# Preadministration of Hydrogen-Rich Water Protects Against Lipopolysaccharide-Induced Sepsis and Attenuates Liver Injury : Shock

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Basic Science Aspects

# Preadministration of Hydrogen-Rich Water Protects Against Lipopolysaccharide-Induced Sepsis and Attenuates Liver Injury

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## Abstract

Despite significant advances in antibiotic therapy and intensive care, sepsis remains the most common cause of death in intensive care units. We previously reported that molecular hydrogen ($H_2$) acts as a therapeutic and preventive antioxidant. Here, we show that preadministration of $H_2$-dissolved water (HW) suppresses lipopolysaccharide (LPS)-induced endotoxin shock in mice. Drinking HW for 3 days before LPS injection prolonged survival in a mouse model of sepsis. The $H_2$ concentration immediately increased in the liver but not in the kidney after drinking HW. The protective effects of the preadministration of HW on LPS-induced liver injury were examined. Twenty-four hours after LPS injection, preadministration of HW reduced the increase in both apoptosis and oxidative stress. Moreover, preadministration of HW enhanced LPS-induced expression of heme oxyganase-1 and reduced endothelin-1 expression. These results indicate the therapeutic potential of HW in preventing acute injury of the liver with attenuation of an increase in oxidative stress. HW is likely to trigger adaptive responses against oxidative stress.

## INTRODUCTION

Sepsis is defined as a clinical syndrome caused by an amplified and dysregulated inflammatory host response to infection. The critical syndrome is characterized by hyperthermia or hypothermia, tachypnea, tachycardia, changes in white blood cell count, positive fluid balance with edema, hemodynamic changes, and organ dysfunction (1). There are several potential sources of reactive oxygen species (ROS) in sepsis including mitochondrial electron transport chain dysfunction and xanthine oxidase activation as a result of ischemia and reperfusion, respiratory burst associated with neutrophil activation, and arachidonic acid metabolism (2). Gram-negative bacterial endotoxins, or lipopolysaccharides (LPS), are released into the circulation from sites of bacterial infection and induce secretion of proinflammatory cytokines, primarily tumor necrosis factor-α (TNF-α), interleukin (IL) 1-β, and IL-6 (3). Since LPS administration in mice represents an established *in vivo* model of sepsis with severe hepatic dysfunction, mouse models of acute liver disease during sepsis are used (4).

The heme oxygenase (HO) system is one of the key regulators of cellular redox homeostasis responding to *ROS* via *HO-1* induction and its transcription is regulated by nuclear factor erythroid 2-related factor 2 (Nrf2) (5). HO-1 functions in the heme salvage pathway, removing the pro-oxidant free heme by converting it to bilirubin, a known antioxidant molecule, and carbon monoxide (CO), a potent vasodilator. CO negatively regulates endothelin 1 (ET-1) at the message level in endothelial cells (6). ET-1 is a potent endogenous vasoconstrictor and plays an important role in the pathophysiology of endotoxin shock and liver dysfunction (7). Previous studies have shown that ET-1 stimulates the production of ROS, primarily superoxide anions, leading to the development of oxidative stress, which is associated with increased lipid peroxidation (8).

We previously reported that molecular hydrogen ($H_2$) selectively reduces ROS, hydroxyl radical, and peroxynitrite (9), and inhalation of $H_2$ gas markedly suppresses ischemia/reperfusion (I/R) injury of

multiple organs [10]. $H_2$ administration reduces inflammation in experimental animal models of disease induced by LPS with decreased levels of proinflammatory cytokines [11, 12]. Hydrogen-saturated drinking is safer and more convenient than inhaling $H_2$ gas, which is explosive when the concentration in air is greater than 4%. Many studies have shown that drinking $H_2$-dissolved water (HW) can reduce oxidative stress in several organs including the brain, lung, liver, kidney, and intestine [10]. In a rat model of Parkinson disease, drinking HW was found to be more effective than inhaling $H_2$ gas [13]. Furthermore, after drinking HW, the $H_2$ concentration in the brain was too low to be detected using a conventional hydrogen sensor [9]. These findings indicate that $H_2$ in HW potentially protects various organs around the gastrointestinal tract with a higher and detectable $H_2$ concentration, which further ameliorates neurodegeneration.

HW may be useful in preventing or minimizing diabetes, cardiovascular disease, and stroke. However, there are insufficient studies on the beneficial effects of drinking HW on a daily basis. The present *in vivo* study indicates that administration of HW before LPS injection sufficiently attenuates liver injury in mice. Preadministration of HW after LPS injection prolonged survival and reduced oxidative stress in the liver with increased expression of HO-1 and reduced ET-1 expression.

# MATERIALS AND METHODS

## Animals

C57BL/6J male mice (weighing 19–22 g, specific pathogen-free) were purchased from CLEA Japan Inc (Tokyo, Japan). Mice were housed at 20°C to 22°C with a 12-h light/dark cycle and provided with sterile food and water. All efforts were made to minimize the number of animals used and their suffering during experimental procedures. All protocols for animal use and experiments followed the Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985). All study protocols were reviewed and approved by the Animal Care Committee of the Tokyo Metropolitan Institute of Gerontology. Animals were sacrificed at predetermined endpoints using asphyxiation by CO2 or via exsanguination under deep anesthesia with combined anesthetic agents according to a previously described method [14]. In brief, three different anesthetic agents were mixed and administered by intraperitoneal (i.p.) injection in the mice. The mice were administered 0.75 mg/kg of medetomidine hydrochloride (Domitol, Meiji Seika Pharma, Tokyo, Japan), 4 mg/kg of midazolam (Dormicum, Astellas Pharma, Tokyo, Japan), and 5 mg/kg butorphanol (Vetorphale, Meiji Seika Pharma). Following induction of sepsis, animals were checked twice daily for 3 days. Moribund animals were sacrificed using humane endpoints.

## Administration of HW to a mouse model of sepsis

HW was prepared using a previously described method [15]. In brief, $H_2$ gas (grade 1, Iwatani, Tokyo, Japan) was dissolved in reverse osmosis water under high pressure (0.4 MPa) to a super saturated level in a stainless-steel tank (Unicontrols, Tokyo, Japan). Saturated HW was poured into closed glass vessels equipped with an outlet line containing two ball bearings, which prevented the water from being degassed. Approximately 80% saturated $H_2$-water (640 μM) at 0.1 MPa RT was used as HW. During preparation of HW, we carefully monitored $H_2$ concentration in room air by $H_2$ sensor with alarm for safety. Water obtained by degassing $H_2$ from HW with gentle stirring overnight was used as a control. Mice were given water freely and the vessel was freshly refilled with HW or control water at 6:00 PM every day.

To determine whether HW ameliorates LPS-induced death, 7-week-old mice were randomly assigned into four groups: control water 3 days before and after LPS injection (Ctl group), HW 3 days before and after LPS injection (HW group), HW 3 days before and control water after LPS injection (preHW group), and control water 3 days before and HW after LPS injection (post-HW group). Mice drinking control water and HW 3 days before saline injection were used as a negative control, respectively (Fig. 1A). LPS (O127:B8, Sigma-Aldrich, St. Louis, Mo) was dissolved in saline and injected i.p. at a dose of 30 mg/kg.

Fig. 1:
Preadministration of HW improves the survival rate and body temperature in a mouse model of sepsis.A, Schematic diagram of the protocol for the administration of HW in a mouse model of sepsis. Mice were administered control water 3 days before and after LPS injection (Ctl group), HW 3 days before and after LPS injection (HW group), HW 3 days before and control water 3 days after LPS injection (preHW group), and control water 3 days before and HW 3 days after LPS injection (post-HW group). B, The Kaplan–Meier curve representing the survival rate of mice in the sepsis model. After LPS injection (LPS [+]), survival rates were significantly improved in HW, preHW groups. * $P < 0.05$ versus Ctl group. Note that there is not a significant difference between HW and preHW groups. C, Amounts of daily drinking water. Noteworthy, amounts of drinking water decreased substantially and immediately after LPS injection. D, Thermographic images of the body temperature of mice 24 h after LPS injection. E, Quantitative analysis of the body temperature of mice 24 h after LPS injection. *** $P < 0.001$ versus LPS (+) Ctl group. HW indicates hydrogen-dissolved water; LPS, lipopolysaccharide.

To determine whether preadministration of HW attenuates LPS-induced liver injury, 7-week-old mice were randomly assigned into four groups: control water 3 days before saline injection, preadministration of HW 3 days before saline injection, control water 3 days before LPS injection, and preadministration of HW 3 days before LPS injection. After the injection, all mice were given control water freely. Their blood for biochemistry and tissues for histochemical studies and protein analysis were obtained at 24 h after the injection.

## Thermal images

To determine whether HW ameliorates LPS-induced hypothermia, 7-week-old mice were randomly assigned into three groups described above: Ctl group, HW group, and preHW group. Mice drinking control water and HW without LPS injection were used as negative control, respectively. Thermal images were obtained from mice prior to and 24 h after LPS injection using a thermographic camera (FLIR, Wilsonville, Ore).

## LPS activity

To determine whether $H_2$ directly affect LPS activity, LPS was dissolved in control water and HW, respectively, and the activity was examined using E-TOXATE kit (Sigma-Aldrich).

## Measurement of $H_2$ concentration in the liver and kidney

The $H_2$ concentration was sequentially measured in the liver and kidney using a needle-type $H_2$ sensor (Unisense, Aarhus N, Denmark). The tip of the sensor was indwelled into the liver or kidney under anesthesia with the aforementioned mixed anesthetic agents. First, the $H_2$ concentration was

monitored for 7 min after oral administration of control water (400 μL) with a feeding needle. After subsequent oral administration of HW (400 μL), the $H_2$ concentration was further monitored.

## Serum TNF-α and IL-6

Seven-week-old mice were randomly assigned into three groups: control water without LPS injection, control water 3 days before, and after LPS injection and HW 3 days before and control water after LPS injection (preHW group). At the indicated time, blood was collected from the eye. Serum TNF-α and IL-6 concentrations were determined using enzyme-linked immunosorbent assay kits in accordance with the manufacturer's instructions (R&D Systems, Minneapolis, Minn).

## Serum biochemistry

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined according to the manufacturer's protocol (Wako, Osaka, Japan). Serum lactate dehydrogenase (LDH) activity was determined using a kit in accordance with the manufacturer's instructions (Nittobo, Tokyo, Japan).

## TdT-mediated dUTP nick end labeling (TUNEL) assay

Apoptosis in the liver was determined in formalin-fixed, paraffin-embedded liver tissue samples, which were cut in 4 μm sections. Following deparaffinization and rehydration, sections were further processed according to the manufacturer's protocol (TdT-mediated dUTP nick end labelling (TUNEL) Apoptosis Detection kit, Millipore, Billerica, Mass). Final color reaction was achieved using 3,3′-diaminobenzidine (DAB) chromogenic substrate (Sigma-Aldrich). The numbers of positive cells in randomly selected 10 fields of view were counted at a final magnification of ×200 for each section using a light microscope.

## Immunohistochemistry

8-hydroxy-2′-deoxyguanosine (8-OHdG) and 4-hydroxy-2-nonenal (4-HNE) were stained in formalin-fixed, paraffin-embedded liver tissues, which were cut in 4 μm sections. Sections were deparaffinized, rehydrated, and microwaved in 10 mM citric acid (pH 6.0) at boiling temperature for 5 min. Endogenous peroxidase was inhibited by 0.3% $H_2O_2$ in PBS at RT for 20 min. Sections were blocked with goat serum and incubated overnight at 4°C with primary monoclonal antibodies against 8-OHdG (2.5 μg/mL, JaICA, Fukuroi, Japan) and 4-HNE (25 μg/mL, JaICA). After washing, sections were incubated with biotinylated secondary antibody against mouse IgG (1:300, Dako, Glostrup, Denmark) at RT for 60 min, and further incubated with horseradish peroxidase (1:100 in ABC kit, Vector lab, Burlingame, Calif) at RT for 30 min. DAB chromogenic substrate was used to achieve a color reaction. To quantify immunointensity of each section, 10 fields of view were selected randomly and photographed at a final magnification of ×200 using a light microscope. The obtained images were converted into gray scale and their level of density was obtained by using ImageJ software (National Institutes of Health, Bethesda, Md).

HO-1 was stained in liver tissues fixed with 4% paraformaldehyde (Wako), which were cryoprotected with sucrose, frozen, and cut in 5 μm sections. Sections were incubated with 10 mM citric acid (p*H* 6.0) at boiling temperature for 5 min. Endogenous peroxidase was inhibited by 0.3% $H_2O_2$ in PBS at RT for 20 min. Sections were blocked with goat serum and incubated overnight at 4°C with primary polyclonal rabbit antibody against HO-1 (1:100, Proteintech, Chicago, Ill). After washing, sections were incubated with biotinylated secondary antibody against rabbit IgG (1:150, Vector Lab) at RT for 60

min, and further incubated with horseradish peroxidase (1:100 in ABC kit) at RT for 30 min. DAB chromogenic substrate was used to achieve a color reaction. The numbers of positive cells in randomly selected 10 fields of view were counted at a final magnification of ×200 for each section using a light microscope.

ET-1 was stained in liver tissue snap frozen in liquid nitrogen, which were cut in 5 μm sections, and fixed in acetone at −20°C for 20 min. Sections were incubated with 10 mM citric acid (p*H* 6.0) at boiling temperature for 5 min. Endogenous peroxidase was inhibited by 0.3% $H_2O_2$ at RT in PBS for 20 min. Sections were blocked with goat serum and incubated overnight at 4°C with primary polyclonal rabbit antibody against ET-1 (1:100, IBL, Fujioka, Japan). After washing, sections were incubated with biotinylated secondary antibody against rabbit IgG (1:150) at RT for 60 min, and further incubated with horseradish peroxidase (1:100 in ABC kit) at RT for 30 min. DAB chromogenic substrate was used to achieve a color reaction. To quantify immunointensity of each section, 10 sinusoids were selected randomly and photographed at a final magnification of ×200 using a light microscope. The obtained images were converted into gray scale and their level of density in sinusoidal endothelial cells was obtained by using ImageJ software.

## Western blot analysis

Liver tissues were homogenized in RIPA buffer and centrifuged (15,000 *g* at 4°C for 20 min), and the supernatants were collected and stored at −80°C. Denatured proteins (10 μg in each lane) were separated on a 10% acrylamide gel and electrotransferred onto a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked with skim milk and incubated at 4°C overnight with primary polyclonal rabbit antibody against HO-1 (1:500). After washing, membranes were incubated with peroxidase-conjugated anti-rabbit IgG (1:10,000) at RT for 1 h. Protein bands were detected using an enhanced chemiluminescence kit (ECL prime, GE Healthcare, Chicago, Ill) and visualized using an exposure and quantitation system (LAS-3000 mini, FUJI film, Tokyo, Japan). As a normalization control, the membranes were reprobed for 3-phosphate dehydrogenase (GAPDH) and exposed to polyclonal rabbit antibody against GAPDH (1:1,000, Cell Signaling, Danvers, Mass).

## Statistical analysis

Statistical analyses were performed using SPSS software (version 22.0; SPSS, Chicago, Ill). The survival rates were analyzed with log-rank test. All values are presented as means ± standard deviation. Statistically significant difference between the groups was determined by a one-way ANOVA followed by Dunnett test. Results were considered significant at $P < 0.05$.

# RESULTS

## Preadministration of HW prolonged survival in a mouse model of sepsis

To determine whether HW ameliorates LPS-induced sepsis, the survival rate was compared between the Ctl and HW groups (Fig. 1A). Treatment with HW significantly prolonged survival at 72 h (57.7% vs. 26.9%; $^{*}P < 0.05$) (Fig. 1B). The amount of drinking water substantially decreased after LPS injection, resulting in no significant difference between the Ctl and HW groups (Fig. 1C), suggesting that drinking HW before i.p. injection may suffice in prolonging survival. The survival rate of mice administered HW 3 days before and control water 3 days after LPS injection (preHW group) was examined (Fig. 1A). Preadministration of HW for 3 days significantly improved the survival rate (50.0% vs. 26.9%; $^{*}P < 0.05$) (Fig. 1B), while postadministration of HW for 3 days after LPS injection (post-HW) did not improve the survival rate (28.6% vs. 26.9%; not significant). There is not a

significant difference between HW and preHW groups. The body temperature of mice 24 h after LPS injection indicated that preadministration of HW ameliorated LPS-induced hypothermia (Fig. 1, D and E).

To exclude the possibility of direct inactivation of LPS by $H_2$, we examined LPS activity in water with or without $H_2$, and found that the activities were not different between them, within 0.013 to 0.04 endotoxin units/mL, indicating that $H_2$ does not inactivate LPS directly.

## Secretion of proinflammatory cytokines was not prevented by preadministration of HW

To determine whether preadministration of HW attenuates LPS-induced secretion of proinflammatory cytokines, serum TNF-α, and IL-6 levels were measured. TNF-α levels increased dramatically in mice drinking control water 1 h after LPS injection (Fig. 2A), which was not reduced in mice pretreated with HW (preHW group). Differences were not observed at 6 and 12 h after LPS injection. Similarly, preadministration of HW did not attenuate an LPS-induced increase of IL-6 (Fig. 2B).

F2-12

Fig. 2:
Preadministration of HW does not markedly ameliorate an elevation in inflammatory cytokines.(A) TNF-α and (B) IL-6 concentrations in serum were measured at 1, 6, and 12 h after LPS injection. HW was preadministered for 3 days before LPS injection (preHW). The number of animals used in each group is 8. $^{***}P<0.001$ versus LPS (+), preHW (−) group. HW indicates hydrogen-dissolved water; IL, interleukin; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

## Drinking HW elevates the concentration of $H_2$ in the liver

The liver and kidney are two major organs that are susceptible to LPS toxicity during sepsis [16, 17]. The $H_2$ concentration was sequentially monitored in each organ using a needle-type $H_2$ sensor. After administration of 400 μL HW containing 640 μM $H_2$ with a feeding needle, the $H_2$ concentration in the liver was immediately elevated, with a peak of approximately 16 μM $H_2$ (Fig. 3A). The $H_2$ concentration was not observed to be elevated in the kidney after HW administration (Fig. 3B). These results suggest the possibility that $H_2$ derived from HW directly and effectively reduces susceptibility of the liver to LPS.

F3-12

Fig. 3:
$H_2$ concentration in the kidney and liver after oral administration of HW.HW was administered 7 min after oral administration of control water with a feeding needle. The tip of the $H_2$ sensor was indwelled into the liver (A) or kidney (B) under anesthesia. $H_2$ indicates hydrogen; HW, $H_2$-dissolved water.

## Preadministration of HW attenuates LPS-induced liver injury

To determine whether preadministration of HW attenuates LPS-induced liver injury, liver tissues were stained with hematoxylin and eosin 24 h after LPS injection. Nuclear condensation, fragmentation, and blood congestion were observed in the liver of mice drinking control water (Ctl group), whereas liver injury was suppressed in mice pretreated with HW (preHW group) (Fig. 4, A, C, and D).

Apoptotic cell death was examined using the TUNEL assay 24 h after LPS injection. Preadministration of HW resulted in a reduction of the increase of TUNEL-positive cells in the liver (Fig. 4, B and E). In mice drinking control water, LPS injection resulted in an increase in ALT, AST, and LDH levels, whereas preadministration of HW significantly attenuated their increase (Fig. 5). These results indicate that preadministration of HW sufficiently attenuates LPS-induced cellular and functional injury of the liver.

Fig. 4:
Preadministration of HW improved septic liver injury.(A, C, and D) Hematoxylin and eosin staining of the liver 24 h after LPS injection. Black arrow heads indicate nuclear condensation and fragmentation in hepatocytes (upper in A). Black arrows indicate blood congestion (lower in A). Quantitative analysis of blood congestion area in vessels in the liver 24 h after LPS injection (C). Percent area of vascular congestion was calculated as a blood cell-occupied area per cross-section area of vessel in the liver ($0.5\ mm^2$). Quantitative analysis of hepatocytes with nuclear condensation and fragmentation in the liver 24 h after LPS injection (D). Percent positive cells per total cells in field of view are shown. (B, E) Apoptotic cells in the liver visualized by the TUNEL assay 24 h after LPS injection. Preadministration of HW for 3 days before LPS injection (preHW) resulted in decreased liver injury and TUNEL-positive cells (B). Quantitative analysis of TUNEL-positive cells (E). Number of positive cells per total cells in the field of view is shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus LPS (+), preHW (−) group. HW indicates hydrogen-dissolved water; LPS, lipopolysaccharide; TUNEL, TdT-mediated dUTP nick end labeling.

Fig. 5:
Preadministration of HW improved hepatic function.Serum biomarkers of hepatic function, AST, ALT, and LDH were measured 24 h after LPS injection. HW was preadministered for 3 days before LPS injection (preHW). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus LPS (+), preHW (−) group. ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; HW, hydrogen-dissolved water; LDH, lactate dehydrogenase; LPS, lipopolysaccharide.

## Preadministration of HW attenuates LPS-induced oxidative stress in the liver

Several studies have reported that treatment with $H_2$ reduces oxidative stress in both organs and cultured cells [10]. We examined the levels of two oxidative stress markers, 4-HNE and 8-OHdG, by immunohistochemical staining. Twenty-four hours after LPS injection, the number of 4-HNE and 8-OHdG-positive cells was dramatically increased in the liver of mice drinking control water (Fig. 6, Supplement Tables 1 and 2, https://links.lww.com/SHK/A515). Preadministration of HW resulted in fewer 4-HNE and 8-OHdG-positive cells, indicating that preadministration of HW reduces LPS-induced oxidative stress in the liver.

Fig. 6:
Preadministration of HW decreased LPS-induced oxidative stress in the liver.Immunohistochemical staining of the oxidative stress markers 8-OHdG and 4-HNE in the liver 24 h after LPS injection. A, Preadministration of HW for 3 days before LPS injection (preHW) resulted in decreased immunostaining of both 8-OHdG and 4-HNE. B

and C, Quantitative analysis of immunointensity of 8-OHdG and 4-HNE. * $P$ <0.05, ** $P$ <0.01, *** $P$ <0.001 versus LPS (+), preHW (−) group. 4-HNE indicates 4-hydroxy-2-nonenal; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; HW, hydrogen-dissolved water; LDH, lactate dehydrogenase; LPS, lipopolysaccharide.

## Preadministration of HW enhances HO-1 expression and attenuates ET-1 expression in the liver

The HO system is one of the key regulators of cellular redox homeostasis. We examined HO-1 expression in the liver by immunohistochemical staining. The number of HO-1-positive cells was observed to increase in the liver of mice drinking control water (Fig. 7, A and B and Supplement Table 3, https://links.lww.com/SHK/A515). Western blot of liver extracts revealed that LPS injection resulted in an increase in HO-1 expression (Fig. 7, C and D). Preadministration of HW significantly enhanced HO-1 expression, suggesting that increased HO-1 reduces the accumulation of LPS-induced oxidative stress in the liver.

F7-12

Fig. 7:

Preadministration of HW increased LPS-induced HO-1 expression.A, Immunohistochemical staining of HO-1 in the liver 24 h after LPS injection. B, Quantitative analysis of HO-1-positive cells in the liver. Percent positive cells per total cells in the field of view is shown. * $P$ <0.05. C, Immunoblots of HO-1 in the liver 24 h after LPS injection. GAPDH was used as an internal control protein. D, The expression levels of HO-1 are quantified by the intensity of each protein immunoblot and normalized to the intensity of samples derived from mice untreated with both LPS and preHW. Pre-administration of HW for 3 days before LPS injection (preHW) resulted in further elevation of LPS-induced HO-1 expression. * $P$ <0.05, ** $P$ <0.01 versus LPS (+), preHW (−) group. GAPDH indicates 3-phosphate dehydrogenase; HO, heme oxygenase; HW, hydrogen-dissolved water; LPS, lipopolysaccharide.

CO, a HO-1 product, negatively regulates ET-1, a potent endogenous vasoconstrictor, which is highly expressed in the septic liver and stimulates the production of ROS. ET-1 expression was examined in the liver of mice 24 h after LPS injection. Immunohistochemical staining of the liver with an ET-1-specific antibody revealed an increase in ET-1 expression in endothelium after LPS injection, whereas preadministration of HW significantly reduced the increase (Fig. 8 and Supplement Table 4, https://links.lww.com/SHK/A515).

F8-12

Fig. 8:

Preadministration of HW decreased LPS-induced ET-1 expression.A, Immunohistochemical staining of ET-1 in the liver 24 h after LPS injection. HW was preadministered for 3 days before LPS injection (preHW). B, Quantitative analysis of immunointensity of ET-1 in hepatic endothelium. * $P$ <0.05, ** $P$ <0.01 versus LPS (+), preHW (−) group. ET-1 indicates endothelin; HW, hydrogen-dissolved water; LPS, lipopolysaccharide.

# DISCUSSION

The present study demonstrates that preadministration of HW is sufficient to prolong survival in a mouse model of sepsis (Fig. 1). After LPS injection, preadministration of HW attenuated liver injury

with a decrease in oxidative stress; however, an early LPS-induced increase of proinflammatory cytokines was not attenuated (Fig. 2). This indicates that preadministration of $H_2$ does not affect an immediate, early, amplified, and dysregulated immunological response.

$H_2$ administrations by inhalation of $H_2$ gas, injection of $H_2$-dissolved saline, and drinking of HW have been shown to prevent LPS-induced sepsis [18–21]. However, a target site(s) of action and a target molecule(s) for the physiological function of $H_2$ remain unclear. The liver and kidney are two major organs that are highly susceptible to LPS toxicity during septic shock in mice [16, 17]. In the present study, HW administration was shown to induce an elevation of the $H_2$ concentration in the liver (16 μM), whereas $H_2$ was not detected in the kidney (Fig. 3). The concentration of $H_2$ has been reported to be elevated in the liver (10–20 μM) after HW administration in rats, whereas a modest elevation was observed in the kidneys (approximately 0.3 μM) [22]. Our results and those of the aforementioned studies indicate that the $H_2$ concentration in the kidney is too low to prevent direct cellular damage. It can be assumed that one of the major target sites of $H_2$ derived from HW administration is the liver. In a previous study, sepsis-induced liver dysfunction was found to be strongly associated with mortality [23].

The survival rate of LPS-induced sepsis in a mouse model was prolonged by HW preadministration, indicating that $H_2$ derived from HW does not directly reduce LPS-induced ROS (Fig. 6). Indeed, HW postadministration during higher production of ROS did not improve the survival rate (Fig. 1). On the other hand, inhalation of $H_2$ gas and administration of $H_2$-loaded eye drops for I/R injury exerted the effect when $H_2$ was inside the brain and eyeball at the onset of reperfusion [24], thereby directly reducing a toxic ROS, ·OH, which is produced during I/R. Different mechanisms underlying the protective effects of $H_2$ between inhalation of $H_2$ gas and drinking of HW can be assumed.

Interestingly, Sato et al. [25] reported that preadministration of HW in mice prevented *in vitro* ROS formation in the brain. In their study, brain sections were prepared after freely drinking HW and ROS formation was subsequently measured during hypoxia and reoxygenation *in vitro*. ROS formation was found to be suppressed in the sections from HW-treated mice. In the present study, preadministration of HW resulted in improved hepatic injury (Figs. 4 and 5) with suppression of oxidative stress markers and enhanced HO-1 expression (Fig. 7). The increased HO-1 most likely resulted in an amelioration of LPS-induced 8-OHdG and 4-HNE accumulation in the liver. Upregulation of HO-1 with hemin was reported to prevent D-galactosamine and LPS-induced hepatic injury in rats [26]. HO-1 expression is transcriptionally regulated by nuclear factor erythroid 2-related factor 2. Kawamura et al. [27] reported that inhalation of $H_2$ gas during exposure to hyperoxia improves blood oxygenation, reduces inflammation, and induces HO-1 expression in the lung via Nrf2 activation. Moreover, $H_2$ regulated endothelial injury and the inflammatory response via Nrf2-mediated HO-1 levels, suggesting that $H_2$ may suppress excessive inflammatory responses and endothelial injury via the Nrf2/HO-1 pathway [28]. Preadministration of HW may potentially enhance antioxidative activities via the adaptive response. Drinking HW resulted in the upregulation of genes for oxidoreduction-related proteins in the rat liver [29]. To understand the precise mechanisms underlying the protective effects of HW-preadministration, we need to investigate physiological changes before and/or just after LPS injection.

The LPS-induced decrease in body temperature and congestion in the liver were significantly improved by preadministration of HW (Figs. 1 and 4), indicating that HW improves a decrease in blood flow. Furthermore, the LPS-induced increase of the endogenous vasoconstrictor, ET-1, in the liver was ameliorated by the preadministration of HW (Fig. 8). Previous studies suggest that HW may improve vascular endothelial function. Flow-mediated dilation of the brachial artery was shown to

improve after administration of HW [30]. In our recent study, $H_2$-containing buffer was found to reduce hepatic reperfusion injury via the maintenance of portal venous flow and ameliorated ET-1 elevation in the liver [31]. The observation of HO-1-deficient mice suggests that the lack of HO-1 allows for a sustained induction of ET-1 message in the liver [32]. However, CO, a HO-1 product, negatively regulates ET-1 at the transcription level in endothelial cells [6]. An increase of HO-1 induced by preadministration of HW is assumed to result in reduced ET-1 expression in the liver.

Despite significant advances in antibiotic therapy and intensive care, sepsis remains the most common cause of death in intensive care units, with in-hospital mortality rates reported to be between 20% and 30% in the United States [33, 34]. The present study demonstrated that preadministration of HW is useful in preventing or minimizing the risk of sepsis. Clinical trials on drinking HW on a daily basis in patients with sepsis are urgently required. However, LPS is not a reliable surrogate for human sepsis [35]. Further investigations whether the preadministration of HW improves septic shock in the model of polymicrobial sepsis should be addressed.

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## REFERENCES

1. Vincent JL, Martinez EO, Silva E. Evolving concepts in sepsis definitions. *Crit Care Nurs Clin North Am* 2011; 23 1:29–39.

- Cited Here |
- PubMed | CrossRef

2. Macdonald J, Galley HF, Webster NR. Oxidative stress and gene expression in sepsis. *Br J Anaesth* 2003; 90 2:221–232.

- Cited Here |
- View Full Text | PubMed | CrossRef

3. Li L, Liu Y, Chen HZ, Li FW, Wu JF, Zhang HK, He JP, Xing YZ, Chen Y, Wang WJ, et al. Impeding the interaction between Nur77 and p38 reduces LPS-induced inflammation. *Nat Chem Biol* 2015; 11 5:339–346.

- Cited Here |
- PubMed

4. Baranova IN, Souza AC, Bocharov AV, Vishnyakova TG, Hu X, Vaisman BL, Amar MJ, Chen Z, Kost Y, Remaley AT, et al. Human SR-BI and SR-BII potentiate lipopolysaccharide-induced inflammation and acute liver and kidney injury in mice. *J Immunol* 2016; 196 7:3135–3147.

- Cited Here |
- PubMed | CrossRef

5. Paine A, Eiz-Vesper B, Blasczyk R, Immenschuh S. Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. *Biochem Pharmacol* 2010; 80 12:1895–1903.

- Cited Here |
- View Full Text | PubMed | CrossRef

6. George EM, Cockrell K, Aranay M, Csongradi E, Stec DE, Granger JP. Induction of heme oxygenase 1 attenuates placental ischemia-induced hypertension. *Hypertension* 2011; 57 5:941–948.

- Cited Here |
- View Full Text | PubMed | CrossRef

7. Liu A, Fang H, Wei W, Kan C, Xie C, Dahmen U, Dirsch O. G-CSF pretreatment aggravates LPS-associated microcirculatory dysfunction and acute liver injury after partial hepatectomy in rats. *Histochem Cell Biol* 2014; 142 6:667–676.

- Cited Here |
- PubMed | CrossRef

8. Viel EC, Benkirane K, Javeshghani D, Touyz RM, Schiffrin EL. Xanthine oxidase and mitochondria contribute to vascular superoxide anion generation in DOCA-salt hypertensive rats. *Am J Physiol Heart Circ Physiol* 2008; 295 1:H281–H288.

- Cited Here |
- PubMed | CrossRef

9. Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med* 2007; 13 6:688–694.

- Cited Here |
- PubMed | CrossRef

10. Ichihara M, Sobue S, Ito M, Ito M, Hirayama M, Ohno K. Beneficial biological effects and the underlying mechanisms of molecular hydrogen—comprehensive review of 321 original articles. *Med Gas Res* 2015; 5:12.

- Cited Here

11. Itoh T, Hamada N, Terazawa R, Ito M, Ohno K, Ichihara M, Nozawa Y, Ito M. Molecular hydrogen inhibits lipopolysaccharide/interferon gamma-induced nitric oxide production through modulation of signal transduction in macrophages. *Biochem Biophys Res Commun* 2011; 411 1:143–149.

- Cited Here |
- PubMed | CrossRef

12. Xie K, Yu Y, Huang Y, Zheng L, Li J, Chen H, Han H, Hou L, Gong G, Wang G. Molecular hydrogen ameliorates lipopolysaccharide-induced acute lung injury in mice through reducing inflammation and apoptosis. *Shock* 2012; 37 5:548–555.

- Cited Here |
- View Full Text | PubMed

13. Ito M, Hirayama M, Yamai K, Goto S, Ito M, Ichihara M, Ohno K. Drinking hydrogen water and intermittent hydrogen gas exposure, but not lactulose or continuous hydrogen gas exposure, prevent 6-hydorxydopamine-induced Parkinson's disease in rats. *Med Gas Res* 2012; 2 1:15.

- Cited Here

14. Kawai S, Takagi Y, Kaneko S, Kurosawa T. Effect of three types of mixed anesthetic agents alternate to ketamine in mice. *Exp Anim* 2011; 60 5:481–487.

- Cited Here |
- PubMed | CrossRef

15. Ohsawa I, Nishimaki K, Yamagata K, Ishikawa M, Ohta S. Consumption of hydrogen water prevents atherosclerosis in apolipoprotein E knockout mice. *Biochem Biophys Res Commun* 2008; 377 4:1195–1198.

- Cited Here |
- PubMed | CrossRef

16. Doi K, Leelahavanichkul A, Yuen PS, Star RA. Animal models of sepsis and sepsis-induced kidney injury. *J Clin Invest* 2009; 119 10:2868–2878.

- Cited Here |
- View Full Text | PubMed | CrossRef

17. Jirillo E, Caccavo D, Magrone T, Piccigallo E, Amati L, Lembo A, Kalis C, Gumenscheimer M. The role of the liver in the response to LPS: experimental and clinical findings. *J Endotoxin Res* 2002; 8 5:319–327.

- Cited Here |
- PubMed | CrossRef

18. Xu XF, Zhang J. Saturated hydrogen saline attenuates endotoxin-induced acute liver dysfunction in rats. *Physiol Res* 2013; 62 4:395–403.

- Cited Here |
- PubMed | CrossRef

19. Sun H, Chen L, Zhou W, Hu L, Li L, Tu Q, Chang Y, Liu Q, Sun X, Wu M, et al. The protective role of hydrogen-rich saline in experimental liver injury in mice. *J Hepatol* 2011; 54 3:471–480.

- Cited Here |
- PubMed | CrossRef

20. Zhang Y, Liu Y, Zhang J. Saturated hydrogen saline attenuates endotoxininduced lung dysfunction. *J Surg Res* 2015; 198 1:41–49.

- Cited Here |
- PubMed | CrossRef

21. Zhang J, Wu Q, Song S, Wan Y, Zhang R, Tai M, Liu C. Effect of hydrogen-rich water on acute peritonitis of rat models. *Int Immunopharmacol* 2014; 21 1:94–101.

- Cited Here |
- View Full Text | PubMed | CrossRef

22. Sobue S, Yamai K, Ito M, Ohno K, Ito M, Iwamoto T, Qiao S, Ohkuwa T, Ichihara M. Simultaneous oral and inhalational intake of molecular hydrogen additively suppresses signaling pathways in rodents. *Mol Cell Biochem* 2015; 403 (1–2):231–241.

- Cited Here |
- PubMed | CrossRef

23. Minter RM, Bi X, Ben-Josef G, Wang T, Hu B, Arbabi S, Hemmila MR, Wang SC, Remick DG, Su GL. LPS-binding protein mediates LPS-induced liver injury and mortality in the setting of biliary obstruction. *Am J Physiol Gastrointest Liver Physiol* 2009; 296 1:G45–G54.

- Cited Here |
- PubMed | CrossRef

24. Oharazawa H, Igarashi T, Yokota T, Fujii H, Suzuki H, Machide M, Takahashi H, Ohta S, Ohsawa I. Protection of the retina by rapid diffusion of hydrogen: administration of hydrogen-loaded eye drops in retinal ischemia-reperfusion injury. *Invest Ophthalmol Vis Sci* 2010; 51 1:487–492.

- Cited Here |
- PubMed | CrossRef

25. Sato Y, Kajiyama S, Amano A, Kondo Y, Sasaki T, Handa S, Takahashi R, Fukui M, Hasegawa G, Nakamura N, et al. Hydrogen-rich pure water prevents superoxide formation in brain slices of vitamin C-depleted SMP30/GNL knockout mice. *Biochem Biophys Res Commun* 2008; 375 3:346–350.

- Cited Here |
- PubMed | CrossRef

26. Wen T, Wu ZM, Liu Y, Tan YF, Ren F, Wu H. Upregulation of heme oxygenase-1 with hemin prevents D-galactosamine and lipopolysaccharide-induced acute hepatic injury in rats. *Toxicology* 2007; 237 (1–3):184–193.

- Cited Here |
- PubMed | CrossRef

27. Kawamura T, Wakabayashi N, Shigemura N, Huang CS, Masutani K, Tanaka Y, Noda K, Peng X, Takahashi T, Billiar TR, et al. Hydrogen gas reduces hyperoxic lung injury via the Nrf2 pathway in vivo. *Am J Physiol Lung Cell Mol Physiol* 2013; 304 10:L646–L656.

- Cited Here |
- PubMed | CrossRef

28. Chen H, Xie K, Han H, Li Y, Liu L, Yang T, Yu Y. Molecular hydrogen protects mice against polymicrobial sepsis by ameliorating endothelial dysfunction via an Nrf2/HO-1 signaling pathway. *Int Immunopharmacol* 2015; 28 1:643–654.

- Cited Here |
- PubMed | CrossRef

29. Nakai Y, Sato B, Ushiama S, Okada S, Abe K, Arai S. Hepatic oxidoreduction-related genes are upregulated by administration of hydrogen-saturated drinking water. *Biosci Biotechnol Biochem* 2011; 75 4:774–776.

- Cited Here |
- PubMed | CrossRef

30. Sakai T, Sato B, Hara K, Hara Y, Naritomi Y, Koyanagi S, Hara H, Nagao T, Ishibashi T. Consumption of water containing over 3.5 mg of dissolved hydrogen could improve vascular

endothelial function. *Vasc Health Risk Manag* 2014; 10:591–597.

- Cited Here |
- PubMed

31. Shimada S, Wakayama K, Fukai M, Shimamura T, Ishikawa T, Fukumori D, Shibata M, Yamashita K, Kimura T, Todo S, et al. Hydrogen gas ameliorates hepatic reperfusion injury after prolonged cold preservation in isolated perfused rat liver. *Artif Organs* 2016; 40 12:1128–1136.

- Cited Here |
- View Full Text | PubMed | CrossRef

32. Wiesel P, Patel AP, DiFonzo N, Marria PB, Sim CU, Pellacani A, Maemura K, LeBlanc BW, Marino K, Doerschuk CM, et al. Endotoxin-induced mortality is related to increased oxidative stress and end-organ dysfunction, not refractory hypotension, in heme oxygenase-1-deficient mice. *Circulation* 2000; 102 24:3015–3022.

- Cited Here |
- View Full Text | PubMed | CrossRef

33. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med* 2001; 29 7:1303–1310.

- Cited Here |
- View Full Text | PubMed | CrossRef

34. Kumar G, Kumar N, Taneja A, Kaleekal T, Tarima S, McGinley E, Jimenez E, Mohan A, Khan RA, Whittle J, et al. Nationwide trends of severe sepsis in the 21st century (2000–2007). *Chest* 2011; 140 5:1223–1231.

- Cited Here |
- View Full Text | PubMed | CrossRef

35. Buras JA, Holzmann B, Sitkovsky M. Animal models of sepsis: setting the stage. *Nat Rev Drug Discov* 2005; 4 10:854–865.

- Cited Here |
- PubMed | CrossRef

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**Keywords:**

Endotoxin; heme oxygenase-1; inflammation; molecular hydrogen; oxidative stress

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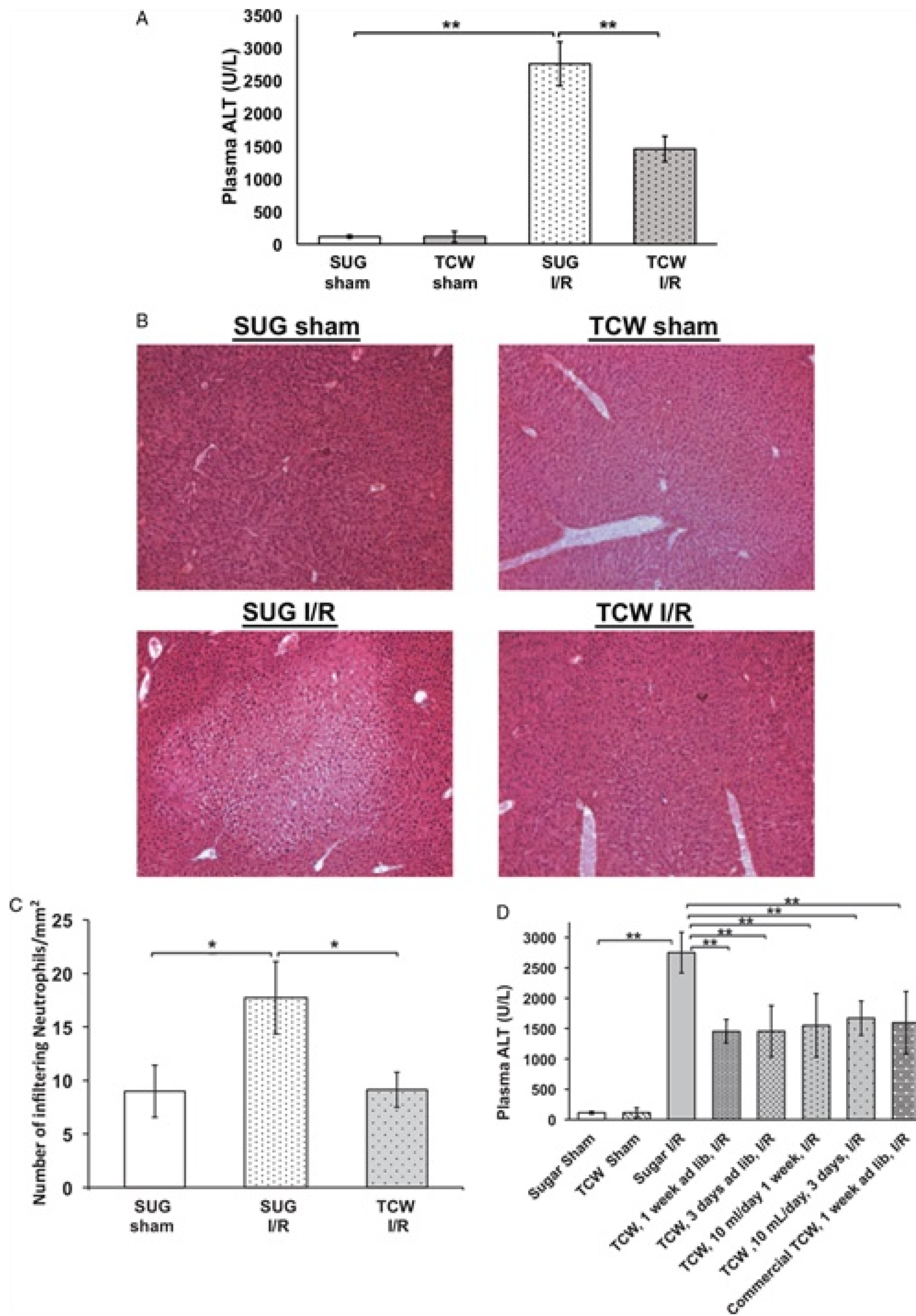


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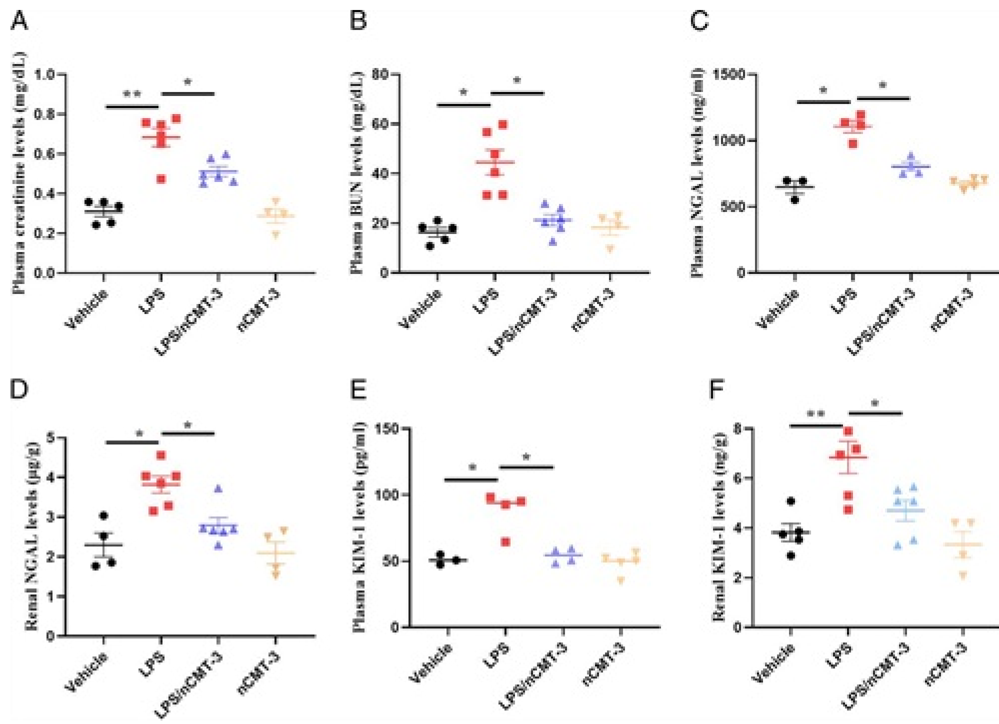
- 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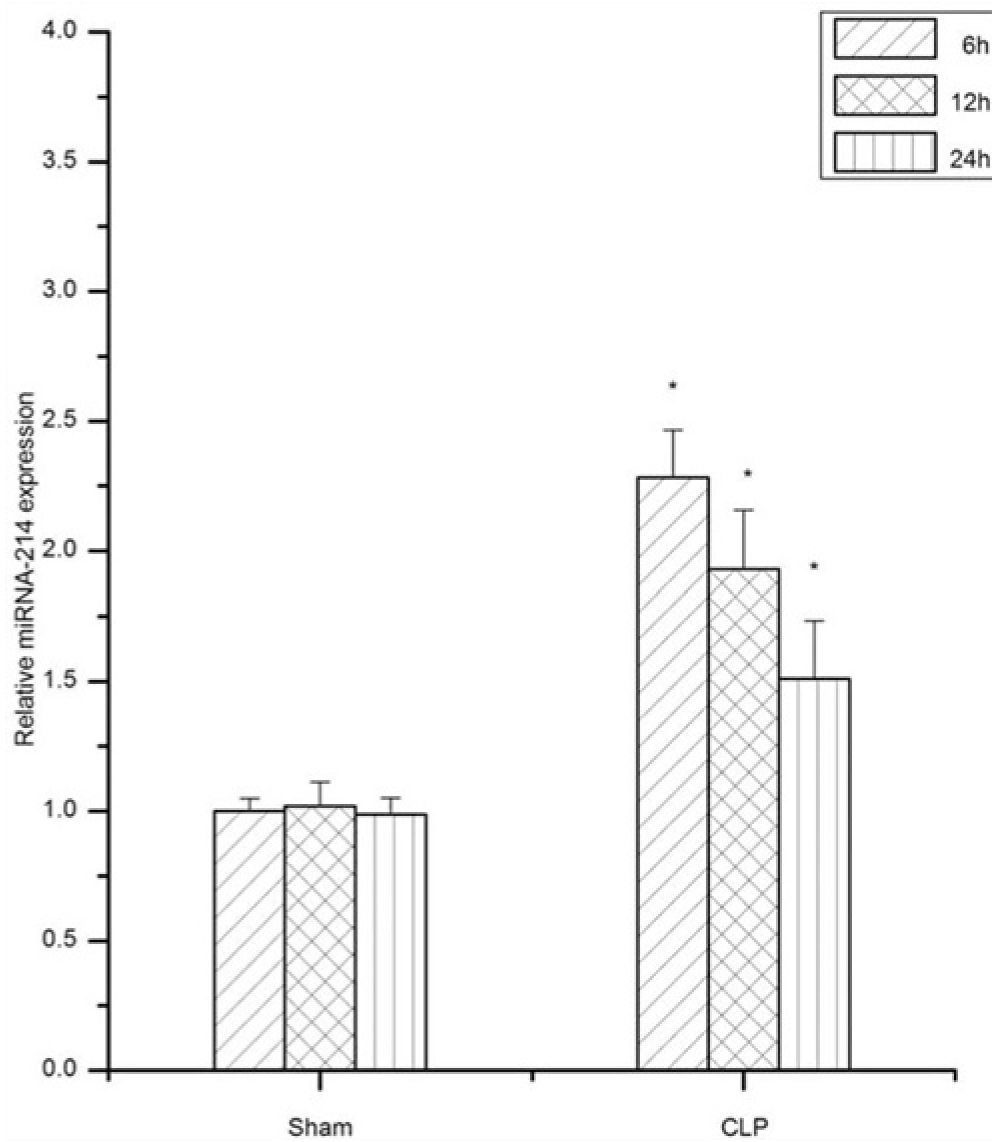
- 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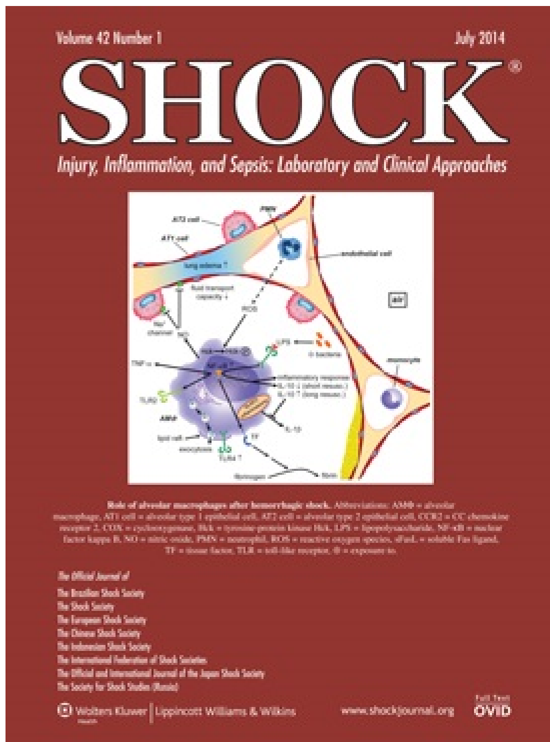
- 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- | Name | GenBank | Forward | Reverse |
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  | IL-6 | NM_031168 | CCGGAGAGGAGACTTCACAG | CAGAATTGCCATTGCACAAC |
  | KC | NM_008176 | GCTGGGATTCACCTCAAGAA | ACAGGTGCCATCAGAGCAGT |
  | MIP-2 | NM_009140 | CCCTGGTTCAGAAAATCATCCA | GCTCCTCCTTTCCAGGTCAGT |
  | COX-2 | NM_011198 | CTCAGCCAGGCAGCAAATC | ACATTCCCCACGGTTTTGAC |
  | iNOS | NM_010927 | GCAGGTCGAGGACTATTTCTTTCA | GAGCACGCTGAGTACCTCATTG |
  | β-actin | NM_007393 | CGTGAAAAGATGACCCAGATCA | TGGTACGACCAGAGGCATACAG |

  COX-2 indicates cyclooxygenase-2; IL, interleukin; iNOS, inducible nitric oxide synthase; KC, keratinocyte-derived chemokine; MIP-2, macrophage inflammatory protein 2.

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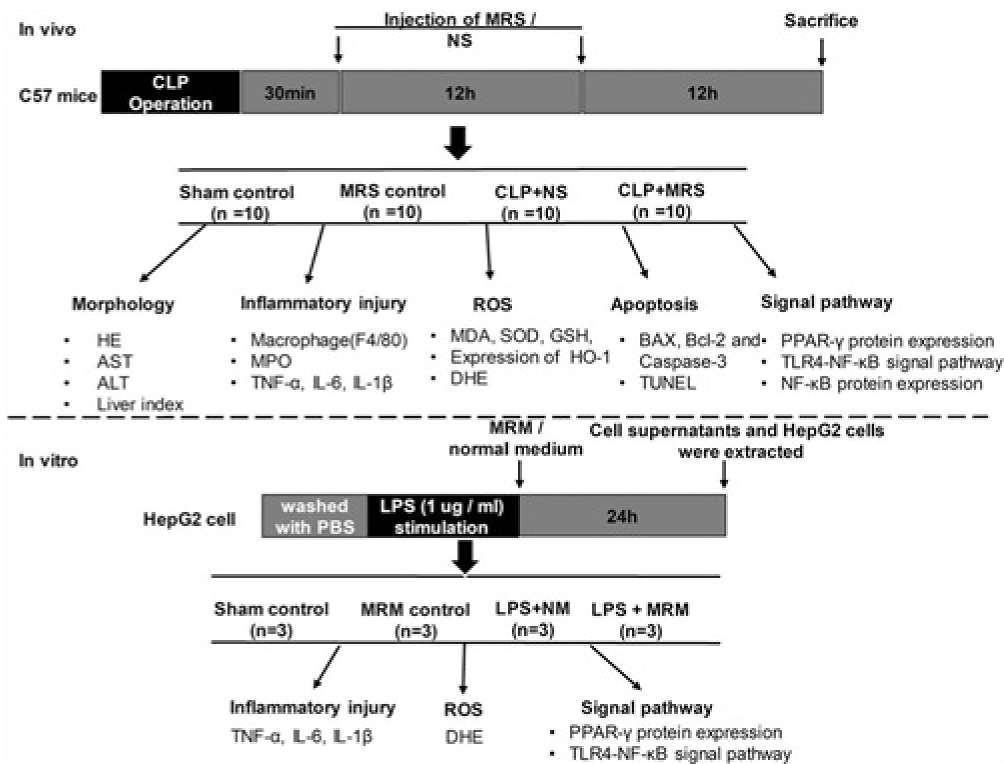
- 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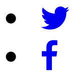
- 

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