours after CLP or sham operation. The values are expressed as mean ± SEM (n = 6 per group). * $P < 0.05$ vs. Sham group; † $P < 0.05$ vs. Moderate CLP group; ‡ $P < 0.05$ vs. Severe CLP group. SOD: superoxide dismutase; CAT: catalase; HMGB1: High-mobility group box 1 protein; CLP: cecal ligation and puncture; H_2: hydrogen.

FIG. 6

H_2 treatment up-regulated the activities of lung antioxidant enzymes, and

reduced the levels of lung oxidative product and inflammatory cytokine in septic mice with moderate or severe CLP. (A): Lung SOD activity; **(B):** Lung CAT activity; **(C):** Lung 8-iso-PGF2α level; **(D):** Lung HMGB1 level. H_2 treatment was given by exposure to 2% H_2 for 60 minutes starting at 1 and 6 hours after CLP or sham operation respectively. The lungs were harvested for measuring these indicators at 24 hours after CLP or sham operation. The values are expressed as mean ± SEM (n = 6 per group). $^{*}P < 0.05$ vs. Sham group; $^{\dagger}P < 0.05$ vs. Moderate CLP group; $^{\ddagger}P < 0.05$ vs. Severe CLP group. SOD: superoxide dismutase; CAT: catalase; HMGB1: High-mobility group box 1 protein; U/mg.protein: unit per milligram protein; CLP: cecal ligation and puncture; H_2: hydrogen.

Fig. 7

H_2 treatment up-regulated the activities of liver antioxidant enzymes, and reduced the levels of liver oxidative product and inflammatory cytokine in septic mice with moderate or severe CLP. H_2 treatment was given by exposure to 2% H_2 for 60 minutes starting at 1 and 6 hours after CLP or sham operation respectively. The liver was harvested for measuring these indicators at 24 hours after CLP or sham operation. The values are expressed as mean ± SEM (n = 6 per group). $^{*}P < 0.05$ vs. Sham group; $^{\dagger}P < 0.05$ vs. Moderate CLP group; $^{\ddagger}P < 0.05$ vs. Severe CLP group. SOD: superoxide dismutase; CAT: catalase; HMGB1: High-mobility group box 1 protein; U/mg.protein: unit per milligram protein; CLP: cecal ligation and puncture; H_2: hydrogen.

FIG. 8

H_2 treatment up-regulated the activities of kidney antioxidant enzymes, and reduced the levels of kidney oxidative product and inflammatory cytokine in septic mice with moderate or severe CLP. H_2 treatment was given by exposure to 2% H_2 for 60 minutes starting at 1 and 6 hours after CLP or sham operation respectively. The kidneys were harvested for measuring these indicators at 24 hours after CLP or sham operation. The values are expressed as mean ± SEM (n = 6 per group). $^{*}P < 0.05$ vs. Sham group; $^{\dagger}P < 0.05$ vs. Moderate CLP group; $^{\ddagger}P < 0.05$ vs. Severe CLP group. SOD: superoxide dismutase; CAT: catalase; HMGB1: High-mobility group box 1 protein; U/mg.protein: unit per milligram protein. CLP: cecal ligation and puncture; H_2: hydrogen.

TABLE LEGENDS

Table 1

H_2 inhalation at a 2% or 4% concentration had no significant effects on pH, P_aO_2 and P_aCO_2 in mice with or without sepsis during the treatment. CLP: cecal ligation and puncture; H_2: hydrogen.

FIGURES

Fig. 1.

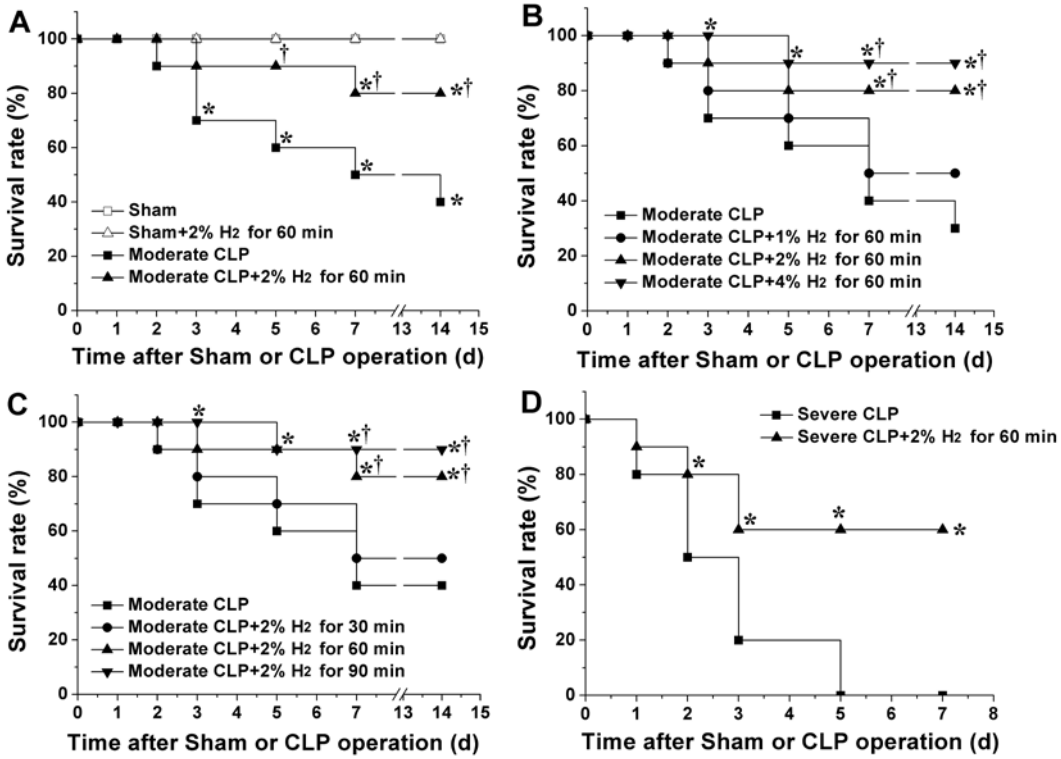

Fig. 2.

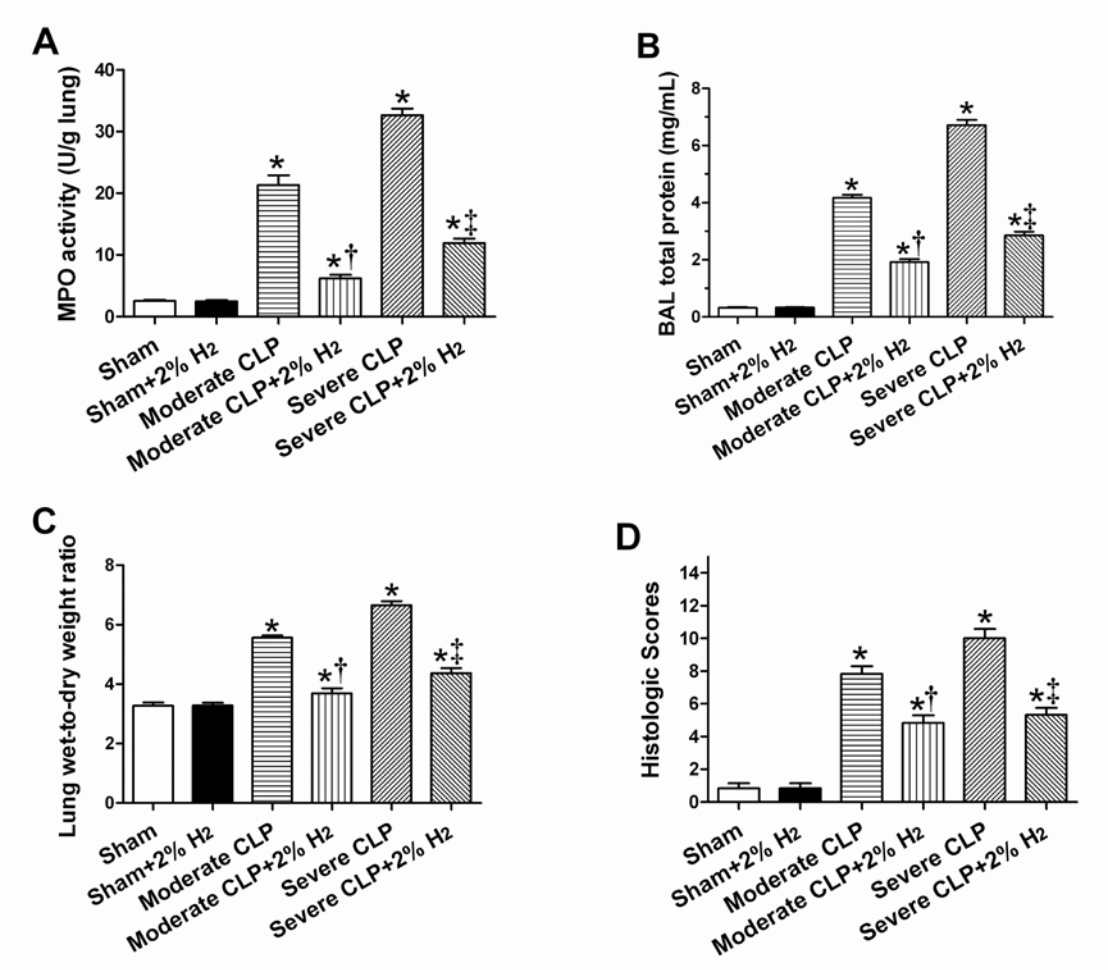
A
MPO activity (U/g lung)
40
30
20
10
0
B
BAL total protein (mg/mL)
8
6
4
2
0
C
Lung wet-to-dry weight ratio
8
6
4
2
0
D
Histologic Scores
14
12
10
8
6
4
2
0
Sham
Sham+2% H2
Moderate CLP
Moderate CLP+2% H2
Severe CLP
Severe CLP+2% H2

Fig. 3.

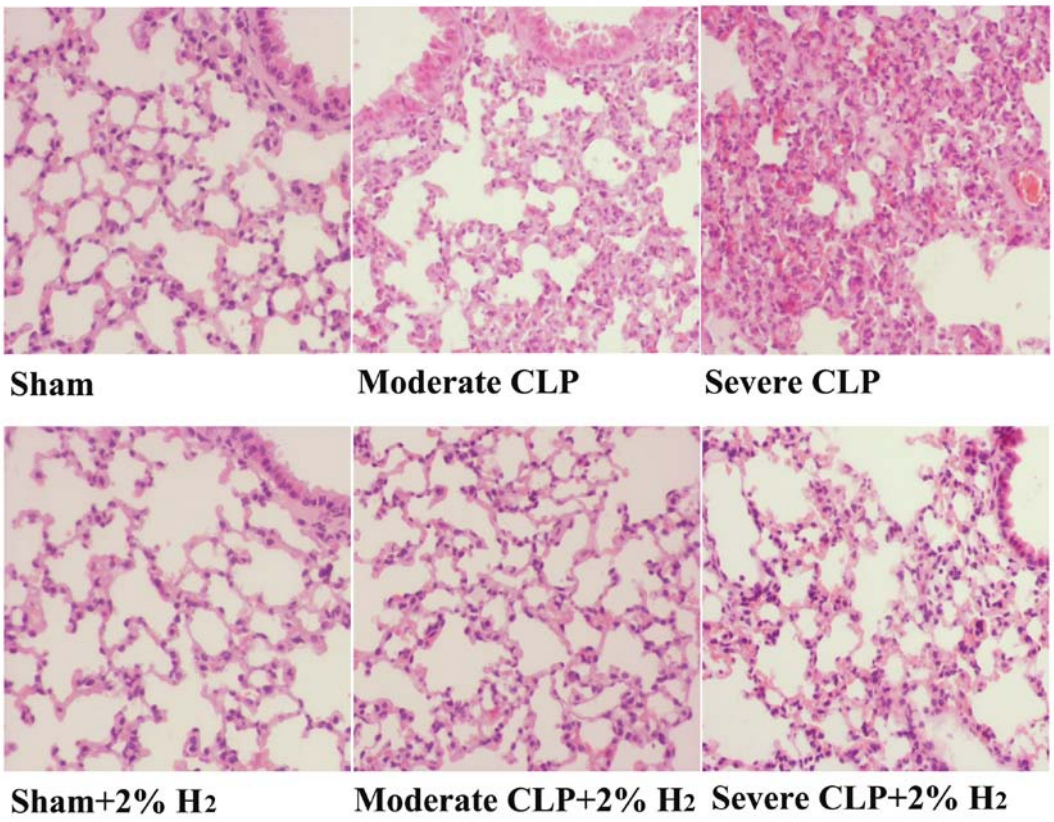
Sham
Moderate CLP
Severe CLP
Sham+2% H2
Moderate CLP+2% H2
Severe CLP+2% H2

FIG. 4.

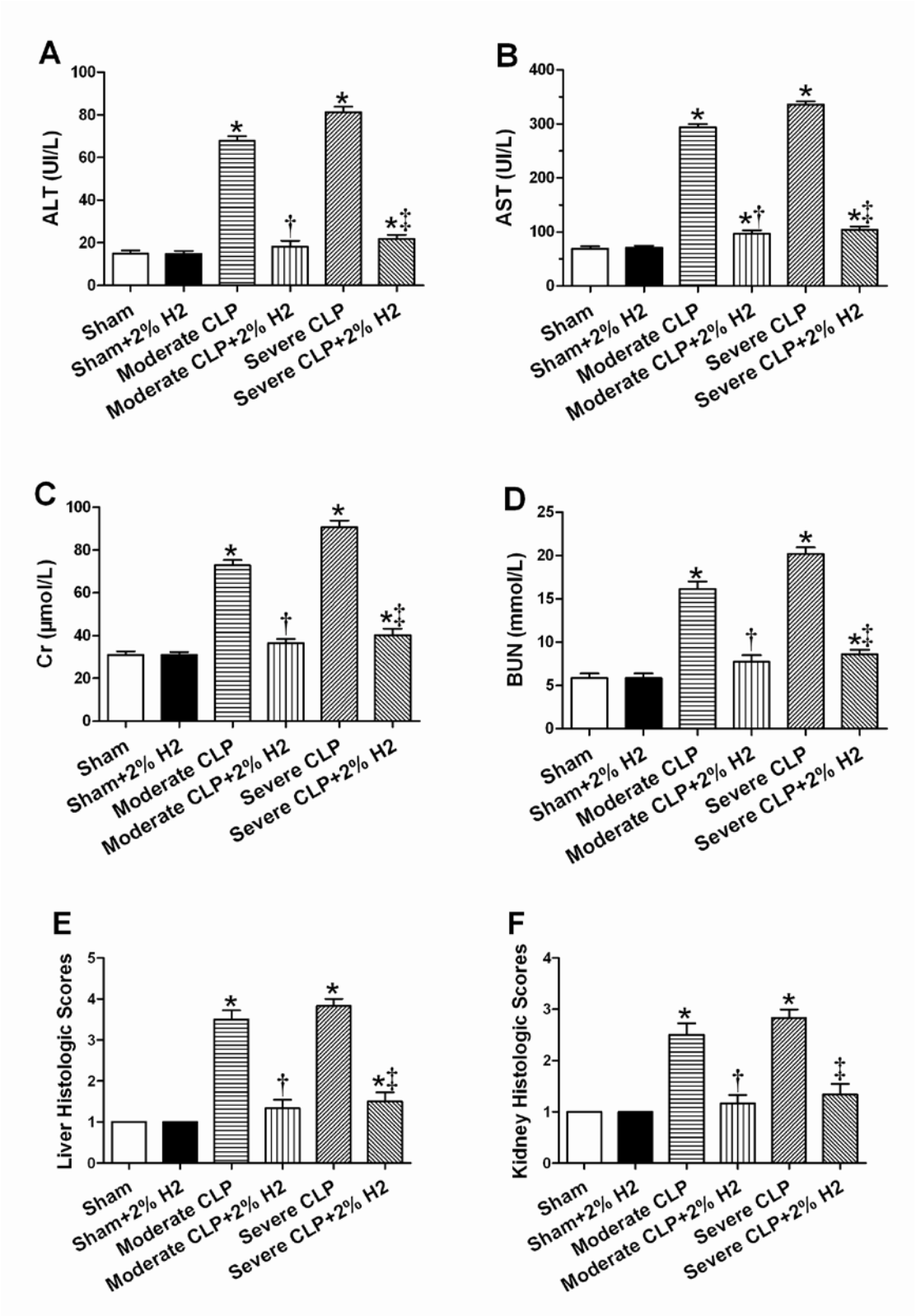
A
ALT (UI/L)
100
80
60
40
20
0
B
AST (UI/L)
400
300
200
100
0
C
Cr (μmol/L)
100
80
60
40
20
0
D
BUN (mmol/L)
25
20
15
10
5
0
E
Liver Histologic Scores
5
4
3
2
1
0
F
Kidney Histologic Scores
4
3
2
1
0
Sham
Sham+2% H2
Moderate CLP
Moderate CLP+2% H2
Severe CLP
Severe CLP+2% H2

Fig. 5.

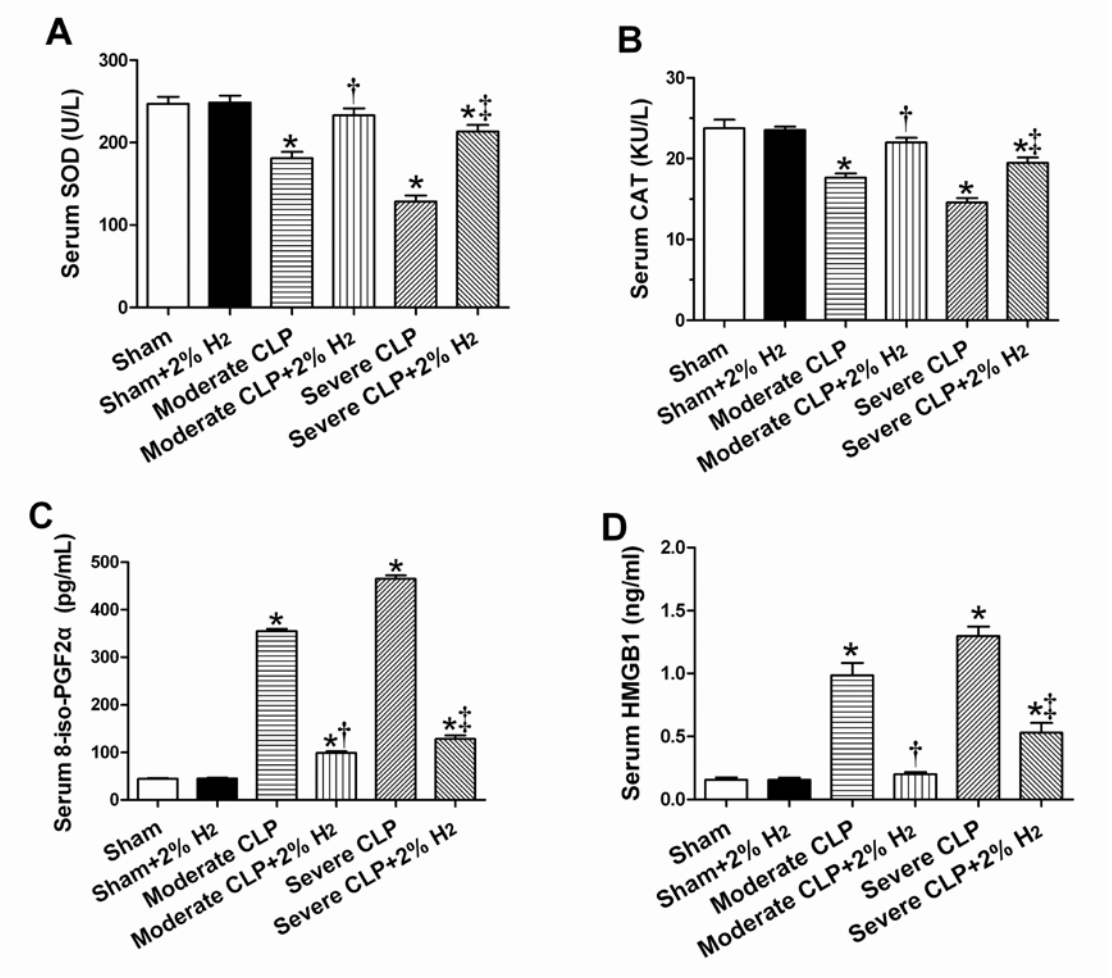
A
Serum SOD (U/L)
300
200
100
0
B
Serum CAT (KU/L)
30
20
10
0
C
Serum 8-iso-PGF2α (pg/mL)
500
400
300
200
100
0
D
Serum HMGB1 (ng/ml)
2.0
1.5
1.0
0.5
0.0
Sham
Sham+2% H2
Moderate CLP
Moderate CLP+2% H2
Severe CLP
Severe CLP+2% H2

Fig. 6.

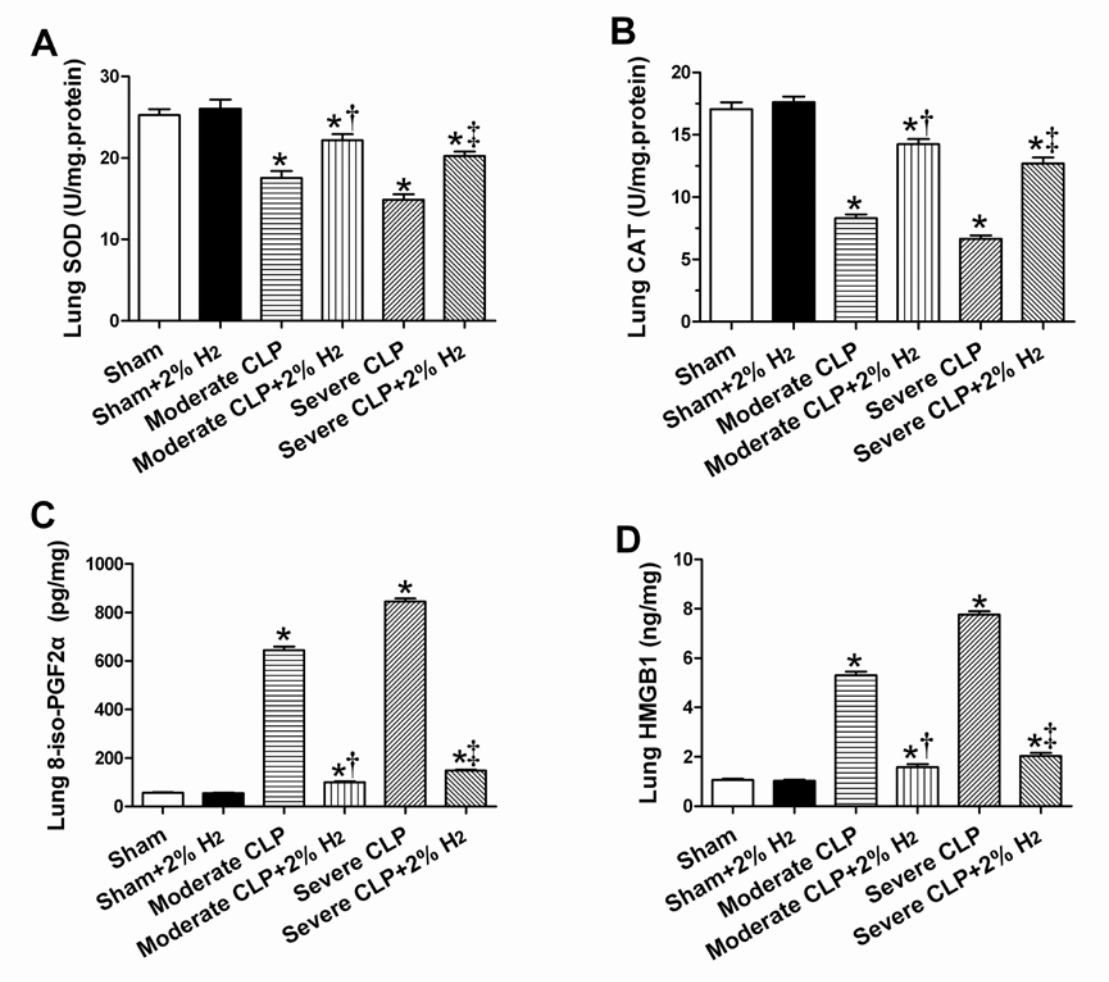
A
Lung SOD (U/mg.protein)
30
20
10
0
B
Lung CAT (U/mg.protein)
20
15
10
5
0
C
Lung 8-iso-PGF2α (pg/mg)
1000
800
600
400
200
0
D
Lung HMGB1 (ng/mg)
10
8
6
4
2
0
Sham
Sham+2% H2
Moderate CLP
Moderate CLP+2% H2
Severe CLP
Severe CLP+2% H2

FIG. 7.

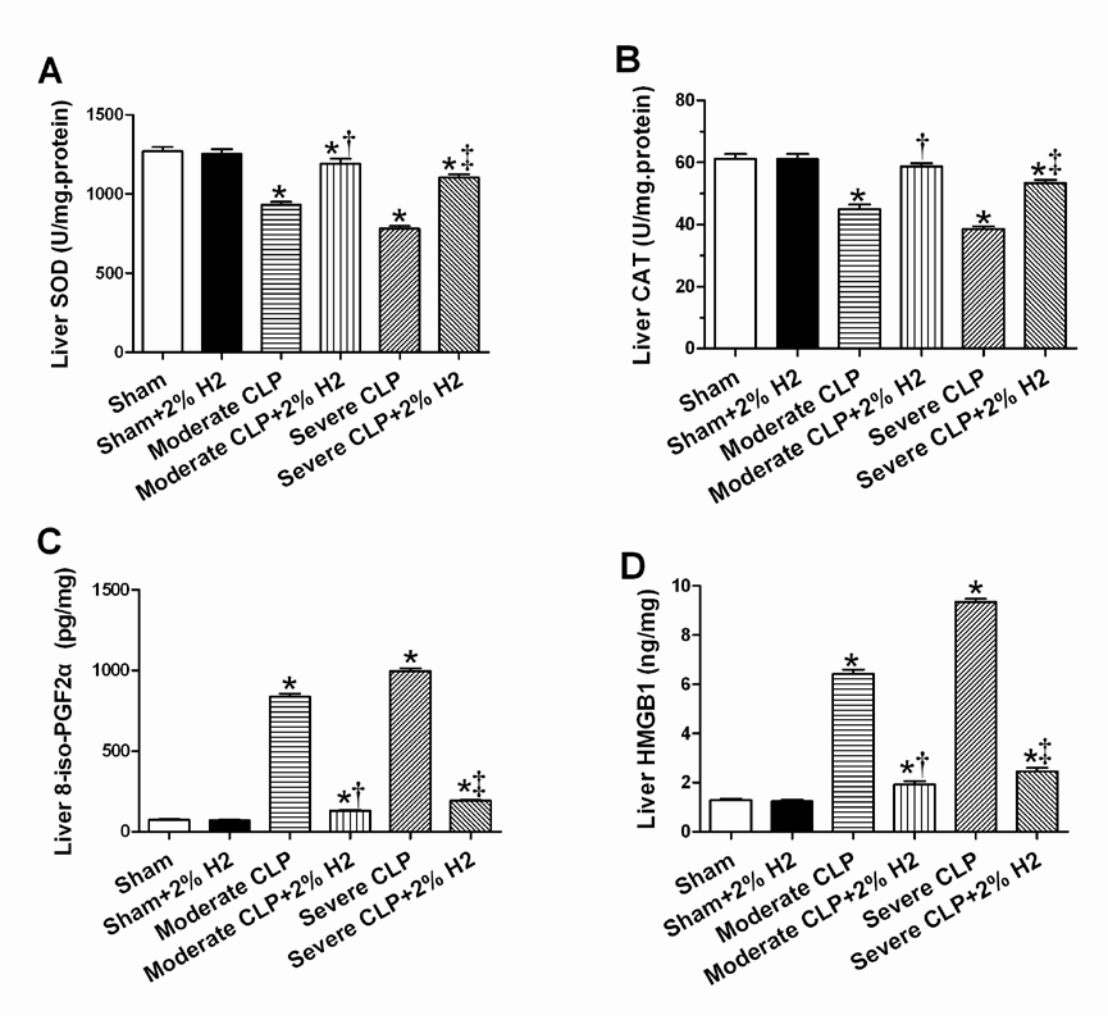
A
Liver SOD (U/mg.protein)
1500
1000
500
0
Sham
Sham+2% H2
Moderate CLP
Moderate CLP+2% H2
Severe CLP
Severe CLP+2% H2
B
Liver CAT (U/mg.protein)
80
60
40
20
0
C
Liver 8-iso-PGF2α (pg/mg)
D
Liver HMGB1 (ng/mg)
10
8
6
4
2

FIG. 8.

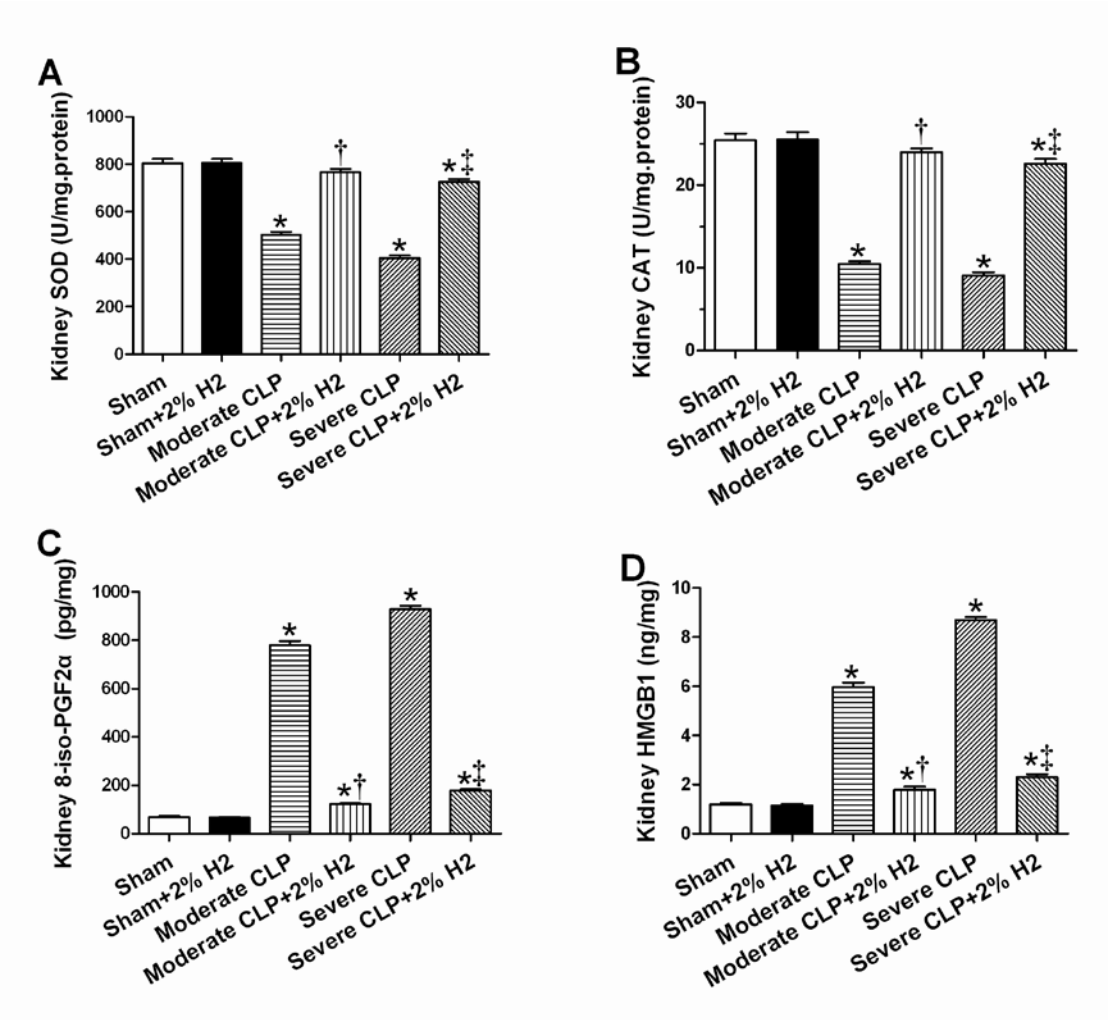
A
Kidney SOD (U/mg.protein)
1000
800
600
400
200
0
Sham
Sham+2% H2
Moderate CLP
Moderate CLP+2% H2
Severe CLP
Severe CLP+2% H2
B
Kidney CAT (U/mg.protein)
30
20
10
0
C
Kidney 8-iso-PGF2α (pg/mg)
D
Kidney HMGB1 (ng/mg)
10
8
6
4
2

Table. 1

H_2 inhalation at a 2% or 4% concentration had no significant effects on pH, P_aO_2 and P_aCO_2 in mice with or without sepsis during the treatment.

Group	pH	P_aO_2	P_aCO_2
Sham	7.41±0.12	96.52±3.12	35.71±1.38
Sham+2% H_2	7.40±0.13	96.49±2.87	35.62±1.52
Sham+4% H_2	7.40±0.15	95.72±3.83	36.11±1.61
Moderate CLP	7.39±0.16	95.89±3.76	35.38±1.53
Moderate CLP+2% H_2	7.41±0.18	96.93±3.62	36.29±1.72
Moderate CLP+4% H_2	7.40±0.17	95.34±3.72	36.81±1.54
Severe CLP	7.39±0.21	95.76±3.81	35.41±1.81
Severe CLP+2% H_2	7.40±0.19	96.86±3.98	36.78±1.63
Severe CLP+4% H_2	7.40±0.23	95.67±4.11	37.12±1.92