COMBINATION THERAPY WITH MOLECULAR HYDROGEN AND HYPEROXIA IN A MURINE MODEL OF POLYMICROBIAL SEPSIS

Keliang Xie,* Wenzheng Fu,† Weibin Xing,‡ Ailin Li,§ Hongguang Chen,* Huanzhi Han,* Yonghao Yu,* and Guolin Wang*

*Department of Anesthesiology, General Hospital of Tianjin Medical University; †Department of Colorectal and Anal Surgery, Tianjin Union Medical Center, Tianjin People’s Hospital, Tianjin; ‡Department of Dermatology, Cangzhou People’s Hospital, Hebei Province, Hebei; and §Department of Neurosurgery, Tianjin Huanhu Hospital, Tianjin, People’s Republic of China

Received 19 Jul 2012; first review completed 7 Aug 2012; accepted in final form 21 Sep 2012

ABSTRACT—Sepsis is the most common cause of death in intensive care units. Some studies have found that hyperoxia may be beneficial to sepsis. However, the clinical use of hyperoxia is hindered by concerns that it could exacerbate organ injury by increasing free radical formation. Recently, it has been suggested that molecular hydrogen (H₂) at low concentration can exert a therapeutic antioxidant activity and effectively protect against sepsis by reducing oxidative stress. Therefore, we hypothesized that combination therapy with H₂ and hyperoxia might afford more potent therapeutic strategies for sepsis. In the present study, we found that inhalation of H₂ (2%) or hyperoxia (98%) alone improved the 14-day survival rate of septic mice with moderate cecal ligation and puncture (CLP) from 40% to 80% or 70%, respectively. However, combination therapy with H₂ and hyperoxia could increase the 14-day survival rate of moderate CLP mice to 100% and improve the 7-day survival rate of severe CLP mice from 0% to 70%. Moreover, moderate CLP mice showed significant organ damage characterized by the increases in lung myeloperoxidase activity, lung wet-to-dry weight ratio, protein concentration in bronchoalveolar lavage, serum biochemical parameters (alanine aminotransferase, aspartate aminotransferase, creatinine, and blood urea nitrogen), and organ histopathological scores (lung, liver, and kidney), as well as the decrease in Paco₂/Fio₂ ratio at 24 h, which was attenuated by either H₂ or hyperoxia alone. However, combination therapy with H₂ and hyperoxia had a more beneficial effect against lung, liver, and kidney damage of moderate or severe CLP mice. Furthermore, we found that the beneficial effect of this combination therapy was associated with the decreased levels of oxidative product (8-iso-prostaglandin F₂α), increased activities of antioxidant enzymes (superoxide dismutase and catalase), and anti-inflammatory cytokine (interleukin 10), and reduced levels of proinflammatory cytokines (high-mobility group box 1 and tumor necrosis factor α) in serum and tissues. Therefore, combination therapy with H₂ and hyperoxia provides enhanced therapeutic efficacy via both antioxidant and anti-inflammatory mechanisms and might be potentially a clinically feasible approach for sepsis.

KEYWORDS—Sepsis, organ damage, molecular hydrogen, hyperoxia, antioxidant enzyme, high-mobility group box 1

INTRODUCTION

Despite substantial advances in antibiotic therapy and intensive care, sepsis remains the most common cause of death in intensive care units, with a mortality of 30% to 50%, which is often accompanied by multiple organ dysfunction (1, 2). In the United States, there are 751,000 cases of severe sepsis each year, with a total annual cost of $16.7 billion and a progressive increase in its incidence over time of 8.7% (1, 2). It is exceedingly difficult to develop effective therapeutic interventions that reduce so high mortality because the factors responsible for sepsis are not fully understood (3). Therefore, there is considerable interest in exploring an effective therapy for patients with sepsis.

Oxygen is often used in critically ill patients. Early goal-directed therapy for sepsis aims to balance tissue oxygen (O₂) delivery and demand. Our and other studies have found that hyperoxia has a beneficial effect against sepsis and sepsis-associated multiple organ damage (4–8). Yet, the clinical use of hyperoxia is hindered by concerns that it may exacerbate organ damage by increasing free radical formation. Oxidative stress plays an essential role in the pathogenesis of sepsis, and overproduction of reactive oxygen species (ROS) can exacerbate organ damage (9, 10). Recently, it has been suggested that a low concentration of molecular hydrogen (H₂) exerts a therapeutic antioxidant activity by selectively reducing hydroxyl radicals (•OH, the most cytotoxic ROS) and peroxynitrite (ONOO⁻) in vitro and also effectively protects against many diseases in vivo (11–15). Furthermore, our studies have
demonstrated that 2% or 4% H\textsubscript{2} can alleviate organ injury and improve survival rate of animals with sepsis via reducing oxidative stress and inflammation (16, 17).

Therefore, these findings strongly indicate that combination therapy with H\textsubscript{2} and hyperoxia may afford more potent therapeutic strategies for sepsis. It is well known that cecal ligation and puncture (CLP) can cause lethal peritonitis and sepsis, which is accompanied by multiple organ dysfunction (18). Thus, the present study was designed to investigate whether combination therapy with H\textsubscript{2} and hyperoxia could produce enhanced efficacy in a murine model of moderate or severe CLP. In addition, the roles of oxidative stress and inflammatory cytokines in the protective effect were studied. Our results may establish a clinically applicable strategy for the treatment of sepsis and provide a new avenue for the use of therapeutic gas in patients.

**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 the Military Medical Science Academy of the PLA. Animals were housed at 20°C to 22°C with a 12-h light-dark cycle. Laboratory mice were acclimatized for 8 h before the experiments. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University and performed in accordance with the National Institutes of Health guidelines for the use of experimental animals.

**CLP model**

Sepsis was induced by CLP model performed as described previously (16, 18, 19). Briefly, the animals were anesthetized 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 hock and a single through-and-through perforation of the ligated segment. For severe CLP (100% lethality), we ligated the distal three quarters of cecum and made a single puncture with a 20-gauge needle; for moderate CLP (30%–40% survival), we ligated the distal one half of 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 abdomen and closed the incision using a sterile 6-0 silk suture. Finally, we administered subcutaneously 1 mL of prewarmed sterile saline (pyrogen-free 0.9% NaCl, 37°C) for fluid resuscitation. Animals with sham operation underwent the same procedure without CLP.

**Molecular hydrogen and/or hyperoxia treatment**

The animals were put in a sealed Plexiglas chamber with inflow and outflow outlets (16, 17). Molecular hydrogen, O\textsubscript{2}, or N\textsubscript{2} was supplied through a gas flowmeter, respectively, and delivered into the chamber through a tube at a rate of 4 L/min. The concentrations of O\textsubscript{2} and N\textsubscript{2} were monitored continuously with a gas analyzer (Medical Gas Analyzer LB-2, Model 40 M; Beckman, Fullerton, Calif) and a commercially available detector (Hy Alerta Handheld Detector Model 500; H\textsubscript{2} Scan, Valencia, Calif), respectively. Carbon dioxide was removed from the chamber gases with Baralyme (Chemetron Medical Division, Allied Healthcare Products, Inc., St. Louis, Mo). The mixed gases were maintained at the predetermined level (2% H\textsubscript{2}, 21% O\textsubscript{2}, and 77% N\textsubscript{2}; 0% H\textsubscript{2}, 98% O\textsubscript{2}, and 2% N\textsubscript{2}; 2% H\textsubscript{2}, 98% O\textsubscript{2}, and 0% N\textsubscript{2}) during the treatment. The animals without H\textsubscript{2} or hyperoxia treatment were exposed to room air in the chamber. Food and water were available ad libitum during the treatment.

**Experimental design**

Experiment 1: Effects of H\textsubscript{2} and/or hyperoxia treatment on the survival rate of septic mice with moderate or severe CLP—Effects of H\textsubscript{2} and/or hyperoxia treatment on the survival rate of septic mice with moderate CLP. One hundred fifty animals were randomly divided into five groups (n = 30 per group); sham, moderate CLP, moderate CLP + O\textsubscript{2}, moderate CLP + H\textsubscript{2}, and moderate CLP + H\textsubscript{2} + O\textsubscript{2} groups. The treatment concentrations of hydrogen and hyperoxia were determined based on our previous studies and preliminary observations (8, 16). The animals in the moderate CLP + O\textsubscript{2} and moderate CLP + H\textsubscript{2} groups were exposed to 98% O\textsubscript{2} or 2% H\textsubscript{2} for 3 h starting at 1 and 6 h after moderate CLP operation, respectively. The animals in the moderate CLP + H\textsubscript{2} + O\textsubscript{2} group were exposed to 98% O\textsubscript{2} and 2% H\textsubscript{2} at the same time points. 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 H\textsubscript{2} and hyperoxia treatment on the survival rate of septic mice with severe CLP. Based on the above experiments, 60 animals were randomly divided into two groups (n = 30 per group): severe CLP and severe CLP + H\textsubscript{2} + O\textsubscript{2} groups. The animals in both groups were exposed to severe CLP operation. The animals in the severe CLP + H\textsubscript{2} + O\textsubscript{2} group were exposed to 98% O\textsubscript{2} and 2% H\textsubscript{2} for 3 h starting at 1 and 6 h 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 days 1, 2, 3, 5, and 7 after CLP operation.

Experiment 2: Effects of H\textsubscript{2} and/or hyperoxia treatment on sepsis-associated organ injury in mice with moderate or severe CLP—Additional 42 animals were used in this experiment and were assigned to seven groups (n = 6 per group): sham, moderate CLP, moderate CLP + O\textsubscript{2}, moderate CLP + H\textsubscript{2}, moderate CLP + H\textsubscript{2} + O\textsubscript{2}, severe CLP, and severe CLP + H\textsubscript{2} + O\textsubscript{2} groups. The detailed experimental protocols were the same as described above. At 24 h after CLP or sham operation, the proinflammatory cytokines (high-mobility group box 1 [HMGB1] and tumor necrosis factor α [TNF-α]), anti-inflammatory cytokine (interleukin 10 [IL-10]), antioxidant enzymes (superoxide dismutase [SOD] and catalase [CAT]), and oxidative product (8-iso-prostaglandin F\textsubscript{2α} [8-iso-PGF\textsubscript{2α}; Cayman Chemical, Ann Arbor, Mich]) in serum, lung, liver, and kidney tissues were measured.

**Oxygenation index analysis**

To evaluate the oxygenation capability of lung, the ratio of O\textsubscript{2} tension to inspired O\textsubscript{2} fraction (Pat\textsubscript{O\textsubscript{2}}/FIO\textsubscript{2}) was calculated. At 24 h of CLP or sham operation, animals were anesthetized and given endotracheal intubation with a 20-gauge catheter. They were subjected to mechanical ventilation with pure O\textsubscript{2} at 7 mL/kg. The respiratory rate was 120 breaths/min. The animals were ventilated for 15 min before blood gas sampling. The arterial blood was obtained from carotid artery and measured with a GEM Premier 3000 gas analyzer (Instrumentation Laboratory, Milan, Italy).

**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. The supernatant from lung homogenate was prepared for detecting the activity of MPO, an indicator of neutrophil infiltration, which was measured as previously reported (16, 20). The 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.

**BAL and total protein assay**

Animals were subjected to BAL for collecting BAL fluid by the methods described previously (16, 21). Two volumes of 0.5 mL of PBS (pH 7.4) were instilled, gently aspirated, pooled, and reaspirated. Lavage samples were centrifuged at 1,500g for 10 min at 4°C. The supernatant was stored at −20°C. The total protein concentration in BAL was determined by using a standard commercial kit (Bio-Rad Laboratories, Hercules, Calif).

**Organ histologic examination**

Organ samples were collected at 24 h after CLP or sham operation for observing morphologic alteration. 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-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 damage was used (16, 22). In addition, according to...
the scoring standard in our recent articles (8, 16), the degree of liver and kidney injury was also graded.

**Antioxidant 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 3,000g for 15 min at 4°C, aliquoted, and stored at −80°C. The tissue homogenates were prepared in chilled PBS (0.1 M, pH 7.4) and centrifuged at 10,000g for 10 min at 4°C. 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). All spectrophotometric readings were performed using a spectrophotometer (DU 640B; Beckman). The tissue protein concentration was determined by a standard commercial kit (Bio-Rad Laboratories, Hercules, Calif).

**Detection of 8-iso-PGF2α**

The serum and tissue homogenates (lung, liver, and kidney) obtained above were also used for detecting the 8-iso-PGF2α level. 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 (23). The levels of serum and tissue 8-iso-PGF2α were detected by specific enzyme-linked immunosorbent assay kits (8-iso-PGF2α; Ann Arbor, Mich) using a microplate reader (CA 94089; Molecular Devices, Sunnyvale, Calif).

**Detection of inflammatory cytokines**

The serum and tissue homogenates (lung, liver, and kidney) obtained above were also used for detecting the levels of proinflammatory cytokines (HMGB1 and TNF-α) and anti-inflammatory cytokine (IL-10). The levels of inflammatory cytokines were detected by specific enzyme-linked immunosorbent assay kits (TNF-α and IL-10 from R&D Systems, Minneapolis, Minn; HMGB1 from IBL, Hamburg, Germany) with a microplate reader (CA 94089; Molecular Devices).

**Statistical analysis**

The survival rates are expressed as percentage. The measurement data are expressed as mean ± SD. The analysis of survival rates was tested by Fisher exact probability method. The intergroup differences of the rest data were tested by one-way analysis of variance followed by least significant difference (LSD)-t test for multiple comparisons. The statistical analysis was performed with SPSS 16.0 software (SPSS Inc., Chicago, Ill). In all tests, P < 0.05 was considered statistically significant.

**RESULTS**

**Changes of arterial blood gas during the treatment**

In the present study, we investigated the levels of arterial pH, PaO2, and PaCO2 in mice with or without CLP operation during the treatment. The arterial blood gas was conducted at 1.5 h after onset of the first treatment (2.5 h after CLP or sham operation) using a GEM Premier 3000 gas analyzer (Instrumentation Laboratory). The levels of PaO2 in the moderate CLP + O2, moderate CLP + H2 + O2, and severe CLP + H2 + O2 groups were 415.2 ± 18.4, 410.7 ± 19.3, and 412.4 ± 23.8 mmHg, respectively. The PaO2 level in the moderate CLP + H2 group was 97.23 ± 8.42 mmHg, whereas the levels of PaO2 in the sham, moderate CLP, and severe CLP groups were 96.38 ± 7.56, 95.87 ± 8.34, and 95.76 ± 10.31 mmHg, respectively. There were no differences in the levels of arterial pH and PaO2 among all groups (data not shown). The results demonstrate that hyperoxia exposure can significantly increase the PaO2 level, and 2% H2 has no marked effects on the PaO2 level in moderate or severe CLP mice during the treatment.

**Combination therapy with H2 and hyperoxia improved the survival rate of moderate or severe CLP mice**

In this study, we investigated the effects of H2 and/or hyperoxia treatment on the survival rates of septic mice with moderate or severe CLP. The 14-day survival rate of moderate CLP mice was 40% (P < 0.05 vs. sham group, n = 30 per group; Fig. 1A). We found that either 98% O2 or 2% H2 exposure for 3 h starting at 1 and 6 h after CLP operation, respectively, improved the 14-day survival rate of moderate CLP mice to 70% or 80% (P < 0.05 vs. moderate CLP group, n = 30 per group; Fig. 1A). Furthermore, combination therapy with 2% H2 and 98% O2 increased the 14-day survival rate of moderate CLP mice to 100% (P < 0.05 vs. moderate CLP group, n = 30 per group; Fig. 1A). In addition, combination therapy with 2% H2 and 98% O2 improved the 7-day survival rate of severe CLP mice from 0% to 70% (P < 0.05, n = 30 per group; Fig. 1B). The above data suggest that combination therapy with H2 and hyperoxia can improve the survival rate of septic mice with moderate or severe CLP in a synergistic manner.

**Combination therapy with H2 and hyperoxia attenuated acute organ injury in moderate or severe CLP mice**

Moderate and severe CLP mice appeared to have profound acute lung injury, which was assessed by MPO activity, W/D ratio, protein concentration in BAL, histopathology, and PaO2/FiO2 ratio. Moderate and severe CLP mice showed the 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; Figs. 2 and 4), as well as the decrease in PaO2/FiO2 ratio (P < 0.05 vs. sham group, n = 6 per...
group; Fig. 2). These abnormal changes in the moderate CLP mice were attenuated by 98% O2 or 2% H2 treatment alone (P < 0.05 vs. moderate CLP, n = 6 per group; Figs. 2 and 4). Furthermore, these abnormal changes in the moderate and severe CLP mice were more significantly ameliorated by combination therapy with 98% O2 and 2% H2 (Figs. 2 and 4).

Moreover, moderate and severe CLP mice appeared to have significant liver and kidney damage at 24 h, which was assessed by serum biochemical parameters (alanine aminotransferase, aspartate aminotransferase, creatinine, and blood urea nitrogen) and histopathology. Moderate and severe CLP mice showed a significant increase in the levels of serum alanine aminotransferase, aspartate aminotransferase, creatinine, and blood urea nitrogen, as well as liver and kidney histologic scores (P < 0.05 vs. sham group, n = 6 per group; Figs. 3 and 4). These abnormal changes in the moderate CLP mice were attenuated by either 98% O2 or 2% H2 treatment alone (P < 0.05 vs. moderate CLP, n = 6 per group; Figs. 3 and 4). Furthermore, these abnormal changes in the moderate and severe CLP mice were more significantly ameliorated by combination therapy with 98% O2 and 2% H2 (Figs. 3 and 4). In addition, the histopathological changes of lung, liver, and kidney are shown in the Figure, Supplemental Digital Content 1, at http://links.lww.com/SHK/A145, which shows the effects of H2 and/or O2 on organ histopathological changes in septic mice with moderate or severe CLP. The lung, liver, and kidney were stained with hematoxylin-eosin at 24 h after CLP or sham operation (original magnification ×20). These results demonstrate that combination therapy with H2 and hyperoxia has an enhanced efficacy against multiple organ damage in moderate and severe sepsis.

**Combination therapy with H2 and hyperoxia prevented the abnormal changes of antioxidant enzymatic activities, oxidative product, and inflammatory cytokines in moderate or severe CLP mice**

The activities of antioxidant enzymes (SOD and CAT), the levels of oxidative product 8-iso-PGF2α, and the levels of proinflammatory cytokines (HMGB1 and TNF-α) and anti-inflammatory cytokine IL-10 in serum and tissues (lung, liver, and kidney) of all animals were observed at 24 h after CLP or sham operation. Our results showed that the decreased activities of antioxidant enzymes (SOD and CAT) as well as the increased levels of oxidative product (8-iso-PGF2α) and inflammatory cytokines (HMGB1, TNF-α, and IL-10) in serum and tissues (lung, liver, and kidney) occurred to mice with moderate or severe CLP (P < 0.05 vs. sham group, n = 6 per group; Figs. 5–8). Combination therapy with 2% H2 and 98% O2 significantly increased the SOD and CAT activities and decreased the 8-iso-PGF2α level in serum and tissues (lung, liver, and kidney) of septic mice with moderate or severe CLP (P < 0.05, n = 6 per group; Figs. 5–8). In addition, Combination therapy with H2 and O2 significantly decreased the HMGB1 and TNF-α levels as well as increased the IL-10 level in serum and tissues (lung, liver, and kidney) of septic mice with moderate or severe CLP (P < 0.05, n = 6 per group; Figs. 5–8). These data suggest that combination therapy with H2 and hyperoxia provides more beneficial effects on sepsis.
and sepsis-associated organ damage, which are associated with the decreased levels of oxidative product and proinflammatory cytokines and the increased levels of antioxidant enzymes and anti-inflammatory cytokine in serum and tissues.

**DISCUSSION**

In the present study, we found that (1) either H₂ or hyperoxia treatment alone improved the survival rate of septic mice with moderate CLP, whereas combination therapy with H₂ and hyperoxia could synergistically increase the survival rate of septic mice with moderate and severe CLP, which was greater than treatment with either gas alone; (2) either H₂ or hyperoxia treatment alone protected against the lung, liver, and kidney damage of moderate CLP mice, whereas combination therapy with H₂ and hyperoxia provided cumulative protection against these organ damage of moderate and severe CLP mice; (3) the beneficial effects of combination therapy with H₂ and hyperoxia on sepsis and sepsis-associated organ injury were associated with the decreased levels of oxidative stress and proinflammatory cytokines as well as the increased activities of antioxidant enzymes and anti-inflammatory cytokine in serum and tissues.

Cecal ligation and puncture is considered to be a clinically relevant model for studying the pathogenesis and treatment of sepsis (18). It can cause lethal peritonitis and sepsis due to a polymicrobial infection, which is accompanied by multiple organ damage. Therefore, the present study was designed to investigate the therapeutic effects of H₂ and/or hyperoxia on sepsis in mice with moderate or severe CLP. In this study,
we successfully produced the moderate or severe CLP model. Moderate CLP caused a 40% survival rate and moderate organ damage, whereas severe CLP caused 100% mortality and severe organ damage. In the present investigation, we observed the increase in lung MPO activity, W/D weight ratio, protein concentration in BAL, $PaO_2/FIO_2$ ratio, and histopathological injury, indicating that CLP causes significant acute lung injury. Furthermore, we found that the increase in serum biochemical parameters and histopathological injury for liver and kidney occurred to moderate or severe CLP mice, demonstrating that

**FIG. 6.** Effects of combination therapy with $H_2$ and $O_2$ on the levels of antioxidant enzymes, oxidative product, and inflammatory cytokines in lungs of septic mice with moderate or severe CLP. The lungs were harvested for measuring these indicators at 24 h after CLP or sham operation. The values are expressed as mean ± SD (n = 6 per group). *$P < 0.05$ vs. sham group, †$P < 0.05$ vs. moderate CLP group, ‡$P < 0.05$ vs. severe CLP group.

**FIG. 7.** Effects of combination therapy with $H_2$ and $O_2$ on the levels of antioxidant enzymes, oxidative product, and inflammatory cytokines in liver of septic mice with moderate or severe CLP. The liver was harvested for measuring these indicators at 24 h after CLP or sham operation. The values are expressed as mean ± SD (n = 6 per group). *$P < 0.05$ vs. sham group, †$P < 0.05$ vs. moderate CLP group, ‡$P < 0.05$ vs. severe CLP group.
CLP leads to significant liver and kidney injury. Thus, it is critical to develop effective strategies for treatment of organ damage in septic patients.

Oxygen therapy is widely used in clinical practice as a mainstay of supportive treatment for patients experiencing hypoxemia and critical illness. It is well known that early goal-directed therapy for sepsis or septic shock aims to balance $O_2$ delivery and demand. In recent years, some animal studies have shown that hyperoxia exposure can improve organ function and survival rate in several models of shock or sepsis (4-8, 24, 25). In addition, we also find that $100\% O_2$ exposure for 2 and 3 h starting at 4 and 12 h, respectively, after zymosan injection benefits the outcome of mice with sterile sepsis (8). It is believed that the improved tissue oxygenation and decreased systemic inflammatory response play an essential role in the protection of hyperoxia treatment (4-8).

However, hyperoxia treatment can induce the production of ROS, which is considered to be associated with $O_2$ toxicity (9, 10). Therefore, the use of hyperoxia is hindered in critically ill patients by concerns that it may exacerbate organ damage by increasing free radical formation.

Molecular hydrogen has been used in medical applications to prevent decompression sickness in deep divers for safety profiles. Recently, several studies demonstrate that $H_2$ exerts a therapeutic antioxidant activity by selectively reducing •OH and $\text{ONOO}^-\text{O}_2$ and effectively protecting against many diseases, suggesting that $H_2$ has potential as an antioxidant for preventive and therapeutic applications (11-15). Our recent studies have shown that $H_2$ treatment has a beneficial effect on sepsis and sepsis-induced organ injury in a concentration- and time-dependent manner (16, 17). Because of the selective nature of •OH and $\text{ONOO}^-\text{O}_2$ inactivation, it is believed that $H_2$ treatment does not eliminate superoxide anions ($O_2^-\text{O}_2$) or hydrogen peroxide (11). Therefore, $H_2$ therapy might spare the innate immune system (macrophages and neutrophils), which requires these ROS ($O_2^-\text{O}_2$ and $H_2\text{O}_2$) to kill some types of phagocytosed bacteria. In addition, $H_2$ is continuously produced in the body by colonic bacteria and circulates in the blood under normal conditions (26). In the present study, we indeed found that combination therapy with $H_2$ and hyperoxia could produce a synergistic protective effect against sepsis and sepsis-associated multiple organ damage.

To further investigate the possible mechanism, we studied the effects of $H_2$ and/or hyperoxia treatment on oxidant and antioxidant system in moderate and severe CLP mice. In rodent sepsis induced by CLP, the activities of SOD, CAT, and glutathione peroxidase in serum and tissues are significantly decreased during the early and late phases, suggesting that sepsis sets up an environment favorable for oxidative stress (27). On the other hand, the detection of products of lipid peroxidation has been widely used to estimate the overall status of oxidative stress. In this study, we observed the decrease in SOD and CAT activities as well as the increase in oxidation product 8-iso-PGF2α in serum, lung, liver, and kidney at 24 h after moderate or severe CLP operation. We further found that combination therapy with $H_2$ and hyperoxia significantly improved the CAT and SOD activities and decreased the 8-iso-PGF2α level in serum and tissues. These results suggest that...
the decrease in oxidative damage and the increase in endogenous antioxidant enzymatic activities may attribute to the protection of H₂ and hyperoxia treatment.

It is also believed that the uncontrolled and exaggerated inflammatory response plays a major role in the pathogenesis of sepsis. The inflammatory cytokines include early inflammatory cytokines such as proinflammatory cytokines, TNF-α and IL-6, and anti-inflammatory cytokine, IL-10, as well as the late inflammatory cytokine, HMGB1 (16, 28). Many researchers discover that a ubiquitous protein HMGB1, which is released by activated macrophages/monocytes, functions as a late mediator of lethal endotoxemia and sepsis (28, 29). Many studies have found that HMGB1 is a necessary and sufficient mediator of lethal organ damage in murine CLP sepsis (28, 29). Pharmacologic agents that reduce circulating HMGB1 levels, such as ethyl pyruvate, provide significant protection against polymicrobial sepsis lethality (30). On the other hand, administration of recombinant HMGB1 to mice recapitulates many clinical signs of sepsis, including fever, intestinal barrier dysfunction, and organ injury (29). Here, we found that combination therapy with H₂ and hyperoxia significantly reduced the HMGB1 and TNF-α levels as well as increased the IL-10 level in serum and tissues of moderate or severe CLP mice and thereby protected against the development of lethal organ damage.

Molecular hydrogen is neither explosive nor dangerous at a low concentration (<4.1% in O₂). Mixed gas therapy with H₂ and hyperoxia is a novel, safe, and potent approach for preventing organ damage and mortality in sepsis. Combination therapy with H₂ and hyperoxia may be a more effective therapeutic strategy for septic patients (31, 32). This study supports the clinical application of therapeutic medical gases and may justify a new avenue of investigation in therapeutic gas use in critically ill patients.

REFERENCES