Hydrogen gas alleviates acute alcohol-induced liver injury by inhibiting JNK activation

YAXING ZHANG1, MINGMIN BI2,3, ZIFENG CHEN4,5, MIN DAI1, GE ZHOU1, YUXUAN HU1, HONGZHI YANG1 and WEIBING GUAN1

1Department of Traditional Chinese Medicine, The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong 510630; 2Department of Otorhinolaryngology, The Seventh Affiliated Hospital, Sun Yat-sen University, Shenzhen, Guangdong 518107; 3Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, Guangdong 510080; 4Biofeedback Laboratory and 5Department of Biomedical Engineering, Xinhua College of Sun Yat-sen University, Guangzhou, Guangdong 510520, P.R. China

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Abstract. Binge alcohol drinking is fast becoming a global health concern, with the liver among the first organ involved and the one afflicted with the greatest degree of injury. Oxidative stress, alterations in hepatic metabolism, immunity and inflammation have all been reported to contribute to the development of alcoholic liver disease (ALD). Hydrogen gas (H2) serves a key role in the modulation of hepatic redox, immune and inflammatory homeostasis. However, the effects of treatment using intraperitoneal injection of H2 on ALD remain unexplored. Therefore, the aim of the present study was to investigate the effects and underlying mechanism of intraperitoneal injection of H2 on acute alcohol-induced liver injury in a mouse model. H2 was administered by daily intraperitoneal injections (1.0 ml/100 g) for 4 days. On day 4, the mice received H2 after fasting for 5.5 h. After 30 min, the mice were administered with 33% (v/v) ethanol at a cumulative dose of 4.5 g/kg body weight by four equally divided gavages at 20-min intervals. Blood and liver tissues were collected at 16 h after the first ethanol gavage. Subsequently, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride and total cholesterol (TC) levels were analyzed using an Automatic Clinical Analyzer. Hepatic JNK activity and GAPDH levels were examined by western blotting. It was observed that acute ethanol gavage induced liver injury, as indicated by significantly increased serum ALT and AST levels, which were effectively decreased by H2 at 16 h after the first ethanol gavage. In addition, H2 treatment reduced serum TC levels in the Alcohol+H2 group when compared with those in Alcohol group. Mechanistically, H2 attenuated hepatic JNK phosphorylation induced by acute ethanol gavage. Therefore, the results of the present study demonstrated that treatment with exogenous H2 by intraperitoneal injection may alleviate acute alcohol-induced liver injury by inhibiting hepatic JNK activation, which may represent a novel therapeutic strategy for ALD.

Introduction

Excessive alcohol intake, as result of binge drinking as well as chronic alcohol consumption of ≥40 g per day, is becoming a global healthcare concern (1,2). Globally, ~2 billion individuals consume alcohol regularly, where >75 million are diagnosed with disorders associated with alcohol abuse and are at risk of developing alcohol-associated liver diseases (3). The liver typically sustains the earliest and the greatest degree of tissue injury caused by excessive drinking, since it is the primary site of ethanol metabolism (1,4). Excessive alcohol consumption causes alcoholic liver disease (ALD), which is characterized by a wide spectrum of hepatic pathologies, from reversible fatty liver (simple steatosis) to acute alcoholic hepatitis, chronic fibrosis and cirrhosis and superimposed hepatocellular carcinoma (HCC) (2,5,6). Mechanistically, pathogenesis of ALD includes genetic susceptibility, oxidative stress, hepatic metabolism alteration and steatosis, acetaldehyde-mediated toxicity, cytokine- and chemokine-induced inflammation, alterations in immunity and dysbiosis, epigenetic changes and modifications in the regenerative process (2,6-8). Alcohol abstinence achieved by psychosomatic intervention is at present the best treatment for all stages of ALD (2). However, drinking discontinuation may be difficult, for example, the cheap price of hard liquor, easy accessibility to alcohol, and alcohol advertisement make it very difficult to prevent the increase in ALD (9). Therefore, new strategies for alleviating alcohol-induced liver injury remain to be in demand.

Hydrogen gas (H2) is the lightest and diffusible gas molecule that has been shown to confer potent antioxidant and
anti-inflammatory properties (10,11). It can be absorbed into the blood circulation, such that it reaches the target organ either by blood circulation or free diffusion (11). Therefore, treatments involving exogenous H₂, including breathing H₂ gas, injection with H₂-rich saline and drinking H₂-rich water, may protect against excessive oxidative stress- and inflammation-related liver damage, including liver injury induced by drugs, sepsis, bile duct ligation, ischemia/reperfusion (I/R), CO₂ pneumoperitoneum and chronic intermittent hypoxia, in addition to non-alcoholic fatty liver disease (NAFLD) (12-17). Furthermore, drinking H₂-rich water has been indicated to protect against chronic ethanol-induced hepatotoxicity (18). These previous observations suggest that H₂ serves an important role in modulating hepatic redox, immune and inflammatory homeostasis (11).

Previous studies have demonstrated that supplementation with exogenous H₂ by intraperitoneal injection may improve lipopolysaccharide (LPS)-induced cardiac dysfunction, isoproterenol-induced cardiac hypertrophy, pressure overload-induced vascular hypertrophy, and cardiopulmonary cerebral resuscitation in a cardiac arrest rabbit model (19-23). These were mediated by the suppression of excessive oxidative stress and inflammatory responses (19-23). However, the effects of intraperitoneal injection of H₂ on ALD remain unclear.

Therefore, the aim of the present study was to investigate the effect of H₂ intraperitoneal injection on acute alcohol-induced liver injury in a mouse model and to elucidate the potential underlying mechanisms.

**Materials and methods**

**Drugs.** In total, 99.999% H₂ (Dalian Special Gases Co., Ltd.) was injected into a vacuumed aseptic soft plastic infusion bag (100 ml; Hebei Tiancheng Pharmaceutical Co., Ltd.) from a seamless steel gas cylinder under sterile conditions, as previously described (Fig. 1) (20-23). Anhydrous ethanol (Guangzhou Chemical Reagent Factory) was dissolved in double-distilled water to obtain 33% (v/v) ethanol (0.26 g/ml) (24).

**Animal model of acute alcohol-induced liver injury and treatment protocol.** Male C57BL/6J mice (Animal license no. 44007200061823) were purchased from the Guangdong Medical Laboratory Animal Center (Foshan, China). A total of 28 mice aged 8–10 weeks were used in this study. All animals were housed in a temperature-controlled animal facility (21-24°C) with a 12-hour light-dark cycle, and the animals had access to rodent chow and water ad libitum (20). All mice were provided with humane care according to the Principles of Laboratory Animal Care formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals published by the NIH (8th Edition, Revised 2011) (20,25). All animal procedures were approved by the Institutional Animal Care and Use Committee of Zhongshan School of Medicine, Sun Yat-sen University (Guangzhou, China).

Mice were randomly assigned into the following four groups (n=7 mice per group): i) Control; ii) Alcohol; iii) Alcohol+H₂; and iv) H₂. In the Alcohol+H₂ and H₂ groups, H₂ was administered daily at the dose of 1.0 ml/100 g by intraperitoneal injection for 4 days. On day 4, mice in each group were fasted for 6 h before the mice in the Alcohol and Alcohol+H₂ groups were orally administered with 33% (v/v) ethanol at a cumulative dose of 4.5 g/kg body weight (17.3 ml/kg body weight) by four equally divided gavages at 20-min intervals (24). Mice in the Alcohol+H₂ and H₂ groups were administered with an intraperitoneal injection (1.0 ml/100 g) of H₂ 30 min before the first ethanol administration (20). In addition, mice in the Control and H₂ groups received the same volume of double-distilled water (17.3 ml/kg body weight) by four equally divided gavages at 20 min intervals. The animals were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) after 16 h of the first ethanol gavage, cardiac puncture was performed when the animals reached the surgical plane of anesthesia and ~0.4-0.5 ml blood was collected from each mouse (26-28). Euthanasia was then performed by cervical dislocation in a state of deep anesthesia before liver tissues were extracted (Fig. 2) (24). The body weight of animals in each group was recorded at the baseline (Control, 25.19±1.58 g; Alcohol, 24.63±1.42 g; Alcohol+H₂, 25.17±1.15 g; H₂, 23.9±0.70 g) and after intervention (Control, 24.90±1.36 g; Alcohol, 25.16±1.53 g; Alcohol+H₂, 25.00±0.93 g; H₂, 23.71±0.63 g).

**Biochemical analysis.** Blood biochemical analysis was performed as previously described (29). Briefly, the blood samples were centrifuged at the speed of 986 x g for 10 min at 4°C to separate the serum. Levels of liver function markers alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum were analyzed using an Automatic Clinical Analyzer (Hitachi 7600; Hitachi High-Technologies Corporation) at the Department
of Clinical Laboratory, The Third Affiliated Hospital of Sun Yat-sen University.

Western blotting. JNK antibody (cat. no. 9252S), phosphorylated (p-) JNK antibody (cat. no. 9255S), anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. 7074S) and anti-mouse IgG HRP-conjugated secondary antibody (cat. no. 7076S) were purchased from Cell Signaling Technology, Inc. GAPDH antibody (cat. no. MB001) was obtained from Bioworld Technology, Inc. BSA (cat. no. ST023-200g) was purchased from Beyotime Institute of Biotechnology.

Western blotting was performed as previously described (30). The proteins were transferred onto polyvinylidene fluoride membranes (EMD Millipore). Membranes were incubated in blocking buffer (1X TBST with 5% BSA) for 30 min at room temperature. For antibody incubations, membranes were incubated with primary antibodies in antibody dilution buffer (1X TBST with 5% BSA) with gentle agitation overnight at 4°C (p-JNK and JNK: 1:2,000; GAPDH: 1:10,000), and with secondary antibodies in antibody dilution buffer (1X TBST with 5% BSA) with gentle agitation for 1 h at room temperature (anti-rabbit IgG HRP-conjugated secondary antibody and anti-mouse IgG HRP-conjugated secondary antibody: 1:2,000). Immobilon™ Western Chemiluminescent HRP Substrate (ECL; cat. no. WBKLS0100, EMD Millipore) was used to reveal the bands using the ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories, Inc.). Image J software (Version 1.52; National Institutes of Health) was used for estimating the ‘IntDen’ value of the western blot bands for quantification.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc.). Data were expressed as the mean ± SD. For biochemical analysis, n=7 mice in each group. For western blotting, n=5 mice in each group. Statistical analysis was performed by one-way ANOVA.
followed by Bonferroni’s post hoc test. P<0.05 was considered to indicate statistically significant differences.

Results

**H₂ treatment prevents acute alcohol-induced liver damage.** To investigate the effects of H₂ on alcohol-induced liver injury, an acute alcohol-induced hepatotoxicity mouse model was established as previously described (24). This model was shown to closely mimic excessive ethanol consumption in humans in terms of blood alcohol levels, behavioral and physiological effects (24). Compared with those in the Control group, serum ALT and AST levels in the Alcohol group were significantly higher following acute alcohol gavage (Fig. 3), whilst intraperitoneal injection of H₂ significantly prevented these elevations in serum ALT and AST levels in mice following alcohol gavage (Fig. 3). However, H₂ alone exerted no effects on serum ALT and AST levels compared with those in the Control group (Fig. 3). Therefore, these observations suggest that intraperitoneal injection of H₂ effectively alleviated acute alcohol-induced liver injury in mice.

**Effects of H₂ on serum lipid levels in acute alcohol-induced liver injury.** Long-term chronic alcohol consumption may lead to disruptions in lipid metabolism (6). Therefore, the serum levels of TC and TG were next examined. Although acute alcohol treatment slightly increased serum TC levels, there was no significant difference between those in the Control and Alcohol groups (Fig. 4A). Serum TC levels in the Alcohol+H₂ group were significantly lower compared with those in the Alcohol group (Fig. 4A). However, there were no significant differences in TG levels among the four groups (Fig. 4B). These data suggest that intraperitoneal injection of H₂ may modulate blood TC levels in an acute alcohol-induced liver injury mice model.

**H₂ alleviates acute alcohol-induced hepatic JNK activation.** Acute ethanol loading causes oxidative stress and activates cell-death signaling through the JNK pathway in the liver (31). In addition, JNK is a key mediator in hepatic steatosis, where it regulates transcription factor activity associated with lipid metabolism (32,33). In the present study, the upregulation of serum ALT and AST levels after ethanol treatment indicated hepatocytes damage. Therefore, the role of hepatic JNK activation in the potentially protective effects of H₂ against acute alcohol-induced liver injury was examined. Western blotting data demonstrated that acute ethanol treatment significantly increased hepatic JNK phosphorylation, which was significantly prevented by intraperitoneal injection with H₂ (Fig. 5). Therefore, these data suggest that intraperitoneal injection with H₂ conferred protective effects against acute alcohol-induced liver injury by preventing hepatic JNK activation.
and dysfunction (20,21), and display neuroprotective effects in

Previous studies found that intraperitoneal injection of H
gas can alleviate vascular remodeling (23), cardiac hypertrophy

The present study was undertaken to determine the effect of

Extensive investigations have been shown to be an able

In 1975 (37). In 2007, H
to alleviate skin tumors

H
demonstrated to act as a therapeutic antioxidant by selectively

Increased hepatic expression of Nrf2 (42) and PPAR-

Additionally, JNK may suppress hepatic peroxisome

which is a transcription factor and a positive regulator of

Acute alcohol feeding activates cytochrome P450 2E1

Acute alcohol feeding activates cytochrome P450 2E1

Figure 5. Effects on H
to hepatic JNK activation after ethanol feeding. (A) Representative western blotting images of p-JNK and JNK and GAPDH. (B) Quantification of p-JNK/total JNK ratio. The averages of the p-JNK/JNK ratio in the Control group were set as ‘1’, and all values were normalized to ‘1’. n=5 mice in each group. **P<0.01 vs. Control; #P<0.05 and ***P<0.001 vs. Alcohol.

Discussion

Inflammatory and cytokine signaling, oxidative stress and mitochondrial dysfunction, alterations in hepatic metabolism and hepatocyte cell death, and abnormalities in immunity and dysbiosis are all key to the pathogenesis of liver diseases, including ALD and NAFLD (2,34-36). Therefore, strategies preventing fatty liver disease progression were previously developed using combinations of naturally occurring compounds products in animal models. For example, the combination of docosahexaenoic acid and the antioxidant hydroxytyrosol was demonstrated to prevent high-fat diet-induced liver steatosis, by inhibiting oxidative stress, mitochondrial dysfunction and inflammation associated with steatosis (34,35). H
demonstrated to act as a therapeutic antioxidant by selectively reducing cytotoxic hydroxyl radical levels to improve focal I/R-induced brain injury in rats (38). Since then, H
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as intestinal bacterial overgrowth) and qualitative (such as colonic Firmicutes and Bacteroidetes levels) changes in the gut microbiota, and pathological bacterial translocation are fundamental for the pathogenesis of ALD (8,52). A number of these intestinal microbiota are affected by alcohol, which express hydrogenases and act as the main producers of endogenous H₂ in humans and animals (10,20,52,53). Endogenous H₂ is crucial for hepatic redox homeostasis, glucose and lipid homeostasis, in addition to immune and inflammatory homeostasis (11,54‑57). Therefore, it remains of importance to investigate the effects of endogenous H₂ in the pathogenesis of ALD.

In summary, findings of the present study indicated that intraperitoneal injection with exogenous H₂ attenuated acute alcohol‑induced liver injury in mice by inhibiting hepatic JNK activation. Therefore, it would be possible to treat alcohol‑induced liver injury with H₂, such as drinking H₂‑rich alcohol or H₂‑rich water, where H₂ can be a potentially useful natural agent for the treatment of ALD.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ, WG, MD and HY conceived and designed the experiments. YZ, GZ, ZC, MB and YH performed the experiments and analyzed the data. YZ drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat‑sen University (IACUC code no: 2018‑057).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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