

## HYDROGEN GAS IMPROVES SURVIVAL RATE AND ORGAN DAMAGE IN ZYMOSAN-INDUCED GENERALIZED INFLAMMATION MODEL

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**ABSTRACT**—Sepsis/multiple organ dysfunction syndrome is the leading cause of death in critically ill patients. Recently, it has been suggested that hydrogen gas (H<sub>2</sub>) exerts a therapeutic antioxidant activity by selectively reducing hydroxyl radical (•OH, the most cytotoxic reactive oxygen species). We have found that H<sub>2</sub> inhalation significantly improved the survival rate and organ damage of septic mice with moderate or severe cecal ligation and puncture. In the present study, we investigated the effects of 2% H<sub>2</sub> treatment on survival rate and organ damage in zymosan (ZY)-induced generalized inflammation model. Here, we found that 2% H<sub>2</sub> inhalation for 60 min starting at 1 and 6 h after ZY injection, respectively, significantly improved the 14-day survival rate of ZY-challenged mice from 10% to 70%. Furthermore, ZY-challenged mice showed significant multiple organ damage characterized by the increase in serum biochemical parameters (aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, and creatinine), as well as lung, liver, and kidney histopathological scores at 24 h after ZY injection, which was significantly attenuated by 2% H<sub>2</sub> treatment. In addition, we found that the beneficial effects of H<sub>2</sub> treatment on ZY-induced organ damage were associated with the decreased levels of oxidative product, increased activities of antioxidant enzyme, and reduced levels of early and late proinflammatory cytokines in serum and tissues. In conclusion, this study provides evidence that H<sub>2</sub> treatment protects against multiple organ damages in ZY-induced generalized inflammation model, suggesting the potential use of H<sub>2</sub> as a therapeutic agent in the therapy of conditions associated with inflammation-related multiple organ dysfunction syndrome.

**KEYWORDS**—Sepsis, multiple organ dysfunction syndrome/failure, reactive oxygen species, inflammatory cytokines, antioxidant enzyme, hydrogen gas

**ABBREVIATIONS**—8-iso-PGF<sub>2</sub>α-8-iso-prostaglandin F<sub>2</sub>α; ALT—alanine aminotransferase; AST—aspartate aminotransferase; BUN—blood urea nitrogen; CLP—cecal ligation and puncture; Cr—creatinine; H<sub>2</sub>—hydrogen; H<sub>2</sub>O<sub>2</sub>—hydrogen peroxide; HMGB1—high-mobility group box 1; MODS—multiple organ dysfunction syndrome; NS—normal saline; •OH—hydroxyl radical; ROS—reactive oxygen species; SOD—superoxide dismutase; ZY—zymosan

### INTRODUCTION

Multiple organ dysfunction syndrome (MODS) as one of the most challenging clinical problems is the leading cause of death in critically ill patients (1). Multiple organ dysfunction syndrome is defined as the progressive deterioration of function, which occurs in several organs or systems in patients with severe sepsis, septic shock, shock, multiple trauma, severe burns, or pancreatitis, and so on (2). Because the mechanisms responsible for its pathology are not fully understood (3), it has been very difficult to develop effective therapeutic measures for patients with MODS.

Many animal and human 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/MODS (4). Recently, some researchers

have found that hydrogen gas (H<sub>2</sub>) exerts a therapeutic antioxidant activity by selectively reducing hydroxyl radical (•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 (5–11). Our recent study has shown that H<sub>2</sub> inhalation starting at 1 and 6 h after cecal ligation and puncture (CLP) operation, respectively, significantly improved the survival rate and multiple organ damage of moderately or severely septic mice in a concentration- and time-dependent manner (12). Furthermore, we found that the beneficial effects of H<sub>2</sub> 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 tissues (12). These findings strongly indicate that H<sub>2</sub> treatment may provide a beneficial effect on MODS.

The zymosan (ZY)-induced generalized inflammation model has been widely used in other research groups (13) as well by our group (14) because ZY, a substance derived from the cell wall of the yeast *Saccharomyces cerevisiae*, can lead to systemic inflammation by inducing a wide range of inflammatory mediators (15). This model is also used in many experimental studies for MODS (15). Therefore, the aim of

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the present study was to investigate the ability of H<sub>2</sub> to reduce multiple organ damage in ZY-induced generalized inflammation model.

## MATERIALS AND METHODS

### Animals

Male ICR (imprinting control region) mice (specific pathogen-free) provided by the Laboratory Animal Center of Fourth Military Medical University, aged 6 to 8 weeks and weighed 20 to 25 g, were used in all experiments. Animals were housed at 20° to 22°C with a 12-h light-dark cycle. Animals were fed standard chow and water *ad libitum*. 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 guidelines for the use of experimental animals.

### Zymosan-induced generalized inflammation model

Zymosan (Sigma Chemical Co, St Louis, Mo) solution was prepared in isotonic sodium chloride solution (normal saline [NS]) to a final concentration of 25 mg/mL and was sterilized at 100°C for 80 min. All suspensions were freshly made before use. Generalized inflammation was induced by an aseptic i.p. injection of ZY at a dose of 1 g/kg of body weight (BW) (14, 15). The same volume of NS was injected through the same route as the control.

### Hydrogen gas treatment

The animals were put in a sealed Plexiglas chamber with inflow and outflow outlets. Hydrogen gas 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 H<sub>2</sub> in the chamber was continuously monitored with a commercially available detector (Hy Alerta Handheld Detector Model 500; H2scan, Valencia, Calif) and maintained at 2% during the treatment. 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). Carbon dioxide was removed from the chamber gases with Baralyme (Chemetron Medical Division, Allied Healthcare Products, Inc., St. Louis, Mo). The animals without H<sub>2</sub> treatment were exposed to room air in the chamber. The room and chamber temperature was maintained at 20° to 22°C. Food and water were available *ad libitum* during the treatment.

### Experimental design

**Experiment 1: Effects of H<sub>2</sub> treatment on the survival rate in ZY-challenged mice**—One hundred twenty animals were randomly divided into four groups (n = 30 per group): NS, NS + H<sub>2</sub>, ZY, and ZY + H<sub>2</sub> groups. The animals in the NS + H<sub>2</sub> and ZY + H<sub>2</sub> groups were exposed to 2% H<sub>2</sub> for 60 min starting at 1 and 6 h after NS or ZY injection, respectively. As a control, the animals from the NS and ZY groups were exposed to room air at the same time points. The survival rate was observed on days 1, 2, 3, 5, 7, and 14 after NS or ZY injection. In addition, arterial blood gas was conducted at 0.5 h after the onset of H<sub>2</sub> inhalation (1.5 h after NS or ZY injection) in all groups.

**Experiment 2: Effects of 2% H<sub>2</sub> treatment on serum biochemical parameters and organ histopathology in ZY-challenged mice**—To further confirm the effects of 2% H<sub>2</sub> treatment on ZY-challenged mice, we examined serum biochemical parameters and organ histopathology. Twenty-four animals were used in this experiment and were assigned to four groups (n = 6 per group). The grouping method and experimental protocols were the same as described above. At 24 h after NS or ZY injection, all the animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the blood samples and organs were collected for detecting serum biochemical parameters and organ histopathology.

**Experiment 3: Effects of 2% H<sub>2</sub> treatment on inflammatory cytokines and oxidant and antioxidant system in ZY-challenged mice**—Additional 24 animals were used in this experiment and were assigned to four groups (n = 6 per group). The grouping method and experimental protocols were the same as experiment 1. At 24 h after NS or ZY injection, the early and late inflammatory cytokines (TNF- $\alpha$  and HMGB1), antioxidant enzyme (superoxide dismutase [SOD]), and oxidative product (8-iso-prostaglandin F<sub>2</sub> $\alpha$  [8-iso-PGF<sub>2</sub> $\alpha$ ]) in serum, lung, liver, and kidney were measured.

### Arterial blood gas analysis

The arterial blood gas analysis was conducted with a GEM Premier 3000 gas analyzer (Instrumentation Laboratory, Milan, Italy).

### Serum biochemical parameters assay

The serum was separated, aliquoted, and stored at -80°C until assayed (12, 14, 16). The samples were evaluated with a biochemistry autoanalyzer (Hitachi Autoanalyzer 7150; Hitachi, Tokyo, Japan) to measure serum levels of alanine aminotransferase (ALT, in international unit [IU] per liter), aspartate aminotransferase (AST, in IU/L), blood urea nitrogen (BUN, in mmol/L), and creatinine (Cr, in  $\mu$ mol/L).

### Organ histological examination

The lung, liver, and kidney were removed immediately, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 4- to 6- $\mu$ m thickness. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin. Based on the scoring standard in our previous studies (14, 16), the histological slides were blindly read and scored by two experienced pathologists.

### Detection of SOD activity

The activities of SOD were measured using commercial kits purchased from Cayman Chemical Company (Ann Arbor, Mich). According to the manufacturer's instructions and our previous studies (12, 14, 16), total SOD activity was assayed. 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-PGF<sub>2</sub> $\alpha$

Measurement of 8-iso-PGF<sub>2</sub> $\alpha$ , free radical-catalyzed products of arachidonic acid, can offer a reliable approach for quantitative measurement of oxidative stress status *in vivo* (17). The levels of serum and tissue 8-iso-PGF<sub>2</sub> $\alpha$  were detected by specific enzyme-linked immunosorbent assay kits (8-iso-PGF<sub>2</sub> $\alpha$ ; Cayman Chemical Company) using a microplate reader (CA 94089; Molecular Devices, Sunnyvale, Calif) (14, 16). All standards and samples were run in duplicate.

### Detection of inflammatory cytokines

The levels of serum and tissue TNF- $\alpha$  and HMGB1 were detected by specific enzyme-linked immunosorbent assay kits (TNF- $\alpha$ ; R&D Systems Inc, Minneapolis, Minn; HMGB1; IBL, Hamburg, Germany) with a microplate reader (CA 94089; Molecular Devices) (12, 14, 16). All standards and samples were run in duplicate.

### Statistical analysis

The survival rates are expressed as percentage. The measurement data are expressed as mean  $\pm$  SEM. The analysis of survival rates was tested by Fisher exact probability method. The intergroup differences of the rest 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 (SPSS Inc, Chicago, Ill). In all tests, *P* < 0.05 was considered statistically significant.

## RESULTS

### 2% H<sub>2</sub> inhalation had no significant effects on arterial pH, PaO<sub>2</sub>, and PaCO<sub>2</sub> in ZY-challenged mice

In the present study, we investigated the effects of H<sub>2</sub> inhalation on arterial pH, PaO<sub>2</sub>, and PaCO<sub>2</sub> in ZY-challenged mice at 0.5 h after the onset of H<sub>2</sub> inhalation (1.5 h after CLP or sham operation). There were no differences in the levels of arterial pH, PaO<sub>2</sub>, and PaCO<sub>2</sub> among all groups. The levels of pH are 7.41  $\pm$  0.14, 7.42  $\pm$  0.13, 7.41  $\pm$  0.17, and 7.40  $\pm$  0.16 in the NS, NS + H<sub>2</sub>, ZY, and ZY + H<sub>2</sub> groups, respectively. The levels of PaO<sub>2</sub> are 95.54  $\pm$  3.74, 96.19  $\pm$  3.57, 95.23  $\pm$  3.42, and 96.38  $\pm$  3.63 mmHg, whereas the levels of PaCO<sub>2</sub> are 35.41  $\pm$  1.62, 35.38  $\pm$  1.46, 36.18  $\pm$  1.73, and 36.52  $\pm$  1.58 mmHg in the NS, NS + H<sub>2</sub>, ZY, and ZY + H<sub>2</sub> groups, respectively. The results demonstrate that 2% H<sub>2</sub> inhalation has no significant effects on arterial pH, PaO<sub>2</sub>, and PaCO<sub>2</sub> in ZY-challenged mice.

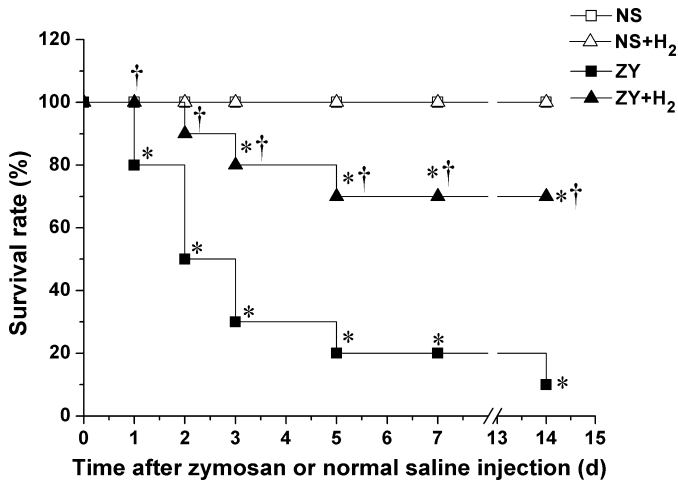


FIG. 1. 2% H<sub>2</sub> treatment improved the survival rate of ZY-challenged mice. The mice were treated with or without 2% H<sub>2</sub> inhalation for 60 min starting at 1 and 6 h after NS or ZY injection, respectively. The values are expressed as survival percentage (n = 30 per group). \*P < 0.05 vs. NS group; †P < 0.05 vs. ZY group.

### H<sub>2</sub> treatment improved the survival rate in ZY-challenged mice

The 14-day survival rate of ZY-challenged mice was 10% ( $P < 0.05$  vs. NS group, n = 30 per group; Fig. 1). Inhalation of 2% H<sub>2</sub> for 60 min starting at 1 and 6 h after ZY injection, respectively, improved the 14-day survival rate of ZY-challenged mice from 10% to 70% ( $P < 0.05$  vs. ZY group, n = 30 per group; Fig. 1). In addition, all the mice of NS and NS + H<sub>2</sub> groups survived during the experiment. The above data suggest that H<sub>2</sub> treatment can improve the survival rate of ZY-challenged mice.

### H<sub>2</sub> treatment improved organ histopathological scores in ZY-challenged mice

At 24 h after ZY or NS injection, the animals in all groups were killed for histopathological analysis. According to the scoring standard in our previous studies (14, 16), the histopathological changes in lung, liver, and kidney were scored using a scale of 1 to 4. As shown in the Fig. 2, the histopathological scores for lung, liver, and kidney in the ZY group were 3 to 3.5, much higher than those in the NS group ( $P < 0.05$ ; n = 6 per group). With 2% H<sub>2</sub> treatment, the histopathological scores for lung, liver, and kidney were decreased significantly ( $P < 0.05$ , ZY + H<sub>2</sub> group vs. ZY group, n = 6 per group).

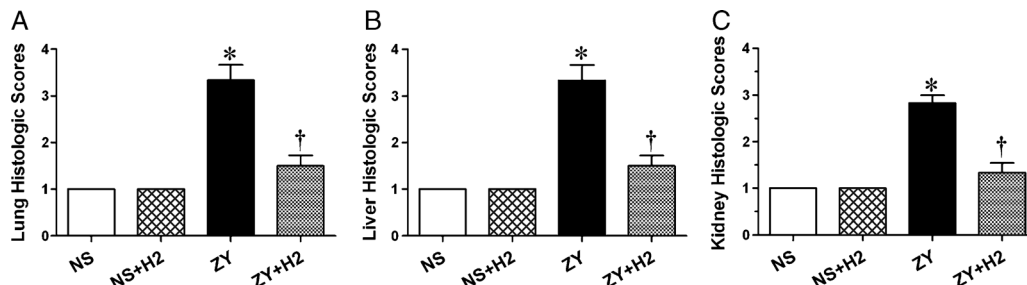


FIG. 2. 2% H<sub>2</sub> treatment improved organ histopathological scores in ZY-challenged mice. The mice were treated with or without 2% H<sub>2</sub> inhalation for 60 min starting at 1 and 6 h after NS or ZY injection, respectively. At 24 h after NS or ZY injection, all animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the organ samples were collected for measuring the histopathological scores. The values are expressed as means  $\pm$  SEM (n = 6 per group). \*P < 0.05 vs. NS group; †P < 0.05 vs. ZY group.

These data demonstrate that ZY-challenged mice had significant organ damage at 24 h after ZY injection, which is significantly attenuated by 2% H<sub>2</sub> treatment, suggesting that H<sub>2</sub> treatment has a beneficial effect on ZY-induced multiple organ damage.

### H<sub>2</sub> treatment improved serum biochemical parameters in ZY-challenged mice

As seen in Fig. 3, the ZY-challenged mice had significantly impaired liver and kidney function at 24 h after CLP operation, which was assessed by serum biochemical parameters for liver and kidney function (ALT, AST, Cr, and BUN). The ZY-challenged mice showed a significant increase in the levels of serum ALT, AST, Cr, and BUN ( $P < 0.05$ , ZY group vs. NS group, n = 6 per group), which were significantly attenuated by 2% H<sub>2</sub> treatment (Fig. 3). These data demonstrate that H<sub>2</sub> treatment has a beneficial effect on ZY-induced organ dysfunction.

### H<sub>2</sub> treatment prevent the abnormal changes of oxidant and antioxidant system in ZY-challenged mice

At 24 h after ZY or NS injection, the activities of antioxidant enzyme SOD and levels of oxidative product 8-iso-PGF<sub>2</sub> $\alpha$  in serum, lung, liver, and kidney of all animals were observed. Our results showed that the decrease in SOD activities and the increase in 8-iso-PGF<sub>2</sub> $\alpha$  levels in serum, lung, liver, and kidney occurred in mice with ZY injection ( $P < 0.05$  vs. NS group, n = 6 per group; Figs. 4–7). Treatment with 2% H<sub>2</sub> increased the SOD activities and decreased 8-iso-PGF<sub>2</sub> $\alpha$  levels in serum and these organs of ZY-challenged mice ( $P < 0.05$ , n = 6 per group; Figs. 4–7). No statistically significant differences in the activities of SOD as well as the levels of 8-iso-PGF<sub>2</sub> $\alpha$  in serum and these organs were present between the NS and NS + H<sub>2</sub> groups ( $P > 0.05$ , n = 6 per group; Figs. 4–7).

These data suggest that H<sub>2</sub> treatment provides beneficial effects on ZY-induced multiple organ damage, which are associated with the decreased levels of oxidative product and increased activities of antioxidant enzyme in serum and tissues.

### H<sub>2</sub> treatment reduced the levels of early and late inflammatory cytokines in ZY-challenged mice

In the present study, we also investigated the effects of H<sub>2</sub> treatment on early and late inflammatory cytokines (TNF- $\alpha$  and HMGB1) in serum and tissues of ZY-challenged mice. The

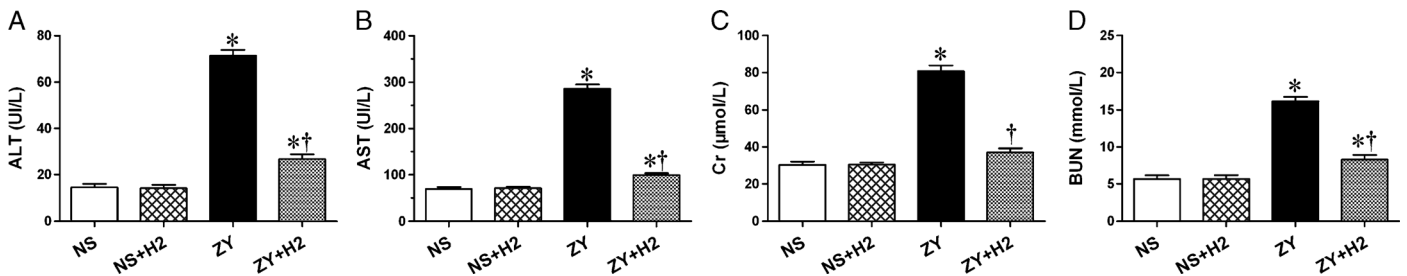


FIG. 3. 2% H<sub>2</sub> treatment improved serum biochemical parameters in ZY-challenged mice. The mice were treated with or without 2% H<sub>2</sub> inhalation for 60 min starting at 1 and 6 h after NS or ZY injection, respectively. At 24 h after NS or ZY injection, all the animals were anesthetized, and the blood samples were collected for detecting serum biochemical parameters. The values represent mean ± SEM (n = 6 per group). \*P < 0.05 vs. NS group; †P < 0.05 vs. ZY group.

levels of TNF- $\alpha$  and HMGB1 in serum, lung, liver, and kidney were significantly increased in ZY-challenged mice at 24 h after ZY injection, which were attenuated by 2% H<sub>2</sub> treatment ( $P < 0.05$  vs. NS group, n = 6 per group; Figs. 4–7). These data suggest that the protective effects of H<sub>2</sub> treatment on ZY-induced multiple organ damage are also associated with the decreased levels of early and late inflammatory cytokines in serum and tissues.

## DISCUSSION

The present study demonstrated that (a) 2% H<sub>2</sub> inhalation for 60 min starting at 1 and 6 h after ZY injection, respectively, significantly improved the 14-day survival rate of ZY-challenged mice; (b) ZY-challenged mice showed significant organ injuries characterized by the increase in AST, ALT, Cr, BUN, and organ histopathological scores at 24 h after ZY injection, which was significantly attenuated by 2% H<sub>2</sub> treatment; (c) the beneficial effects of H<sub>2</sub> treatment on ZY-induced organ injury were associated with the decreased levels of oxidative product 8-iso-PGF2 $\alpha$ , increased activities of antioxidant enzyme SOD, and reduced levels of inflammatory cytokines TNF- $\alpha$  and HMGB1 in serum and tissues.

Zymosan, a substance derived from the cell wall of the yeast *S. cerevisiae*, can lead to systemic inflammation by inducing a wide range of inflammatory mediators (15). Based on our previous studies and other studies, i.p. injection of a high dose of ZY (0.8–1.0 g/kg BW) can induce a generalized inflammation model in rats or mice, which is accompanied by multiple organ damage (13–16). We found that ZY (1.0 g/kg BW, i.p. injection) successfully induced sterile inflammation model in mice characterized by the decrease in survival rates

of mice, histopathological injury, organ dysfunction, and abnormally decreased tissue oxygenation (14, 16). In the present study, we also found that these changes were present in ZY-challenged mice.

Sepsis, when accompanied by multiple organ failure, contributes to be the leading cause of death in the intensive care unit (1). 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 and MODS (4). An excessive production of ROS contributes to an overwhelming inflammatory response and tissue injury (18). In excess, ROS and their by-products could exacerbate organ damage and thus overall clinical outcome (18). It is well known that ROS include many types such as superoxide anion,  $\bullet$ OH, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and so on (11). Despite their cytotoxic effects, superoxide anion and H<sub>2</sub>O<sub>2</sub> play important physiological roles at low concentrations: they function as regulatory signaling molecules that are involved in numerous signal transduction cascades and also regulate biological processes such as apoptosis, cell proliferation, and differentiation (11, 19). At higher concentrations, H<sub>2</sub>O<sub>2</sub> is converted into hypochlorous acid by myeloperoxidase; hypochlorous acid defends against bacterial invasion (20). In addition, some endogenous antioxidant enzymes can scavenge H<sub>2</sub>O<sub>2</sub> and superoxide anion *in vivo* (12). However,  $\bullet$ OH is the strongest of the oxidant species and reacts indiscriminately with nucleic acids, lipids, and proteins (11). There is no known detoxification system for  $\bullet$ OH *in vivo* (11). Therefore, scavenging  $\bullet$ OH is a critical antioxidant process, which may be a good and critical measure for treating sepsis/MODS.

Interestingly, recent studies demonstrate that H<sub>2</sub> exerts a therapeutic antioxidant activity by selectively reducing  $\bullet$ OH

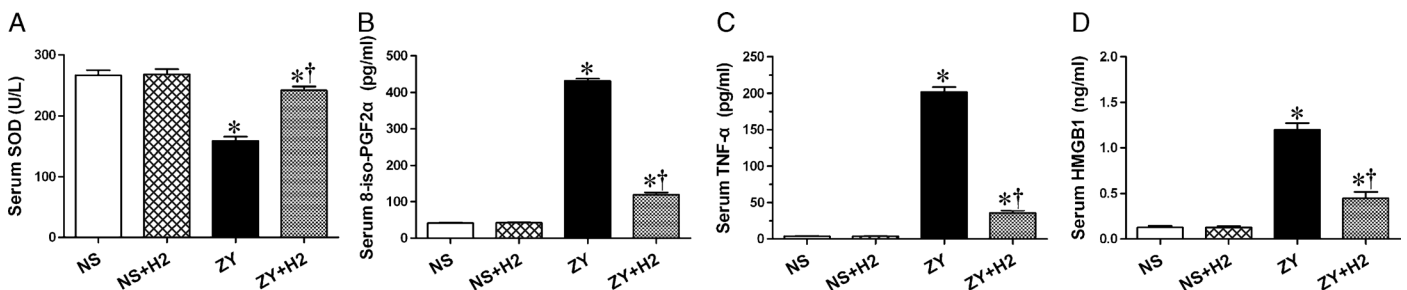


FIG. 4. 2% H<sub>2</sub> treatment upregulated the activities of serum antioxidant enzyme and reduced the levels of serum oxidative product and inflammatory cytokines in ZY-challenged mice. A, Serum SOD activity, (B) serum 8-iso-PGF2 $\alpha$  level, (C) serum TNF- $\alpha$  level, (D) serum HMGB1 level. The mice were treated with or without 2% H<sub>2</sub> inhalation for 60 min starting at 1 and 6 h after NS or ZY injection, respectively. The serum was harvested for measuring these indicators at 24 h after NS or ZY injection. The values are expressed as mean ± SEM (n = 6 per group). \*P < 0.05 vs. NS group; †P < 0.05 vs. ZY group.



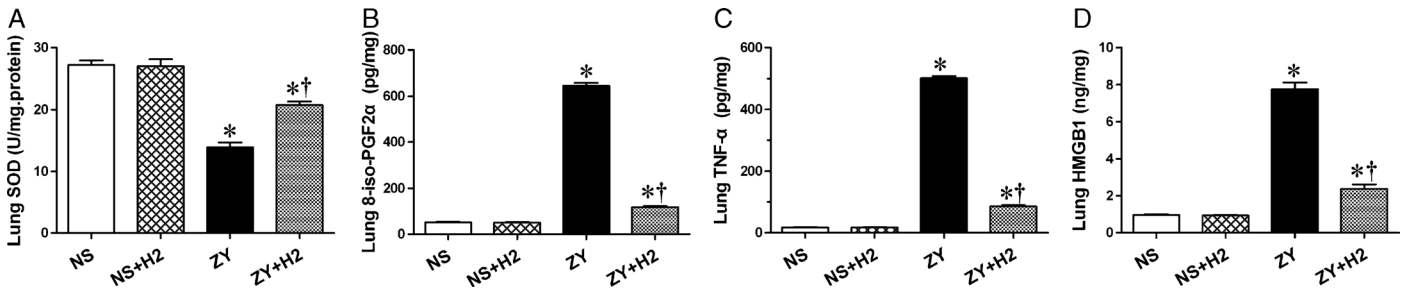


FIG. 5. 2% H<sub>2</sub> treatment upregulated the activities of lung antioxidant enzyme and reduced the levels of lung oxidative product and inflammatory cytokines in ZY-challenged mice. A, Lung SOD activity, (B) lung 8-iso-PGF2 $\alpha$  level, (C) lung TNF- $\alpha$  level, (D) lung HMGB1 level. The mice were treated with or without 2% H<sub>2</sub> inhalation for 60 min starting at 1 and 6 h after NS or ZY injection, respectively. The lungs were harvested for measuring these indicators at 24 h after NS or ZY injection. The values are expressed as mean  $\pm$  SEM (n = 6 per group). \*P < 0.05 vs. NS group; †P < 0.05 vs. ZY group.

(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, suggesting that H<sub>2</sub> has potential as an antioxidant for preventive and therapeutic applications (5–11). Our recent study has also shown that H<sub>2</sub> treatment starting at 1 and 6 h after CLP operation significantly improves the long-term survival rate of moderately or severely septic mice in a concentration- and time-dependent manner (12). Furthermore, we have found that 2% H<sub>2</sub> treatment significantly attenuates 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 h after CLP operation (12). These findings strongly indicate that H<sub>2</sub> treatment may become a good measure for treating patients with MODS.

Our previous study has found that the protective effects of H<sub>2</sub> treatment on septic mice are time- and concentration-dependent (12). Based on our previous studies and preliminary studies (12), the present study was designed to investigate the effects of 2% H<sub>2</sub> inhalation for 60 min starting at 1 and 6 h after ZY injection, respectively, on ZY-challenged mice. Here, we found that 2% H<sub>2</sub> treatment starting at 1 and 6 h after ZY injection significantly improved the long-term survival rate of ZY-challenged mice. Furthermore, we found that 2% H<sub>2</sub> treatment significantly attenuated ZY-induced lung, liver, and kidney injuries through observing the indicators including serum biochemical parameters (AST, ALT, Cr, and BUN) and organ histopathological scores at 24 h after ZY injection. The above results demonstrate

that H<sub>2</sub> treatment has a beneficial effect on multiple organ dysfunction/failure in the ZY-induced generalized inflammation model.

To further investigate the possible mechanism, we studied the effects of H<sub>2</sub> treatment on oxidant and antioxidant system in ZY-challenged mice. In our previous studies, we have found that the activities of SOD, catalase, and glutathione peroxidase in serum and tissues are significantly decreased during the early and late stages, indicating that ZY sets up an environment favorable for oxidative stress (14, 16). The detection of products of lipid peroxidation has been widely used to estimate the overall status of oxidative stress (17). In the present study, we observed the decreased activities of SOD and the increased levels of oxidative product 8-iso-PGF2 $\alpha$  in serum, lung, liver, and kidney at 24 h after ZY injection. We further showed that 2% H<sub>2</sub> treatment significantly improved the activities of SOD and decreased the levels of 8-iso-PGF2 $\alpha$  in these organs and serum. These results suggest that the decrease in oxidative damage and the increase in endogenous antioxidant enzymatic activities in serum and tissues may attribute to the protection of H<sub>2</sub> treatment, which is similar with our previous study (12).

It is also believed that the uncontrolled and exaggerated inflammatory response plays a major role in the pathogenesis of sepsis/MODS (3). The inflammatory cytokines include early inflammatory cytokines such as proinflammatory cytokines, TNF- $\alpha$  and IL-6, and anti-inflammatory cytokine, IL-10, as well as the late inflammatory cytokine HMGB1 (21, 22). The early and late inflammatory cytokines can interact and facilitate the organ dysfunction and injury in sepsis/MODS (23). Recently, some studies have found that HMGB1 is a necessary

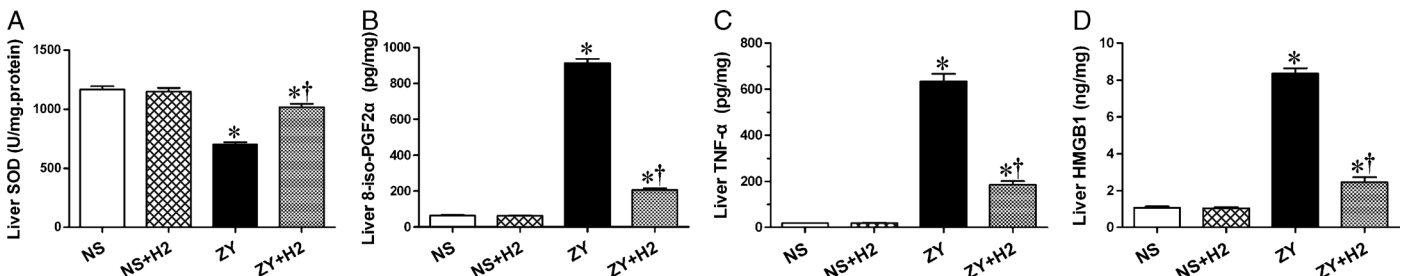


FIG. 6. 2% H<sub>2</sub> treatment upregulated the activities of liver antioxidant enzyme and reduced the levels of liver oxidative product and inflammatory cytokines in ZY-challenged mice. A, Liver SOD activity, (B) liver 8-iso-PGF2 $\alpha$  level, (C) liver TNF- $\alpha$  level, (D) liver HMGB1 level. The mice were treated with or without 2% H<sub>2</sub> inhalation for 60 min starting at 1 and 6 h after NS or ZY injection, respectively. The liver was harvested for measuring these indicators at 24 h after NS or ZY injection. The values are expressed as mean  $\pm$  SEM (n = 6 per group). \*P < 0.05 vs. NS group; †P < 0.05 vs. ZY group.

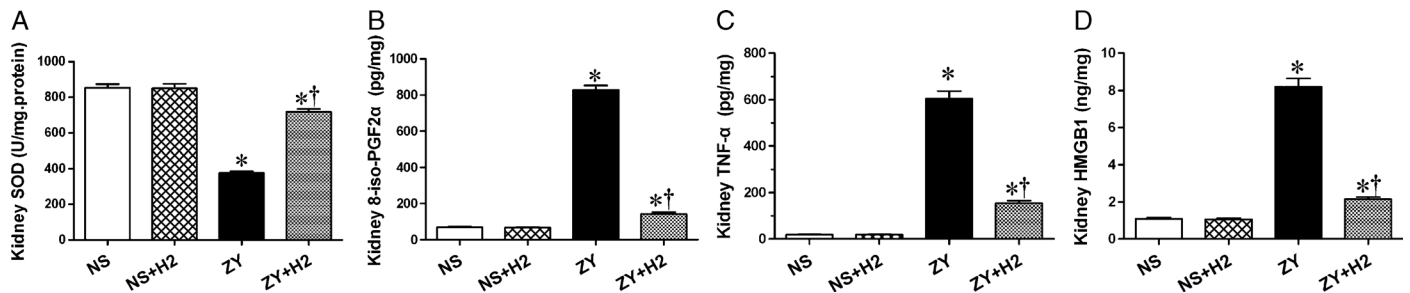


FIG. 7. 2% H<sub>2</sub> treatment upregulated the activities of kidney antioxidant enzyme and reduced the levels of kidney oxidative product and inflammatory cytokines in ZY-challenged mice. A, Kidney SOD activity, (B) kidney 8-iso-PGF<sub>2</sub>α level, (C) kidney TNF-α level, (D) kidney HMGB1 level. The mice were treated with or without 2% H<sub>2</sub> inhalation for 60 min starting at 1 and 6 h after NS or ZY injection, respectively. The kidneys were harvested for measuring these indicators at 24 h after NS or ZY injection. The values are expressed as mean ± SEM (n = 6 per group). \*P < 0.05 vs. NS group; †P < 0.05 vs. ZY group.

and sufficient mediator of lethal organ damage in murine CLP sepsis (23, 24). Our previous studies also demonstrated that HMGB1 contributed to organ damage in the ZY-induced generalized inflammation model (14, 16). In the present study, we found that ZY-challenged mice showed the significant increase in TNF-α and HMGB1 in serum, lung, liver, and kidney, which was significantly attenuated by 2% H<sub>2</sub> treatment. These data suggest that the protective effects of decrease in H<sub>2</sub> treatment on ZY-challenged mice are associated with the decrease in early and late proinflammatory cytokines in serum and tissues, which is similar with our previous study (12).

The present and our previous studies have shown that inhaled H<sub>2</sub> at therapeutic dose has no adverse effects on the saturation level of arterial oxygen (SpO<sub>2</sub>) or hemodynamic parameters (11). Furthermore, H<sub>2</sub> is neither explosive nor dangerous at a concentration of less than 4.7% in air, which has been proved by 17-year-long studies on cells, mice, monkeys, and deep-sea divers (COMEX HYDRA program, Marseille, France). Moreover, H<sub>2</sub> as a potential antioxidant has certain unique properties (11): (a) unlike most known antioxidants, which are unable to successfully target organelles, H<sub>2</sub> is permeable to cell membranes and can target organelles, including the cytosol, mitochondria, and nuclei; (b) despite the moderate reduction activity of H<sub>2</sub>, its rapid gaseous diffusion might make it highly effective for reducing cytotoxic radicals; (c) it is likely that H<sub>2</sub> 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 can affect essential defensive mechanisms). Ohsawa et al. (11) found that H<sub>2</sub> directly reacted with free radical species such as •OH *in vitro*. However, the detailed mechanisms are unclear *in vivo*. Further studies will reveal the mechanisms by which H<sub>2</sub> protects cells and tissues against oxidative stress *in vivo*.

Zymosan has been shown to lead to bacterial translocation and even systemic bacteremia, which is improved with antibiotics (25). In the present study, the failure to test for an infectious component in the ZY model is a limitation of our study.

In conclusion, our findings in a model of ZY-induced inflammation support is in agreement with our recent observations (12), the potential use of H<sub>2</sub> as a therapeutic agent in

the therapy of conditions associated with inflammation and oxidation-related multiple organ dysfunction. We propose that H<sub>2</sub>, one of the most well-known molecules, could be widely used in medical applications as a safe and effective antioxidant with minimal adverse effects.

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