The protective role of hydrogen-rich saline against liver injury caused by acetaminophen in mice

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Abstract: Background: Acetaminophen (AP) overdose causes acute liver injury inducing the formation of reactive oxygen and nitrogen species. Hydrogen-rich saline (HS) has a protective role for injuries by selectively reducing hydroxyl radical and peroxynitrite as hydrogen. This study evaluated the protective effects of HS on acetaminophen-induced liver injury in mice. Methods: Forty-two mice were divided randomly into three groups: sham, AP and AP+HS groups. The sham group received a single dose of NS (500 mg/kg) intraperitoneal injection. In the AP and AP+HS groups, liver injury was induced by intraperitoneal injection of 500 mg/kg AP. The AP+HS group received HS (6 ml/kg) every 3 hours after AP administration. All animals were killed 24 hours after AP administration. Blood samples and liver tissues were harvested to determine liver injury, inflammatory reaction, oxidative stress parameters, mitochondrial damage and histopathological analyses. Results: In acetaminophen-induced liver injury in mice, HS can significantly decrease the levels of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and inhibit the generations of tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and decrease the contents of the myeloperoxidase (MPO), malondialdehyde (MDA), and increase the reservation of glutathione (GSH). Additionally, HS can also markedly reduce the degree of hepatocyte necrosis and attenuate mitochondrial damage. Conclusions: HS has a protective effect against acetaminophen-induced liver injury in mice.

Keywords: Hydrogen-rich saline, acetaminophen, liver injury

Introduction

Drug-induced liver injury (DILI), which is mainly induced by the drug itself or its metabolic products, may cause liver failure and even lead to human death. Acetaminophen (AP), is a commonly-used analgesic and antipyretic drug that is a major cause of DILI in the United States, northern Europe, and Australia [1, 2]. In the United States, acetaminophen-induced liver injury (AILI) accounts for 39% of all cases [1], while the percentage due to this cause is only 3.99% in China [3].

The mechanism of AILI has been studied for several decades. At therapeutic doses, most of an administered dose of AP is conjugated with glucuronic acid or sulfate and forms nontoxic metabolites and excretes [4]. Only a small fraction is metabolized by cytochrome P450 enzymes to form the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) [4]. Glutathione (GSH), an endogenous antioxidant and detoxifier, initially traps and conjugates with NAPQI to form the GSH adduct to excrete [4-6]. However, at supra-therapeutic doses of AP, both the glucuronidation and sulfation pathways become saturated, the GSH pool is exhausted, and NAPQI is produced abundantly [7]. NAPQI formation leads to the continuous production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) as well [8, 9]. Excessive production of ROS and RNS further aggravates oxidative stress [10, 11] and mitochondrial damage [4, 10]. The damage mitochondria can also generate oxidant stress and produce plenty of ROS and RNS such as hydroxyl radicals (•OH) and peroxynitrite (ONOO⁻) [10], leading ultimately to hepatic necrosis.

ROS and RNS play a central role in the necrosis of hepatocytes in AILI [8, 10, 12], and diverse
antioxidants have been effectively shown to protect against acetaminophen hepatotoxicity [13]. Hydrogen (H₂), a new antioxidant, can react with cytotoxic ROS and RNS to protect against oxidative damage [14]. However, hydrogen-rich saline (HS) has many advantages over H₂, being safe and convenient but providing the same effect as H₂. Due to its antioxidative properties, the protective effect of HS has been proved for damage in the brain and heart, intestinal, renal ischemia-reperfusion injury [15-18], as well as all kinds of liver injuries [19-21]. However, it is still unclear whether HS has any effect in AP-induced liver injury.

Therefore, we designed this experimental study to determine the protective effects of HS in AILI, and to reveal the clinical potential of HS for preventive and therapeutic anti-oxidative applications.

Materials and methods

**Experimental preparation of materials**

HS was provided by the Department of Nautical Medicine of Second Military Medical University. The detailed production method of HS was described in our previous reports [20]. MPO assay reagent was supplied by the Nanjing Jiangcheng Bioengineering Institute (Nanjing, China). Mouse malondialdehyde (MDA) and GSH enzyme-linked immunosorbent assay (ELISA) kits, and AP were purchased from Hefei Bomei Biotechnology (Hefei, China). Mouse tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) ELISA kits came from MultiSciences (Hangzhou, China). Trizol reagent was purchased from Takara Biotechnology (Dalian, China). The primers of mouse gene TNF-α, IL-6, and Glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Generay Biotech (Shanghai, China). The SybrGreenPCR master mix and cDNA synthesis kit came from Xinghan Science and Technology (Shanghai, China).

**Animals**

Male BALB/C mice (20-25 g) were obtained from the Research Center of Chinese Academy of Sciences in Shanghai, China. Forty-two mice were divided randomly and equally into three groups: normal saline (NS), AP, and AP+HS groups. They were kept in an appropriate temperature- and humidity-controlled facility with a 12 h light/dark cycle and free access to food and water. Mice were fasted overnight (12-16 h) prior to administration of AP (purity > 98%, 25 g/L) dissolving in hot saline water before each experiment after acclimation for 7 days. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Second Military Medical University (Shanghai, China).

**Measurement of plasma ALT, AST and ALP**

Activities of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were determined by an automated procedure in the Department of Inspection, Eastern Hepatobiliary Surgery Hospital (Shanghai, China).

**Measurement of hepatic oxidant stress parameters**

Levels of MPO were measured in accordance with the instructions of the manufacturer. Levels of MDA and GSH were measured with a commercial ELISA kit in accordance with instructions of the manufacturer.

**Measurement of plasma TNF-α and IL-6**

Levels of plasma TNF-α and IL-6 were measured with a commercial ELISA kit in accordance with the instructions of the manufacturer.

**Measurement of liver tissue TNF-α and IL-6 mRNAs**

Total liver tissue RNA was isolated with Trizol reagent in accordance with the manufacturer’s instructions. Two microliters of RNA (1000 µg/mL) with 18 µL of cDNA master mix was tran-
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scribed using the cDNA synthesis kit for reverse transcription. For reverse transcription polymerase chain reaction (RT-PCR), we used forward and reverse primers (Table 1) in combination with SybrGreenqPCR master mix. Two microliters of cDNA was subjected to 35 cycles of PCR amplification. GAPDH was used as the internal control. RT-PCR analysis was performed using a Mx3000P real time PCR system. The minimum of the NS group was defined as unit 1.0 in the process of calculation of relative mRNA quantification. Data were analyzed using the real time calculator 1.1 software.

The analysis of hepatic mitochondrial injury

Hepatocyte mitochondria were examined by transmission electron microscopy. Liver specimens were fixed with 2.5% glutaraldehyde and embedded in Spurr’s resin. Thin sections were double-stained with lead and uranyl acetate, and were then observed with a Hitachi JEM-1230 transmission electron microscope at 75 kV.

Histopathological analysis of hepatocellular necrosis

A small portion of the left lobe from liver tissues was placed in cassettes and fixed with 4% neutral formalin. Specimens were dehydrated and embedded in paraffin. Tissue sections of 4 µm were stained with hematoxylin and eosin (H&E) to allow assessment of overall tissue morphology and injury grade. The percentage of the degree of hepatocellular necrosis was estimated under a light microscope of 100 magnifications by two investigators blinded.

Statistical analysis

All results are expressed as mean ± standard deviation. Differences between the experimental and control groups were assessed by analysis of variance (ANOVA) using SPSS 16.0 software (SPSS, Inc.). A P-value of less than 0.05 was considered statistically significant.

Results

Effects of HS on plasma ALT, AST, and ALP in mice with AILI

Effects of HS on liver function markers in mice with AILI are shown in Figure 1. Plasma ALT, AST, and ALP levels of the AP group were significantly higher than those of the NS group. These

Figure 1. Effects of HS treatment on plasma ALT, AST and ALP levels in mice with AILI. Blood samples were collected at 24 h after AILI. Liver injury levels of ALT (A), AST (B), and ALP (C) were determined in plasma. NS, mice were treated with NS; AP, mice were treated with AP; AP+HS, mice were treated with HS and exposed to AP. Data are expressed as mean ± SD, n = 14. *P < 0.05, **P < 0.01.

Figure 2. Effect of treatment with HS on hepatic MPO, MDA, and GSH activity in mice with AILI. Liver MPO (A), MDA (B), and GSH (C) were assessed 24 h after AILI. NS, mice were treated with NS; AP, mice were treated with AP; AP+HS, mice were treated with HS and exposed to AP. Data were expressed as mean ± SD, n = 14. *P < 0.05, **P < 0.01.
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Effects of HS on MPO, GSH, and MDA in mice with AILI

Effects of HS on liver oxidative damage in mice with AILI are shown in Figure 2. Hepatic tissue MPO and MDA levels of the AP group were significantly higher than those of the NS group. However, the liver GSH level decreased significantly in the AP group compared with the NS group. This was increased significantly in the AP+HS group than in the AP group.

Effects of HS on plasma TNF-α and IL-6 in mice with AILI

Effects of HS on the levels of TNF-α and IL-6 in mice with AILI are shown in Figure 3. Plasma TNF-α and IL-6 levels in the AP group were significantly higher than in the NS group. These were significantly lower in the AP+HS group than in the AP group.

Effects of HS on expression of TNF-α and IL-6 mRNAs in mice with AILI

Effects of HS on hepatic TNF-α and IL-6 mRNA levels in mice with AILI are shown in Figure 4. Mouse hepatic TNF-α and IL-6 mRNA levels were significantly higher in the AP group compared with those of the NS group. These levels were significantly lower in the AP+HS group than in the AP group.

Effects of HS on mitochondrial morphological changes in mice with AILI

As shown in Figure 5, the NS group (Figure 5, NS) showed mitochondria is rich and regular shape. In the AP (Figure 5, AP) group revealed that mitochondria was swollen, and mitochondrial cristae was broken or disappeared. However, in the AP+HS (Figure 5, AP+HS) group, the degree of mitochondrial injury was alleviated obviously.

Effects of HS on histopathology in mice with AILI

Liver samples were assessed semi-quantitatively by visual inspection of the sections under a light microscope [22, 23] (Figure 6). The NS group (Figure 6A, NS) showed no abnormality in liver histology, hepatocyte plates were normal, and sinusoidal narrowing or congestion was not observed. The AP group (Figure 6A, AP) revealed a remarkable centrilobular (zone III) hepatic necrosis and liver tissue congestion and swelling, and the hepatic lobule sinus clearance was significantly enlarged. In the AP+HS group (Figure 6A, AP+HS), the area of cellular necrosis was markedly smaller and the hepatocytes appeared more normal in morphology.
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when compared with the AP group. Moreover, the area of hepatocellular necrosis was calculated as shown in Figure 6B. The degree of hepatocellular necrosis in the AP group was significantly higher than in the NS group. These were significantly lower in the AP+HS group than in the AP group.

Discussion

AP is the most widely used over-the-counter painkiller. However, it is also the leading cause of drug-induced acute liver injury in the world [2]. During an AP overdose, the production of ROS and RNS is abnormally elevated in the process of NAPQI formation, resulting in damage to DNA, protein, and lipids [8, 24, 25]. The model of AILI was successfully established, based on the evaluation of plasma enzyme levels such as ALT and AST [24].

The inflammation and injury to hepatocytes usually results in leakage of ALT and AST from the hepatocellular plasma membrane into the bloodstream [24]. In the present study, plasma ALT and AST were significantly higher in the AP group compared with the NS group, and markedly lower in the AP+HS group than in the AP group. According to the Drug Hepatotoxicity Steering Committee of 2005, the ratio of serum ALT to serum ALP has been designated as the R value. Hepatocellular DILI is defined as $R \geq 5$ and $ALT \geq 3ULN$ (the upper limit of normal), cholestasis as $R \leq 2$ and $ALP \geq 2ULN$, and “mixed” as $2 < R < 5$ and $ALT \geq 3ULN$ and $ALP \geq 2ULN$ [25]. In the present study, the R of the AP group was $R > 5$ and $ALT > 3ULN$, indicating that the AILI in mice was hepatocellular DILI. HS treatment in AILI was associated with lower levels of plasma ALT, AST, and ALP, indicating stabilization of liver cell membrane permeability and demonstrating the protective effect of liver function. Furthermore, histopathological examination of liver tissue results showed that with HS treatment hepatocyte morphology was maintained and the degree of hepatocyte necrosis was markedly lower, compared with AP group.

A large number of studies have shown that high doses of AP can produce ROS and RNS and inhibit the function of mitochondria [26], resulting in hepatocyte damage [27-30]. Phagocytes such as Kupffer cells infiltrating the liver can

![Figure 5. Electron microscopic features of hepatic mitochondria in mice with AILI. NS, mice were treated with NS; AP, mice were treated with AP; AP+HS, mice were treated with HS and exposed to AP. Arrows point to areas of mitochondrial damage.](image-url)
recognize damaged hepatocytes and release abundant amounts of ROS and RNS and many proinflammatory cytokines, including TNF-α and IL-6 [5, 31, 32]. The release of TNF-α was found to be responsible for the hepatic injury of AP [31, 33]. Liver regeneration and hepatoprotection required the cytokine IL-6, however, over expression of IL-6 can cause liver injury [34, 35]. In addition, the mRNA expressions of TNF-α and IL-6 in liver tissue were elevated in mice injected with AP [36-38]. In the present study, we measured the levels of plasma TNF-α and IL-6 to evaluate the systemic inflammatory status. Our results showed that TNF-α and IL-6 levels and their mRNA expression in liver tissue were markedly higher in the AP group compared with the NS group. This suggested that these cytokines were involved in hepatotoxicity induced by AP. However, TNF-α and IL-6 were obviously less in the AP+HS group compared with the AP group, indicating that HS may have had a protective effect against AILI by attenuating the production of ROS and RNS, suppressing the infiltration of inflammatory cells and the release of cytokines such as TNF-α and IL-6.

MPO is an important oxygen-dependent enzyme in neutrophils, and if released into local tissue or the systemic circulation can induce oxidative stress, with variable degrees of cytotoxicity [39]. MPO and lipid peroxidation can also generate ROS, such as fatty acid hydroperoxides and alkoxy radicals [10]. MDA originating from lipid peroxidation is a good indicator of oxidative stress [40], which is closely related to AP-induced tissue damage. In the present study, we also observed significantly higher levels of MPO and MDA in the liver tissues of the AP group compared with the NS group, while these levels were lower in the AP+HS group compared with the AP group.

Figure 6. Effect of HS on the pathology in mice with AILI. H&E staining was assessed 24 hour after AILI. Representative histopathological liver sections are shown (A). The percentage of the degree of hepatocellular necrosis was estimated under a light microscope of 100 magnifications (B). NS, mice were treated with NS; AP, mice were treated with AP; AP+HS, mice were treated with HS and exposed to AP. Data were expressed as mean ± SD, n = 14. **P < 0.01. Arrows point to areas of tissue damage. CV: central vein.
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These might be due to HS scavenging of ROS and RNS. Therefore, it was suggested that HS may have potent beneficial effects by suppressing lipid peroxidation and oxidative stress.

GSH is a scavenger of toxic metabolites, including NAPQI which is the metabolite of AP [41]. GSH is also an important constituent of intracellular protective mechanisms against various noxious stimuli, including like oxidative stress and inflammatory reaction [42]. In our experiment, AP administration led to remarkably lower GSH level compared with the NS group. However, GSH was significantly higher in the AP+HS group compared with the AP group. Therefore, the hepatoprotection of HS against AP toxicity may be through the restoration of GSH levels.

The importance of mitochondrial dysfunction in the pathophysiology of AP hepatotoxicity is supported by the protective effect of cyclosporine in vivo [43]. The formation of ROS and RNS can cause mitochondrial membrane permeabilization and mitochondrial dysfunction in rodent models [10, 44], leading to cell necrosis ultimately. Our data showed that HS treatment after AP administration alleviated the degree of mitochondrial injury obviously via reducing mitochondrial oxidative stress and suppressing the production of free radicals.

Conclusions

AP is a widely used as an over-the-counter non-prescription drug. Yet, the hepatic GSH pool is consumed quickly during an AP overdose. The formation of NAPQI generates generous amounts of ROS and RNS and causes oxidative stress to lead to liver injury. Kupffer cells swallow damaged hepatocytes and release abundant amounts of ROS and RNS and many cytokines. The production of ROS and RNS aggravates mitochondrial damage and hinders the restoration of GSH, promoting hepatic necrosis. The experiment took BALB/C male mice as the research object to research the protective role in AILI. The study proved that HS can reduce plasma ALT, AST and ALP levels, inhibit oxidative stress, reduce inflammation, improve mitochondrial structure and function, and effectively alleviate the severity of liver cell necrosis.

In addition, I would like to specifically declare that: All the authors declare that have no conflict of interest and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Second Military Medical University (Shanghai, China).

Disclosure of conflict of interest

None.

Abbreviations

AP, acetaminophen; HS, hydrogen-rich saline; NS, normal saline; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; MPO, myeloperoxidase; MDA, malondialdehyde; GSH, glutathione; DILI, drug-induced liver injury; AILI, acetaminophen-induced liver injury; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin; ULN, the upper limit of normal.

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