5-HMF Attenuates Liver Fibrosis in CCl4-Plus-Alcohol-Induced Mice by Suppression of Oxidative Stress

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Summary The aim of this study was to investigate the effects of 5-hydroxymethyl-2-fural (5-HMF) on liver fibrosis induced by carbon tetrachloride (CCl4) and alcohol. Male ICR mice were treated with CCl4 dissolved in olive oil (10% v/v, 2.5 μg/L) intraperitoneally (i.p.), and given at a dose of 2.5×10^−3 mg/kg B.W. twice a week for 7 wk. Concurrently, mice received drinking water with or without alcohol. The mice in treatment groups and positive control group were gavaged with 5-HMF (7.5, 15, and 30 mg/kg B.W.) or Huganpian (350 mg/kg B.W.) daily starting in the fourth week and lasting for 4 wk. The blood samples were analyzed for biochemical markers of hepatic injury and tissue samples were subjected for estimation of liver antioxidants and histopathological studies. The concentrations of HA (hyaluronic acid), LN (laminin), CIV (collagen type IV), and MDA (malondialdehyde), as well as the serum levels of ALT (alanine aminotransferase) and AST (aspartate aminotransferase) were markedly reduced by 5-HMF. On the other hand, enzymatic antioxidants SOD (superoxide dismutase), CAT (catalase) and GSH-Px (glutathione peroxidase) were markedly elevated in liver tissue treated with 5-HMF. Histopathological examination revealed that 5-HMF treatment noticeably prevented hepatocyte apoptosis, fatty degeneration and inflammatory cell infiltration on liver fibrosis induced by CCl4 and alcohol. Hoechst 33258 staining also revealed hepatocyte apoptosis. 5-HMF could exert protective effects against liver injury and reduce liver fibrosis induced by CCl4 and alcohol in mice.

Key Words 5-HMF, carbon tetrachloride, liver fibrosis, oxidative stress

Liver fibrosis is a wound-healing response to chronic liver injury caused by a variety of etiological factors including viruses, alcohol and drug abuse, metabolic syndrome, and autoimmune diseases (1). Eventually, fibrosis can progress into cirrhosis. Without liver transplantation, cirrhosis often leads to the death of the patient (2). It is now generally accepted that liver fibrosis is associated with activation of hepatic stellate cells (HSCs). Physiologically, HSCs are quiescent and mainly responsible for the uptake, storage and delivery of retinoid (3). Upon liver injury, a variety of factors such as cytokines, chemokine or reactive oxygen species (ROS) induce the trans-differentiation of HSC from a quiescent state into myofibroblast-like cells with the appearance of smooth muscle α-actin (α-SMA) and loss of cellular retinoid storage, finally resulting in excess production and deposition of extracellular matrix (ECM) components as signs of hepatic fibrosis (4). To date, there are no effective treatments for patients with liver fibrosis, so a better understanding of pathways that regulate fibrosis has great clinical potential (5).

Carbon tetrachloride (CCl4) is a well-known hepatotoxin that is widely used to induce toxic hepatic injuries in experimental animal models (6, 7). In the rodent model of alcoholic liver disease (ALD), alcohol alone fails to induce significant liver fibrosis regardless of the duration of alcohol consumption. Therefore, some rodent models of ALD have been developed in which a secondary agent such as CCl4 has been used concomitantly with chronic alcohol treatment (8). The alcohol plus CCl4 model is in accordance with the “two-hit” theory of ALD. In the present study, experimental alcoholic liver fibrosis was induced by alcohol administration together with a low dose of CCl4 in mice. It has been reported that hepatic histopathologic changes in a CCl4/alcohol-induced fibrosis model were similar to those found in human fibrosis (8, 9). Hepatic fibrosis is characterized by the excessive deposition of extracellular matrix (ECM) proteins, such as hyaluronic acid (HA), laminin (LN) and type IV collagen (CIV), which leads to severe pathophysiological disturbances, including remodeling of the liver architecture, development of intrahepatic shunts, liver insufficiency, portal hypertension, esophageal varices, ascites and encephalopathic coma. Despite the high incidence of hepatic fibrosis worldwide, no generally accepted anti-fibrogenic therapy is available (10–12). Presently, many studies are assessing potential...
5-HMF (5-hydroxymethylfurfural, C_5H_6O_3), a product of the famous Maillard reaction, is mainly generated by acid-catalysed thermal dehydration of fructose and identified as a flavoring substance in a wide variety of heat-processed products (shown in Fig. 1) (14). Interestingly, it is also found in plants, foods and beverages that contain carbohydrates, such as the roots of *Polygonum multiflorum*, dried fruits, bread, fruit juices, milk and coffee (15, 16). In the last decades, there has been intense debate concerning 5-HMF’s toxicity, mutagenicity, and carcinogenicity (17). Therefore, the content of 5-HMF in honey, beer, and coffee has been strictly limited (18, 19). Despite the previous concern over the dangers of 5-HMF in recent years a theory for the biological activity of 5-HMF was accepted. The beneficial roles of 5-HMF include the inhibition of red blood cell sickling, the amelioration of hemorheology and antioxidant activity, cytoprotective, anti-myocardial ischemia and improvement of hemorheology effects (20–23).

Previous studies reported that 5-HMF can protect alcohol-induced liver damage as evidenced mainly by the restoration of the enzymatic antioxidants CAT (catalase), GSH-Px (glutathione peroxidase) and SOD (superoxide dismutase), together with lowered serum and liver lipid peroxidation, and reduced TNF-α and IL-1β levels. Moreover, 5-HMF also can ameliorate lipid degradation and hepatocyte apoptosis (24).

Hence, the aim of the present study was to investigate the effects of 5-HMF on markers of liver function in the serum, antioxidant enzyme levels and hepatic histopathology of the liver in mice with CCl4-induced hepato-toxicity and to further explore the possible mechanisms involved.

**EXPERIMENTAL SECTION**

**Chemicals and reagents.** 5-HMF (purity >98% by HPLC method) was isolated from the fruits of *Schizia chinensis* as described in a previous work (24). Huganpian (positive control), a liver protectant, which mainly contains *Radix bupleuri*, *Oriental wormwood*, *Isatis root*, and *S. chinensis* were purchased from Zhejiang Medicine Co. Ltd. (batch No. 091103, Zhejiang, China).

Commercial assay kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) were purchased from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Mouse hyaluronic acid (HA), type IV collagen (CIV), and laminin (LN) ELISA kits were purchased from R&D Systems (Minneapolis, MN). Other chemicals, such as carbon tetrachloride (CCl4) and alcohol (>99.7%), were obtained from Beijing Chemical Reagent Factory.

**Animals.** Male ICR mice, 18–22 g, were provided by Experimental Animal Holding of Jilin University with Certificate of Quality No. of SCXK (Jl) 2011-0004 (Changchun, China). All animals were housed under specific standard laboratory conditions for 1 wk, including a temperature-controlled environment (25 ±2 °C), a relative humidity of 50 ±5%, and a regular 12 h light/dark cycle. All animals were fed with a standard chow diet and water ad libitum. All the procedures were in strict accordance with Chinese legislation on the use and care of laboratory animals.

The experiments were conducted according to the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006). All experimental procedures were approved by the ethical committee for laboratory animals of Jilin Agricultural University.

**Experimental design.** Mice were randomly divided into six groups (10 mice per group), a normal control group, a fibrotic model group, a positive control group (Huganpian) and three treatment groups. The treatment groups were administered 5-HMF by gastric intubation. Mice received drinking water with or without 5% alcohol, which was gradually increased to 10%, at which level it continued to the end. Mice were treated with CCl4 dissolved in olive oil (10% v/v, 2.5 μg/L) intraperitoneally (i.p.), and given at a dose of 2.5×10^-5 mg/kg B.W. twice a week for 7 wk. The mice in the normal control group received only olive oil. The treatment was started at the beginning of the fourth week and lasted for 4 wk.

In the positive control group, mice were given Huganpian at a dose of 350 mg/kg B.W. (suspended in 5% carboxymethyl cellulose) by gavage per day. In 5-HMF treated groups, mice were given 7.5, 15, and 30 mg/kg B.W. (suspended in 5% carboxymethyl cellulose) by gavage per day. Other groups received an equal volume of vehicle as a control. The body weight was recorded.
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twice a week before 5-HMF treatment, then recorded every day. In the seventh week, all animals were fasted but given free access to water for 12 h, and subsequently sacrificed.

Blood samples were collected by the retrobulbar vessels and allowed to clot for 45 min at room temperature. After standing for 1 h, the serum was separated by centrifugation (3,500 rpm, 10 min, and twice) and stored in a −80°C freezer before analysis. The liver was removed and washed with saline solution. A small piece of tissue was cut off from the same part of the left lobe of the liver in each mouse and fixed in 10% buffered formalin solution for histopathological analysis. The remaining liver tissues were stored at −80°C for hepatic homogenate preparation.

Assay for serum AST and ALT. Serum was used for the spectrophotometric determination of AST and ALT using commercially available diagnostic kits purchased from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China) as previously described (25). In brief, the samples were transferred into a new 96-well plate containing substrates or buffer solution. After incubation at 37°C, the plate was incubated for an additional time after adding color developing agent and the absorbance at 510 or 520 nm was measured. The final data are represented as U/L.

Assay for serum HA, LN and CIV. Serum levels of HA, LN and CIV were determined by using the commercially available ELISA kits provided from R&D Systems according to the manufacturer’s instructions and the results are presented as ng/mL serum. Briefly, prepared reagent, samples and standards were combined, antibodies were labeled with enzyme, and reaction occurred for 60 min at 37°C. After the addition of stopping solution, measurement and calculation of the OD value occurred within 10 min.

Assay for hepatic antioxidant activities. Antioxidant activity assays were performed as previously described (26). In brief, liver samples were homogenized in 9 volumes of ice-cold 0.9% NaCl. The suspension was then centrifuged twice at 3,500 rpm for 10 min at 4°C, and the supernatant was used for the measurement. Levels of CAT, GSH-Px and SOD in liver homogenates were measured by commercial kits according to the manufacturer’s instructions (Nanjing Jiancheng Institute of Biotechnology). The concentration of malondialdehyde (MDA) was assayed by monitoring thiobarbituric acid reactive substance formation as described by Draper and Hadley (27). The amount of protein was measured using the Bradford assay (28).

Histopathological examination. A small piece of tissue was cut off from the same part of the left lobe of the liver in each mouse and fixed in 10% buffered formalin solution for histopathological analysis. The remaining liver tissues were stored at −80°C for hepatic homogenate preparation.

Assay for hepatic apoptosis. Paraffin sections (5 μm) were stained with Hoechst 33258 staining. First for dewaxing and hydration, subsequent stained with Hoechst 33258, finally sealing piece. Change and apoptosis in the liver tissues were observed by fluorescence microscope.

Statistical analysis. All data are expressed as the mean ± SD. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunn’s multiple comparison tests using SPSS 17.0 software (SPSS Inc., Chicago, IL). The value of $p<0.05$ was considered statistically significant. The Nonparametric Test (Ridit analysis) was used for the histological examination comparison.

RESULTS

Effect of 5-HMF on body weight

The animals were acclimatized for 1 wk before initiation of the experiment; the body weight had a marked elevation (shown in Fig. 2). Compared to the control group, the body weight significantly decreased after administration of CCl4. However, the treatment with 5-HMF improved the reduction in body weight of those

Fig. 2. Effect of 5-HMF on body weight in mice.
mice treated with CCl₄/alcohol.

**Effects of 5-HMF on serum ALT and AST**

The leakage of ALT and AST into the blood indirectly reflects liver failure caused by alcohol-induced hepatotoxicity (29, 30). As shown in Fig. 3, the results showed that serum ALT and AST were elevated and had a significant difference when compared with the control group, indicating the liver fibrosis model had been established successfully. 5-HMF treatment (15 and 30 mg/kg) and Huganpian treatment significantly decreased ALT and AST when compared with the liver model group.

**Effects of 5-HMF on serum HA, LN and CIV**

Levels of HA, LN and CIV in serum are the important indices reflecting the degree of liver fibrosis (31). As shown in Fig. 4, at the end of the experiment, the serum levels of HA, LN and CIV in mice were significantly increased in the fibrotic model group compared with the normal control group. The level of HA was significantly decreased in all treatment groups. However, the levels of LN and CIV in mice were significantly decreased in the treatment group (7.5 and 15 mg/kg) compared with the model group.

**Effects of 5-HMF on CAT, GSH-Px, SOD and MDA in hepatic homogenate**

Oxidative stress is one of the mechanisms of alcohol-induced liver injury. To eliminate oxidative stress, a number of enzymatic and non-enzymatic mechanisms have evolved to protect against reactive oxygen species.
(ROS) resulting from oxidative stress in alcoholic liver injury. These antioxidant enzymes include CAT, GSH-Px, SOD and MDA (shown in Fig. 5).

In the fibrotic model group, CAT activity was decreased when compared to the normal control group. However, treatment with 5-HMF at the dose of 15 and 30 mg/kg completely prevented a decrease in CAT activity. A dose-dependent effect was found in CAT activity between the middle (15 mg/kg) and high (30 mg/kg) 5-HMF treatment groups. The oxidative stress induced by CCl4/alcohol caused a significant decrease in the level of GSH-Px, a key intracellular enzymatic antioxidant, in comparison with the normal control group. The treatment of 5-HMF at 15 and 30 mg/kg prevented the reduction of GSH-Px level.

SOD is an antioxidant enzyme that can scavenge the lipid peroxide radical. MDA is an end product of the break-down of polyunsaturated fatty acids and related esters, and its formation is an index of lipid peroxidation in many organ homogenates. SOD and MDA levels in hepatic homogenate were measured, and the results showed that CCl4/alcohol induced the decline of the antioxidant activity of SOD and the increase of the pro-oxidant activity of MDA. As shown, treatment with Huganpian significantly inhibited the changes in the levels of SOD and MDA. Compared to the model group, the hepatic SOD activities in the 5-HMF groups were elevated by 28.72%, 30.23% and 37.91%. Meanwhile, the level of MDA was decreased by 15.5%, 19.42% and 26.07%, respectively.

Histopathology

H&E staining for sections of the normal control group showed structural integrity, regular hepatic cords with central veins, and an absence of inflammation. However, the CCl4/alcohol-induced liver injury model group showed significant changes in hepatic architecture and vacuolar fat, and mild inflammatory cell infiltration. Nevertheless, the CCl4/alcohol induction with 5-HMF treatment group showed that 5-HMF could significantly alleviate CCl4/alcohol-induced alterations. The high dose of 5-HMF exerted a protective effect against cellular swelling and hepatocyte apoptosis. The low dose of 5-HMF group showed only minor fat vacuoles, quite similar to the controls (shown in Fig. 6).

Based on a microscopic examination, the severe hepatic fibrosis induced by CCl4/alcohol was markedly reduced by treatment with 5-HMF. These data correlate with the results of the serum aminotransferase and hepatic antioxidant enzyme levels.

Masson staining is a classic method, shows that collagen fiber technology can display well when used with appropriate collagen fibers, and has a good visual contrast. The relative content of the degree of liver collagen was assessed with Masson staining so as to reflect the degree of liver fibrogenesis. The hepatic histopathological examination in the control group displayed normal structure and no pathological changes. In the model group, there was a large infiltration of inflammatory cells.
cells, necrosis and fibrosis. With the different doses and administration duration of 5-HMF (7.5, 15, and 30 mg/kg), collagenous fibers were decreased compared to the fibrotic model group (shown in Fig. 6).

The liver pathological classification and grading

Systems for grading and staging incorporate the view that necroinflammation is not only a measure of severity but also of ongoing disease activity and the parameter most potentially responsive to therapy. As shown in Table 1, the pathological change in liver is mainly hepatic steatosis and occurs mainly in the central veins.

The fibrotic model group presented significant liver injury relative to the normal group. The positive group and 5-HMF treatment groups exhibited an alleviated steatosis state to different degrees.

**Effects of 5-HMF on apoptosis in hepatic tissues**

Stained with Hoechst 33258, the normal control group did not display the blue fluorescent nuclei, indicating no apoptosis (shown in Fig. 7A); the fibrotic model group exhibited a large number of evenly distributed blue fluorescence nuclei, the nucleus structure was broken, and the fluorescence intensity was stronger. In the treatment of 5-HMF at 15 and 30 mg/kg groups, the number of blue fluorescent nuclei decreased, indicating 5-HMF could significantly inhibit cell apoptosis. The percentage of apoptosis within groups (shown in Fig. 7B) correlates with the results of the Hoechst 33258 staining.

**DISCUSSION**

CCl₄ is one of the xenobiotics that has been reported to induce acute and chronic tissue injuries (33) through bioactivation of the phase I cytochrome P450 system to form reactive metabolic trichloromethyl radicals (·CCl₃) and peroxy trichloromethyl radicals (·OOCCl₃). These free radicals can covalently bind to macromolecules such as proteins, lipids and nucleic acids. The double allylic hydrogen bonds of polyunsaturated fatty acid (PUFA) are susceptible to abstraction by free radicals; CCl₄ exposure induces an increase in lipoperoxide and free peroxide radical concentrations that are highly reactive and cause injury or necrosis (34). The biotransformation of CCl₄ utilizes CYP2E1, leading to a decrease in CYP2E1 expression (35).
Hepatic fibrosis is a major complication in chronic liver disease, leading to the risk of cirrhosis and ultimately to hepatic dysfunction and hepatocellular carcinoma. Biologically, Liver failure (HF) is defined as the wound-healing process that occurs as a result of a wide range of inflammatory reactions in the liver (36–38). There are numerous environmental toxins for chronic liver disease, including cholestasis, circulatory disturbances, autoimmune and nutrition disorders, environmental toxins and the use of particular medicines; however, the two primary causes of liver fibrosis have been identified as infections caused by the hepatitis virus and alcoholism. Treating the cause of the liver disease may lead to fibrosis reversal. At present, the only curative treatment for advanced liver fibrosis or cirrhosis is organ transplantation, which, however, is limited by the availability of a donor organ. Therefore, it is urgent to develop an approach for the prevention or treatment of hepatic fibrosis.

Chronic inflammation leads to continuous hepatocyte damage and subsequently hepatic fibrosis (39, 40). The response is generalized, with features common to multiple organ systems. In the liver, a variety of different types of injury lead to inflammation and fibrogenesis, implying a common pathogenesis. A CCl4-induced hepatic injury is a widely used experimental model for anti-inflammatory and hepatoprotective drug screening, promoting hepatic pathology similar to that observed in humans (41–43). Alcohol was the key mediator to induce liver injury, whilst CCl4 accelerated the progression of liver injury by increasing lipid accumulation (44) and enhancing oxidation stress (45, 46). The present paper used the CCl4/alcohol-injury hepatic model to analyze the 5-HMF therapeutic potential in preventing chronic hepatotoxicity by attenuating the fibrosis in the liver.

In the present study, we successfully established a hepatic fibrosis model by intraperitoneal injection of 10% CCl4/olive oil at a dose of 2.5×10^{-5} mg/kg B.W. for 7 wk in mice. Our findings indicated that the body weight significantly decreased after administration of CCl4, and that 5-HMF reversed the decline of body weight with mice having better mental state when compared to the liver model group. In the present study, the serum levels of the hepatic enzymes AST and ALT were increased, reflecting the hepatocellular damage in the CCl4/alcohol-induced injury model. However, treatment with 5-HMF for 4 wk lowered the AST and ALT levels of CCl4/alcohol-exposed mice.

Hepatic fibrosis is characterized by extensive deposition of ECM proteins, such as collagen types I, III, and IV, laminin and hyaluronic acid (hyaluronan) (47). In
recent years, these serum markers related to elevated ECM synthesis have been identified as possible useful indicators of liver fibrosis. Levels of serological markers (HA, CIV and LN) clearly indicated that HSC was activated by CCl4/alcohol-induced liver injury. In addition, levels of HA, CIV and LN were measured, which were expected to increase as a result of remodeling and recurrent scarring in liver fibrogenesis (31). In our mouse model, along with chronic alcohol and CCl4 administration, there was a significant progressive increase in the serum levels of HA, LN, and CIV. 5-HMF markedly decreased the serum levels of HA, LN, CIV, suggesting that the extract has hepatoprotective effects and the mechanism of 5-HMF in reducing hepatic fibrosis may be closely related to inactivation of HSC.

In the present study, the liver model group significantly exhibited an evident inflammatory process, as well as necrosis and fibrosis. However, a reduction of inflammatory infiltrates, liver necrosis, and fibrosis was observed after the 5-HMF treatment. Therefore, 5-HMF treatment caused a remarkable reduction of hepatic damage, corroborating the results of previous studies (24). The results of the present study demonstrated that there was a significant elevation in MDA content in the liver tissues of model group mice. This enhanced lipid peroxidation led to tissue damage and the failure of antioxidant defense mechanisms. In the present study, there was a significant elevation in liver MDA content in the liver model group and a significant reduction in the group treated with 5-HMF, which suggests enhanced lipid peroxidation leading to tissue damage and the failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals. Therefore, since 5-HMF treatment reduced MDA levels, it probably exhibits an antioxidant action. In this study, it has been shown that 5-HMF can protect CCl4/alcohol-induced liver damage as evidenced mainly by the restoration of the enzymatic antioxidants CAT, GSH-P-x and SOD together with lowered serum and liver lipid peroxidation.

In addition, these effects on liver function correlate with the histopathological changes observed from the microscopic examination of 5-HMF-treated animals. Centrilobular hepatic necrosis, fatty changes, ballooning degeneration and infiltrating lymphocytes were found in mice exposed to CCl4/alcohol. Treatment with 5-HMF prevented these histopathological changes. There was an increased number of Hoechst-positive cells in the fibrotic model group but 5-HMF reduced the number of Hoechst-positive cells and attenuated apoptosis in hepatocytes. Thus, our results suggest that attenuating the elevation of certain markers of liver failure may be the mechanism of protective action of 5-HMF against CCl4/alcohol-induced hepatotoxicity.

In conclusion, the findings from the present investigation clearly demonstrated that 5-HMF has hepatoprotective effects against CCl4/alcohol-induced hepatotoxicity in mice. We propose that the observed enhancement of antioxidant enzymes and reduction in malondialdehyde are the major mechanisms of action of 5-HMF in the prevention of CCl4/alcohol-induced liver fibrosis. However, additional work is required to establish the efficacy of 5-HMF as a potent anti-hepatic fibrosis drug.

The present investigation showed that the fibrosis area, inflammatory infiltration, steatosis and levels of HA, CIV, LN, SOD, ALT and AST were decreased significantly in the 5-HMF-treated group compared to the fibrotic model group. However, treatment with 5-HMF lead to a decrease in MDA level compared to the fibrotic model group. These results suggest that 5-HMF is a potential therapeutic agent for liver fibrosis.

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