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EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

The protective role of hydrogen-rich saline in experimental liver injury in mice

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Background & Aims: Reactive oxygen species (ROS) are considered to play a prominent causative role in the development of various hepatic disorders. Antioxidants have been effectively demonstrated to protect against hepatic damage. Hydrogen (H_2), a new antioxidant, was reported to selectively reduce the strongest oxidants, such as hydroxyl radicals (·OH) and peroxynitrite ($ONOO^-$), without disturbing metabolic oxidation–reduction reactions or disrupting ROS involved in cell signaling. In place of H_2 gas, hydrogen-rich saline (HS) may be more suitable for clinical application. We herein aim to verify its protective effects in experimental models of liver injury.

Methods: H_2 concentration in vivo was detected by hydrogen microelectrode for the first time. Liver damage, ROS accumulation, cytokine levels, and apoptotic protein expression were, respectively, evaluated after GalN/LPS, CCl_4, and DEN challenge. Simultaneously, CCl_4-induced hepatic cirrhosis and DEN-induced hepatocyte proliferation were measured.

Results: HS significantly increased hydrogen concentration in liver and kidney tissues. As a result, acute liver injury, hepatic cirrhosis, and hepatocyte proliferation were reduced through the quenching of detrimental ROS. Activity of pro-apoptotic players, such as JNK and caspase-3, were also inhibited.

Conclusions: HS could protect against liver injury and also inhibit the processes leading to liver cirrhosis and hepatocyte compensatory proliferation.

Keywords: Hydrogen-rich saline; Acute hepatic failure; Hepatic cirrhosis; Hepatocyte proliferation; Reactive oxygen species; Inflammation; Apoptosis; JNK.
Received 8 January 2010; received in revised form 18 August 2010; accepted 31 August 2010

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Abbreviations: ROS, reactive oxygen species; ·OH, hydroxyl radicals; $ONOO^-$, peroxynitrite; HS, hydrogen-rich saline; NS, normal saline; GalN, D-galactosamine; LPS, lipopolysaccharide; CCl_4, carbon tetrachloride; DEN, diethylnitrosamine; AHF, acute hepatic failure; H_2, hydrogen; O_2^-, superoxide anion radical; H_2O_2, hydrogen peroxide; NO·, nitric oxide; IHC, immunohistochemical; DHE, dihydroethidine; HSC, hepatic stellate cells; RC, regular chow.

Introduction

Acute hepatic failure (AHF) is defined as the rapid onset of severe hepatocellular dysfunction with poor prognosis. It frequently results from hepatitis virus infection, the induction of drugs and toxins, or hepatic ischemia–reperfusion injury. Oxidative stress has been regarded as a major contributor to the development of various hepatic disorders including acute hepatic failure, hepatic fibrosis, and hepatic cancer [1–3]. Moreover, it also represents an imbalance between the production of ROS and the activity of antioxidant defense systems [4]. Earlier reports have demonstrated that antioxidants were effective in protecting against hepatic damage by inhibiting free radical generation or scavenging for free radicals generated by other biochemical reactions [5,6].

Molecular hydrogen (H_2), the lightest and most abundant chemical element, has been defined as a novel antioxidant, which selectively quenches detrimental ROS, such as ·OH and $ONOO^-$, while maintaining metabolic oxidation–reduction reaction and other less potent ROS, such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and Nitric oxide (NO·) [7]. Hydrogen acts as a reductant for molecules that are strongly pro-oxidant [8,9]. Unlike most known antioxidants, which are unable to successfully target organelles, hydrogen has advantageous distribution characteristics for its capability to penetrate biomembranes and diffuse into the cytosol, mitochondria, and nucleus [10]. It has been demonstrated that the inhalation of H_2 gas can reduce brain, liver, or heart ischemia–reperfusion injury as well as intestinal graft injury, via its antioxidant effect [7,11–13]. Moreover, inhalation of H_2 gas was more efficacious than a treatment currently approved for cerebral infarction [7]. These findings indicate that the beneficial effects of H_2 could be used for the treatment of hepatic and other diseases. However, in clinical application, inhalation of H_2 gas is not convenient and is dangerous because of its flammable and explosive nature even at a concentration of 4.7% in air.

In contrast to H_2 gas, HS (H_2 saturated in saline) is easily administered and is safe for clinical application. It has been

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reported that HS can prevent or reduce early pathological changes and also lead to long lasting functional improvement in neonatal hypoxia–ischemia rat models [14]. However, it remains unclear whether HS has similar protective effects on acute hepatic injury, and whether it can prevent ROS-induced cell death in inflammation of the liver. In this study, we demonstrated that HS could alleviate liver injury in experimental Galn/LPS, CCl_4, or DEN-induced AHF models, and revealed the clinical potential of HS for preventive and therapeutic anti-oxidative applications.

Materials and methods

Preparation of hydrogen-rich saline

The detailed information for the preparation of HS was described in our previous reports [15].

Animals

Male C57Bl/6 mice (20–25 g) were obtained from the Model Animal Research Center of Nanjing University in Nanjing, China. They were maintained under controlled conditions (25 °C, 55% humidity and 12 h day/night rhythm) and fed standard laboratory food. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Second Military Medical University (Shanghai, China).

Experimental model for hydrogen distribution studies in mice

Eight milliliter per kilogram HS or its control, NS, was injected into mice via the peritoneal cavity. The mice were anesthetized with pentobarbital (0.7 μg/g body weight, i.p.) and placed in supine position. An incision was made on the midline of the abdomen under aseptic conditions. Heparin saline 0.5 ml (50,000 U/L) was injected into the peritoneal cavity. Hydrogen microelectrode (dia. 50 μm) was penetrated into the liver and kidney at a depth of 300 μm.

Mice model of hepatic failure

GalN (Sigma, USA) was administered i.p. at 800 mg/kg followed with lipopolysaccharide treatment (LPS, i.p., 20 μg/kg; Sigma, USA). HS (8 ml/kg) or an equivalent volume of NS as control was given intraperitoneally every 1 h after the administration of GalN/LPS. After stimulation of GalN/LPS (800 vs. 20 μg/kg or 800 vs. 5 μg/kg body weight), survival rates of mice were measured ($n = 15$ each group).

CCl_4 mixed with olive oil (1:19 v/v, 4 ml/kg) was gaged for acute hepatic injury and cirrhosis (3 times/week, 12 weeks) model [16,17].

DEN (100 mg/kg; Sigma, USA) was injected intraperitoneally for acute hepatic injury [18].

Either HS (8 ml/kg) or an equivalent volume of NS as control was given intraperitoneally every 3 h after the administration of CCl_4 or DEN.

Histology of mice liver tissue

IHC analysis was performed with phospho-c-Jun antibody, F4/80 antibody, and α-SMA antibody, using methods as described previously [19].

Measurement of transaminase activities

Activities of serum aminotransferases (ALT and AST) were determined by an automated procedure in the Department of Inspection, Eastern Hepatobiliary Surgery Hospital.

Cytokine measurement in murine serum

Levels of TNF-α and IL-6 were measured with a commercial ELISA kit following the instructions of the manufacturer (Dakewe, Shenzhen, China) (Synergy 2 Multi-Mode Microplate Reader, BioTek, USA).

Analysis of hepatocyte apoptosis

Apoptotic hepatocytes were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Olympus BX51, Olympus, Japan) staining according to manufacturer's recommendations of In Situ Cell Apoptosis Detection kit (Keygen, Nanjing, China) (Synergy 2 Multi-Mode Microplate Reader, BioTek, USA. Caspase-3 activities were measured using fluorometric caspase activity detection kits (Keygen, Nanjing, China) (Synergy 2 Multi-Mode Microplate Reader, BioTek, USA. The assays were performed as recommended by the manufacturer.

Measurement of ROS and GSH

Liver cryosections prepared 5 h after GalN/LPS injection and 48 h after CCl_4 lavage were incubated with 2 mM dihydroethidine hydrochloride for 30 min at 37 °C. Cells staining positive for the oxidized dyes were identified by fluorescence microscopy (Olympus IX70, Olympus, Japan). At the same time, liver homogenates were prepared and analyzed for GSH content with a commercial ELISA kit (Jiancheng, Nanjing, China) according to the protocol provided by the manufacturer.

Analysis of liver fibrogenesis

mRNA was quantified by real-time PCR assay (7300 Real-Time PCR System, Applied Biosystems, USA) using double-stranded DNA-binding dye SYBR green-I (Trkara, Dalian, China), as described previously [20]. The expression of all the target genes was normalized to 18S. The liver sections were stained with picro-sirius red for collagen distribution [21]. The content of hepatic hydroxyproline was determined by using the hydroxyproline kit following the protocol provided by the manufacturer (Genmed, Shanghai, China).

Western blot analysis

The anti-JNK, pJNK, PARP, α-SMA, and GAPDH, monoclonal antibodies were purchased from Neomarker, Santacruz, Kangcheng for Sigma and Cell signaling. Protein concentration was determined by BCA method. Western blotting was performed as previously described [20].

Detection of hepatocytes proliferation

Hepatocyte proliferation was measured by Edu incorporation 72 h after DEN challenge. The assays were performed as recommended by the manufacturer of Edu detection kits (Ribobio, Guangzhou, China) (Olympus IX70, Olympus, Japan).

Statistical analysis

All results were expressed as mean ± standard deviation (SD). Differences between experimental and control groups were assessed by either the analysis of variance (ANOVA) or nonparametric tests, as applicable, using SPSS 16.0 (SPSS, Inc.). Recipient survival was plotted using the Kaplan–Meier method, and the differences between groups were analyzed using the log-rank test. A p-value of less than 0.05 was considered statistically significant.

Results

Intraperitoneal injection of HS significantly increased H_2 concentration in liver and kidney tissues

The H_2 levels in liver and kidney tissues were measured by H_2 microelectrode (Denmark–Unisense). A linear correlation was found between the current value of H_2 microelectrode and hydrogen concentration (H_2 concentration: 0–40 μM, $R^2 = 0.9977$, Fig. 1A). As shown in Fig. 1B and C, concentrations of molecular H_2 peaked approximately 5 min following HS injection in the liver and kidney, and returned to normal levels 40 min later. These results suggest that HS is an ideal tool for molecular H_2 induction, and intraperitoneal administration of HS could efficiently deliver

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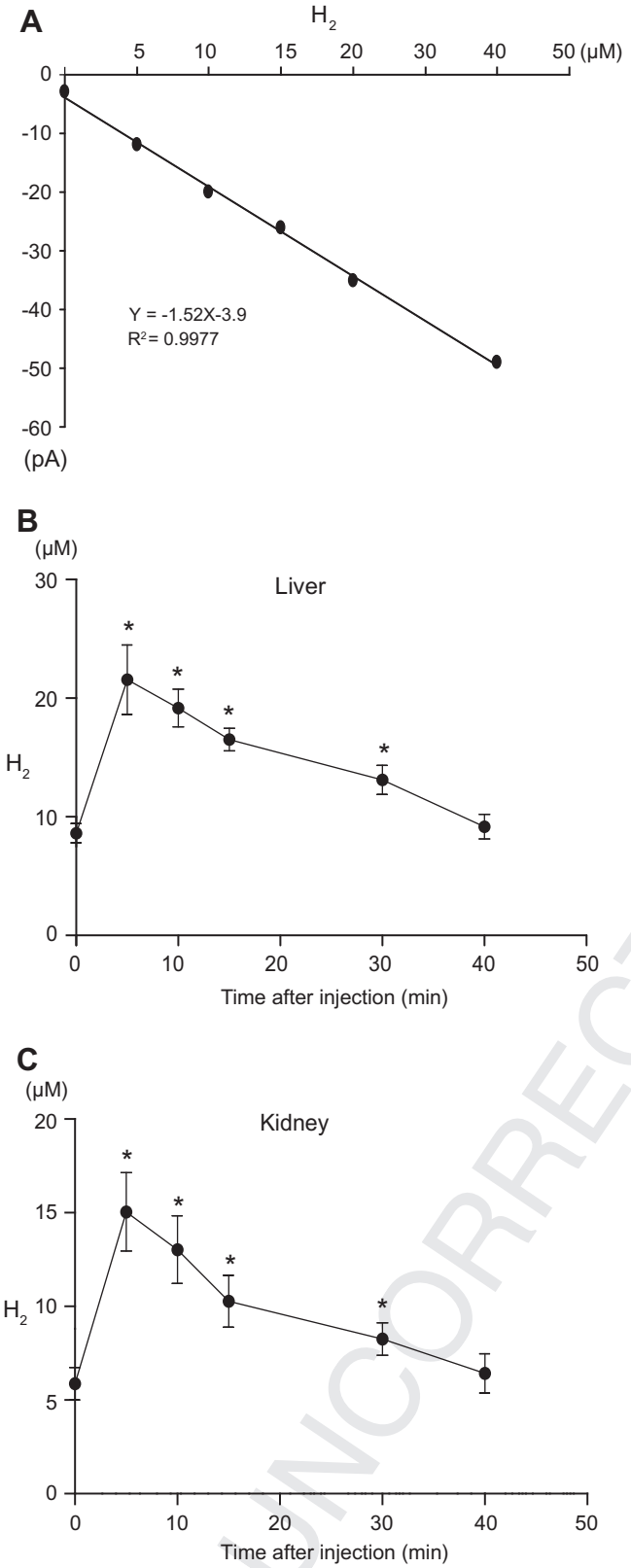

Fig. 1. Concentration of hydrogen in mice abdominal organs and blood samples. (A) The standard curve represents the linear correlation of hydrogen concentration (μM) in saline and the current value (pA). (B and C) H_2 molecules concentration was changed after injected hydrogen-rich saline in mice abdominal organs (liver or kidney, $n = 8$, $^*p < 0.05$ vs. time, 0 min).

hydrogen into the liver and kidney. In addition, we measured the pH values of HS and NS, and found no significant difference in the pH levels of the two solutions (NS, 7.35 ± 0.02 vs. HS, 7.32 ± 0.03).

GalN/LPS-induced liver injury was reduced by HS treatment

The effect of HS was tested in a widely accepted model of fulminant hepatitis, i.e. in the model of GalN/LPS-induced liver injury. HS (8 ml/kg) or an equivalent volume of NS as control was injected every hour after GalN/LPS challenge. Liver injury was strongly reduced as determined by measurement of serum transaminase activities 5 h after GalN/LPS administration (Fig. 2A). Histological examination of liver tissue by H&E staining revealed a prominent preservation in the liver structure of HS-treated animals (Fig. 2B). To characterize the inflammatory infiltration, sections of liver were subjected to immunohistochemical (IHC) staining to identify the presence and distribution of macrophages. As shown in Fig. 2C, GalN/LPS treatment resulted in the accumulation of macrophages in close vicinity to injured hepatocytes. However, the infiltration of macrophages was blunted in AHF mice followed with HS administration. In accordance with histological and biochemical findings, cytokine expression of injury markers was also blunted in HS-treated mice. As shown in Fig. 2D, GalN/LPS-induced increment of pro-inflammation cytokines TNF-α and IL-6 in serum was remarkably prevented by treatment with HS. Furthermore, the mortality in HS treated group with GalN/LPS-induced fulminant hepatic failure was decreased to 46.7% (73% in NS group) at 10 h after GalN/LPS treatment (Fig. 2E, left panel). Similarly, HS also reduced the mortality of high dose GalN/LPS-treated mice (Fig. 2E, right panel).

These data demonstrate a notable improvement in the condition of mice with GalN/LPS-induced acute hepatic failure if followed with HS administration, as compared with that of control mice.

HS reduced ROS-induced pro-apoptotic signaling and hindered the activation of JNK in GalN/LPS-challenged mice

As GalN/LPS-induced liver injury is characterized by apoptosis of hepatocytes, the expression and activity of pro-apoptotic molecules were examined 5 h after GalN/LPS treatment. The purpose is to verify whether HS exerted its protective activity by preventing cell death. As shown in Fig. 3A and B, although the administration of GalN/LPS resulted in a dramatic activation of caspase-3 and cleavage of PARP, these effects were markedly decreased in the presence of HS. Similar results were also observed in liver tissue samples by applying TUNEL-based IHC assay (Fig. 3C).

In the GalN/LPS model, TNF-α-induced ROS generation is the major mediator leading to apoptotic liver injury [22]. To verify whether the protective function of HS resulted from the reduction of ROS accumulation, we assessed the levels of hepatocyte superoxides. Freshly frozen liver sections were stained with dihydroethidine (DHE), whose oxidation gives rise to the fluorescent derivative ethidine [23]. GSH levels of fresh liver tissue were then detected. As expected, the administration of HS remarkably decreased the amount of DHE-positive hepatocytes and increased the levels of GSH (Fig 3D and E). Similarly, serum ALT level (Fig. 3F) was also reduced in GalN/LPS-sensitized mice fed with the antioxidant BHA-supplemented diet. Consistent with this notion, ROS-enhanced JNK activation, which contributed to liver

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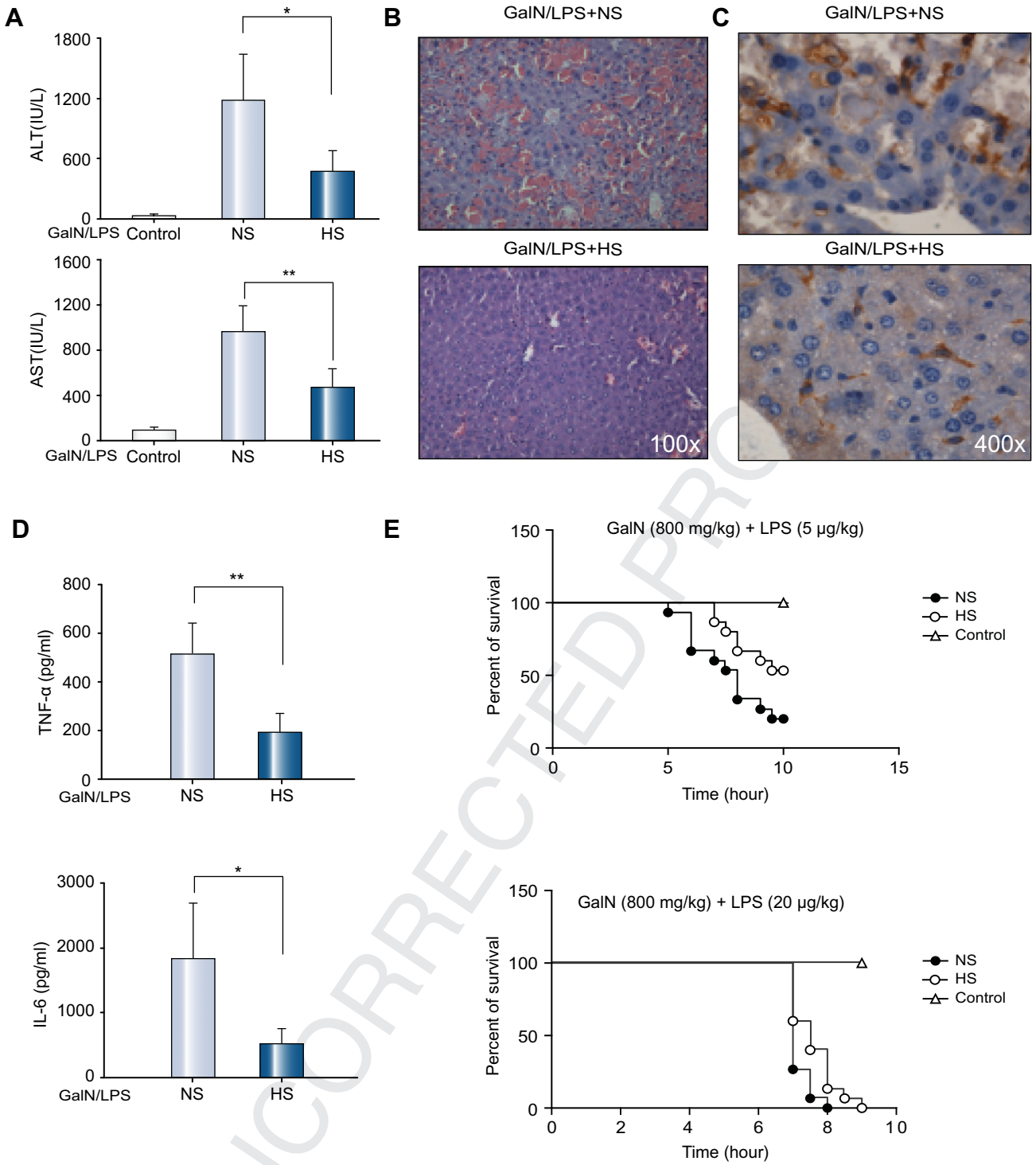

Fig. 2. HS leads to a significant decrease in acute liver injury 5 h after GalN/LPS challenge. (A) Transaminase levels (AST and ALT) of mice ($n = 8$, mean ± SD, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. NS). (B) Hematoxylin–eosin staining of mice liver sections. (C) IHC staining with F4/80 antibody of mice liver sections. (D) TNF-α and IL-6 serum levels of mice were determined by ELISA ($n = 8$, mean ± SD, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. NS). (E) HS reduced the mortalities of low (left graph) and high dose (right graph) GalN/LPS-treated mice (CON group $n = 5$, HS or NS groups $n = 15$, Kaplan–Meyer, log-rank test, $p < 0.05$ HS vs. NS).

failure, was prevented by HS administration (Fig. 3G) or BHA diet (data not shown). These data indicated that HS might exert its anti-apoptotic activity by preventing the effects of oxidative stress and JNK signaling.

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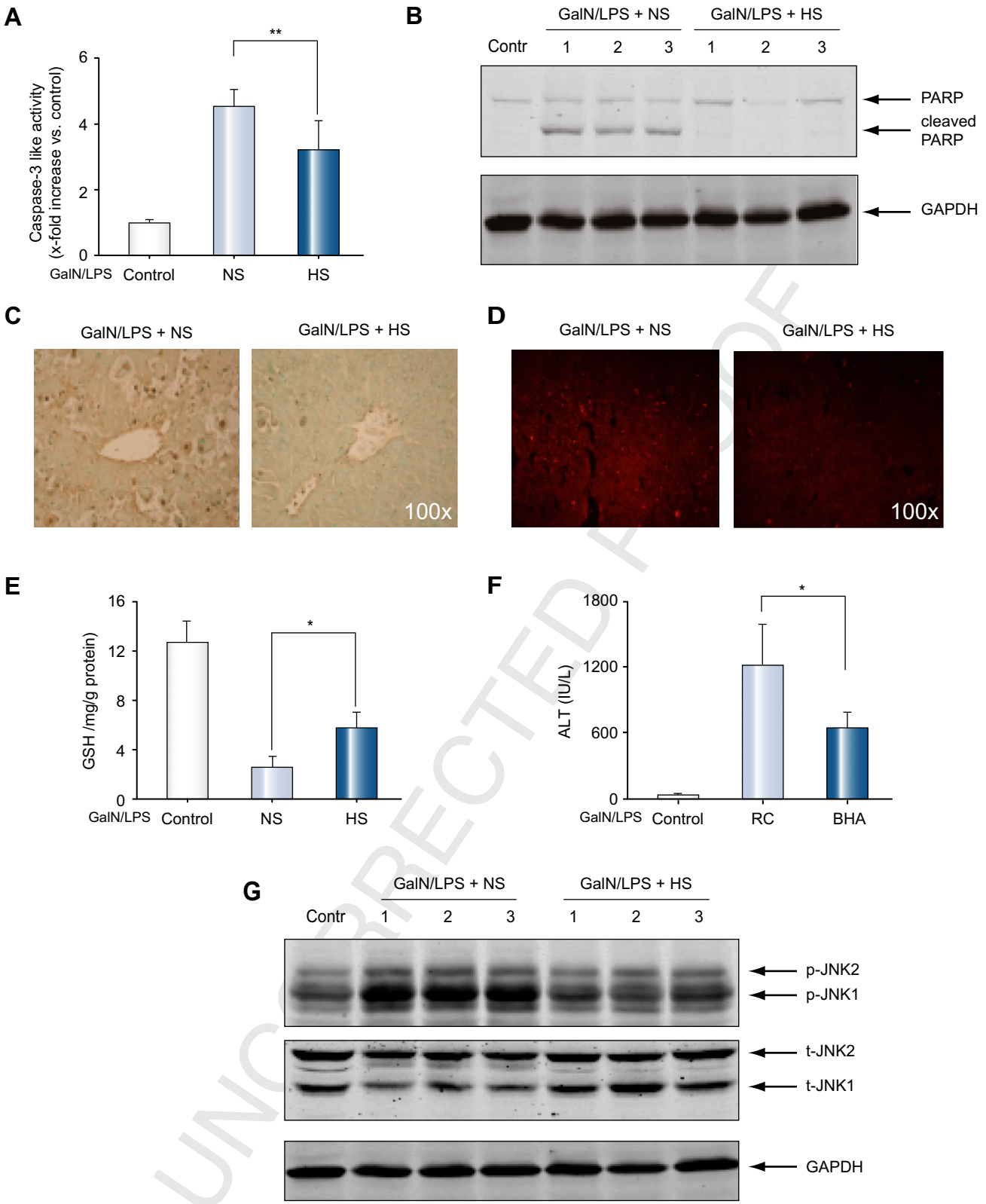

Fig. 3. HS attenuated ROS-induced pro-apoptotic signaling in GalN/LPS challenged mice. (A) Caspase-3 like activity assay, controls were defined as 1.0 ($n = 8$, mean ± SD, $^{**}p < 0.01$ vs. NS). (B) Hepatic expression of PARP, cleaved PARP, GAPDH. (C) Liver histology stained with TUNEL. (D and E) Accumulation of hepatocyte superoxides assessed by staining freshly frozen liver sections with dihydroethidine (DHE) and measuring hepatic GSH levels ($n = 8$, mean ± SD, $^{*}p < 0.05$ vs. NS). (F) Effects of BHA on serum transaminase activities in GalN/LPS-treated mice [$n = 8$, mean ± SD, $^{*}p < 0.05$ vs. RC (regular chow)]. (G) Hepatic expression of t-JNK, p-JNK and GAPDH.

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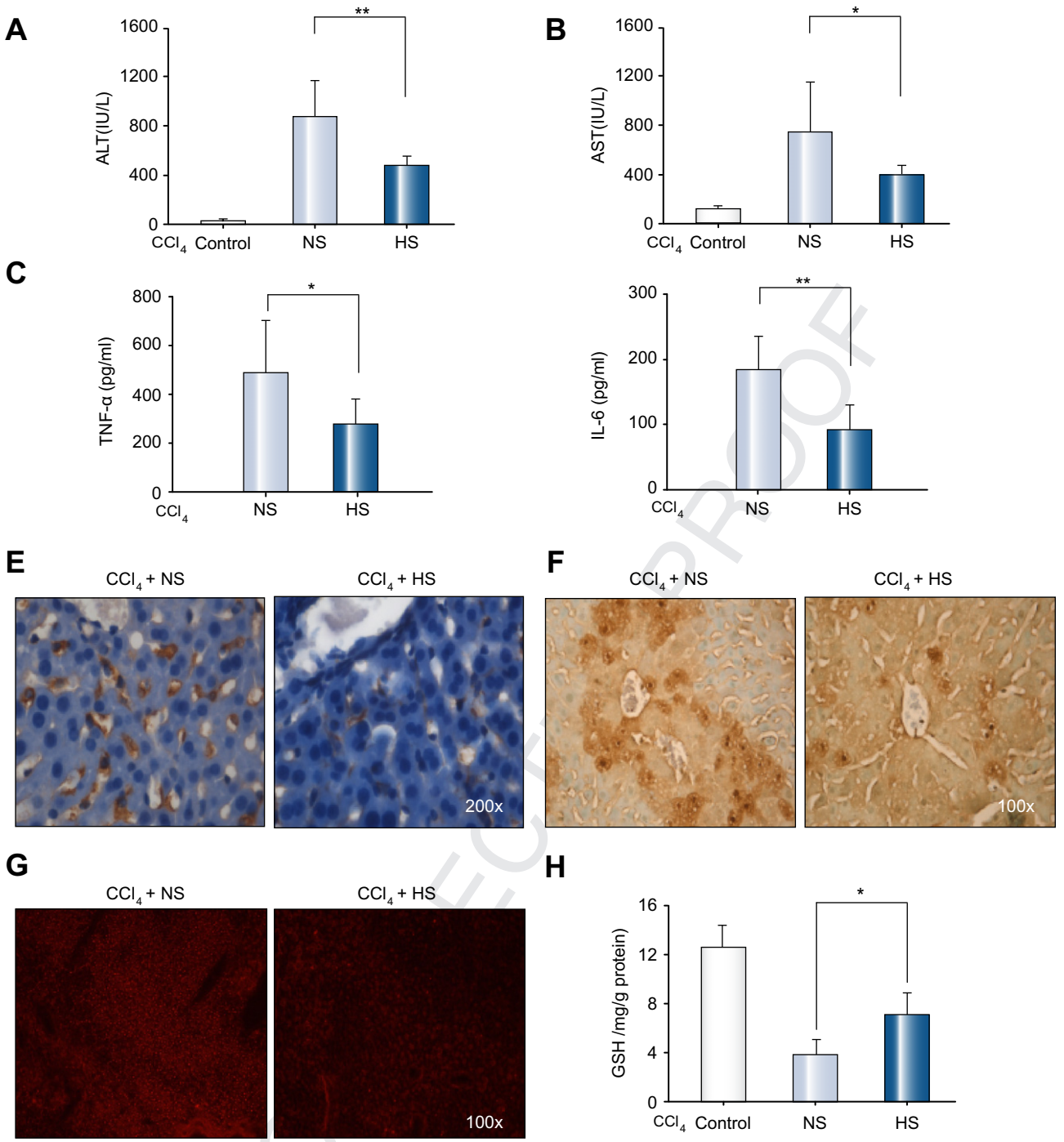

Fig. 4. Role of HS in acute liver injury 12 h after CCl_4 challenge. (A and B) ALT and AST serum levels of mice ($n = 8$, mean ± SD, $^{**}p < 0.01$, $^{*}p < 0.05$, vs. NS). (C and D) TNF-α and IL-6 serum levels of mice determined by ELISA ($n = 8$, mean ± SD, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. NS). (E) IHC staining with F4/80 antibody of mice liver sections. (F) Liver histology stained with TUNEL. (G) and (H) Accumulation hepatocyte superoxides assessed by staining freshly frozen liver sections with dihydroethidine (DHE) and measuring hepatic GSH levels ($n = 8$, mean ± SD, $^{*}p < 0.05$ vs. NS).

HS attenuated acute liver injury in the CCl_4 model of hepatitis

To examine whether HS also controlled ROS accumulation and in turn attenuated liver injury, we injected HS via the peritoneal into CCl_4-treated mice. CCl_4 challenge increased the serum levels of ALT and AST by approximately 24- and 6-fold, respectively. These levels were markedly lowered after administration of HS (Fig. 4A and B). Measurement of serum TNF-α and IL-6 also indicated the protective effects of HS against the release of injury-mediated cytokines (Fig. 4C and D). In addition, IHC and apoptosis analysis revealed a decrease in the amount of macrophage infiltration and TUNEL-positive hepatocytes in the HS-treated group

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(Fig. 4E and F). Furthermore, the reduced accumulation of superoxides and an increase in GSH content were detected in livers of CCl_4-treated mice followed with HS administration (Fig. 4G and H). Taken together, these data indicate that HS could attenuate CCl_4-sensitized acute liver injury.

Chronic CCl_4 treatment-induced hepatic cirrhosis was alleviated in the presence of HS

To investigate whether HS has protective effects against CCl_4-induced chronic liver injury and cirrhosis, collagen deposition, and hepatic stellate cell (HSC) activation were examined between groups treated with CCl_4 plus NS or CCl_4 plus HS injection. After CCl_4 administration, mice were injected intraperitoneally once every day with a single dose of HS. As shown in Fig. 5A and B, sirius red staining and hydroxyproline content gradually increased after chronic CCl_4 treatment, but were significantly reduced in the HS injection group. Western blot and IHC analyses also revealed a similar reduction of α-SMA expression in liver sections (Fig. 5C and D). Furthermore, we examined the mRNA expression of early markers of fibrogenesis, including collagen-α1 (encoded by Col1a1) (Fig. 5E) and α-SMA (encoded by Acta2) (Fig 5F) [24], and observed approximately 50% reduction upon HS injection in the CCl_4 treatment model. These results suggest that HS has a protective capability against CCl_4-induced chronic liver injury and cirrhosis.

HS reduced liver injury and hepatocyte proliferation in DEN-challenged mice

DEN is the chemical procarcinogen that is widely used to induce hepatocarcinogenesis in mouse and rat models. ROS accumulation has been suggested to be a major contributor to DEN-induced HCC by promoting inflammation and stimulating compensatory proliferation [18,25]. As shown in Fig. 6A, serum ALT and AST levels were increased upon DEN administration but reduced after HS injection (HS vs. NS, ALT: 303.40/529.24 IU/L, AST: 237.17/371.64 IU/L). The concentration of the tumor-promoting cytokine IL-6 was also lower in the HS group than in the NS group (Fig. 6B). In addition, IHC analyses revealed that JNK activation was reduced in the DEN plus HS model (Fig. 6C). This was detected by phosphorylation of c-Jun, a specific JNK substrate, which mostly occurred in hepatocytes that were involved in DEN metabolism and ROS production [3]. Interestingly, HS not only reduced acute liver injury, but also inhibited hepatocyte compensatory proliferation. As shown in Fig. 6D, the level of Edu-positive hepatocytes was reduced in HS-treated mice 72 h after DEN administration. Thus, HS has protective capability against DEN-induced acute liver injury and compensatory proliferation.

Discussion

ROS, which include $^{\cdot}OH$, $ONOO^-$, $O_2^{\cdot-}$, H_2O_2, and $NO^{\cdot}$, are important cytotoxic and signaling mediators in the pathophysiology of inflammatory liver diseases [26,27]. Among them, $^{\cdot}OH$ and $ONOO^-$ are much more reactive than others and have been regarded as major cytotoxic mediators of cellular oxidative damage [28–30]. Previous studies have reported that H_2 reacts only with the strongest oxidants ($^{\cdot}OH$ and $ONOO^-$), which is advantageous for medical procedures, since H_2 is mild enough not to disturb metabolic oxidation reduction reactions or disrupt ROS involved in cell signaling—unlike some antioxidant supplements with strong reductive reactivity [7]. We now demonstrated that hydrogen-saturated saline also prevents ROS accumulation, cytokine production, and cell death in various types of liver injury.

Gas chromatography-based technology has been successfully applied to examine the concentration of H_2 in blood, and it has been reported that dissolved H_2 in arterial and venous blood was increased by the inhalation of H_2 or the administration of H_2-water [31]. It was supposed that the elevated H_2 level in serum might lead to the incorporation of H_2 into organs, and thus plays a pivotal protective role in oxidative stress-induced tissue damage. The facts that H_2 protected mitochondria and nuclear DNA and that the amount of H_2 dissolved in venous blood was less than that in artery blood provided indirect evidences that H_2 could penetrate most membranes and diffuse into organelles. However, there is a lack of direct evidence in vivo that the concentration of H_2 was enhanced after H_2 inhalation or HS administration.

To verify whether the injection of HS could increase the organ levels of H_2, a real time dynamic method with glass-based H_2 microelectrode was developed to accurately, continuously, and directly monitor the concentration of H_2 in abdominal organs for the first time. After HS injection, H_2 concentration in the liver and kidney reached a peak 5 min later and gradually decreased to normal levels after 40 min. The arterial/venous blood pH was also measured after HS administration, and no significant difference was observed between the HS and NS groups (Supplementary Fig. 1), which suggested that HS treatment has no effect on the blood PH. To our knowledge, it is a direct evidence of the diffusion of H_2 in the organs. These data also indicate that it is realizable to prevent ROS accumulation by intraperitoneal administration of HS in the organs, such as the liver and kidney.

Oxidative stress activates various kinds of apoptotic signaling pathways, among which we focused particularly on JNK. This is due to a number of recent reports which have shown that JNK activation, following oxidative stress, induces apoptosis via activation of c-Jun, through the caspase-dependent mitochondria pathway in the liver. In a model of fulminant liver failure (GalN/LPS), a disease that is associated with many complications and high mortality, administration of HS resulted in a marked reduction of liver injury. ROS down regulation by HS or antioxidant BHA, as shown here, led to reduced apoptotic activity (PARP cleavage and caspase-3 activation) as well as decreased inflammatory cytokine release and tissue damage after GalN/LPS challenge. Importantly, phosphorylation, and consequent activation of the pro-apoptotic kinase JNK, was blocked after HS administration (Fig. 3G) or BHA induction (data not shown). This indicated that HS may be exerting its protective role by preventing the activation of the ROS-JNK-caspase-3 pathway. Moreover, the accumulation of Kuffer cells in the liver (Fig. 2C) was also attenuated after HS injection, which may lead to further decrease of inflammatory cytokine (such as TNF-α, IL-6) production and release.

CCl_4 and DEN-sensitized AHF are the other two settings where ROS accumulation was thought to be responsible for liver damage [18,25,32,33]. As shown in Figs. 4 and 6, HS resulted in a similar beneficial outcome as seen in the model of GalN/LPS-induced liver damage by scavenging for ROS and inhibiting the activation of its downstream JNK pathway (data not shown). The level of

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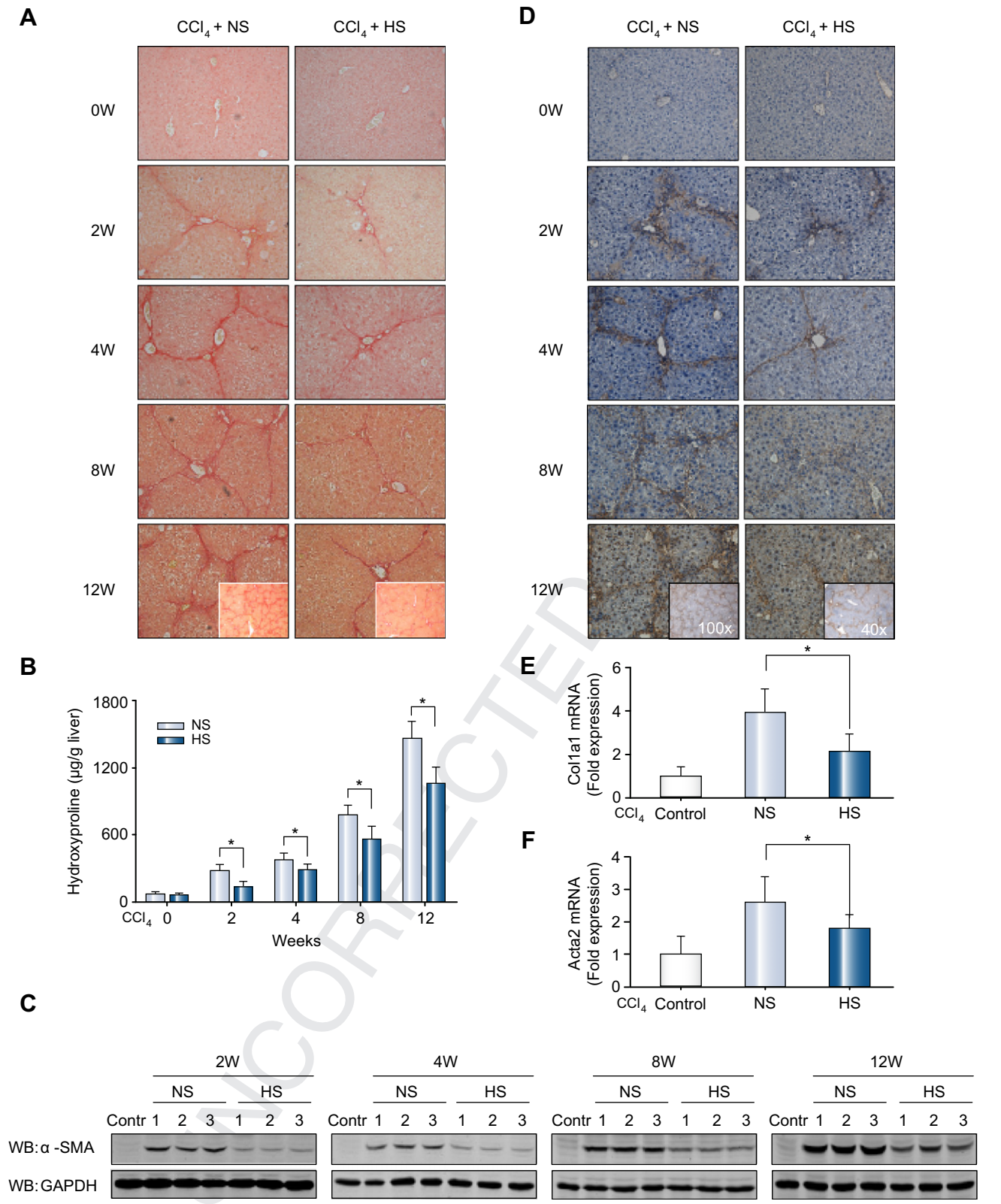

Fig. 5. HS reduced hepatic cirrhosis 12 W after CCl_4 treatment. (A and B) Collagen deposition was evaluated by sirius red staining and hydroxyproline measurement 2, 4, 8, and 12 W after CCl_4 challenge. (C and D) Expression of a-SMA was determined by Western blot analysis and IHC. (E and F) Hepatic levels of Col1a1 (E), Acta2 (F) mRNA were measured by qPCR in the HS group ($n = 8$) and NS group ($n = 8$) 72 h after CCl_4 challenge and gene expression in control group was arbitrarily assigned the value of 1 ($^*p < 0.05$ vs. NS).

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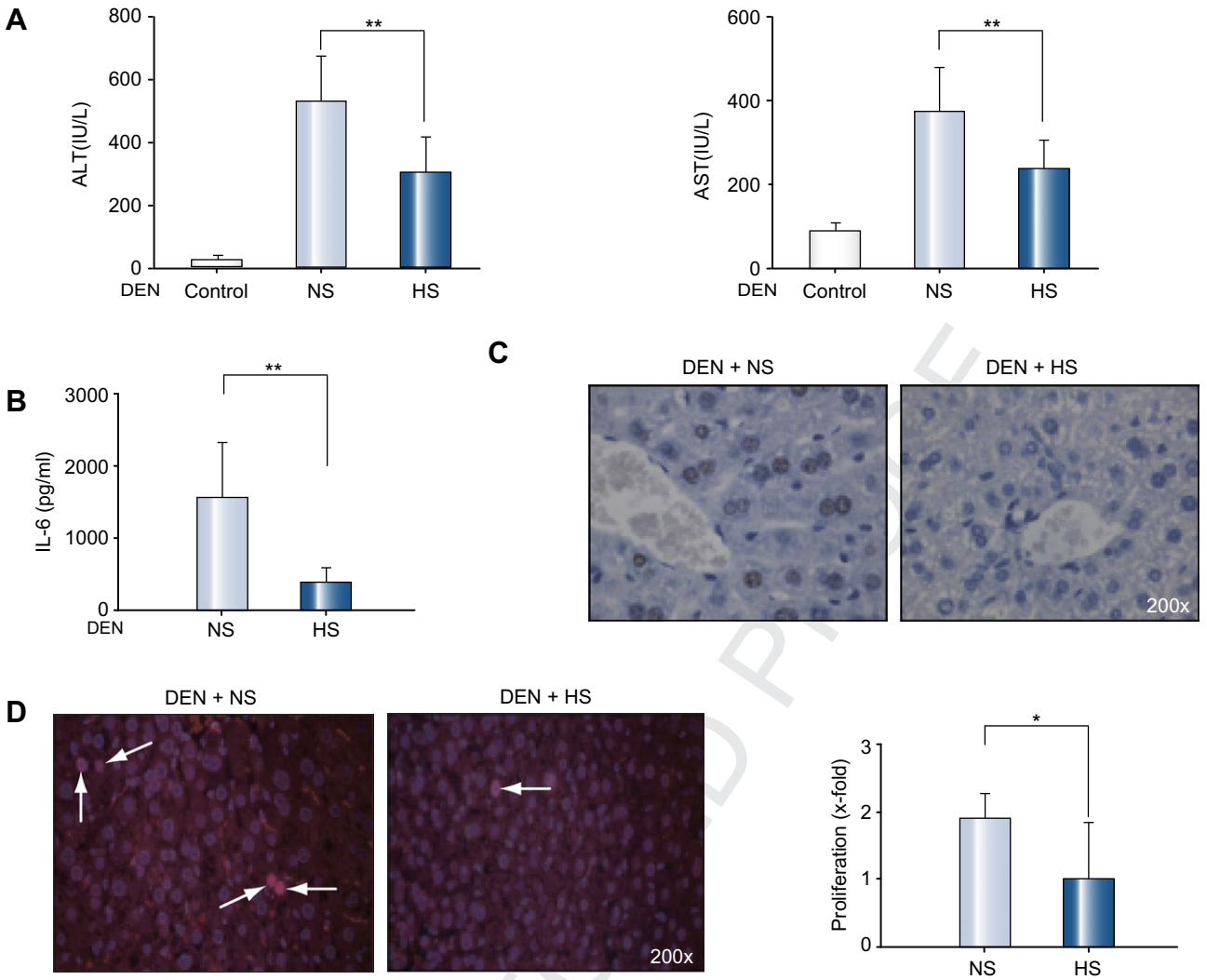

Fig. 6. Effects of HS treatment in acute liver injury after DEN challenge. (A) ALT and AST levels in serum were determined 48 h after DEN injection. ($n = 8$, mean ± SD, $^{**}p < 0.01$ vs. NS). (B) Role of HS on serum IL-6 4 h after DEN challenge. ($n = 8$, mean ± SD, $^{**}p < 0.01$ vs. NS). (C) Expression of phospho-c-Jun in DEN-treated livers. (D) Hepatocyte proliferation (upper panel) was measured by Edu incorporation at 72 h and quantified by counting five randomly chosen high-power fields ($^{*}p < 0.05$).

serum transaminases and the concentration of inflammatory cytokines in serum were lower in the HS group than in its counterpart NS group. Histopathological findings also demonstrate the HS protective effects to AHF.

Liver cirrhosis is a common scarring response to all forms of chronic liver injury and is always associated with inflammation that contributes to fibrogenesis. The use of antioxidants, such as SAMe and vitamin E, has been reported to successfully delay the progress of hepatic cirrhosis and reduce liver damage [34]. In line with this notion, the effect of HS in the model of CCl_4-induced chronic liver damage was observed. Both the collagen deposition and nodule number were inhibited in the presence of HS. It is the first report of the protective role HS can play in chronic liver injury, and suggests that HS could be used to prevent and retard fibrogenesis in medical application. Further studies with other models of cirrhosis are warranted.

A causal link between ROS accumulation and cancer has been proposed. Previous results obtained in a mouse model in which HCC was induced by the chemical procarcinogen DEN suggest that DEN-induced oxidative stress leads to hepatocyte death, cytokine release, compensatory proliferation, and eventually, HCC development [35]. We now showed that HS could reduce transaminase activities and inflammatory cytokine (IL-6) production in DEN-induced liver injury. IL-6 is a multifunctional cytokine, which is largely responsible for compensatory hepatocyte proliferation that has a critical role in DEN-induced hepatocarcinogenesis [36]. Indeed, we also found the remarkable reduction in DEN-induced hepatocyte proliferation in the HS group (Fig 6B). Further investigation on the contribution of HS to the development of HCC should be performed.

In conclusion, we herein presented a novel antioxidant-HS, which is easier and safer to apply than H2 gas, and could selectively remove ROS. We examined the impact of HS in the inflammatory models of GalN/LPS, CCl_4, and DEN challenge, respectively. HS attenuates liver injury and also inhibits the processes leading to liver cirrhosis and hepatocyte compensatory proliferation. This reveals the potential application of HS to target oxidative stress and alleviates liver injury clinically.

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Financial support

This research was supported by the projects from the National Natural Science Foundation of China (Nos. 30921006, and 2008ZX10002), and the China Key Basic Research Program (No. 90713032, 06MA154, 2006AA02A249, 06Z059, and 08Z20), and is supported by the Grants from Shanghai (Nos. 09CG33, 10QA1408700).

Acknowledgements

The authors thank Dr. Atsunori Nakao from University of Pittsburgh Medical Center for his helpful discussions and suggestions in the preparation of this paper. The authors also thank DongPing Hu, LinNa Guo, and Dan Cao for technical assistances.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2010.08.011.

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Please cite this article in press as: Sun H et al. The protective role of hydrogen-rich saline in experimental liver injury in mice. J Hepatol (2010), doi:10.1016/j.jhep.2010.08.011