For Peer Review

Hydrogen-Rich Saline Protects against Liver Injury in Rats with Obstructive Jaundice

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Liver International</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>LIVint-09-00708.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Original Articles</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>05-Mar-2010</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Liu, Qu; Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Department of Special Treatment Shen, Wei-feng; Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Department of Special Treatment Sun, Han-Yong; Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Department of Hepatic Surgery Fan, Dan-feng; Faculty of Naval Medicine, Second Military Medical University, Department of Diving Medicine Nakao, Atsunori; University of Pittsburgh Medical Center, Department of Surgery and Thomas E Starzl Transplantation Institute Cai, Jian-mei; Faculty of Naval Medicine, Second Military Medical University, Department of Diving Medicine Yan, Ge; Changhai Hospital, Second Military Medical University, Department of General Surgery Zhou, Weiping; Second Military Medical University, The 3rd Department of Hepatic Surgery, Eastern Hepatobiliary Surgery Hospital Shen, Rong-xi; Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Department of Special Treatment Yang, Jiaemei; Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Department of Special Treatment Sun, Xue-jun; Faculty of Naval Medicine, Second Military Medical University, Department of Diving Medicine</td>
</tr>
<tr>
<td>Keywords:</td>
<td>Hydrogen-rich saline, inflammation, oxidative stress, liver injury, antioxidant, obstructive jaundice</td>
</tr>
</tbody>
</table>
Hydrogen-Rich Saline Protects against Liver Injury in Rats with Obstructive Jaundice

Qu Liu1*, Wei-Feng Shen1*, Han-Yong Sun2*, Dan-Feng Fan3, Atsunori Nakao4, Jian-Mei Cai5, Ge Yan5, Wei-Ping Zhou2, Rong-Xi Shen1, Jia-Mei Yang†, Xue-Jun Sun3†

1 Department of Special Treatment, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China.
2 Department of Hepatic Surgery III, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China.
3 Department of Diving Medicine, Faculty of Naval Medicine, Second Military Medical University, Shanghai 200433, China.
4 Department of Surgery and Thomas E Starzl Transplantation Institute, University of Pittsburgh Medical Center, Pittsburgh, PA.
5 Department of General Surg, Changhai Hospital, Second Military Medical University, Shanghai, 200433, China.

* These authors contributed equally to this work.

† Corresponding authors:
Jia-Mei Yang, MD.
Department of Special Treatment, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China.
Tel: (86-21) 8187-5598
Fax: (86-21) 6556-8652
E-mail: docjmy@yahoo.com.cn

Xue-jun Sun, MD, PhD.
Department of Diving Medicine, Faculty of Naval Medicine, Second Military Medical University, Shanghai 200433, China.
Tel: (86-21) 8187-1141
Fax: (86-21) 6549-2382
E-mail: sunxjk@hotmail.com
**Abstract: Background** Hydrogen selectively reduces levels of hydroxyl radicals and alleviates acute oxidative stress in many models. Hydrogen-rich saline provides a high concentration of hydrogen that can be easily and safely applied. **Aims** In this study, we investigated the effects of hydrogen-rich saline on the prevention of liver injury induced by obstructive jaundice in rats. **Methods** Male Sprague-Dawley rats (n = 56) were divided randomly into four experimental groups: sham operated, bile duct ligation (BDL) plus saline treatment (5 ml/kg, i.p.), BDL plus low-dose hydrogen-rich saline treatment (5 ml/kg, i.p.), and BDL plus high dose hydrogen-rich saline treatment (10 ml/kg, i.p.). **Results** The liver damage was evaluated microscopically 10 days after BDL. Serum ALT and AST levels, tissue MDA content, MPO activity, TNF-α, IL-1β, IL-6, and HMGB1 levels were all increased significantly by BDL. Hydrogen-rich saline reduced levels of these markers and relieved morphological liver injury. Additionally, hydrogen-rich saline markedly increased the activities of antioxidant enzymes SOD and CAT and downregulated ERK1/2 activation. **Conclusions** Hydrogen-rich saline attenuates BDL-induced liver damage, possibly by reduction of inflammation and oxidative stress and inhibition of the ERK1/2 pathway.

**Keywords:** Hydrogen-rich saline, inflammation, oxidative stress, liver injury, antioxidant, obstructive jaundice.

**Abbreviations:** OJ, obstructive jaundice; ROS, reactive oxygen species; H2, hydrogen; HS, hydrogen-rich saline; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; MDA, malondialdehyde; MPO, myeloperoxidase; SOD, superoxide dismutase; CAT, catalase;
BDL, bile duct ligation; TBil, total bilirubin; DBil, direct bilirubin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HE, hematoxylin and eosin; LAL, limulus amebocyte lysate; TBA, thiobarbituric acid; HMGB1, high-mobility group box 1; ERK, extracellular signal-regulated protein kinase.
Introduction

Obstructive jaundice (OJ) is a frequently observed condition caused by occlusion of the common bile duct or its tributaries. Surgical, endoscopic, and interventional radiographic decompression are the principal treatments of biliary obstruction, but decompression alone may not be sufficient to prevent the development of life threatening complications such as endotoxemia, systemic inflammatory response, liver injury, and multiple organ dysfunction that carry a high risk for mortality [1, 2]. The mechanisms responsible for the pathogenesis of cholestatic liver injury from acute biliary obstruction remain largely unknown, although intrahepatic accumulation of reactive oxygen species (ROS) is thought to be an important cause. Experimental and clinical studies have demonstrated the pivotal role of oxidative stress associated with overproduction of ROS, which may cause lipid peroxidation and disturb the integrity of cellular membranes in the promotion of hepatic injury in obstructive jaundice [3-5]. In addition, inflammation is known to contribute to the development of cholestatic liver injury in humans. Increased production of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) has also been implicated [6-8]. However, effective pharmacological approaches to protect liver function have not been introduced into practice and new treatments for obstructive jaundices are needed.

Molecular hydrogen (H₂) possesses antioxidative effects through selective reduction of the levels of hydroxyl radical [9]. Previous studies have demonstrated that H₂ in the form of gas or dissolved in water can suppress tissue injuries in brain, liver, heart and intestine caused by oxidative stress following ischemia-reperfusion [10-14]. Hydrogen also suppresses the inflammation induced in tissue-destructive diseases such as colitis, hepatitis, and
transplantation-induced intestinal graft injury [15-17].

Hydrogen would be more selective than other anti-oxidative drugs if it could be applied in clinical practice. However, in clinical application, inhalation of H\(_2\) gas is not convenient and is dangerous because of its flammable and explosive nature. On the other hand, hydrogen-rich saline (H\(_2\) gas saturated physiological saline; HS) can be easily and safely applied. In this study, we used peritoneal injection of HS into animals to avoid losing hydrogen into the environment. In addition, this also allowed accurate dosing and application of a high concentration of hydrogen. We found that HS treatment ameliorated liver injury by reducing oxidative stress and the inflammatory response in rats with obstructive jaundice.
Materials and methods

Animals

Adult male Sprague–Dawley rats, weighing 220–250 g, were provided by the Experimental Animal Center of the Second Military Medical University. Rats were fed with standard rodent chow and tap water ad libitum under a natural day/night cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Second Military Medical University (Shanghai, China).

Drugs and materials

Hydrogen-rich saline was prepared as described previously [12]. Briefly, hydrogen was dissolved in physiological saline for 6 h under high pressure (0.4 MPa) to a supersaturated level using a hydrogen-rich water producing apparatus constructed in our department. The saturated hydrogen saline was stored under atmospheric pressure at 4 ºC in an aluminum bag with no dead volume and was sterilized by gamma radiation. The hydrogen level in the saline was measured by gas chromatography using the method described by Ohsawa et al. [9]. The average saline hydrogen level was 0.86 mmol/L. HS was freshly prepared every week to ensure that a concentration of more than 0.6 mmol/L was maintained.

Malondialdehyde (MDA) and myeloperoxidase (MPO) assay reagents were purchased from the Nanjing Jiangcheng Bioengineering Institute (Nanjing, China). TNF-α, IL-1β, and IL-6 Enzyme-Linked Immunosorbent Assay (ELISA) kits were purchased from Invitrogen (Carlsbad, CA). Superoxide dismutase (SOD) and catalase (CAT) assay kits were purchased from Cayman Chemical Company (Ann Arbor, MI). The limulus amebocyte lysate (LAL) assay was purchased
from Biowhittaker Inc (Walkersville, MD). The naphthol ASD-chloroacetate esterase kit was purchased from Sigma-Aldrich (St Louis, MO). The BCA protein assay kit was supplied by Pierce Biotechnology (Rockford, IL). Mouse polyclonal anti-phosphorylated-extracellular signal-regulated protein kinase (pERK) 1/2, ERK1/2, and monoclonal anti-GAPDH antibodies were products of Cell Signaling Technology, Inc. (Beverly, MA). Secondary goat anti-mouse and rabbit antibodies were obtained from Calbiochem (La Jolla, CA). The Envision TM detection kit was purchased from Gene Tech, Inc. (Shanghai, China).

Experimental protocol

Rats were randomly divided into four groups each containing 14 animals. Group 1 animals (Sham plus saline) underwent a sham operation and were treated with normal saline (NS). Group 2 animals (BDL+ HS 5ml/kg) had common bile duct ligation (BDL) and were treated with NS. Animals in group 3 (BDL+HS 5ml/kg) and group 4 (BDL+HS 10ml/kg) had BDL and were treated with 5 ml/kg HS or 10 ml/kg HS, respectively.

Rats were fasted for 12 h with water ad libitum before the operation. Each rat was weighed and anesthetized with pentobarbital (50 mg/kg) intraperitoneally. Following a midline incision, the common bile duct was exposed and a double-ligature with 5-0 silk suture was performed and the bile duct was sectioned between the ligatures. In the sham-operated animals, the common bile duct was freed from surrounding soft tissue without ligation. A two-layer running suture was used for abdominal closure with 4-0 dixon and 2-0 nylon. Physiological saline (5 ml/kg) or HS (5 ml/kg and 10 ml/kg) was administered intraperitoneally daily at 10:00 AM, beginning 1 day after the ligature and for 10 days thereafter. The animals were sacrificed 10 days after BDL.
Sample collection

The animals were anesthetized and a second laparotomy was performed. After blood samples were drawn from the inferior vena cava, the liver was carefully dissected from its attachment and totally excised. The blood samples were kept at 4°C for biochemical analyses of total bilirubin (TBil), direct bilirubin (DBil), alanine aminotransferase (ALT), aspartate aminotransferase (AST), endotoxin, and HMGB1 levels. The left lobe of the liver was excised and flushed with physiological saline and then cut into three pieces, of which two pieces were immediately frozen in liquid nitrogen and stored at -80°C for the measurement of MDA, MPO and the protein levels of TNF-α, IL-1β, IL-6, and HMGB1, and the third piece was fixed in 40 g/L paraformaldehyde for histological analyses.

Histopathologic observations and immunohistological staining

To investigate whether HS treatment protects the liver against cholestatic injury, all paraffin-embedded liver tissues were stained with hematoxylin and eosin (H&E). Ductal proliferation was scored by an experienced hepatopathologist in a blinded fashion according to the method described by Alvaro et al. [18]. Neutrophils that accumulated in the liver were stained using the naphthol AS-D chloroacetate esterase technique for chloroacetate esterase activity. Neutrophils, identified by positive staining and morphology, were counted in 20 randomly selected fields at microscope magnification 400×. All cell counts were performed in a blinded fashion. The data were expressed as the number of polymorphonuclear neutrophils per high power field (PMNs/HPF). Immunohistochemistry for pERK1/2 (1:200 diluted) was performed on paraffin-embedded liver sections according to manufacturer’s protocol (Gene Tech).
**Blood biochemistry**

To biochemically verify the results of the histological examination, we measured serum ALT, AST, TB, and DB levels in each experimental group. After clotting, a blood sample was centrifuged at 3000 rpm for 5 min at 4°C. The top, clear layer was centrifuged again under the same conditions to prepare serum. ALT, AST, and bilirubin activities were measured using an autoanalyzer (HITACHI 7600-020, Japan).

**Determination of the levels of plasma endotoxin**

Endotoxin concentrations were measured by the chromogenic LAL assay, as previously described [19]. Each sample was measured in duplicate and the results are expressed as the mean of the two tests.

**Determination of MDA, MPO, SOD, and CAT in liver tissue**

Hepatic MDA levels were assessed spectrophotometrically according to the procedure described by Ohsawa et al. [9]. The MPO activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance occurring in the redox reaction of \( \text{H}_2\text{O}_2 \) (460 nm, 37°C). Values are expressed as MPO U/g wet weight. SOD activity was assayed by detecting superoxide radicals generated by xanthine oxidase and hypoxanthine based on the manufacturer’s protocol (Cayman Chemical Company). The reaction was monitored at 450 nm and one unit of SOD activity was defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical. The CAT activity was assayed by measuring the reduction of hydrogen peroxide at 540 nm.
and one unit was defined as the amount of enzyme that would cause the formation of 1.0 nmol of formaldehyde per minute at 25°C. All assays were conducted in duplicate. Protein content in the sample was determined using a BCA protein assay kit and the concentrations are expressed as pg/ml.

**Determination of TNF-α, IL-1β, and IL-6 protein levels in liver tissue**

Liver tissues were collected, weighed, and washed in NS and then homogenized immediately in 10 volumes of NS at 4°C. After centrifugation, supernatants were collected and stored at -80°C. Levels of TNF-α, IL-1β, and IL-6 were measured with a commercial ELISA kit (Biosource, Camarillo, CA) according to the instructions of the manufacturer. The absorbance was read on a microplate reader (Denley Dragon, Wellscan MK 3, Thermo, Finland) and the concentrations were calculated based on a standard curve.

**Detection of high-mobility group box 1 (HMGB1)**

The levels of HMGB1 were determined in serum and tissue homogenates using an ELISA kit from Abcom (Cambridge, MA). All standards and samples were run in duplicate.

**Western blots**

Liver tissues stored at -80°C were homogenized on ice in five volumes of the lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1% NP-40, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1mM EDTA, 1 mM EGTA, 1 μg/ml leupeptin, and 1 mM PMSF. The lysate was centrifuged at 15,000 g for 10 minutes at 4°C and the supernatant was used for western blot analysis of
phosphorylated and total ERK1/2 (1:1000) according to the manufacturer’s recommended method (Cell Signaling Technology).

Statistical analysis

All data are expressed as means ± SD. The differences among experimental groups were detected by one-way analysis of variance. The survival data was analyzed by log-rank test. The difference of morbidity and amount of ascites was detected by Chi-square test. Between groups, variance was determined using the LSD-t test. A P value of less than 0.05 was considered statistically significant.
Results

Body weight

Bile duct ligation (BDL) animals treated with NS gradually lost body weight after BDL. HS treatment significantly reduced this weight loss. There was no disparity in body weight between the four groups in the first 6 days after BDL. After day 7, the BDL group had significantly lower body weight compared to sham-treated animals. In contrast, rats that received HS injection after BDL weighed more than BDL rats treated with regular saline (Fig. 1A, *\( P < 0.05 \), **\( P < 0.01 \)).

Animal survival

BDL results in severe liver damage and we observed a 57.1% survival rate at 10 days after BDL in control BDL group treated with normal saline (NS). The survival rate after BDL in both experimental groups treated with HS (5 ml/kg and 10 mg/kg) was significantly higher; 85.7% of animals survived more than 10 days, suggesting that HS increased the resistance against BDL-induced liver injury (Fig. 1B, \( P < 0.05 \)). There was no statistical difference in survival between groups treated with different doses of HS (Kaplan Meyer and Log-rank test).

Amount of ascites

At day 10, ascites were found in 50% of animals in BDL treated with NS (34.75 ± 8.40 ml) but in only two animals treated with HS (5 ml/kg dose, 4.8 ml; 10ml/kg dose, 9 ml). No ascites were observed in the sham-operated group. However, differences among these three groups were not significant (n=8–12; Chi-square test; \( P > 0.05 \)).

Histopathological examination by H&E staining
H&E stained liver sections of sham-operated rats had a normal hepatic architecture (Fig. 2A and B). After 10 days, the liver tissues of BDL rats treated with saline were severely damaged, with severe edema and extensive inflammatory cell infiltration. Histopathological examination also showed bile epithelial cell proliferation in BDL rats was significantly higher than in sham-operated rats (Fig. 2C and D). Cholestatic liver damage was significantly reduced by HS treatment. Moderate hepatic cell edema, necrosis, and neutrophil cell infiltration were seen in HS treated groups (Fig. 2E, F, G and H) and the increased ductular proliferation observed after bile duct ligation was significantly diminished after hydrogen treatment (Fig. 3A; \( P < 0.01 \)). There was no significant difference in ductular proliferation between HS dose groups (\( P > 0.05 \)). We investigated the accumulation of neutrophils in the liver using a neutrophil esterase stain. As shown in Fig. 3B, the livers of BDL rats contained significantly more neutrophils than those from untreated rats (\( P < 0.01 \)). Moreover, quantification of the inflammatory infiltrate in liver tissues showed that with HS treatment decreased the number of neutrophils per HPF significantly from 26.0 ± 5.8 PMNs/HPF to 12.4 ± 3.5 and 16.1 ± 4.2 PMNs/HPF (Fig. 3B; \( P < 0.01 \)).

Blood biochemistry

Ligation of the common bile duct significantly elevated serum total bilirubin levels by more than 258 fold compared to sham-treated animals, suggesting that significant cholestasis was induced in this model (Fig. 4A). Bilirubin levels in rats treated with HS after BDL were not different from those in NS-treated animals, indicating that the degree of cholestasis was similar in all experimental groups (Fig. 4A, B). BDL caused substantial hepatocellular injury as indicated by a more than 3-fold increase in liver enzymes; however, HS treatment significantly reduced
BDL-induced liver damage (Fig. 4C, D; $P < 0.01$ vs. BDL + NS group). Administration of 5 ml/kg of HS considerably suppressed the release of ALT and AST from the liver by 60.03% and 57.81%, respectively, compared with NS-treated rats. The treatment with 10 ml/kg HS decreased cholestasis-induced ALT and AST levels by 66.89% and 64.88%, respectively (Fig. 4C, D; $P < 0.01$ vs. BDL + NS group).

**Plasma endotoxin in BDL rats**

Endotoxin concentrations in blood collected from portal vein were measured. Jaundiced animals had significantly higher endotoxin concentrations (1.35±0.22 EU/ml) than sham-operated animals (0.26±0.06 EU/ml) ($P < 0.01$). When ligation of common bile duct was followed by HS treatment, plasma endotoxin concentrations were significantly reduced compared with NS group (5 ml/kg HS, 0.71±0.14 EU/ml; 10 mg/kg HS, 0.74±0.16 EU/ml; $P < 0.01$).

**MDA, MPO, SOD, and CAT in liver tissues**

The potential anti-oxidative properties of HS were determined by the measurement of tissue MDA levels. MDA, a marker of lipid peroxidation, was significantly increased 10 days after BDL in liver tissues when compared with sham-operated rats. In contrast, HS treatment dramatically suppressed the production of MDA in rats with OJ (Fig. 5A; $P < 0.01$). MPO activity, an indicator of neutrophil infiltration, is shown for each group of animals in Figure 5B. Compared with the sham-operated group, MPO activity in the BDL group was significantly increased ($P < 0.01$). However, administration of 5 ml/kg or 10 ml/kg HS reduced the hepatic levels of MPO in BDL rats by 65.63% and 75.56%, respectively. Our results also showed that the increase of the
oxidation products MDA was accompanied by a significant decrease in activities of the antioxidant enzymes SOD and CAT in the liver of BDL rats, whereas treatment with HS markedly increased the hepatic SOD and CAT activities in BDL rats (Fig. 5C, D; P < 0.01).

**TNF-α, IL-1β and IL-6 levels in liver issue**

TNF-α, IL-1β, and IL-6 concentrations in the liver were significantly elevated in the livers of the BDL animals when compared with those in sham-operated animals. HS administration markedly lowered OJ-induced elevation of hepatic TNF-α, IL-1β and IL-6 concentrations (Fig. 6; P < 0.01).

**HMGB1 levels in serum and liver tissue**

As shown in Figure 7, at 10 days after BDL or sham operation, the levels of inflammatory mediator HMGB1 were determined in serum and liver of all animals. HMGB1 levels were higher in serum and liver of rats with BDL than in sham operated animals. Treatment with HS significantly attenuated HMGB1 levels in serum and liver of BDL rats. The differences in the levels of HMGB1 between HS dose groups were not significant.

**HS treatment decreases the activation of inflammatory signaling pathways following BDL**

Immunohistochemical and western blot analyses demonstrated that ERK1/2 was less activated in the liver tissues obtained from HS-treated rats than in those from NS-treated rats (Fig. 8). These results indicate that HS administration inhibited the ERK1/2 signaling pathway that is known to contribute to the development of cholestatic liver injury.
Discussion

Our study demonstrated that hydrogen-rich saline treatment significantly ameliorated hepatic injury in cholestatic rats. Intraperitoneal administration of hydrogen-rich saline effectively diminished the liver damage induced by obstructive jaundice, attenuated the induction of proinflammatory cytokines and neutrophil infiltration into the liver tissue, and was associated with a decrease of lipid peroxidation in liver. Biliary obstruction is associated with an intense state of oxidative stress that promotes hepatic injury. Obstructive jaundice also alters the activities of antioxidant enzymes, resulting in the increased production of superoxide anion, hydrogen peroxide, and hydroxyl radical [20]. Hydroxyl radical, the most reactive product of ROS, indiscriminately reacts with nucleic acids, lipids, and proteins, causing DNA fragmentation and lipid oxidation [21]. Thus, the accelerated generation of ROS may, at least in part, have an important role in the pathogenesis of hepatic injury associated with obstructive jaundice. No endogenous enzymatic pathway is known to neutralize hydroxyl radical [9]. Therefore, we hypothesized that therapeutic targeting of hydroxyl radicals may ameliorate OJ-induced hepatic injury. Hydrogen therapy may have several advantages over current pharmacological therapies, as it is highly diffusible and could potentially reach the subcellular structure of the mitochondria and nuclei, the primary site of ROS production and DNA damage, which are notoriously difficult to target pharmacologically [9]. Hydrogen scavenging of hydroxyl radicals would preserve mitochondrial membrane potential, maintain ATP synthesis, prevent DNA damage, and decrease lipid peroxidation.

In this study, MDA, which is the end product of oxidative injury and an indicator of lipid peroxidation, rapidly increased in the hepatic tissues in the BDL rats. The MDA levels were significantly decreased after hydrogen-rich saline treatment. We also observed that the activities of
SOD and CAT were significantly decreased in liver tissues 10 days after BDL when compared to sham-operated animals. Hydrogen treatment markedly increased the hepatic SOD and CAT activities in BDL rats. These data suggest that the protective effects of HS are associated with the abated production of hepatic oxidative products and improved endogenous antioxidant potential.

Several findings confirm that neutrophil recruitment constitutes a rate-limiting step in cholestasis-induced liver injury [22, 23]. Activated neutrophils accumulate in inflammatory sites and release ROS and these toxic substances can damage vascular endothelial cells and hepatic parenchymal cells either directly or by inducing proinflammatory cytokines, resulting in a decrease in liver function [24, 25]. In our study, HS decreased MPO levels, a marker of neutrophil recruitment, by 65.63% compared to OJ animals treated with saline, suggesting that HS reduces neutrophil recruitment to cholestatic liver, protecting against hepatocellular damage.

In association with neutrophil accumulation, a molecular inflammatory response is also involved in the pathogenesis of hepatic damage after BDL. Many studies have shown that OJ induces disruption of the intestinal mucosal barrier, allowing translocation of bacteria and endotoxin into the circulation, which may trigger a systemic inflammatory response and result in multiple organ dysfunction syndromes, including acute liver injury [26-28]. Hydrogen treatment that maintains intestinal mucosal permeability and improvement in intestinal barrier function could alleviate endoxemia and have important systemic benefits [14, 15]. Moreover, proinflammatory cytokines TNF-α, IL-1β, and IL-6, released by activated Kupffer cells and neutrophils, exert a considerable amplifying effect on hepatic inflammatory response and cause severe hepatic tissue damage. The severity of cholestatic liver injury is correlated with TNF-α, IL-1β, and IL-6 activity [28, 29] and a close link between inflammation and oxidative stress is
recognized [30-32]. In the present study, HS significantly reduced these proinflammatory
cytokines in cholestatic liver, suggesting that the preventive effect of HS on liver injury may be
mediated by suppression of the excessive hepatic inflammatory response and its cascade induced
by OJ.

HMGB1, secreted by activated monocytes/macrophages, neutrophils, and endothelial cells
and released passively from necrotic cells, acts as an endogenous danger signal and inflammatory
mediator [33, 34]. HMGB1 exerts its pro-inflammatory effects through activating endothelial cells
and promoting the release of multiple pro-inflammatory cytokines from phagocytes [35, 36].
HMGB1 was initially identified as a late mediator of lethal systemic inflammation in mice. In
experimental models of endotoxemia, increases in HMGB1 levels are delayed by 12 to 18 hours
compared with the early pro-inflammatory cytokines IL-1 and TNF-α [37, 38]. Ample evidence
now exists to indicate the HMGB1 also acts as an early inflammatory mediator in ischemia [39,
40], hemorrhagic shock [41], and noninfectious hepatitis [42]. HMGB1 blockade significantly
reduced organ damage and improved survival even with delayed treatment in various animal
models [37, 38, 43, 44]. Moreover, recombinant HMGB1 induced an endotoxemia-like state,
worsened tissue injury, and was lethal to mice when administered in large amounts [38, 39]. Both
in vitro and in vivo, endotoxins and inflammatory mediators induced by endotoxin induced the
release of HMGB1 [37, 45]. Here we showed that obstructive jaundice resulted in translocation of
gut-derived endotoxin, release of pro-inflammatory cytokines, and markedly enhanced the
accumulation of HMGB1 in serum and liver. When endotoxin levels in serum and
pro-inflammatory cytokines levels in tissue were attenuated by HS treatment, serum and tissue
HMGB1 levels significantly decreased, suggesting that the up-regulation of HMGB1 after BDL
might be due to the direct and/or indirect modulation of endotoxin.

MAPKs are proposed to play a key role in intracellular signaling cascades in normal and pathogenetic conditions [46, 47]. ERK1/2 is one of the major members of MAPKs and is associated with cellular oxidative stress, inflammation, and proliferation [46, 47]. ERK1/2 can be activated by lipopolysaccharide, leading to the production of inflammatory mediators such as TNF-α, IL-6, and nitrous oxide [48, 49]. Furthermore, inhibition of ERK1/2 can effectively attenuate organ dysfunction and inhibit inflammatory reaction [50, 51]. We speculate that the inhibition of TNF-α and IL-6 that we observe in HS-treated animals may be achieved by the interruption of ERK1/2 signaling pathway. We found that ERK1/2 phosphorylation was significantly inhibited in HS-treated animals, which might contribute to hydrogen-mediated hepatic protective effect.

In conclusion, the results of this study demonstrate that HS ameliorated BDL-induced liver damage by reducing oxidative stress and inflammatory cascades in liver tissue. Furthermore, hydrogen treatment appears to decrease mitochondrial stress, apoptosis, and autophagy in the liver during cholestasis (Q. Liu, unpublished date). Although the exact mechanism involved in the protective role of HS remains to be determined, due to its safety, efficacy, and convenience, peritoneal injection of HS should be considered as a potential therapy for liver damage caused by obstructive jaundice.
References:


Figure legends

Fig. 1. Body weights and survival rates of rats in each of the four treatment groups. A) Significant body weight loss in rats was observed after 7 days of BDL, persisting until d10 when animals were sacrificed. In contrast, rats receiving HS (5 and 10 ml/Kg) injection showed no loss in body weight. Data are expressed as means ± SD. *P <0.05, **P < 0.01. B) Hydrogen rich saline significantly enhanced BDL animal survival (n=14; Kaplan–Meyer; log-rank test; *P < 0.05, BDL + HS vs. BDL + NS ).

Fig. 2. Effects of hydrogen-rich saline on cholestatic liver injury indicated by H&E staining. Rats were injected intraperitoneally (i.p.) with NS or HS after BDL. A) and B) Sham-operated rats exhibited a normal hepatic structure. C) and D) Rats subjected to BDL and treated with NS had a severe destruction of the liver with significant hepatic cell edema, inflammatory cell infiltration, and bile epithelial cell proliferation. Treatment with 5 ml/kg (E, F) or 10 ml/kg (G, H) hydrogen-rich saline significantly attenuated cholestatic liver damage.

Fig. 3. Effects of hydrogen-rich saline on ductular proliferation (grade) and neutrophils accumulation. A) Statistical analysis of ductular proliferation (grade). B) Quantitative analysis of neutrophil accumulation. Data are expressed as means ± SD. **P <0.01 vs. Sham; *P < 0.01 vs. BDL + NS group.

Fig. 4. Changes in serum bilirubin and liver enzymes 10 days after bile duct ligation. The levels of A) total bilirubin, B) direct bilirubin, C) alanine aminotransferase (ALT) and D) aspartate
aminotransferase (AST) were determined. Data are expressed as mean ± SD. **P < 0.01 compared with sham group; †P > 0.05, **P < 0.01 compared with BDL + NS group.

Fig. 5. Hepatic malondialdehyde (MDA) levels, hepatic myeloperoxidase (MPO), Superoxide dismutase (SOD) and catalase (CAT) activity. A) Hepatic MDA levels, B) hepatic MPO activity, C) hepatic SOD activity, D) hepatic CAT activity. Data are expressed as means ± SD. **P < 0.01 vs. Sham; **P < 0.01 vs. BDL + NS group.

Fig. 6. Levels of TNF-α, IL-1β and IL-6 in rat hepatic tissues at 10 days after BDL. The levels of TNF-α, IL-1β and IL-6 were determined by ELISA. Data are expressed as mean ± SD. **P < 0.01 compared with sham group; **P < 0.01 compared with BDL+NS group.

Fig. 7. Levels of HMGB1 in serum and hepatic tissues. A) Levels of HMGB1 in serum. B) Levels of HMGB1 in hepatic tissues. Data are expressed as mean ± SD. **P < 0.01 compared with sham group; **P < 0.01 compared with BDL+NS group.

Fig. 8. Hydrogen-rich saline decreases hepatic ERK1/2 phosphorylation in the liver of cholestatic rats. A) Representative immunohistochemical images of activated ERK1/2. B) Western blot analysis demonstrated that ERK1/2 was less activated in the livers obtained from hydrogen-rich saline treated rats than those obtained from NS treated rats. Images are representative of four independent experiments.
For Peer Review

Fig. 1. Body weights and survival rates of rats in each of the four treatment groups. A) Significant body weight loss in rats was observed after 7 days of BDL, persisting until d10 when animals were sacrificed. In contrast, rats receiving HS (5 and 10 ml/Kg) injection showed no loss in body weight. Data are expressed as means ± SD. *P < 0.05, **P < 0.01. B) Hydrogen rich saline significantly enhanced BDL animal survival (n=14; Kaplan–Meyer; log-rank test; *P < 0.05, BDL + HS vs. BDL + NS).

84x115mm (300 x 300 DPI)
For Peer Review

Fig. 2. Effects of hydrogen-rich saline on cholestatic liver injury indicated by H&E staining. Rats were injected intraperitoneally (i.p.) with NS or HS after BDL. A) and B) Sham-operated rats exhibited a normal hepatic structure. C) and D) Rats subjected to BDL and treated with NS had a severe destruction of the liver with significant hepatic cell edema, inflammatory cell infiltration, and bile epithelial cell proliferation. Treatment with 5 ml/kg (E, F) or 10 ml/kg (G, H) hydrogen-rich saline significantly attenuated cholestatic liver damage.

30x47mm (500 x 500 DPI)
Fig. 3. Effects of hydrogen-rich saline on ductular proliferation (grade) and neutrophils accumulation. A) Statistical analysis of ductular proliferation (grade). B) Quantitative analysis of neutrophil accumulation. Data are expressed as means ± SD. +++P < 0.01 vs. Sham; **P < 0.01 vs. BDL + NS group.

71x47mm (500 x 500 DPI)
Fig. 4. Changes in serum bilirubin and liver enzymes 10 days after bile duct ligation. The levels of (a) total bilirubin, (b) direct bilirubin, (c) alanine aminotransferase (ALT) and (d) aspartate aminotransferase (AST) were determined. Data are expressed as mean ± SD. ++P < 0.01 compared with sham group; †P > 0.05, **P < 0.01 compared with BDL + NS group.
Fig. 5. Hepatic malondialdehyde (MDA) levels, hepatic myeloperoxidase (MPO), Superoxide dismutase (SOD) and catalase (CAT) activity. A) Hepatic MDA levels, B) hepatic MPO activity, C) hepatic SOD activity, D) hepatic CAT activity. Data are expressed as means ± SD. ++P <0.01 vs. Sham; **P < 0.01 vs. BDL + NS group.

Sham; **P < 0.01 vs. BDL + NS group.
Fig. 6. Levels of TNF-α, IL-1β and IL-6 in rat hepatic tissues at 10 days after BDL. The levels of TNF-α, IL-1β and IL-6 were determined by ELISA. Data are expressed as mean ± SD. ++P < 0.01 compared with sham group; **P < 0.01 compared with BDL+NS group.
Fig. 7. Levels of HMGB1 in serum and hepatic tissues. A) Levels of HMGB1 in serum. B) Levels of HMGB1 in hepatic tissues. Data are expressed as mean ± SD. **P < 0.01 compared with sham group; ***P < 0.01 compared with BDL+NS group.

71x49mm (500 x 500 DPI)
Fig. 8. Hydrogen-rich saline decreases hepatic ERK1/2 phosphorylation in the liver of cholestatic rats. A) Representative immunohistochemical images of activated ERK1/2. B) Western blot analysis demonstrated that ERK1/2 was less activated in the livers obtained from hydrogen-rich saline treated rats than those obtained from NS treated rats. Images are representative of four independent experiments.

84x87mm (300 x 300 DPI)