

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

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RUNNING TITLE: H2 treatment attenuates zymosan-induced multiple organ damage in mice.

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ABBREVIATIONS---8-iso-PGF2 α , 8-iso-prostaglandin F2 α ; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; CAT, catalase; CLP, cecal ligation and puncture; Cr: creatinine; GSH-Px, glutathione peroxidase; H2, hydrogen; H2O2, hydrogen peroxide; HMGB1, high-mobility group box 1; ICU, intensive care units; MODS, multiple organ dysfunction syndrome; NS, normal saline; •OH: hydroxyl radicals; ROS, reactive oxygen species; SOD, superoxide dismutase; ZY, zymosan.

ABSTRACT----Sepsis/Multiple organ dysfunction syndrome (MODS) is the leading cause of death in critically ill patients. Recently, it has been suggested that hydrogen gas (H₂) exerts a therapeutic antioxidant activity by selectively reducing hydroxyl radical (•OH, the most cytotoxic ROS). We have found that H₂ inhalation significantly improved the survival rate and organ damage of septic mice with moderate or severe cecal ligation and puncture (CLP). In the present study, we investigated the effects of 2% H₂ treatment on survival rate and organ damage in zymosan-induced generalized inflammation model. Here, we found that 2% H₂ inhalation for 60 minutes starting at 1 and 6 hours after zymosan injection, respectively, significantly improved the 14-day survival rate of zymosan-challenged mice from 10% to 70%. Furthermore, zymosan-challenged mice showed significant multiple organ damage characterized by the increase of serum biochemical parameters (AST, ALT, BUN and Cr), as well as lung, liver and kidney histopathological scores at 24 hours after zymosan injection, which was significantly attenuated by 2% H₂ treatment. In addition, we found that the beneficial effects of H₂ treatment on zymosan-induced organ damage were associated with the decreased levels of oxidative product, increased activities of antioxidant enzyme as well as reduced levels of early and late proinflammatory cytokines in serum and tissues. In conclusion, this study provides evidence that H₂ treatment protects against multiple organ damages in zymosan-induced generalized inflammation model, suggesting that the potential use of H₂ as a therapeutic agent in the therapy of conditions associated with inflammation-related MODS.

KEY WORDS----sepsis; multiple organ dysfunction syndrome/failure; reactive oxygen species; inflammatory cytokines; antioxidant enzyme; hydrogen gas

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). MODS 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 H₂ exerts a therapeutic antioxidant activity by selectively reducing hydroxyl radical (\bullet OH, the most cytotoxic ROS) and effectively protects against organ damage such as transient cerebral ischemia, neonatal cerebral hypoxia-ischemia, liver injury, lung injury and myocardial injury induced by ischemia/reperfusion (5-11). Our recent study has shown that H₂ inhalation starting at 1 and 6 hours 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 H₂ 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 H₂ treatment may provide a beneficial effect on MODS.

The zymosan-induced generalized inflammation model has been widely used in other research groups (13) as well by our group (14) because zymosan, 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 the present study was to investigate the ability of H₂ to reduce multiple organ damage in zymosan-induced generalized inflammation model.

MATERIALS AND METHODS

Animals

Male Imprinting Control Region (ICR) mice (Specific Pathogen Free) provided by the Laboratory Animal Center of Fourth Military Medical University, aging 6 to 8 weeks and weighing 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 Fourth Military Medical University, and performed in accordance with the National Institutes of Health (NIH, USA) guidelines for the use of experimental animals.

Zymosan-Induced Generalized Inflammation Model

Zymosan (Sigma Chemical Co., St. Louis, MO, USA) 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 minutes. All suspensions were freshly made before use. Generalized

inflammation was induced by an aseptic intraperitoneal injection of zymosan 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 (H₂) treatment

The animals were put in a sealed plexiglas chamber with inflow and outflow outlets. H₂ 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 H₂ in the chamber was continuously monitored with a commercially available detector (Hy Alerta Handheld Detector Model 500, H2 Scan, Valencia, CA, USA) 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, USA). Carbon dioxide was removed from the chamber gases with baralyme. The animals without H₂ 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 One: Effects of H₂ treatment on the survival rate in zymosan-challenged mice.

120 animals were randomly divided into 4 groups (n = 30 per group): NS, NS+H₂, zymosan (ZY), and ZY+H₂ groups. The animals in the NS+H₂ and ZY+H₂ groups were exposed to 2% H₂

for 60 minutes starting at 1 and 6 hours after NS or zymosan 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 day 1, 2, 3, 5, 7 and 14 after NS or zymosan injection. In addition, arterial blood gas was conducted at 0.5 hour after the onset of H₂ inhalation (1.5 hours after NS or zymosan injection) in all groups.

Experiment Two: Effects of 2% H₂ treatment on serum biochemical parameters and organ histopathology in zymosan-challenged mice

In order to further confirm the effects of 2% H₂ treatment on zymosan-challenged mice, we examined serum biochemical parameters and organ histopathology. 24 animals were used in this experiment and were assigned to 4 groups (n = 6 per group). The grouping method and experimental protocols were the same as described above. At 24 hours after NS or zymosan injection, all the animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), and the blood samples and organs were collected for detecting serum biochemical parameters and organ histopathology.

Experiment Three: Effects of 2% H₂ treatment on inflammatory cytokines as well as oxidant and antioxidant system in zymosan-challenged mice

Additional 24 animals were used in this experiment and were assigned to 4 groups (n = 6 per group). The grouping method and experimental protocols were the same as Experiment One. At 24 hours after NS or zymosan injection, the early and late inflammatory cytokines (TNF- α and high-mobility group box 1 [HMGB1]), antioxidant enzyme (superoxide dismutase [SOD]) and oxidative product (8-iso-prostaglandin F₂ α [8-iso-PGF₂ α]) 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, International Unit per liter), aspartate aminotransferase (AST, International Unit per liter), blood urea nitrogen (BUN, mmol/L) and creatinine (Cr, $\mu\text{mol/L}$).

Organ histologic examination

The lung, liver and kidney were removed immediately, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 4 to 6 μm 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, MI, USA). 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, Fullerton, CA, USA). All assays were conducted in triplicates. The tissue protein concentration was determined by using a standard commercial kit (Bio-Rad Laboratories, Hercules, CA, USA).

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

Measurement of 8-iso-PGF₂α, free radical-catalysed products of arachidonic acid, can offer a reliable approach for quantitative measurement of oxidative stress status in vivo (17). The levels of serum and tissue 8-iso-PGF₂α were detected by specific enzyme-linked immunosorbent assay (ELISA) kits (8-iso-PGF₂α, Ann Arbor, MI, USA) using a microplate reader (CA 94089, Molecular Devices, Sunnyvale, Canada) (14, 16). All standards and samples were run in duplicate.

Detection of inflammatory cytokines

The levels of serum and tissue TNF-α and HMGB1 were detected by specific ELISA kits (TNF-α, R&D Systems Inc., Minneapolis, Minnesota, USA; HMGB1, IBL, Hamburg, Germany) with a microplate reader (CA 94089, Molecular Devices, Sunnyvale, Canada) (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's exact probability method. The inter-group differences of the rest data were tested by one-way ANOVA followed by LSD-*t* Test for multiple comparisons. The statistical analysis was performed with SPSS 16.0 software. In all tests, a *P* value of less than 0.05 was considered statistically significant.

RESULTS

2% H₂ inhalation had no significant effects on arterial pH, PaO₂ and PaCO₂ in zymosan-challenged mice

In the present study, we investigated the effects of H₂ inhalation on arterial pH, PaO₂ and PaCO₂ in zymosan-challenged mice at 0.5 hour after the onset of H₂ inhalation (1.5 hours after CLP or Sham operation). There were no differences in the levels of arterial pH, PaO₂ and PaCO₂ 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+H₂, ZY and ZY+H₂ groups, respectively. The levels of PaO₂ are 95.54 \pm 3.74, 96.19 \pm 3.57, 95.23 \pm 3.42 and 96.38 \pm 3.63, while the levels of PaCO₂ are 35.41 \pm 1.62, 35.38 \pm 1.46, 36.18 \pm 1.73 and 36.52 \pm 1.58 in the NS, NS+H₂, ZY and ZY+H₂ groups, respectively. The results demonstrate that 2% H₂ inhalation has no significant effects on arterial pH, PaO₂ and PaCO₂ in zymosan-challenged mice.

H₂ treatment improved the survival rate in zymosan-challenged mice

The 14-day survival rate of zymosan-challenged mice was 10% ($P < 0.05$ vs. NS group, $n = 30$ per group, **Fig. 1**). 2% H₂ inhalation for 60 minutes starting at 1 and 6 hours after zymosan injection, respectively, improved the 14-day survival rate of zymosan-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+ H₂ groups survived during the experiment. The above data suggest that H₂ treatment can improve the survival rate of zymosan-challenged mice.

H₂ treatment improved organ histopathological scores in zymosan-challenged mice

At 24 h after zymosan 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% H₂ treatment, the histopathological scores for lung, liver and kidney were decreased significantly ($P < 0.05$, ZY+H₂ group vs. ZY group, $n = 6$ per group).

These data demonstrate that zymosan-challenged mice appear significant organ damage at 24 hours after zymosan injection, which is significantly attenuated by 2% H₂ treatment, suggesting that H₂ treatment has a beneficial effect on zymosan-induced multiple organ damage.

H₂ treatment improved serum biochemical parameters in zymosan-challenged mice

As seen in **Fig. 3**, the zymosan-challenged mice appeared significantly impaired liver and kidney function at 24 hours after CLP operation, which was assessed by serum biochemical parameters for liver and kidney function (ALT, AST, Cr and BUN). The zymosan-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% H₂ treatment (**Fig. 3**). These data demonstrate that H₂ treatment has a beneficial effect on zymosan-induced organ dysfunction.

H₂ treatment prevent the abnormal changes of oxidant and antioxidant system in zymosan-challenged mice

At 24 hours after zymosan or NS injection, the activities of antioxidant enzyme SOD and levels of oxidative product 8-iso-PGF2 α in serum, lung, liver and kidney of all animals were observed. Our results showed that the decrease of SOD activities and the increase of 8-iso-PGF2 α levels in serum, lung, liver and kidney occurred to mice with zymosan injection ($P < 0.05$ vs. NS group, $n = 6$ per group, **Figs. 4-7**). Treatment with 2% H₂ increased the SOD activities and decreased 8-iso-PGF2 α levels in serum and these organs of zymosan-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-PGF2 α in serum and these organs were present between the NS and NS+H₂ groups ($P > 0.05$, $n = 6$ per group, **Figs. 4-7**).

These data suggest that H₂ treatment provides beneficial effects on zymosan-induced

multiple organ damage, which are associated with the decreased levels of oxidative product and increased activities of antioxidant enzyme in serum and tissues.

H₂ treatment reduced the levels of early and late inflammatory cytokines in zymosan-challenged mice

In the present study, we also investigated the effects of H₂ treatment on early and late inflammatory cytokines (TNF- α and HMGB1) in serum and tissues of zymosan-challenged mice. The levels of TNF- α and HMGB1 in serum, lung, liver and kidney were significantly increased in zymosan-challenged mice at 24 hours after zymosan injection, which were attenuated by 2% H₂ treatment ($P < 0.05$ vs. NS group, $n = 6$ per group, **Figs. 4-7**). These data suggest that the protective effects of H₂ treatment on zymosan-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 1) 2% H₂ inhalation for 60 minutes starting at 1 and 6 hours after zymosan injection, respectively, significantly improved the 14-day survival rate of zymosan-challenged mice. 2) Zymosan-challenged mice showed significant organ injuries characterized by the increase of AST, ALT, Cr, BUN and organ histopathological scores at 24 hours after zymosan injection, which was significantly attenuated by 2% H₂ treatment. 3) The beneficial effects of H₂ treatment on zymosan-induced organ injury were associated with the decreased levels of oxidative product 8-iso-PGF2 α , increased activities of antioxidant enzyme

SOD, and reduced levels of inflammatory cytokines TNF- α and HMGB1 in serum and tissues.

Zymosan, 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). Based on our previous studies and other studies, intraperitoneal injection of a high dose of zymosan (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 zymosan (1.0 g/kg BW, intraperitoneal injection) successfully induced sterile inflammation model in mice characterized by the decrease of survival rates of mice, histopathological injury, organ dysfunction, abnormally decreased tissue oxygenation (14, 16). In the present study, we also found these changes were present in zymosan-challenged mice.

Sepsis, when accompanied by multiple organ failure, contributes to be the leading cause of death in ICU (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, hydroxyl radicals (\bullet OH), hydrogen peroxide (H_2O_2), and so on (11). Despite their cytotoxic effects, superoxide anion and H_2O_2 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_2O_2 is converted into

hypochlorous acid by myeloperoxidase; hypochlorous acid defends against bacterial invasion (20). In addition, some endogenous antioxidant enzymes can scavenge H_2O_2 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_2 exerts a therapeutic antioxidant activity by selectively reducing hydroxyl radicals ($\bullet OH$, the most cytotoxic ROS) and effectively protected against organ damage such as transient cerebral ischemia, neonatal cerebral hypoxia-ischemia, liver injury, lung injury and myocardial injury induced by ischemia/reperfusion, suggesting that H_2 has potential as an antioxidant for preventive and therapeutic applications (5-11). Our recent study has also shown that H_2 treatment starting at 1 and 6 hours 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_2 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 hours after CLP operation (12). These findings strongly indicate that H_2 treatment maybe become a good measure for treating patients with MODS.

Our previous study has found that the protective effects of H_2 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_2 inhalation for 60

minutes starting at 1 and 6 hours after zymosan injection, respectively, on zymosan-challenged mice. Here, we found that 2% H₂ treatment starting at 1 and 6 hours after zymosan injection significantly improved the long-term survival rate of zymosan-challenged mice. Furthermore, we found that 2% H₂ treatment significantly attenuated zymosan-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 hours after zymosan injection. The above results demonstrate that H₂ treatment has a beneficial effect on multiple organ dysfunction/failure in the zymosan-induced generalized inflammation model.

To further investigate the possible mechanism, we studied the effects of H₂ treatment on oxidant and antioxidant system in zymosan-challenged mice. In our previous studies, we have found that the activities of SOD, CAT and GSH-Px in serum and tissues are significantly decreased during the early and late stages, indicating that zymosan 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 α in serum, lung, liver, and kidney at 24 hours after zymosan injection. We further showed that 2% H₂ treatment significantly improved the activities of SOD and decreased the levels of 8-iso-PGF2 α in these organs and serum. These results suggest that the decrease of oxidative damage and the increase of endogenous antioxidant enzymatic activities in serum and tissues may attribute to the protection of H₂ 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 pro-inflammatory cytokines TNF- α , 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 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 zymosan-induced generalized inflammation model (14, 16). In the present study, we found that zymosan-challenged mice showed the significant increase of TNF- α and HMGB1 in serum, lung, liver and kidney, which was significantly attenuated by 2% H₂ treatment. These data suggest that the protective effects of decrease of H₂ treatment on zymosan-challenged mice are associated with the decrease of 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 H₂ at therapeutic dose has no adverse effects on the saturation level of arterial oxygen (SpO₂) or hemodynamic parameters (11). Furthermore, H₂ 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). Moreover, H₂ as a potential antioxidant has certain unique properties (11): 1) unlike most known antioxidants, which are unable to successfully target organelles, H₂ is permeable to cell membranes and can target organelles, including the cytosol, mitochondria and nuclei. 2) Despite the moderate reduction activity of H₂, its rapid

gaseous diffusion might make it highly effective for reducing cytotoxic radicals. 3) It is likely that H₂ 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 H₂ 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 H₂ 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 zymosan model is a limitation of our study.

In conclusion, our findings in a model of zymosan-induced inflammation support, in agreement with our recent observations (12), the potential use of H₂ as a therapeutic agent in the therapy of conditions associated with inflammation and oxidation-related multiple organ dysfunction. We propose that H₂, one of the most well-known molecules, could be widely used in medical applications as a safe and effective antioxidant with minimal side effects.

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FIGURE LEGENDS

FIG. 1

2% H₂ treatment improved the survival rate of zymosan-challenged mice. The mice were treated with or without 2% H₂ inhalation for 60 minutes starting at 1 and 6 hours after NS or zymosan 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. NS: normal saline; ZY: zymosan; H₂: hydrogen.

FIG. 2

2% H₂ treatment improved organ histopathological scores in zymosan-challenged mice.

The mice were treated with or without 2% H₂ inhalation for 60 minutes starting at 1 and 6 hours after NS or zymosan injection, respectively. At 24 hours after NS or zymosan injection, all animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), and the organ samples were collected for measuring the histopathological scores. The values are expressed as means ± SEM (n = 6 per group). * $P < 0.05$ vs. NS group; † $P < 0.05$ vs. ZY group. NS: normal saline; ZY: zymosan; H₂: hydrogen.

FIG. 3

2% H₂ treatment improved serum biochemical parameters in zymosan-challenged mice.

The mice were treated with or without 2% H₂ inhalation for 60 minutes starting at 1 and 6 hours after NS or zymosan injection, respectively. At 24 hours after NS or zymosan injection, all the

animals were anesthetized, and the blood samples were collected for detecting serum biochemical parameters. The values represent mean \pm SEM (n = 6 per group). * $P < 0.05$ vs. NS group; † $P < 0.05$ vs. ZY group. ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; Cr: creatinine; UI/L: International Unit per liter; NS: normal saline; ZY: zymosan; H2: hydrogen.

FIG. 4

2% H2 treatment up-regulated the activities of serum antioxidant enzyme, and reduced the levels of serum oxidative product and inflammatory cytokines in zymosan-challenged mice.

(A): Serum SOD activity; (B): Serum 8-iso-PGF2 α level; (C): Serum TNF- α level ; (D): Serum HMGB1 level. The mice were treated with or without 2% H₂ inhalation for 60 minutes starting at 1 and 6 hours after NS or zymosan injection, respectively. The serum was harvested for measuring these indicators at 24 hours after NS or zymosan 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. SOD: superoxide dismutase; 8-iso-PGF2 α : 8-iso-prostaglandin F2 α ; HMGB1: High-mobility group box 1 protein; NS: normal saline; ZY: zymosan; H2: hydrogen.

FIG. 5

2% H2 treatment up-regulated the activities of lung antioxidant enzyme, and reduced the levels of lung oxidative product and inflammatory cytokines in zymosan-challenged mice.

(A): Lung SOD activity; (B): Lung 8-iso-PGF2 α level; (C): Lung TNF- α level ; (D): Lung

HMGB1 level. The mice were treated with or without 2% H₂ inhalation for 60 minutes starting at 1 and 6 hours after NS or zymosan injection, respectively. The lungs were harvested for measuring these indicators at 24 hours after NS or zymosan injection. The values are expressed as mean ± SEM (n = 6 per group). * *P* < 0.05 vs. NS group; † *P* < 0.05 vs. ZY group. SOD: superoxide dismutase; 8-iso-PGF2α: 8-iso-prostaglandin F2α; HMGB1: High-mobility group box 1 protein; U/mg.protein: unit per milligram protein; NS: normal saline; ZY: zymosan; H₂: hydrogen.

FIG. 6

2% H₂ treatment up-regulated the activities of liver antioxidant enzyme, and reduced the levels of liver oxidative product and inflammatory cytokines in zymosan-challenged mice.

(A): Liver SOD activity; **(B):** Liver 8-iso-PGF2α level; **(C):** Liver TNF-α level ; **(D):** Liver HMGB1 level. The mice were treated with or without 2% H₂ inhalation for 60 minutes starting at 1 and 6 hours after NS or zymosan injection, respectively. The liver was harvested for measuring these indicators at 24 hours after NS or zymosan injection. The values are expressed as mean ± SEM (n = 6 per group). * *P* < 0.05 vs. NS group; † *P* < 0.05 vs. ZY group. SOD: superoxide dismutase; 8-iso-PGF2α: 8-iso-prostaglandin F2α; HMGB1: High-mobility group box 1 protein; U/mg.protein: unit per milligram protein; NS: normal saline; ZY: zymosan; H₂: hydrogen.

FIG. 7

2% H₂ treatment up-regulated the activities of Kidney antioxidant enzyme, and reduced the levels of Kidney oxidative product and inflammatory cytokines in zymosan-challenged mice. (A): Kidney SOD activity; (B): Kidney 8-iso-PGF₂ α level; (C): Kidney TNF- α level ; (D): Kidney HMGB1 level. The mice were treated with or without 2% H₂ inhalation for 60 minutes starting at 1 and 6 hours after NS or zymosan injection, respectively. The kidneys were harvested for measuring these indicators at 24 hours after NS or zymosan 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. SOD: superoxide dismutase; 8-iso-PGF₂ α : 8-iso-prostaglandin F₂ α ; HMGB1: High-mobility group box 1 protein; U/mg.protein: unit per milligram protein; NS: normal saline; ZY: zymosan; H₂: hydrogen.

Figure 1

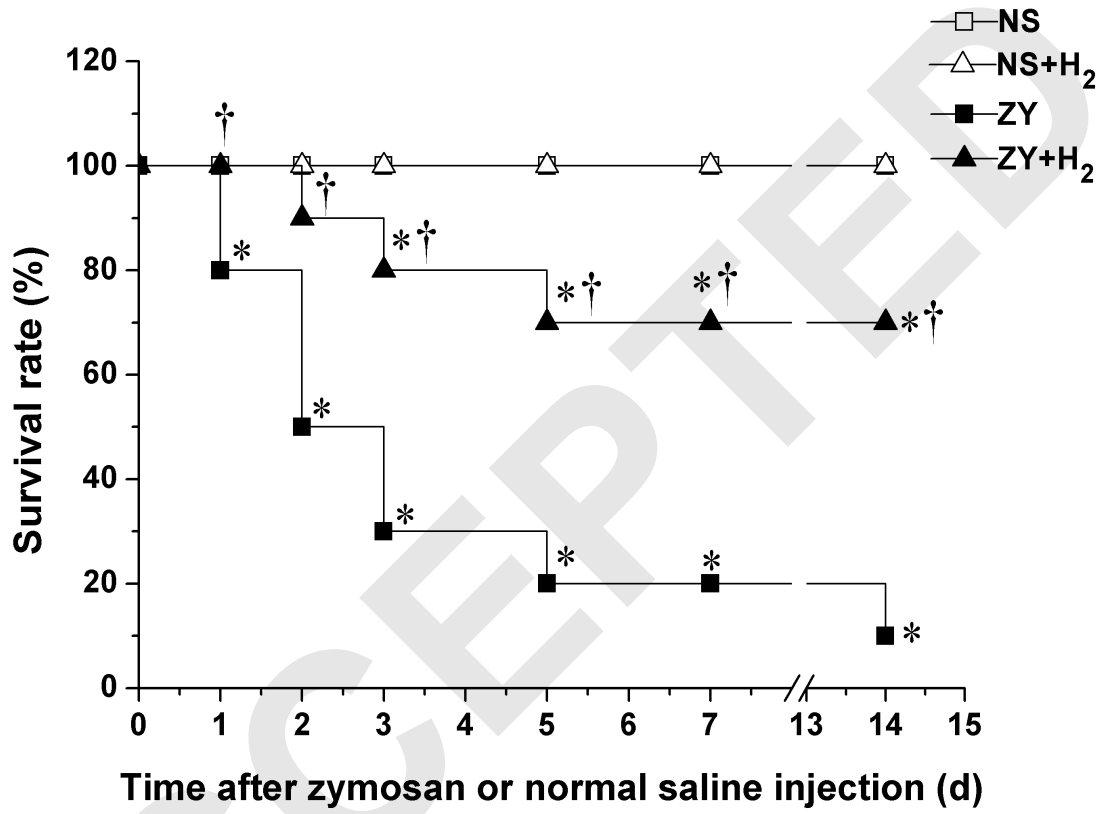


Figure 2

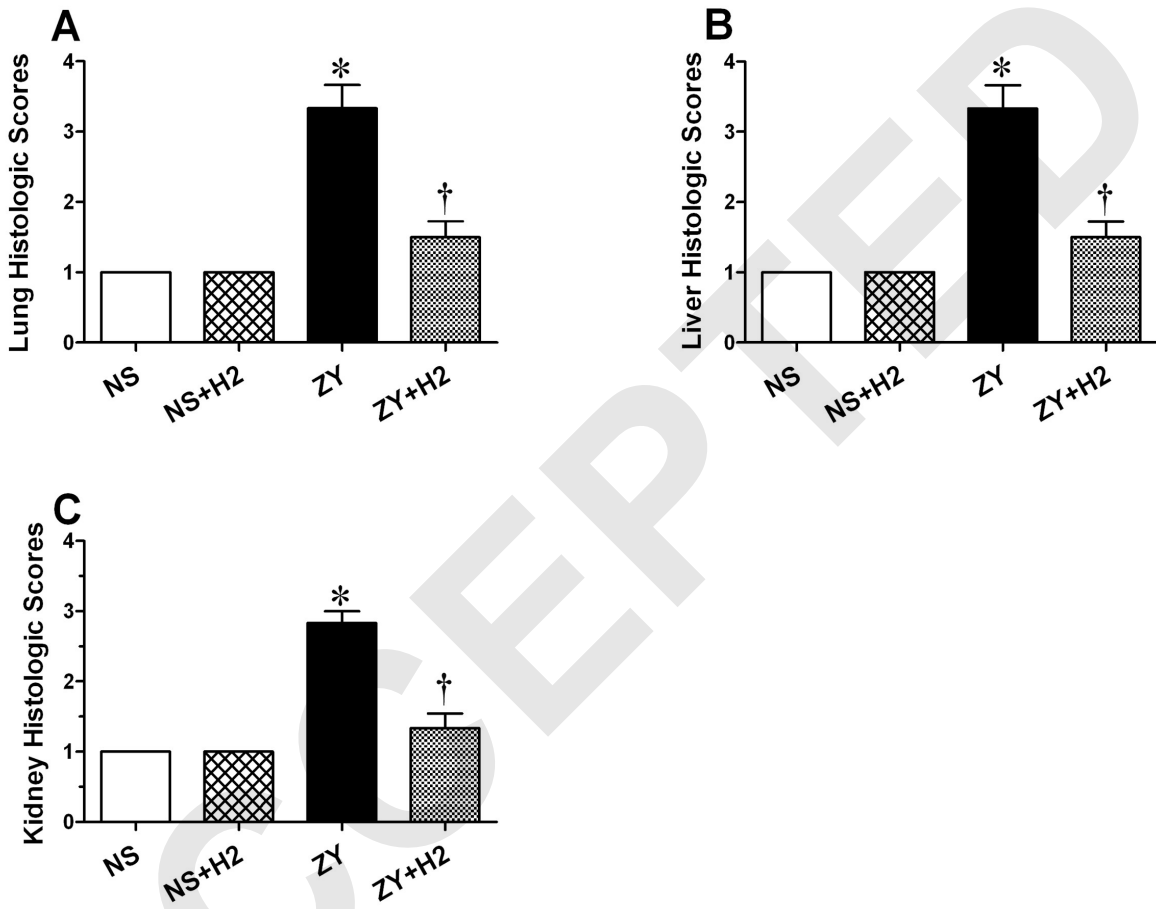


Figure 3

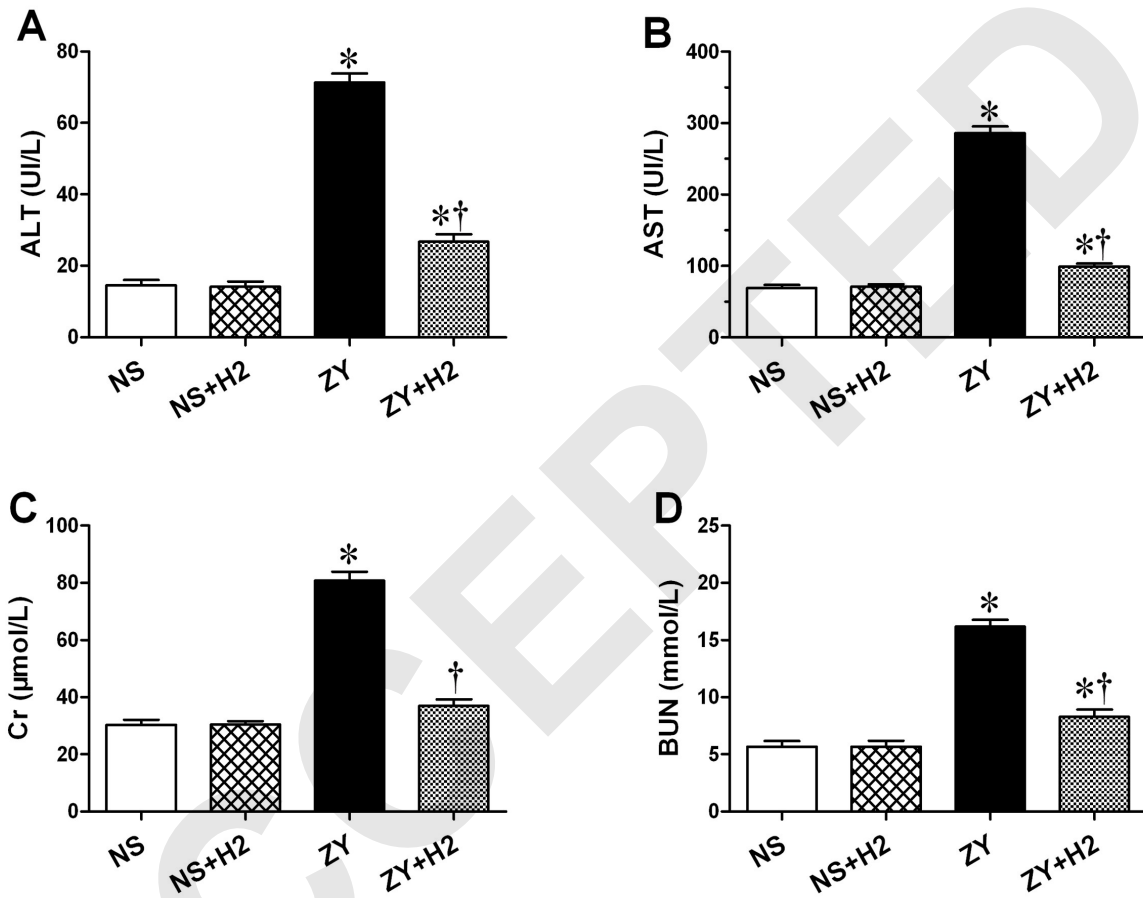


Figure 4

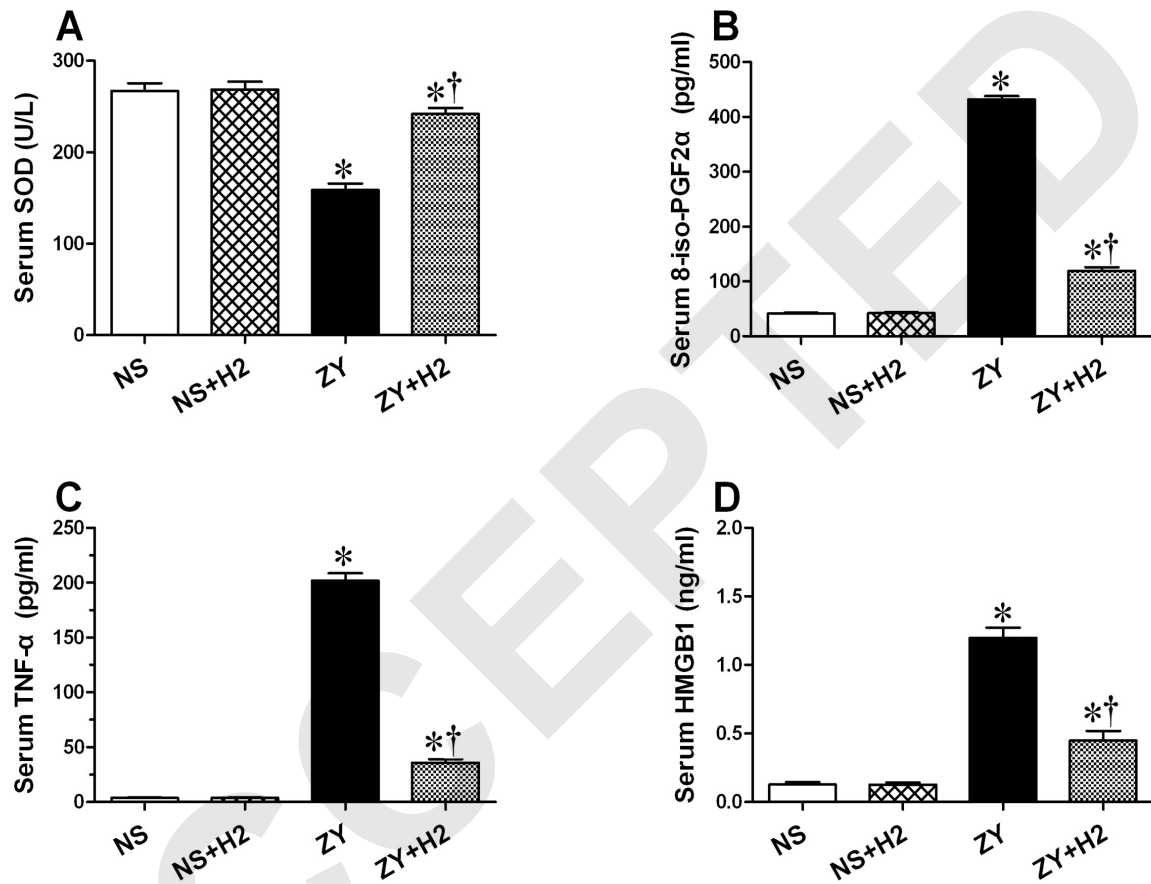


Figure 5

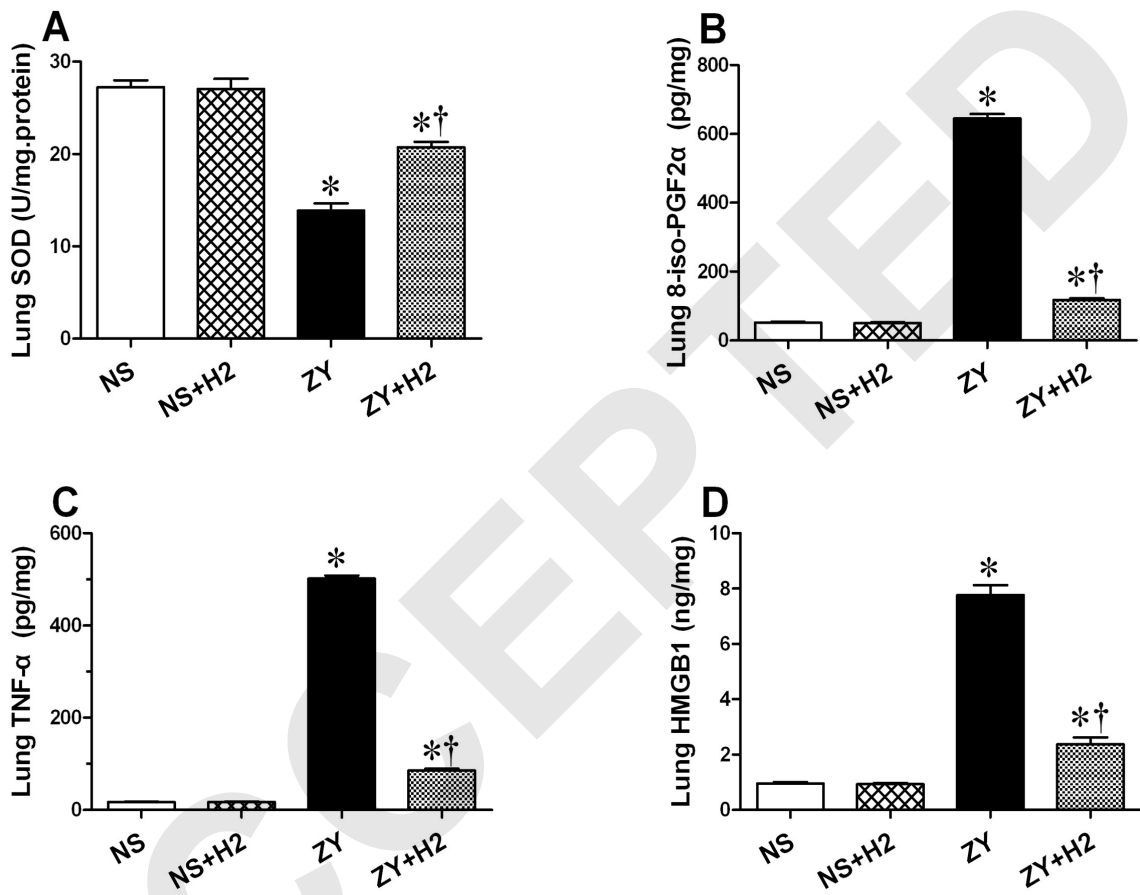


Figure 6

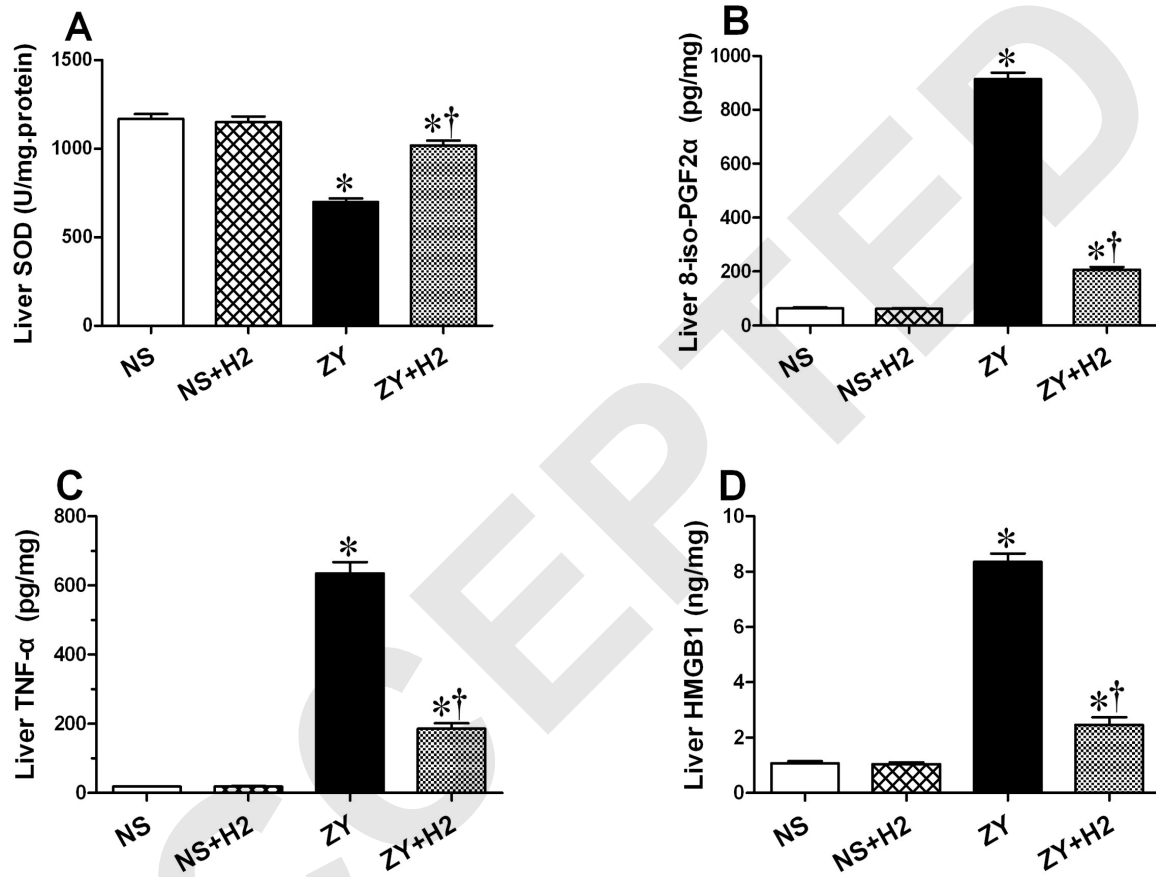


Figure 7

