Original Article
Hydrogen-rich saline attenuates carbon tetrachloride induced acute liver injury and c-Jun N-terminal kinase activation in rats

Xing-Feng Zheng1*, Fang Zhang1*, Xue-Jun Sun2, Atsunori Nakao3, Feng-Yong Yang4, Guang-Qing Wang1, Zhao-Fan Xia1

1Department of Burn Surgery, Changhai Hospital, Second Military Medical University, Shanghai 200433, China; 2Department of Diving Medicine, Second Military Medical University, Shanghai 200433, China; 3Department of Emergency and Critical Care Medicine, Okayama University, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan; 4Intensive Care Unit, The People’s Hospital of Laiwu City, Laiwu 271199, China. *Equal contributors.

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Abstract: Oxidative stress plays critical roles in the pathogenesis of chemically induced liver injury. In this study, the efficacy of hydrogen-rich saline as an antioxidant to protect against acute hepatic injury induced by carbon tetrachloride (CCl4) in rats was investigated. CCl4 administration led to liver injury in rats as shown by pathological observation, hepatic enzyme activities, and bilirubin determination, while hydrogen-rich saline markedly ameliorated liver injury caused by CCl4 toxicity. The hepato-protective effect of hydrogen-rich saline was also verified by survival analysis in CCl4-intoxicated rats. In addition, hydrogen-rich saline alleviated the hepatic apoptosis, as manifested by TUNEL analysis and caspase-3 activity measurement. Furthermore, hydrogen-rich saline attenuated the elevation of malondialdehyde (MDA) and protein carbonyl and also the reduction of glutathione caused by CCl4. Moreover, hydrogen-rich saline diminished the increase of TNF-α and phosphorylation of c-Jun N-terminal Kinase (JNK) caused by CCl4. In conclusion, hydrogen-rich saline attenuates CCl4 induced liver injury in rats through anti-oxidative, anti-inflammatory, and anti-apoptotic pathways, which might be mediated by inhibition of JNK activation.

Keywords: Hydrogen-rich saline, carbon tetrachloride, hepatotoxicity, antioxidant, c-Jun N-terminal kinase

Introduction

Drug or chemicals induced hepatic injury is a thorny clinical problem widely concerned by doctors in various Departments. The incidence of drug or chemicals induced hepatotoxicity has risen in recent years [1]. Although many achievements on this question have been gained, it is still unclear of the deep-going mechanisms of drug or chemicals induced hepatotoxicity are the treatment of such liver diseases is a major challenge faced by clinicians [2]. Oxidative stress is considered to play a critical role in the pathophysiological development of liver damage. Over production of free radicals are toxic to hepatocytes and initiate reactive oxygen species (ROS)-mediated cascade reaction causing acute hepatic injury [3].

Carbon tetrachloride (CCl4) is a lipid soluble potent hepatotoxic and well-known compound for producing chemically-induced liver injury [4]. CCl4 generates free radicals causing membrane lipid peroxidation, DNA damage, and eventual liver injury [5, 6]. Earlier reports indicated that anti-oxidant treatment was a potential means of preventing or treating toxic liver injury by reducing free radical generation or scavenging the generated free radicals [7]. A number of antioxidative agents have been experimentally used in the model of CCl4 induced hepatotoxicity [8-11].

Hydrogen, the lightest gas, is routinely used in diving medicine as hydreliox which contains 49% hydrogen [12]. Dole et al. observed that hyperbaric hydrogen treatment caused a marked aggression of the skin tumors in mice [13]. Gharib et al. reported that it was helpful to relieve schistosomiasis-associated chronic liver injury by treating animals in a hydrogen-supplemented hyperbaric chamber [14]. Recently, hy-
Hydrogen gas has been recognized as a potent anti-oxidative substrate as it could selectively scavenge reactive oxygen species (ROS), such as •OH and ONOO- in vitro and the inhalation of non-pressurized hydrogen gas can be effective in models of ischemia-reperfusion injury [15-17]. In addition, hydrogen was proved to be protective in patients with ischemia diseases such as cerebral infarction [18] and hypercholesterolemia [19].

Although these investigations discovered the benefits of hydrogen on various diseases, the effects of hydrogen for chemically induced liver injury have not been clarified. We developed hydrogen-saturated physiological saline, which might be easier and safer than inhalation of hydrogen gas for clinical application. Our previous studies proved that hydrogen-rich saline exerted neuroprotective effects in neonatal hypoxia-ischemia injury [20] and protected the intestinal ischemia-reperfusion injury [21] and the associated acute lung injury [22]. Here, whether the administration of hydrogen-rich saline exerted protective effects in CCl₄ induced acute hepatic injury and its mechanism were investigated.

Materials and methods

Animals

Sprague-Dawley rats (male, weighing 220-250 g) were used in this study. The experimental procedure in this study was conducted according to the guidelines of the Animal Care and Use Committee of the Second Military Medical University in Shanghai, China and the Guiding Principles in the Use of Animals in Toxicology, which were adopted by the Society of Toxicology in 1989.

Experimental design

CCl₄ (Sigma, St. Louis, MO) mixed with olive oil (1:1 v/v, 2 ml/kg) was injected intraperitoneally for hepatic injury model. Control rats were injected intraperitoneally with olive oil (2 ml/kg) instead. Hydrogen-rich saline (5 ml/kg) or nitrogen-rich saline of equivalent volume was given intraperitoneally 10 minutes prior to the injection of CCl₄ and every 3 hours after the administration of CCl₄. Hydrogen-rich saline and nitrogen-rich saline were produced as described in our previous study [21]. After the animals were euthanized, blood and liver tissues were taken for assays.

For survival analysis experiment, rats were injected intraperitoneally with a lethal dose of CCl₄ mixed with olive oil (1:1 v/v, 7.5 ml/kg). The mortality of rats was recorded 48 hours following CCl₄ injection.

Histopathological study of the liver

Histopathological changes were examined by two independent and blinded researchers in liver sections after hematoxylin and eosin (H&E) staining. Ballooned hepatocytes were counted to assess the degree of histological damage in liver injury. For each microscopic section, 10 visual fields with 400× magnification were analysed and the counts of ballooned hepatocytes per visual field were averaged.

Serum biochemistry

Serum aminotransferase (AST) [23], alanine aminotransferase (ALT) [24] and lactate dehydrogenase (LDH) activities [25], serum direct bilirubin (DBIL) and total bilirubin (TBIL) levels [26] were determined by an automatic blood biochemical analyzer (7600-120, Hitachi High-Technologies Corp., Tokyo, Japan) using commercial kits.

Measurement of hepatocyte apoptosis

Apoptotic hepatocytes were assayed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining according to the manufacturer’s recommendations of In Situ Cell Death Detection kit (Roche, Mannheim, Germany) [27]. TUNEL-positive and negative cells were counted in randomly selected fields of view under light microscopy (×100) by two independent researchers. The mean number of TUNEL-positive cells was evaluated as apoptotic index (%) (Number of apoptotic cells/total number of cells ×100).

Caspase-3 activities were determined using fluorometric caspase activity detection kits (Calbiochem, La Jolla, CA) [28]. Tissue lysates were centrifuged at 10,000 g for 10 minutes at 4°C and the supernatant was collected. Cell lysate (50 ul) was added to a black opaque 96 well plate along with an equal volume of reaction buffer with freshly prepared DTT to a final
concentration of 10 mM and incubated for 30 minutes at 37°C. Finally, 10 ul of caspase 3 fluorogenic substrate (DEVD-AFC) was added, and the plate was incubated in the reading chamber of FlexStation® II Microplate Reader (Molecular Devices, Sunnyvale, CA) at 37°C. Fluorescence was continued measured for 1 hour at 5 minutes intervals with a 400 nm excitation and a 505 nm emission filter. Data are expressed as the rate of increase in relative fluorescent units (the slope of the line) between readings at zero time and 1 hour after the addition of substrate per microgram of protein.

Estimation of malondialdehyde (MDA), protein carbonyl and glutathione (GSH) levels

Liver tissues were homogenized in ice-cold 0.15 M KCl and MDA was measured by the TBA reaction method [29]. In brief, samples were mixed with TBA reagent consisting of 0.375% TBA and 15% trichloroacetic acid in 0.25 N HCl. The reaction mixtures were placed in a boiling water bath for 40 minutes. The absorbance of the supernatant following centrifugation at 2000 g for 5 minutes was measured at a wavelength of 532 nm. MDA was calculated by an extinction coefficient of 1.56×10^5/M cm. The results are expressed as nmol/mg protein.

Protein carbonyl contents in liver tissues were determined by spectrophotometric DNPH (2, 4-dinitrophenylhydrazine) assay. Briefly, the homogenate was added with streptomycin and then centrifuged. The supernatants were incubated with DNPH for one hour and then precipitated by TCA. The pellet was washed with ethanol-ethyl acetate and then resuspended in guanidine hydrochloride. After centrifuged the supernatants were incubated at 37°C for 10 minutes. Finally, the absorbance was measured at 366 nm wavelength and protein carbonyl contents were expressed as nmol/mg protein [30].

For GSH content determination, liver tissues were homogenized in 5% 5-sulfosalicylic acid. Samples were added with 0.1 M of sodium phosphate buffer (pH 7.4) containing 5 μM EDTA, 0.6 mM of 5, 5-dithiol-bis (2-nitrobenzoic acid), 0.2 mM NADPH and glutathione reductase and then incubated for 10 minutes at room temperature. The absorbance of the product was measured at a wavelength of 412 nm. GSH content was determined using a standard curve generated from known concentrations of GSH [31]. The results are expressed as nmol/mg protein.

Protein concentration was determined on the homogenates by BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as the standard [32]. All of the analyses above were performed in duplicate.

Serum cytokine determination

TNF-α level in serum were determined by highly sensitive enzyme-linked immunosorbent assay (ELISA) kits from R&D systems according to the manufacturer's instruction.

Western blot analysis of JNK phosphorylation

Liver tissues were homogenization on ice in five volumes of the extraction buffer. All debris and nuclei were removed by centrifugation at 10000 g at 4°C for 10 minutes, and the supernatant was used for Western blot analysis. Detection of phosphorylated and total c-Jun N-terminal kinase (JNK) was done using phospho JNK and JNK antibody (Cell Signaling, Beverly, MA) respectively and horseradish peroxidase-linked secondary anti-rabbit IgG antibody (Calbiochem, La Jolla, CA). Immunoactive protein bands were revealed by ECL chemiluminescence agents (Amersham, Little Chalfont, UK). Densitometry was performed using a gel documentation system (Fluor-S-Multimager and Quantity One Analysis Software, Bio-Rad, Hercules, CA).

Statistical analysis

All data are expressed as the mean ± SEM. Results were analyzed by computerized statistical packages (SPSS). Each mean value was compared by one-way analysis of variance (ANOVA) and Student-Newman-Keuls (SNK) for multiple comparisons as the post hoc test. Survival time was compared by using the log-rank test. P-values less than 0.05 were considered statistically significant.

Results

Impacts of hydrogen-rich saline on survival time of rats intoxicated by CCl₄

The hydrogen-rich saline group had a significant survival advantage over the nitrogen-rich
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Table 1. Effect of hydrogen-rich saline on serum bilirubin and hepatic enzymes activities in CCl4-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CCl4+N2</th>
<th>CCl4+H2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBIL (umol/L)</td>
<td>1.15±0.22</td>
<td>5.71±0.90*</td>
<td>2.84±0.51*</td>
</tr>
<tr>
<td>DBIL (umol/L)</td>
<td>1.02±0.15</td>
<td>5.04±0.82*</td>
<td>2.51±0.49*</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>40.20±2.48</td>
<td>549.31±79.70*</td>
<td>235.15±58.53*</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>99.59±9.23</td>
<td>1395.65±140.92*</td>
<td>693.56±110.34*</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>225.21±31.56</td>
<td>2090.75±388.50</td>
<td>902.19±107.21*</td>
</tr>
</tbody>
</table>

Rats were randomly assigned into three groups and treated as described in Materials and Methods. Blood samples were collected 12 hours after the CCl4 administration. Data are expressed as means ± SEM for triplicate independent experiments (n = 8 per group in each independent experiment). *P < 0.01 vs. the control group; #P < 0.05 vs. the CCl4+nitrogen-rich saline group.

Effects of hydrogen-rich saline on hepatic damage induced by CCl4

Administration of CCl4 to rats significantly increased the serum activities of ALT, AST, LDH and levels of DBIL and TBIL compared to those in control group at 12 hours after CCl4 injection. Treatment of hydrogen-rich saline significantly attenuated the increases of serum enzymes ALT, AST, and LDH activities and serum DBIL and TBIL levels in comparison with those in CCl4 plus nitrogen-rich saline group (Table 1).

No obvious histological change was detected in the liver of control animals, who received vehicle (olive oil) only (Figure 1A). The ballooning of hepatocytes is one of the most obvious histological changes which can be observed in the liver under CCl4 intoxication [33]. A mass of ballooned hepatocytes was observed in the rat liver of nitrogen-rich saline treated group (Figure 1B). Histological analysis showed that treatment with hydrogen-rich saline markedly ameliorated this change of ballooning of hepatocytes induced by CCl4 (Figure 1C, 1D).

Effects of hydrogen-rich saline on hepatocyte apoptosis induced by CCl4

As hepatocytes damage in acute liver injury induced by CCl4 has been attributed to apoptosis in addition to necrosis, we examined apoptotic cells in the liver of intoxicated rats with nitrogen-rich saline or hydrogen-rich saline treatments. By TUNEL staining detection, the number of apoptotic hepatocytes was significantly larger the liver of CCl4 plus nitrogen-rich saline treated rats than that in the control group (Figure 1A, 1B). However, hydrogen-rich saline significantly reduced the increase of apoptotic cells in the liver, comparing with CCl4 plus nitrogen-rich saline treated rats (Figure 2C, 2D).

Furthermore, because caspase-3 plays a key role in various forms of apoptosis, this enzyme activity could be altered by hydrogen-rich saline treatment and was thus investigated further. The results suggested that hydrogen-rich saline administration significantly diminished the hepatic caspase-3 activity elevation induced by CCl4 intoxication (Figure 2E).

Effects of hydrogen-rich saline on oxidative stress in liver induced by CCl4

In order to evaluate the effect of hydrogen-rich saline treatment on CCl4 induced oxidative stress in liver, the levels of MDA, one of the principal products of lipid peroxidation, as well as protein carbonyl, an indicator of protein oxidation were monitored. CCl4 exposure increased hepatic MDA concentration significantly as compared with that in control rats, which is inhibited significantly by hydrogen-rich saline treatment (Figure 3A). Additionally, CCl4 exposure also increased hepatic protein carbonyl level markedly as compared with that in control rats, which is inhibited significantly by hydrogen-rich saline treatment (Figure 3B).

Effects of hydrogen-rich saline on liver GSH and serum TNF-α induced by CCl4

GSH is present in most living cells and participates in a variety of vital cellular reactions. In particular, GSH is a potent antioxidative enzyme protecting cells and is associated with the process of detoxification. Figure 3C illustrates that GSH content significantly decreased in the liver of CCl4 administrated rats compared to that in control rats and hydrogen-rich saline treatment significantly restored the GSH level reduced by CCl4.

The pro-inflammatory cytokine TNF-α was tested in serum and found to play an important role in the inflammatory response induced by CCl4. Serum TNF-α level was significantly increased
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in rats administrated with CCl₄ compared to that in control rats and the hydrogen-rich saline treatment lowered the elevation significantly (Figure 3D).

Effects of hydrogen-rich saline on liver JNK activation induced by CCl₄

In addition, hydrogen-rich saline was detected on liver JNK activation induced by CCl₄. JNK activation was revealed by the ratio of phosphorylated to total forms of JNK. CCl₄ exposure significantly activated JNK in the liver of rats and hydrogen-rich saline treatment reduced the JNK activation markedly as compared with that in the nitrogen-rich saline treated rats (Figure 4).

Discussion

This study demonstrates that hydrogen-rich saline can attenuate liver damage functionally and morphologically and prolong the survival time of rats in the model of CCl₄ induced hepatic injury. Hydrogen-rich saline was found to significantly reduce the elevation of serum hepatic enzymes activities and bilirubin levels, which was consistent with the histopathological findings. Furthermore, hydrogen-rich saline diminished the increase of hepatocytes apoptosis as shown by immunohistochemical findings and caspase-3 activity.

The liver is vulnerable to frequent exposure to environmental toxicants, drugs, and other substances. CCl₄ is a potent toxicant inducing oxidative stress associated hepatic injury. It is metabolized in the liver by the cytochrome P450 oxidase system, producing the reactive trichloromethyl radical (•CCl₃) and the trichloromethyl-peroxyl (•OOCCl₃) radical [34]. The liver injury induced by CCl₄ results from both covalent binding of CCl₄ metabolites and lipid peroxidation. Moreover, •CCl₃ and •OOCCl₃ can be an initiating or triggering factor in the progress of liver injury. The hepatotoxicity of CCl₄ is also magnified by formation of other metabolites or biological molecular. In other words, some secondary mechanisms can be involved in the degenerative effects of the hepatocytes [6]. These secondary processes of CCl₄ toxicity include Kupffer cells activation, inflammatory mediator production, signaling transduction pathway activation, etc.

The production of excessive ROS is also a critical event responsible for the toxicity of CCl₄, including hydrogen peroxide, superoxide, hy-
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hydroxyl radicals, etc. CCl₄-induced hepatotoxicity was significantly inhibited by 5,6,7,8-tetrahydronopterin (NPH₄), which could exert high scavenging activity against superoxide anion radicals [35]. CCl₄-induced hepatotoxicity was enhanced in acatalasemic mice in whom the catalase activity was decreased [36]. In addition, Campo et al. reported that hydroxyl radical formation was significantly elevated in CCl₄-treated rats in comparison with that in control rats.

The hydroxyl radical are known to be the most reactive product of intracellular ROS produced in vivo, which is generated from superoxide anion and hydrogen peroxide by the Haber-Weiss reaction or from hydrogen peroxide by the Fenton reaction [37]. Superoxide anion and hydrogen peroxide can be destroyed by antioxidant enzymes in biological system, such as superoxide dismutase, glutathione-peroxidase et al. [21]. However, no endogenous enzymatic pathway has been known to eliminate hydroxyl radicals. Accordingly, targeting hydroxyl radicals should be vital in alleviation of CCl₄-induced hepatic injury. For example, a hydroxyl radicals and peroxyl radicals scavenger, edaravone, have been demonstrated to have an inhibitory effect on acute liver injury in CCl₄ treated rats [38].

Ohsawa et al. has revealed that hydrogen gas could be used as a therapeutic antioxidant to reduce oxidative stress in ischemia-reperfusion injury. Biochemical research revealed that hydrogen gas may selectively scavenge the hydroxyl radical [15]. Since then, hydrogen at therapeutic concentration has been shown to be beneficial against pitfalls associated with oxidative injury including ischemia-reperfusion organ injury [15-17, 39], sepsis [40], diabetes [41], myocardial infarction [42], and traumatic brain injury [43]. In the current study, hydrogen-rich saline could protect from hepatic damage and reduce oxidative stress induced by CCl₄ as indicated by the marker, MDA, and protein car-

Figure 2. Effect of hydrogen-rich saline treatment on hepatocytes apoptosis in CCl₄-treated rats. Rats were randomly assigned into three groups and treated as described in Materials and Methods. The livers were removed for TUNEL staining or caspase-3 activity determination 12 hours after the CCl₄ administration. Representative photomicrographs of liver sections of rats in (A) the control group, (B) the CCl₄ plus nitrogen-rich saline treated group and (C) the CCl₄ plus hydrogen-rich saline treated group (400×). (D) Apoptotic index of liver sections in rats of the three groups. (E) Hepatic caspase-3 activity in rats of the three groups. Data are expressed as mean ± SEM for triplicate independent experiments (n = 8 per group in each independent experiment). *P < 0.01 vs. the control group; #P < 0.05 vs. the CCl₄+nitrogen-rich saline group.
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ROS also exhibit proinflammatory properties and inflammatory response is another important mechanism in the pathogenesis of severe hepatic injury independent of the initial insult. It is already known for a long time that TNF-α signaling is associated with enhanced generation of ROS. Secondary liver injury following CCl₄ administration can induce inflammatory processes mediated by activated Kupffer cells which release some cytokines including TNF-α [44]. TNF-α plays a critical role in CCl₄-induced liver injury as TNF-α may induce both necrosis and apoptosis of hepatocytes through direct or indirect mechanisms [45]. Our results indicate that administration of hydrogen-rich saline could inhibit the TNF-α elevation and relieve the inflammatory response in the rats with CCl₄-induced liver injury.

Furthermore, we observed that JNK was markedly activated in livers of CCl₄-treated animals, which is consistent with previous reports [46, 47]. JNK is a stress activated protein kinase, which is primarily activated by various environmental stresses including oxidative stresses and pro-inflammatory cytokines. It has been well established that the production of ROS, which act as intracellular signaling molecules in stress response [48], is responsible for the prolonged activation of
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JNK [49]. In addition, JNK has been shown to be activated by TNF-α [50]. JNK plays a critical role in the initiation of apoptotic pathways in oxidative stress induced liver injury [51, 52]. SP600125, an inhibitor of JNK, could protect mice from D-galactosamine/lipopolysaccharide-induced hepatic failure [53]. Therefore, reduction of oxidative stress and TNF-α level induced by hydrogen might contribute to the decrease of JNK activation and the downregulation of JNK activation might be an important signaling component involved in the hepatoprotective effect of hydrogen [54]. The exact mechanism how hydrogen affects this signaling pathway and whether other signaling transduction would be affected remain to be studied in the future. In addition, seeking for the other molecular events except for hydroxyl radical scavenging, on which hydrogen could target, associating with the hepatoprotective effect of hydrogen is what we need to do in the future studies.

Hydrogen, which had been considered to be not active in chemical reaction under physiological conditions, was used to prevent decompression sickness (DCS) in deep divers for safety profiles [55]. Thanks to the efforts of investigations, we know that hydrogen could react with ROS and exert anti-oxidative beneficial effects either in the high pressure or in the normal pressure. However, hydrogen gas is inflammable and can be explosive. This characteristic might limit its clinical application. In contrast, hydrogen-rich saline should be more convenient to be used in practical. Since hydrogen has been used in human beings safely for a long time and it is much cheap and less pollutant for hydrogen production, we speculate that hydrogen-rich saline would be a cost-effective and clinically feasible therapeutic agent.

In conclusion, this study reports the beneficial effect of hydrogen-rich saline on chemically induced hepatotoxicity. Hydrogen-rich saline might protect against CCl₄ induced liver injury through anti-oxidant, anti-inflammatory, and anti-apoptotic pathways, as well as the inhibition of JNK activation. Our findings indicate that in vivo hydrogen-rich saline administration may be useful for the treatment of oxidative stress-induced liver injury.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhao-Fan Xia and Guang-Qing Wang, Department of Burn Surgery, Shanghai Hospital, Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, China. Tel: (86)21-31161821; Fax: (86)21-65589829; E-mail: xiazhaofan_smmu@163.com (ZFX); Tel: (86)21-31161826; Fax: (86)21-65589829; E-mail: guangqing_wang@yahoo.com (GQW)

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