

# Saturated Hydrogen Saline Attenuates Endotoxin-Induced Acute Liver Dysfunction in Rats

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## Summary

To determine the effect of saturated hydrogen saline on lipopolysaccharide (LPS)-induced acute liver dysfunction, rats were divided into control, LPS, and LPS plus saturated hydrogen saline (LPS+H<sub>2</sub>) groups. Treatment with saturated hydrogen saline prolonged the median survival time and reduced liver dysfunction. Moreover, saturated hydrogen saline significantly reduced pathological alterations in liver tissues, the number of ballooned hepatocytes, serum tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-6 levels, and myeloperoxidase (MPO) and malondialdehyde (MDA) levels in liver tissues ( $P < 0.05$ ). Cell apoptosis was detected in liver tissues after LPS treatment, and attenuated by saturated hydrogen saline treatment. Saturated hydrogen saline also decreased phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated Jun kinase (p-JNK), nuclear factor-kappa B (NF- $\kappa$ B), and second mitochondria-derived activator of caspase (Smac) levels, and increased p38 activation ( $P < 0.05$ ). Thus, saturated hydrogen saline may attenuate LPS-induced acute liver dysfunction in rats, possibly by reducing inflammation and cell apoptosis. Mitogen-activated protein kinase (MAPK), NF- $\kappa$ B, and Smac may contribute to saturated hydrogen saline-mediated liver protection.

## Key words

Saturated hydrogen saline • Acute liver dysfunction • LPS

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## Introduction

Liver injury can be induced by various pathogenic factors, including hepatitis viruses, ethanol, drugs, hepatotoxicants, and lipopolysaccharides (LPS). Among these factors, LPS, also known as endotoxins, exert multiple effects on the pathogenesis of progressive liver disease (Crispe 2009). For example, LPS activate effector cells, including monocytes/macrophages, neutrophils, and other non-immune cells (Wright *et al.* 1990), and trigger the release of pro-inflammatory cytokines, which contribute to the induction of the systemic inflammatory response syndrome and liver dysfunction (Jirillo *et al.* 2002, Su 2002). However, the pathological and molecular mechanisms underlying LPS-induced acute liver dysfunction remain poorly understood.

Emerging lines of evidence indicate that saturated hydrogen saline (H<sub>2</sub> saturated in saline), which is easily administrated and is safe for clinical application, can prevent galactosamine (GalN)/LPS-induced liver injury in a rodent model (Sun *et al.* 2009, 2011). Although it is known that saturated hydrogen saline prevents liver injury by decreasing the production of reactive oxygen species (ROS), and by blocking the activity of pro-apoptotic players, such as JNK and caspase-3 (Sun *et al.* 2011), the precise mechanism underlying saturated hydrogen saline-mediated liver protection remains unclear. Therefore, the goal of this study was to investigate the effects of saturated hydrogen saline on LPS-induced acute liver dysfunction in a rat model, and to explore the potential mechanisms involved in this process.

## Materials and Methods

### Reagents

Saturated hydrogen saline was provided by the Department of Diving Medicine, Faculty of Navy Medicine, Second Military Medical University, in Shanghai, China. Polyclonal mouse anti-nuclear factor kappa B (NF- $\kappa$ B) antibody was purchased from Neomarkers (Fremont, CA, USA). Monoclonal rabbit anti-second mitochondria-derived activator of caspase (Smac) antibody was obtained from Santa Cruz (Santa Cruz, CA, USA), and endotoxin (Coli 055:B5) was purchased from Sigma (St. Louis, MO, USA). Myeloperoxidase (MPO) and malondialdehyde (MDA) detection kits were purchased from the NanJing Jiancheng Bioengineering Institute (China). Enzyme-linked immunosorbent assay (ELISA) kits for TNF- $\alpha$  and IL-6 were obtained from Abcam (Cambridge, MA, USA). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit was provided by Roche (Nutley, NJ, USA).

### Animals and treatment

A total of 85 specific pathogen-free (SPF) level male Sprague-Dawley (SD) rats, with a body mass of 250-350 g, were provided by the Laboratory Animal Center at the Affiliated Shengjing Hospital of China Medical University in Shenyang, China. The rats were housed in a controlled environment at 22-25 °C with a 12/12 h light/dark cycle. Animals had free access to food and water. Animals were randomly divided into three groups: control (n=15), LPS (n=35), and LPS+H<sub>2</sub> (n=35). Animals in the LPS group were given a single caudal vein injection of 10 mg/kg LPS, and animals in the control group received the same dose of normal saline using the same method. The saturated hydrogen saline was stored under atmospheric pressure at 4 °C in an aluminum bag without dead volume. Hydrogen-rich saline (H<sub>2</sub>) was freshly prepared every week and stored at 4 °C to ensure a constant concentration of more than 0.6 mM. In the LPS+H<sub>2</sub> group, rats were given 8 ml/kg saturated hydrogen saline by caudal vein injection 20 min prior to LPS treatment, and every 1 h for a continuous 6 h after LPS administration. Twenty rats in the LPS group, and 20 rats in the LPS+H<sub>2</sub> group were maintained for 72 h after the final injection to determine animal survival, while the other rats were anesthetized with an intraperitoneal injection of 20 % urethane (w/v;

3.75 ml/kg body weight) 6 h after the final injection. Samples were collected from these rats for examination. All efforts were made to minimize animal suffering and to reduce the number of animals used. All animal procedures were approved by the Affiliated Shengjing Hospital of China Medical University.

### Determination of liver damage and cytokine concentrations

The degree of liver injury was evaluated by the histological activity index (HAI) score (Knodell *et al.* 1981). Blood samples were collected from the inferior vena cava of rats under anesthesia. After centrifuging at 3000 rpm at 4 °C, blood serum was collected and serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), total bilirubin (TBIL) and direct bilirubin (DBIL) were determined using an automatic analyzer (HITACHI-7600-120, Japan). The levels of MPO and MDA in liver tissues were evaluated using detection kits. The serum levels of tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-6 were measured using ELISA kits according to the manufacturer's instructions.

### Immunohistochemical analysis

The liver was carefully removed from animals 6 h after the final injection. Paraffin-embedded liver tissues were sectioned into 4  $\mu$ m slices, fixed in 4 % paraformaldehyde, deparaffinized, and rehydrated. Samples were incubated with anti-NF- $\kappa$ B or anti-Smac antibodies, followed by incubation with secondary antibodies. Some samples were then stained with hematoxylin and eosin (H&E), mounted, and evaluated under a microscope at a 400x magnification. At least 10 fields were randomly selected. The number of ballooned hepatocytes in each field was counted blindly by two pathologists.

### Transmission electron microscopy (TEM) analysis

TEM analysis was performed as previously described (Qian and Yang 2009). In brief, liver tissues were fixed in 2.5 % glutaraldehyde, and post-fixed in 1 % osmium tetroxide. After dehydration through a series of ethanol gradients, samples were embedded in Epon 812 epoxy resin, sectioned, and double-stained with uranyl acetate and lead citrate. Staining was evaluated using a JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan).

### Caspase 3 activity assay

The enzyme activity of caspase-3 was determined via the spectrophotometric method (Enari *et al.* 1998) using a caspase-3 colorimetric activity assay kit (Chemicon International, Temecula, CA, USA). An emission wavelength of 505 nm and an excitation wavelength of 400 nm were used.

### Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Cell apoptosis was evaluated using a TUNEL kit according to the manufacturer's instructions. TUNEL signals were visualized under a microscope (Olympus, Japan). At least 10 fields were randomly selected, and the apoptotic index (AI %) was calculated as the percentage of TUNEL-positive cells.

### Western blot analysis

Western blot analysis was conducted as previously described (Yang *et al.* 2007). Briefly, total protein was extracted from the liver tissues using whole cell lysis buffer. Protein samples were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, after which proteins were transferred to polyvinylidene fluoride membranes using wet turn membrane method. After blocking, membranes were probed with rabbit anti-rat primary antibody. Immune complexes were detected by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody and an ECL detection system.

### Statistical analysis

The median survival time of animals was estimated by the Kaplan-Meier method. Statistical analyses were performed using SPSS software version 13.0. Data were presented as mean  $\pm$  standard deviation

(SD). Student t-test was performed to compare differences between the two groups. Differences among three groups were determined by analysis of variance (ANOVA), followed by *post-hoc* Student-Newman-Keuls (SNK) pair-wise comparisons when required. P values less than 0.05 were considered statistically significant.

## Results

### Saturated hydrogen saline reduced LPS-induced liver dysfunction

The median survival time for animals in the LPS treatment group was 6 h (95 % confidence interval, 95 % CI, 4.314-5.988). Saturated hydrogen saline significantly prolonged the median survival time of rats in the LPS+H<sub>2</sub> treatment group (8 h, 95 % CI, 3.730-6.280,  $P < 0.05$ ). Within 72 h after LPS treatment, 4 animals died at 12 h, three died at 24 h, one died at 48 h, and the others survived. In the LPS+H<sub>2</sub> treatment group, 2 animals died at 12 h, one died at 24 h, and the others survived until 72 h after treatment. The minimum and maximum survival time was 3 h and 72 h, respectively. As shown in Table 1, LPS administration dramatically elevated the serum levels of TBIL, DBIL, ALT, AST, and LDH ( $P < 0.05$  for all compared to control), whereas LPS plus saturated hydrogen saline treatment remarkably reduced the serum levels of these parameters ( $P < 0.05$  for all compared to LPS treatment alone). This indicates that saturated hydrogen saline treatment may reduce liver injury induced by LPS. Moreover, saturated hydrogen saline greatly decreased the HAI score compared to LPS treatment alone ( $13.83 \pm 0.89$  vs.  $16.10 \pm 1.73$ ,  $P < 0.05$ ). Three components of the HAI score, such as periportal necrosis (PN), portal inflammation (PI), and intra-lobuli hepatitis necrosis (IN), were closely associated with liver inflammation, suggesting that saturated hydrogen saline may reduce liver injury by decreasing inflammation.

**Table 1.** Saturated hydrogen saline improved liver function after LPS injury.

	Control	LPS	LPS+H <sub>2</sub>
TBIL (umol/l)	0.55 $\pm$ 0.21	7.65 $\pm$ 0.88*	2.63 $\pm$ 0.62*#
DBIL (umol/l)	0.18 $\pm$ 0.07	6.13 $\pm$ 0.76*	2.43 $\pm$ 0.54*#
ALT (U/l)	43.84 $\pm$ 4.14	659.81 $\pm$ 82.80*	215.75 $\pm$ 68.47*#
AST (U/l)	143.54 $\pm$ 26.44	1665.65 $\pm$ 130.72*	583.56 $\pm$ 108.39*#
LDH (U/l)	360.21 $\pm$ 86.56	1556.75 $\pm$ 379.50*	922.19 $\pm$ 117.21*#

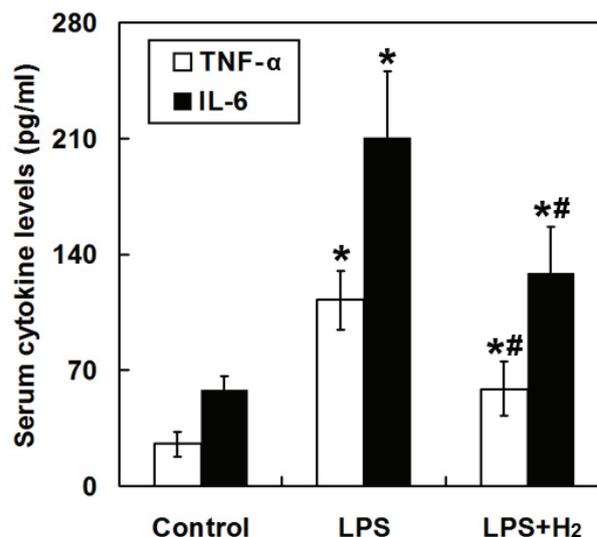
LPS, lipopolysaccharide; H<sub>2</sub>, saturated hydrogen saline; TBIL, total bilirubin; DBIL, direct bilirubin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase. \* $P < 0.05$  compared with control; # $P < 0.05$  compared with LPS.

*Pathological and ultrastructural analysis of saturated hydrogen saline-mediated liver protection in LPS-induced acute liver dysfunction*

As shown in [Supplementary Figure 1A](#) (on-line only), control rats exhibited a normal hepatic lobule structure. A central vein ran through the middle of the lobe and was surrounded by cords of hepatocytes that radiated out in all directions. No necrosis, degradation, or inflammatory cell infiltration were found in the liver of control rats. Liver damage with the appearance of abnormal liver structure, cellular swelling and vacuolation occurred after LPS treatment. Flake necrosis, hemorrhaging, hepatic sinus expansion bleeding, portal area gore, and inflammatory cell infiltration were detected in LPS-treated livers. However, saturated hydrogen saline administration significantly reduced pathological alterations in liver tissues, although slight hepatocyte swelling could still be observed. In addition, saturated hydrogen saline treatment greatly reduced the number of ballooned hepatocytes that were upregulated by LPS injection ( $P < 0.05$ ) ([Supplementary Fig. 1B](#), on-line only). TEM analysis further revealed that chromatin margination and widespread vacuoles appeared in hepatocytes after LPS treatment ([Supplementary Fig. 1C](#), on-line only), which was rarely found in the LPS+H<sub>2</sub> treatment group.

*Saturated hydrogen saline decreased cytokine production*

LPS treatment significantly promoted serum cytokine generation, including TNF- $\alpha$  and IL-6, compared to the control (Fig. 1). However, saturated hydrogen saline statistically reduced the serum levels of both TNF- $\alpha$  and IL-6 in animals that received LPS+H<sub>2</sub>

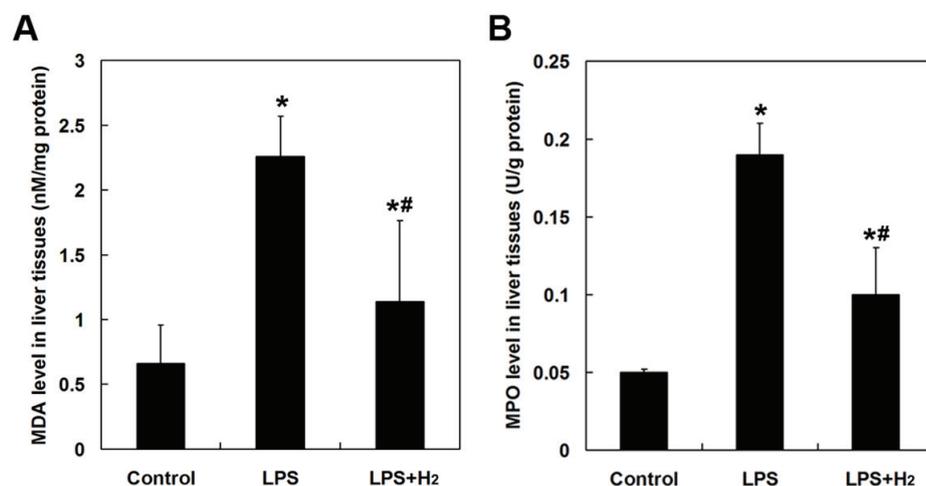


**Fig. 1.** Protective effects of saturated hydrogen saline on LPS-induced acute liver dysfunction by measuring cytokine production, including TNF- $\alpha$  and IL-6. Serum cytokine levels (pg/ml), including TNF- $\alpha$  and IL-6, were evaluated by ELISA. \* $P < 0.05$  compared with control; # $P < 0.05$  compared with LPS.

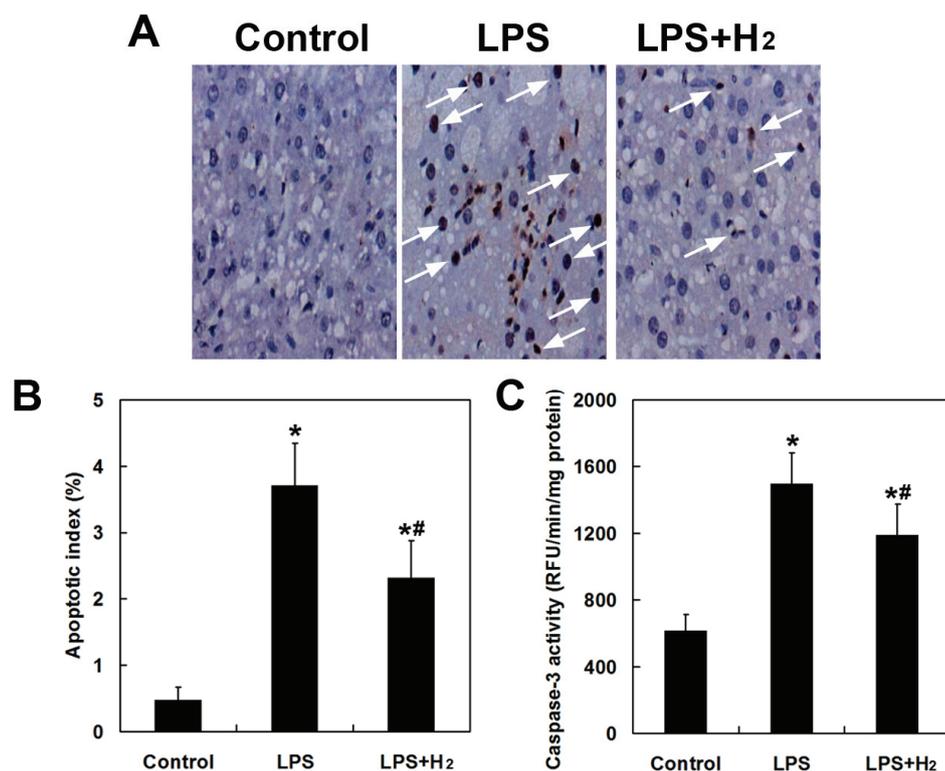
treatment ( $P < 0.05$  compared to LPS).

*Saturated hydrogen saline reduced oxidative stress in livers induced by LPS challenge*

Since MDA and MPO are biomarkers for oxidative stress, we examined MDA and MPO levels in liver tissues in rats after different treatments. As expected, LPS challenge significantly upregulated MDA and MPO expression (Fig. 2,  $P < 0.05$  compared to control), whereas saturated hydrogen saline reduced MDA and MPO levels compared to LPS treatment alone ( $P < 0.05$ ).



**Fig. 2.** Effects of saturated hydrogen saline on MDA and MPO levels in liver tissues. MDA (**A**) and MPO (**B**) levels in liver tissues were evaluated by biomedical analyses using detection kits. \* $P < 0.05$  compared with control; # $P < 0.05$  compared with LPS.



**Fig. 3.** Saturated hydrogen saline reduced apoptosis induction induced by LPS. **(A)** Cell apoptosis was analyzed by a TUNEL assay. Dark brown cells were recognized as TUNEL-positive cells (indicated by arrows). Magnification, 400x. **(B)** The apoptotic index (%) was calculated as described in the Materials and Methods. **(C)** Caspase-3 activity in liver tissues was determined. \* $P < 0.05$  compared with control; # $P < 0.05$  compared with LPS.

#### Saturated hydrogen saline reduced apoptosis induction induced by LPS

LPS induced an increased number of TUNEL-positive cells in liver sections (Fig. 3A,B) ( $P < 0.05$  compared to control). However, saturated hydrogen saline treatment significantly decreased LPS-induced cell apoptosis ( $P < 0.05$  compared to LPS). Moreover, caspase-3 activity in liver tissues was also upregulated upon LPS treatment, and dramatically downregulated after LPS+H<sub>2</sub> treatment (Fig. 3C) ( $P < 0.05$ ), indicating that saturated hydrogen saline may reduce apoptosis induction evoked by LPS.

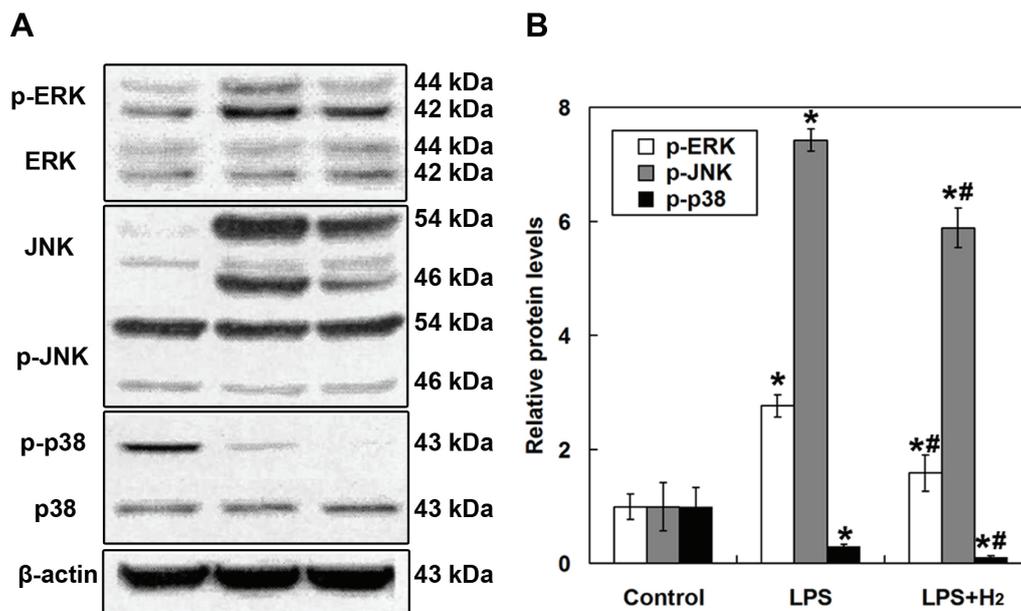
#### Signaling pathways involved in the protective effects of saturated hydrogen saline

To illustrate the mechanism of saturated hydrogen saline-induced liver protection, several molecular signaling pathways were investigated. We first determined the involvement of mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinase (ERK), Jun kinase (JNK), and p38, in this process. As revealed in Figure 4, LPS administration remarkably upregulated the expression of phosphorylated ERK and JNK (p-ERK and p-JNK), but downregulated p-p38 levels ( $P < 0.05$  compared to control). Notably, saturated

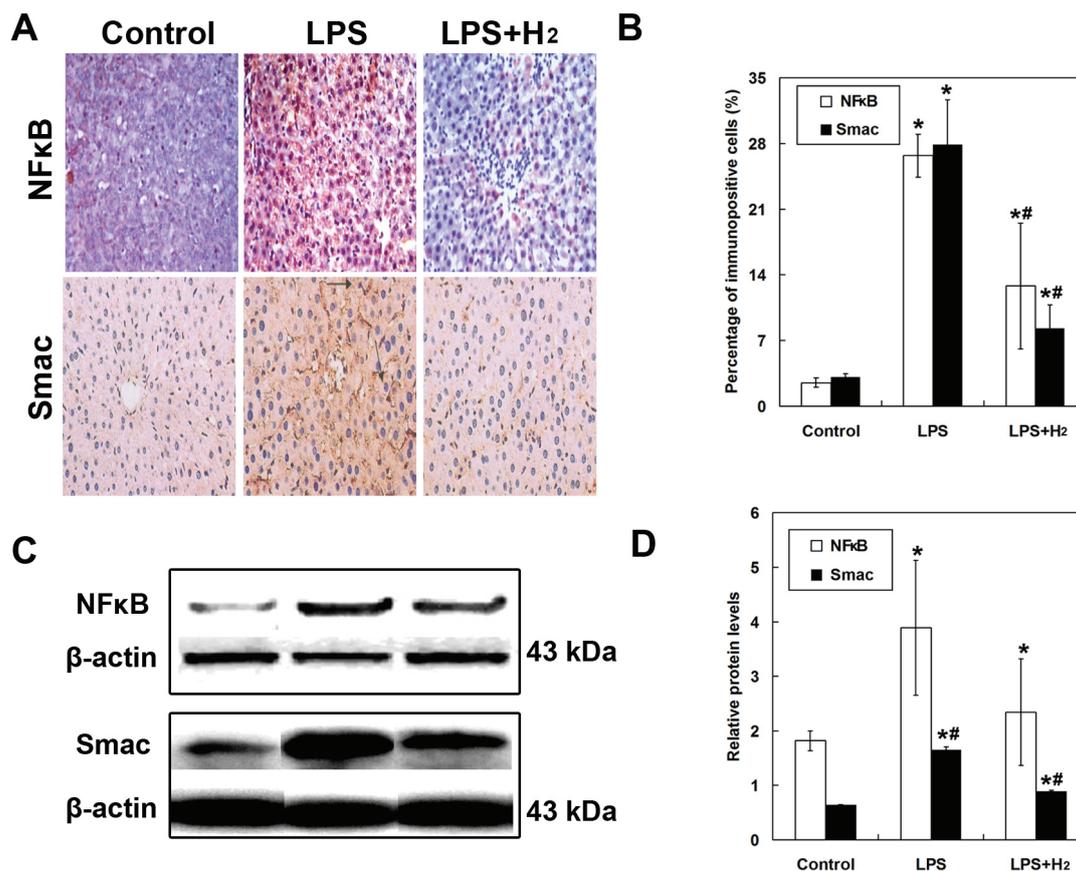
hydrogen saline treatment efficiently decreased the activation of ERK and JNK, while promoting the activation of p38 ( $P < 0.05$  compared to LPS), demonstrating that saturated hydrogen saline may reduce LPS-induced liver damage *via* regulation of MAPK signaling pathways.

#### Involvement of NF- $\kappa$ B and Smac in saturated hydrogen saline-induced liver protection

Considering NF- $\kappa$ B and Smac both play critical roles in the activation and progression of cell apoptosis, we next examined the involvement of these molecules in saturated hydrogen saline-induced liver protection. The basal expression of NF- $\kappa$ B and Smac were relatively low in normal liver tissues. As evident in Figure 5A and 5B, there was enhanced nuclear expression of NF- $\kappa$ B and cytoplasmic expression of Smac in the liver tissues after LPS treatment. Saturated hydrogen saline treatment clearly decreased the expression of NF- $\kappa$ B and Smac compared to LPS administration alone. Western blot analysis further confirmed these findings (Fig. 5C,D). These observations suggest that NF- $\kappa$ B and Smac are involved in saturated hydrogen saline-induced liver protection.



**Fig. 4.** Involvement of MAPK signaling pathways in saturated hydrogen saline-induced liver protection. **(A)** The expression of ERK, p-ERK, JNK, p-JNK, p38, p-p38 in liver tissues after different treatments was determined by Western blotting.  $\beta$ -actin was used as an internal control. **(B)** The relative protein expression (target protein/ $\beta$ -actin) was quantified from three independent experiments. \* $P < 0.05$  compared with control; # $P < 0.05$  compared with LPS.



**Fig. 5.** Involvement of NF- $\kappa$ B and Smac signaling pathways in saturated hydrogen saline-induced liver protection. **(A)** Immunohistochemical analysis of NF- $\kappa$ B and Smac expression in liver sections. Magnification, 400x. **(B)** Quantitative analysis of immunostaining. **(C)** The expression of NF $\kappa$ B and Smac in liver tissues after different treatments was determined by Western blotting.  $\beta$ -actin was used as an internal control. **(D)** The relative protein expression (target protein/ $\beta$ -actin) was quantified from three independent experiments. \* $P < 0.05$  compared with control; # $P < 0.05$  compared with LPS.

## Discussion

The gram-negative bacteria-derived endotoxin, LPS, is a potent inducer of lipid peroxidation (LPO), which is associated with the development of endotoxemia (Morrison and Ulevitch 1978, Yoshikawa *et al.* 1994). LPS has been demonstrated to induce acute liver dysfunction through generation of pro-inflammatory cytokines (Jirillo *et al.* 2002, Su 2002), while the mechanism underlying LPS-challenged liver dysfunction and hepatocyte damage remains unclear. Molecular hydrogen, which reacts with the hydroxyl radical, has been considered a novel antioxidant. Recent studies have reported that saturated hydrogen saline prevents liver injury by decreasing ROS generation and inhibiting apoptosis (Sun *et al.* 2011). In addition, saturated hydrogen saline attenuated bile duct ligation (BDL)-induced liver damage, possibly by reducing inflammation and oxidative stress (Liu *et al.* 2010). Therefore, we speculated that saturated hydrogen saline may have the ability to reduce LPS-induced acute liver dysfunction. We tested this hypothesis using a well-characterized experimental mouse model of LPS-induced shock.

Our results demonstrated that saturated hydrogen saline treatment greatly reduced acute LPS-induced liver dysfunction in rats. Compared to the LPS treatment group, serum levels of the liver function biomarkers, including ALT, AST, TBIL, DBIL and LDH, dramatically decreased after administration of saturated hydrogen saline. This was further confirmed by histological examination, as saturated hydrogen saline attenuated the structural damage, as well as hepatocyte degeneration induced by LPS.

It has been widely accepted that LPS upregulates the production of the pro-inflammatory cytokines IL-6 and TNF- $\alpha$ , which play important pathogenic roles in the regulation of inflammation (Su 2002). We found that saturated hydrogen saline significantly decreased the production of IL-6 and TNF- $\alpha$ , suggesting that saturated hydrogen saline may reduce liver injury by inducing anti-inflammatory responses. In addition, the levels of MDA, an end product of lipid peroxidation, and MPO, an important neutrophil enzyme that can generate aggressive oxidants in LPS-challenged liver, were dramatically elevated upon LPS treatment, which is consistent with previous a report (Zhang *et al.* 2010). Notably, saturated hydrogen saline efficiently reduced MDA and MPO generation, indicating that saturated hydrogen saline can reduce oxidative stress induced by LPS. Activation of

NF- $\kappa$ B occurs in the presence of pro-inflammatory stimuli, and results in the increased expression of pro-inflammatory cytokines and chemokines during liver injury (Nanji *et al.* 1999). In accordance with this report, we found that LPS greatly upregulated NF- $\kappa$ B activity, whereas saturated hydrogen saline decreased NF- $\kappa$ B expression compared to LPS treatment alone ( $P < 0.05$ ).

Since widespread apoptosis of liver cells is mechanistically important for the pathogenesis of end-stage liver disease (Malhi and Gores 2008), we investigated the effects of saturated hydrogen saline on LPS-induced hepatocyte apoptosis. We found that saturated hydrogen saline treatment significantly decreased LPS-triggered cell apoptosis, as indicated by a decreased number of TUNEL-positive cells and reduced caspase-3 activity. Since it has been demonstrated that Smac promotes the proteolytic activation of pro-caspase-3 and upregulates the enzymatic activity of mature caspase-3, which contributes to the progression of apoptosis (Chai *et al.* 2000), we examined the expression levels of Smac in liver tissues. Saturated hydrogen saline treatment notably decreased Smac expression in liver tissues compared to LPS administration, suggesting that saturated hydrogen saline may reduce cell apoptosis *via* downregulation of Smac.

The MAPK cascades are involved in a wide variety of cellular processes. MAPK signaling can be activated through oxidative stress and increased apoptosis receptor expression, and contribute to hepatocellular injury (Schattenberg *et al.* 2006). MAPK pathways are mediated by ERK, JNK, and p38 protein kinases (Johnson and Lapadat 2002), all of which are activated by pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-6 (Liao *et al.* 2001, Lejeune *et al.* 2002, Thirunavukkarasu *et al.* 2006). We found that saturated hydrogen saline treatment efficiently decreased the activation of ERK and JNK, while promoting the activation of p38 ( $P < 0.05$  compared with LPS), suggesting the involvement of MAPK cascades in saturated hydrogen saline-mediated liver protection. Nevertheless, the potential association between the MAPK signaling pathway and oxidative stress or inflammatory responses needs to be further clarified.

Collectively, our present study demonstrates that saturated hydrogen saline can attenuate LPS-induced acute liver dysfunction in rats, possibly by suppressing inflammation and apoptosis. Since some bacterium in the gut can produce H<sub>2</sub>, the induction and control of endogenous H<sub>2</sub> may serve as a novel strategy for the

treatment of oxidative injury. Several molecular signaling pathways, including ERK, JNK and p38, NF- $\kappa$ B, and Smac may contribute to saturated hydrogen saline-mediated liver protection. These findings provide valuable insights for understanding the mechanism of a wide range of LPS-induced liver disease processes in humans, and may help to develop therapeutic approaches for the treatment of these diseases. Future studies are needed to explore the potential correlation between these

pathways and inflammatory responses.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

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