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PROTECTIVE EFFECTS OF HYDROGEN GAS ON MURINE POLYMICROBIAL SEPSIS VIA REDUCING OXIDATIVE STRESS AND HMGB1 RELEASE

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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. Excessive production of reactive oxygen species 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 reactive oxygen species) and effectively protects against organ damage induced by I/R. 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 h 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 activity, wet-to-dry weight ratio, protein concentration in bronchoalveolar lavage, serum biochemical parameters, and organ histopathologic scores at 24 h 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 in serum and tissue. Thus, H_2 inhalation may be an effective therapeutic strategy for patients with sepsis.

KEYWORDS—Sepsis, acute lung injury, organ damage, reactive oxygen species, high-mobility group box 1, antioxidant enzyme, hydrogen gas

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; H_2–hydrogen; H_2O_2–hydrogen peroxide; HMGB1–high-mobility group box 1; 8-iso-PGF2α–8-iso-prostaglandin F2α; MPO–myeloperoxidase; •OH–hydroxyl radicals; ROS–reactive oxygen species; SOD–superoxide dismutase; W/D–wet-to-dry

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, which is a complex, incompletely understood, and often fatal disorder, typically accompanied by multiple organ dysfunction (1, 2). More than 750,000 people become septic each year with a mortality rate of 30% to 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 the high mortality rate. 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 important roles in the pathogenesis of sepsis (4). Therefore, many researchers have focused on reducing the levels of ROS to treat sepsis (4). Hydrogen (H_2) gas has been used in medical applications to prevent decompression sickness in deep-sea 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 I/R (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 because of 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

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antioxidant enzymes and high-mobility group box 1 (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°C to 22°C 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 the Fourth Military Medical University and performed in accordance with the National Institutes of Health (USA) guidelines for the use of experimental animals.

CLP model

We performed CLP as previously described (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. One milliliter of prewarmed sterile saline (pyrogen-free 0.9% NaCl, 37°C) was administered s.c. for fluid resuscitation. Animals with sham operation underwent the same procedure without CLP.

H_2 gas treatment

The animals were put in a sealed plexiglas chamber with inflow and outflow outlets. Hydrogen 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, Fullerton, Calif). 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, Calif) 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 temperatures were maintained at 20°C to 22°C. 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—One hundred twenty animals were randomly divided into four 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 min starting at 1 and 6 h after sham and moderate CLP operations, 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 days 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—One hundred twenty animals were randomly divided into four 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 h after CLP operation, the animals were exposed to different concentrations of H_2 (0%, 1%, 2%, or 4%) for 60 min. The survival rate was observed on days 1, 2, 3, 5, 7, and 14 after CLP operation.

Effects of H_2 treatment for different times on the survival rate of septic mice with moderate CLP—Based on the previous experiments, 2% H_2 treatment was used in this experiment. One hundred twenty animals were randomly divided into four 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 h after CLP operation, the animals were exposed to 2% H_2 for different times (0, 30, 60, or 90 min). The survival rate was observed on days 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 previous experiments, 2% H_2 treatment for 60 min was used in this experiment. Sixty animals were randomly divided into two 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 min at 1 and 6 h after CLP operation. 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 days 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 previous experiments, 2% H_2 treatment for 60 min was used in this experiment. An additional 36 animals were used in this experiment and were assigned to six 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 previously described. 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 h after CLP or sham operation. In addition, we detected the serum biochemical parameters, as well as liver and kidney histopathology at 24 h 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—An additional 36 animals were used in this experiment and were assigned to six groups (n = 6 per group). The grouping method and experimental protocols were the same as those in experiment two. At 24 h after CLP or sham operation, the proinflammatory cytokine (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 h 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°C 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). The *MPO activity* was defined as the quantity of enzyme degrading 1 μmol of peroxide per min at 37°C 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).

Lung 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 h at 80°C and weighed when it was dried.

BAL and total protein assay

Animals were subjected to BAL for collecting BAL fluid by the methods previously described (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 reaspirated. Lavage samples were centrifuged at 1,500*g* for 10 min at 4°C. The supernatant was stored at −20°C. Total protein concentration in BAL was determined by using a standard commercial kit (Bio-Rad Laboratories, Hercules, Calif).

Organ histological examination

Organ samples were taken at 24 h after CLP or sham operation for observing morphological alterations. The samples were fixed with 10% formalin for 6 h 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 histological changes were evaluated by two pathologists who were blinded to the treatment regimen. A scoring system to grade the degree of lung injury was used based on the following histological features: edema, hyperemia and congestion, neutrophil margination and tissue infiltration, intra-alveolar hemorrhage and debris, and cellular hyperplasia. Each feature was graded as absent, mild, moderate, or severe, with a score of 0 to 3. A total score was calculated for each animal (19). In addition, according to the scoring standard in our recently published articles (20, 21), the degree of liver and kidney injury was also graded.

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TABLE 1. **Hydrogen inhalation at a 2% or 4% concentration had no significant effects on pH, Pao_2, and $Paco_2$ in mice with or without sepsis during the treatment**

Group	pH	Pao_2	$Paco_2$
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

Enzymatic activity assay

Blood and organ specimens (lung, liver, and kidney) were collected at 24 h after CLP or sham operation. The serum was separated by centrifugation at 3,000*g* for 15 min at 4°C, aliquoted, and stored at −80°C 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°C for 10 min. The supernatants were collected, aliquoted, and stored at −80°C until the following analysis.

The activities of SOD and CAT were measured using commercial kits purchased from Cayman Chemical Company (Ann Arbor, Mich). 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 min at 25°C. All spectrophotometric readings were performed by using a spectrophotometer (DU 640B; Beckman). All assays were conducted in triplicates. The tissue protein concentration was determined by using a standard commercial kit (Bio-Rad Laboratories, Hercules, Calif).

Detection of 8-iso-PGF2α

The serum and tissue homogenates (lung, liver, and kidney) previously obtained were also used for detecting the level of 8-iso-PGF2α. Measurement of 8-iso-PGF2α, free radical–catalyzed 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 kits (Ann Arbor, Mich) using a microplate reader (CA 94089; Molecular Devices, Sunnyvale, Canada). All standards and samples were run in duplicate.

Detection of HMGB1

The serum and tissue homogenates (lung, liver, and kidney) previously obtained were also used for detecting the level of HMGB1. The levels of serum and tissue HMGB1 were detected by specific enzyme-linked immunosorbent assay 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 percentages. The measurement data are expressed as mean ± SEM. The analysis of survival rates was tested by Fisher exact test probability method. The intergroup differences of the rest of the data were tested by one-way ANOVA followed by least significant difference–*t* test for multiple comparisons. The statistical analysis was performed with SPSS 16.0 software. In all tests, a value of $P < 0.05$ was considered statistically significant.

RESULTS

H_2 inhalation at a 2% or 4% concentration had no significant effects on arterial pH, Pao_2, and $Paco_2$ in mice with or without sepsis during the treatment

In the present study, we investigated the effects of H_2 inhalation on arterial pH, Pao_2, and $Paco_2$ in mice with or without CLP operation during the treatment. The arterial blood gas was conducted at 0.5 h after the onset of H_2 inhalation (1.5 h 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, Pao_2, and $Paco_2$ 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, Pao_2, and $Paco_2$ 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 concentrations or different therapeutic times on the survival rates of septic mice with moderate or severe CLP. The 14-day survival rate of moderate CLP mice was 30% to 40% ($P < 0.05$ vs. sham group, n = 30 per group; Fig. 1). Two percent H_2 inhalation for 60 min starting at 1 and 6 h after CLP operation 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). Figure 1B shows that the protective effects of H_2 treatment on septic mice are concentration dependent. One percent 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 treatments increased the 14-day survival rate of moderate CLP mice to 80% and 90%, respectively ($P < 0.05$ vs. moderate CLP group, n = 30

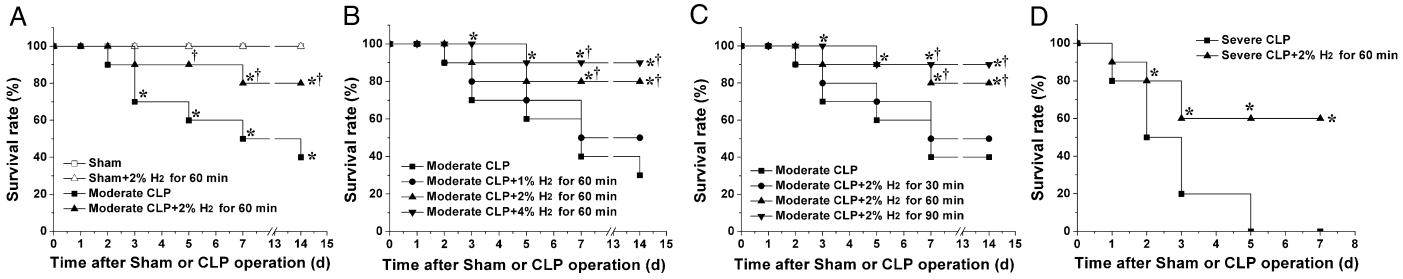

FIG. 1. **Hydrogen treatment improved the survival rate of septic mice with moderate or severe CLP in a concentration- and time-dependent manner**. The values are expressed as survival percentage (n = 30 per group). A, Effects of 2% H_2 treatment for 60 min starting at 1 and 6 h after CLP and sham operations, 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 min starting at 1 and 6 h after CLP operation 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 h after CLP operation 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 min starting at 1 and 6 h after CLP operation on the survival rate of septic mice with severe CLP. $^*P < 0.05$ vs. sham group.

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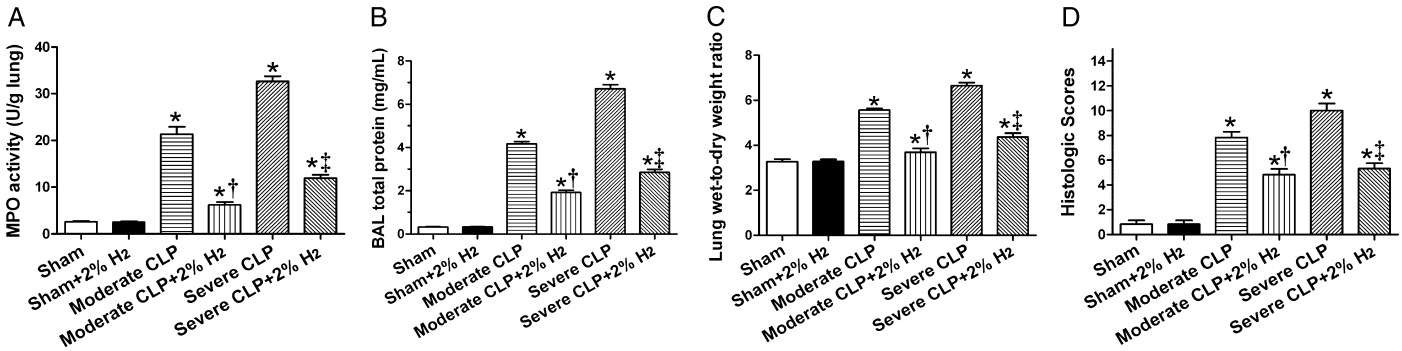

FIG. 2. **Hydrogen treatment attenuated ALI in septic mice with moderate or severe CLP**. A, Lung MPO activity. B, Lung BAL total protein. C, Lung W/D weight ratio. D, Lung histological scores. Hydrogen treatment was given by exposure to 2% H_2 for 60 min starting at 1 and 6 h after CLP and sham operations, respectively. These indicators were measured at 24 h after CLP or sham operation. The values are expressed as means ± 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.

per group; Fig. 1B). Figure 1C shows that the beneficial effects of H_2 treatment on septic mice are time dependent. Two percent H_2 inhalation for 30, 60, and 90 min starting at 1 and 6 h after CLP operation 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 min starting at 1 and 6 h after CLP operation improved the 7-day survival rate of severe CLP mice from 0% to 60% ($P < 0.05$, n = 30 per group; Fig. 1D). The previous 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 Figure 2, moderate and severe CLP mice appeared to have significant acute lung injury (ALI) at 24 h 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 histological 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 histopathologic 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. Two percent 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 seemed significant liver and kidney injury at 24 h after CLP operation,

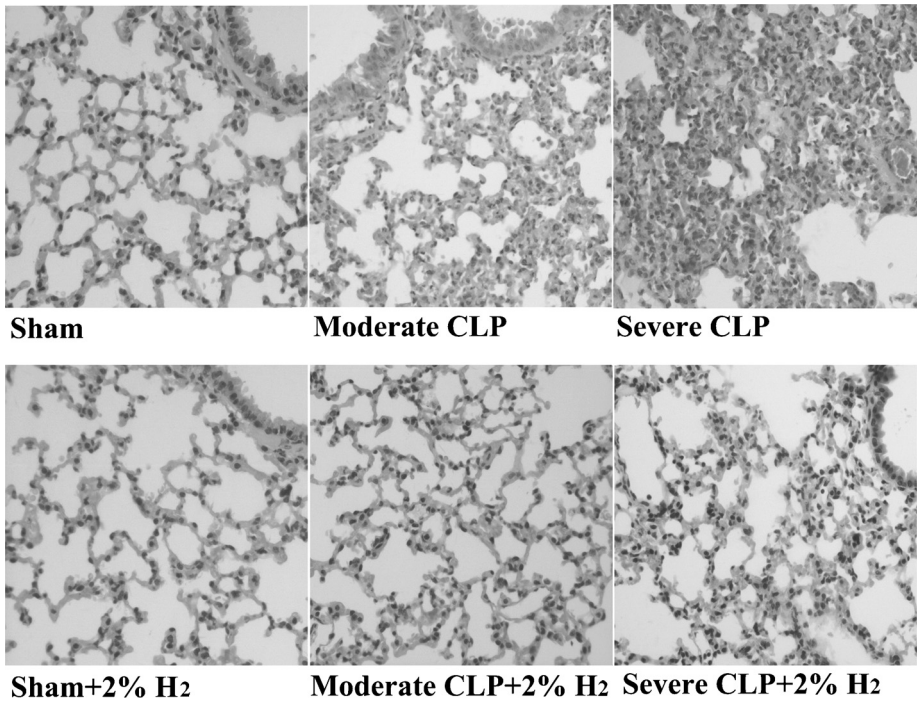

FIG. 3. **Hydrogen treatment attenuated lung histopathologic changes in septic mice with moderate or severe CLP**. Hydrogen treatment was given by exposure to 2% H_2 for 60 min starting at 1 and 6 h after CLP and sham operations, respectively. The lungs were stained with hematoxylin-eosin at 24 h after CLP or sham operation (original magnification ×40).

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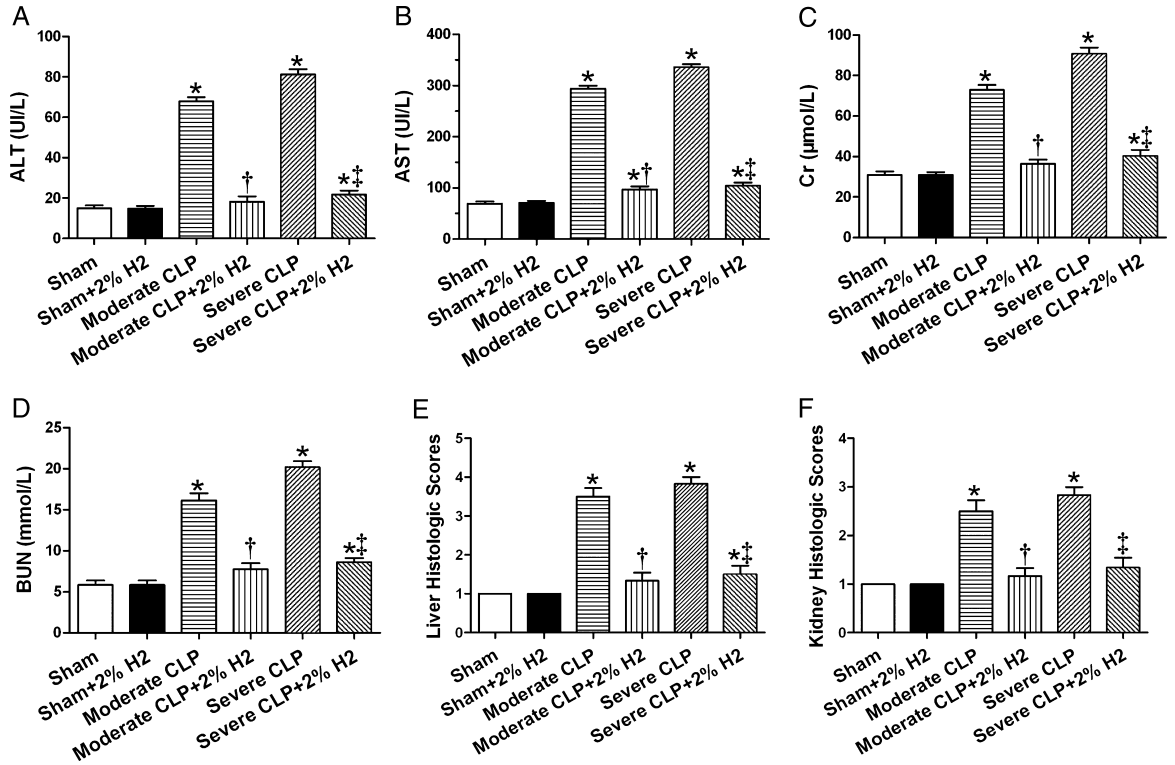

FIG. 4. **Hydrogen treatment attenuated acute liver and kidney injury in septic mice with moderate or severe CLP**. Hydrogen treatment was given by exposure to 2% H_2 for 60 min starting at 1 and 6 h after CLP and sham operations, respectively. These indicators were measured at 24 h after CLP or sham operation. The values are expressed as means ± 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. UI/L–International Unit per liter.

which was assessed by serum biochemical parameters for liver and kidney (alanine aminotransferase [ALT], aspartate aminotransferase [AST], creatinine [Cr], and blood urea nitrogen [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 histological 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 seem to have significant organ damage at 24 h 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 h 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 proinflammatory cytokine HMGB1 (a critical mediator of lethal sepsis) in serum and lung of all animals were observed. Our results showed that the

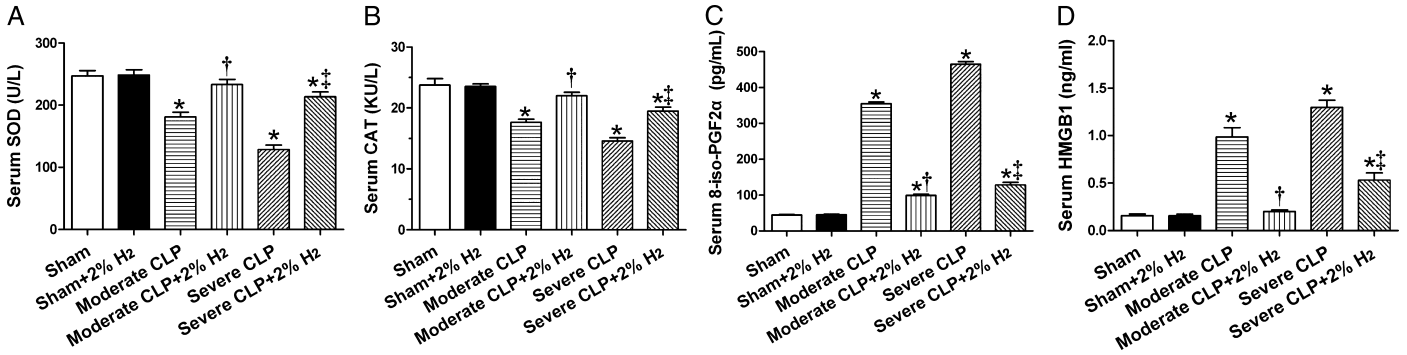

FIG. 5. **Hydrogen treatment upregulated the activities of serum antioxidant enzymes and reduced the levels of serum oxidative product and inflammatory cytokine in septic mice with moderate or severe CLP**. A, Serum SOD activity. B, Serum CAT activity. C, Serum 8-iso-PGF2α level. D, Serum HMGB1 level. Hydrogen treatment was given by exposure to 2% H_2 for 60 min starting at 1 and 6 h after CLP and sham operations, respectively. The serum was harvested for measuring these indicators at 24 h 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.

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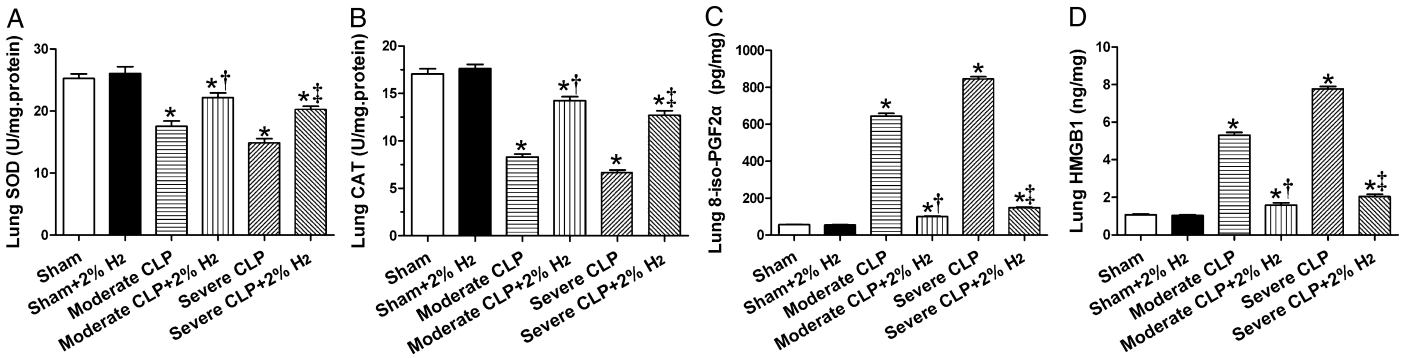

FIG. 6. **Hydrogen treatment upregulated the activities of lung antioxidant enzymes and reduced the levels of lung oxidative product and inflammatory cytokine in septic mice with moderate or severe CLP**. A, Lung SOD activity. B, Lung CAT activity. C, Lung 8-iso-PGF2α level. D, Lung HMGB1 level. Hydrogen treatment was given by exposure to 2% H_2 for 60 min starting at 1 and 6 h after CLP and sham operations, respectively. The lungs were harvested for measuring these indicators at 24 h 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. U/mg protein–unit per milligram protein.

decrease of SOD and CAT activities as well as the increase of 8-iso-PGF2α and HMGB1 levels in serum and lung occurred in mice with moderate or severe CLP ($P < 0.05$ vs. sham group, n = 6 per group; Figs. 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; Figs. 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; Figs. 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 h after CLP or sham operation. The results were similar with those in serum and lung; the detailed data were shown in Figures 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 proinflammatory cytokine HMGB1 in serum and tissue.

DISCUSSION

In the present study, we found that 1) H_2 treatment starting at 1 and 6 h 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 histopathologic scores at 24 h 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). Cecal ligation and puncture can cause lethal peritonitis and sepsis because of 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% to 40% survival rate and moderate organ injury, whereas 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 intensive care units, with a mortality that has remained more than 40% (23). In the present investigation, we also observed the increase of

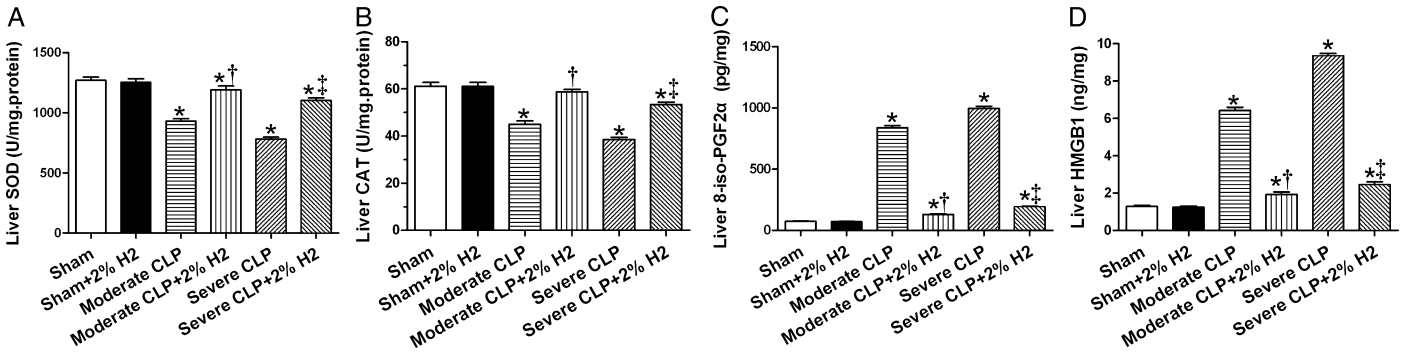

FIG. 7. **Hydrogen treatment upregulated the activities of liver antioxidant enzymes and reduced the levels of liver oxidative product and inflammatory cytokine in septic mice with moderate or severe CLP**. Hydrogen treatment was given by exposure to 2% H_2 for 60 min starting at 1 and 6 h after CLP and sham operations, respectively. The liver was harvested for measuring these indicators at 24 h 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. U/mg protein–unit per milligram protein.

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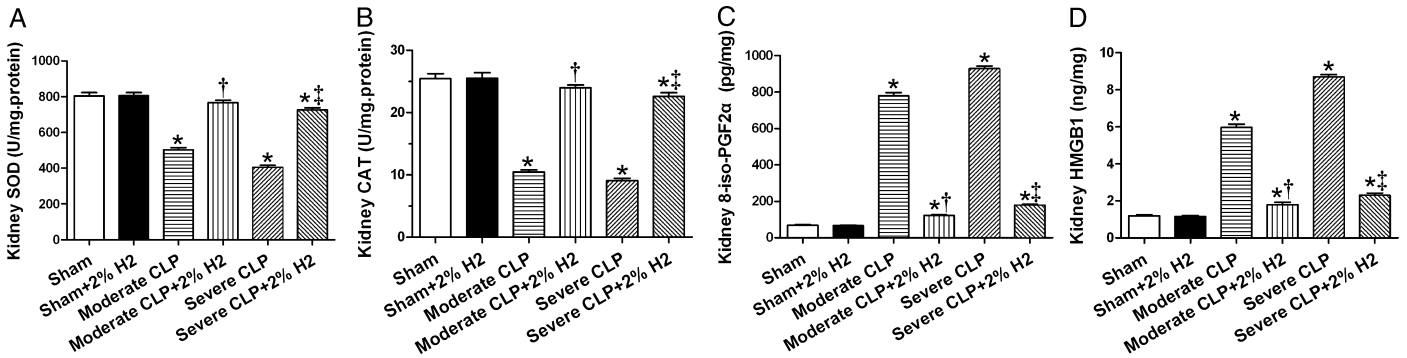

FIG. 8. **Hydrogen treatment upregulated the activities of kidney antioxidant enzymes and reduced the levels of kidney oxidative product and inflammatory cytokine in septic mice with moderate or severe CLP**. Hydrogen treatment was given by exposure to 2% H_2 for 60 min starting at 1 and 6 h after CLP and sham operations, respectively. The kidneys were harvested for measuring these indicators at 24 h 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. U/mg protein–unit per milligram protein.

lung MPO activity, lung W/D weight ratio, and protein concentration in BAL, as well as lung histopathologic injury, indicating that CLP causes significant ALI. In addition, we also found that the increase of serum biochemical parameters and histopathologic injury for liver and kidney occurred in 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 patients with sepsis.

A growing number of studies have found that excessive production of ROS and reduction of antioxidant defense systems play important roles 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 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.

Hydrogen has been used in medical applications to prevent decompression sickness in deep-sea 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 I/R, 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 h 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 histopathologic scores at 24 h after CLP operation. The previous 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 systems in moderate and severe CLP mice. In the rodent sepsis model induced by CLP, the activities of SOD, CAT, and glutathione peroxidase in tissue and serum were significantly decreased during the early and late phases, 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 h 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, whereas 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). Pharmacological agents

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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 concentrations (<4% in air), H_2 is neither explosive nor dangerous, which has been proven through 17-year-long studies on cells, mice, monkeys, and deep-sea divers (COMEX HYDRA program, Marseille, France). Inhaled H_2 at a therapeutic dose has no adverse effects on the saturation level of arterial oxygen (Spo_2) or hemodynamic parameters, and so on (13), which was also proven by the present study. Hydrogen, 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. Hydrogen 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 reactivities, 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 h 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 patients with sepsis because of its ability to rapidly diffuse across membranes.

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