Dihydromyricetin alleviates carbon tetrachloride-induced acute liver injury via JNK-dependent mechanism in mice

Jun Xie, Jie Liu, Tu-Ming Chen, Qing Lan, Qing-Yu Zhang, Bin Liu, Dong Dai, Wei-Dong Zhang, Li-Ping Hu, Run-Zhi Zhu

Abstract

AIM: To assess the effects of dihydromyricetin (DHM) as a hepatoprotective candidate in reducing hepatic injury and accelerating hepatocyte proliferation after carbon tetrachloride (CCH) treatment.

METHODS: C57 BL/6 mice were used in this study. Mice were orally administered with DHM (150 mg/kg) for 4 d after CCl4 treatment. Serum and liver tissue samples were collected on days 1, 2, 3, 5 and 7 after CCl4 treatment. The anti-inflammatory effect of DHM was assessed directly by hepatic histology detection and indirectly by serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, and superoxide dismutase (SOD). Inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α), were detected using ELISA kits. Proliferating cell nuclear antigen (PCNA) staining was used to evaluate the role of DHM in promoting hepatocyte proliferation. Hepatocyte apoptosis was
measured by TUNEL assay. Furthermore, apoptosis proteins Caspases-3, 6, 8, and 9 were detected by Western blot. SP600125 were used to confirm whether DHM regulated liver regeneration through JNK/TNF-α pathways.

RESULTS: DHM showed a strong anti-inflammatory effect on CCl4-induced liver injury in mice. DHM could significantly decrease serum ALT, AST, IL-1β, IL-6 and TNF-α and increase serum albumin, SOD and liver SOD compared to the control group after CCl4 treatment (P < 0.05). PCNA results indicated that DHM could significantly increase the number of positive cells compared to the control (348.9 ± 56.0 vs 107.1 ± 31.4, P < 0.01). Caspase apoptosis detection showed that DHM could reduce the activities of Caspases-8, 3, 6 and 9 compared to the control (P < 0.05). The results of Western blot showed that DHM increased the expression of JNK and decreased TNF-α expression. However, DHM could not affect TNF-α expression after SP600125 treatment. Furthermore, DHM could significantly improve the survival rate of acute liver failure (ALF) mice (73.3% vs 20.0%, P < 0.0001), and SP600125 could inhibit the effect of DHM.

CONCLUSION: These findings demonstrate that DHM alleviates CCl4-induced liver injury, suggesting that DHM is a promising candidate for reversing liver injury and ALF.

Key words: Dihydromyricetin; Liver regeneration; Tumor necrosis factor-α

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Core tip: Our research confirmed that dihydromyricetin (DHM) plays an anti-inflammatory role in the carbon tetrachloride (CCl4) induced acute liver injury mice. It was shown that DHM could alleviate CCl4-induced acute liver injury by reducing apoptosis and accelerating proliferation of hepatocytes. Furthermore, DHM treatment up-regulated Jun kinase expression in liver tissue, and the mice which were injected with SP600125 could not survive in the acute liver failure induced by lethal dose CCl4 injection.

INTRODUCTION

The liver plays a crucial role in the regulation of body metabolism as well as in detoxification. Due to these essential functions, injuries to this organ need to be rapidly and efficiently remedied. Hepatotoxins, such as alcohol, acetaminophen, and carbon tetrachloride (CCl4), induced liver injury, which is characterized by hepatocyte necrosis and dysfunction of the liver. Since the mechanism of CCl4-induced liver injury is very similar to liver diseases, it is commonly used as a hepatotoxin in experimental hepatopathy [1-3].

Dihydromyricetin was isolated from the tender stem and leaves of the Ampelopsis grossedentata species. DHM has numerous pharmacological activities, such as anti-inflammatory, antioxidation and anticarcinogenic effects [4]. In the present study, we aimed to assess the effects of DHM as a hepatoprotective candidate in reducing hepatic injury and accelerating hepatocyte proliferation following CCl4 treatment. The present findings indicate that DHM shows a potent antihepatotoxic activity in recovery of hepatocellular apoptosis and acceleration of liver regeneration during liver injury. A better understanding of DHM-regulated liver regeneration will be important to develop effective interventions to prevent or treat liver disease.

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine. Activation of TNF-receptor family members is considered to play an important role in the pathogenesis and progression of liver disease [5,6]. TNF-α is implicated in hepatocyte apoptosis, but the pathways contributing to initiation and progression of acute liver injury are presently vague [7]. The JNK signaling pathway plays an important role in TNF-dependent acute liver damage [8,9]. JNK has been shown to be involved in liver carcinogenesis and be required for hepatocellular carcinoma (HCC) cell proliferation and hepatocyte proliferation in liver regeneration [10]. In a previous study, we found that CCl4 could increase TNF-α expression in serum and liver tissue, which results in acute liver injury [11]. Furthermore, we found that DHM could up-regulate the activation of JNK, and then decreased the expression of TNF-α in CCl4-induced liver injury mice. In addition, the hepatoprotective role of DHM could be inhibited after blocking the activation of JNK. These results suggest that DHM could be a treatment option for liver injury. We thus assess its therapeutic potential in clinically relevant models of TNF-mediated liver damage and acute liver failure (ALF).

MATERIALS AND METHODS

Animal care and use statement

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 ℃, 12 h/12
Serum AST, ALT, albumin and SOD levels were determined with the commercial assay kits. Serum IL-6, IL-1β and TNF-α levels were determined with ELISA kits according to the manufacturer's instructions.

**Histology and injury grading**

Histology and injury grading were performed as previously described. The degree of necrosis after acute liver injury was evaluated by the degree of necrotic lesions in the parenchyma.

**PCNA staining**

The hepatocyte proliferation was evaluated by PCNA staining. Liver tissues were fixed in neutral buffered formalin for at least 24 h, and then embedded in paraffin to make pathological section. The sections were stained using PCNA antibody and the SABC staining kit according to manufacturer's protocol. After that, sections were observed under a light microscope and the PCNA positive cells were counted in at least 5 fields.

**TUNEL assay**

Cell apoptosis rate was detected using In Situ Cell Death Detection kit-POD according to the manufacturer’s instructions. Briefly, liver tissues were dehydrated and rehydrated by using xylene and a graded series of ethanol, and then incubated for 15 min at 37℃ with proteinase K working solution. After that, the samples were incubated at 37℃ in dark for 1 h with 50 μL Converter-POD per sample for 30 min. Hematoxylin was used to stain the nuclei and the stained cells was analyzed under a light microscope.

**Survival analysis**

One hundred and twenty mice were divided randomly into four groups. Group 1 was administered with a lethal dose of CCl4 (2.6 mg/kg) and served as a control; group 2 was administered with 2.6 mg/kg CCl4 and 2 h later, each mouse was orally treated with 150 mg/kg per day DHM; group 3 was injected intraperitoneally with SP600125, one hour before CCl4 administration, and 2 h later, each mouse was orally treated with 150 mg/kg per day DHM. Survival rates in these groups were recorded every 12 h.

**Western blot analysis**

Liver tissues were homogenized and lysed with RIPA buffer (Beyotime, Jiangsu, China), and the protein concentration in each sample was detected with a BSA assay kit (Beyotime, Jiangsu, China). Proteins were extracted to detect cytochrome C by using the Mitochondria/Cytosol Fractionation Kit. Proteins were electrophoresed on an SDS-PAGE gel, and then transferred onto PVDF membranes. The membranes were then incubated with primary and secondary antibodies. Signal detection was performed by using enhanced ECL chemiluminescence reagents (GE-Enhanced...
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Healthcare, Pittsburgh, United States).

**Evaluation of caspases-3, 8 and 9 activity**

Caspases-3, 6, 8 and 9 activities were determined in liver extracts by measuring proteolytic cleavage of the proluminescent substrates Z-IETD-AMC, AC-DEVD-AMC, AC-VEID-AFC and AC-LEHD-AFC (Calbiochem, La Jolla, CA). The fluorescence was determined based on the amount of released AFC (caspase-8, -6, -9, λex = 400, λem = 505) or AMC (caspase-3, λex = 380, λem = 460). The results are expressed as arbitrary units of fluorescence (AUf) per milligram of protein.

**Statistical analysis**

The data between the CCl4 group and DHM group were compared by t-test, and the survival results were analyzed by log-rank test and presented as Kaplan-Meier survival curves. The statistical methods of this study were reviewed by Qingyu Zhang from Affiliated Hospital of Guangdong Medical College.

**RESULTS**

**DHM protects mice against acute liver injury**

To confirm the role of DHM in protecting mice against acute liver injury, we employed serum ALT, AST, albumin and SOD as indicators. After CCl4 injection, serum ALT and AST were rapidly increased to peak levels on day 2, and then decreased thereafter, while DHM could significantly decrease serum ALT and AST from day 1 to day 5 (Figure 1A and B). The attenuation of serum AST and ALT indicated that DHM possesses a good protective effect on liver function. Serum albumin level is also considered an important indicator for evaluating functional recovery of acutely injured liver. Serum albumin was significantly raised after DHM administration compared to the control (Figure 1C). SOD belongs to active oxygen scavenging enzyme systems, which is regarded as a biomarker to judge the anti-oxidative ability of the liver. We found that the activities of SOD in both serum and liver tissue were enhanced markedly compared to the control after DHM treatment in mice with CCl4-induced liver injury (Figure 1D and E). Furthermore, serum IL-1β was found to be rapidly increased after CCl4 treatment, which was also supported in previous studies[14], whereas DHM administration resulted in significant attenuation from day 1 to day 5 after CCl4 injection (Figure 1F). IL-6 and TNF-α have been identified as attractive targets for liver regeneration. The data showed that DHM could markedly down-regulate IL-6 and TNF-α (Figure 1G and H).

**DHM promotes hepatocyte proliferation from an early phase**

To determine whether DHM could accelerate hepatocyte proliferation from acute phase during liver regeneration, we evaluated the proliferation of hepatocytes by using immunostaining of PCNA in liver sections on day 2 after CCl4 treatment. Compared with the control, DHM significantly increased the number of PCNA positive cells. A great number of PCNA+ hepatocytes were found surrounding the portal area (Figure 2A and B). PCNA+ cells in at least 12 mm2 tissue sections were counted for each mouse, and the data showed that DHM could markedly accelerate hepatocyte proliferation from an acute phase (Figure 2C).

**DHM reduces necrosis and apoptosis of hepatocytes**

The H&E staining and TUNEL assay were used to investigate the effect of DHM on hepatocellular necrosis, inflammation and the apoptosis. Liver sections stained with HE showed that the DHM administrated group demonstrated only moderate necrosis, maintaining normal architecture; the necrotic areas were significantly diminished around the central vein and centrilobular regions compared with the control on day 2 after CCl4 treatment (Figure 3A and B). The results of TUNEL assay demonstrated that DHM significantly decreased the number of apoptotic cells in the section of liver tissue compared with the control on day 2 after CCl4 injection, and only a fewer apoptotic cells were detected in the visual field (Figure 3C and D). At least 12 mm2 tissue sections were counted for each mouse, and the data showed that DHM could significantly reduce hepatocellular apoptosis (Figure 3E).

**DHM effectively reduces the release of cytochrome C from the mitochondria and inhibits caspase activity**

After displaying the effect of DHM in protecting hepatocytes from hepatotoxicity induced by CCl4, we investigated the relationship between DHM and hepatoprotective pathways. As shown in Figure 4A, the release of Cytochrome c and Bax expression in the DHM groups were significantly inhibited compared to the control on day 2 after CCl4 treatment. Similarly, the activities of caspases-8, 3, 6 and 9 in the liver were significantly lower in the DHM group than in the control group on day 2 after CCl4 treatment (Figure 4B-E). These results indicated that DHM could efficiently reduce hepatic injury by inhibiting the activities of Cytochrome C and Caspases-mediated apoptosis pathways.

**TNF-α mediated liver regeneration is regulated by DHM via the JNK pathway**

In this part of experiment, the proteins in liver tissues collected both from liver injury mice and ALF mice were detected by Western blot. The result showed that the content of TNF-α was markedly higher in the control compared to DHM-administered groups after CCl4 treatment, but the level of JNK was promoted significantly in DHM-administered groups (Figure 5A and B). Moreover, TNF-α was not reduced in the ALF mice pretreated with JNK-inhibitor SP600125 (Figure 5B). These data demonstrated that TNF-α mediated...
Figure 1  Dihydromyricetin protects mice against CCl₄-induced liver injury. Mice were treated with CCl₄ (1 mL/kg body weight and 1:3 diluted in corn oil) to induce acute liver injury, and then orally administered with DHM (80 mg/kg body weight and diluted in CMC-Na) 2 h after CCl₄ injection, once per day for 4 d. Control mice were treated with an equal volume of CMC-Na. Subsequently, serum ALT, AST, albumin, SOD, IL-1β, IL-6, TNF-α and liver SOD were measured at indicated time points and determined as described in materials and methods. A: Serum ALT; B: Serum AST; C: Serum Albumin; D: Serum SOD; E: Liver SOD; F: Serum IL-1β; G: Serum IL-6; H: Serum TNF-α. Values represent mean ± SE (n = 6). *P < 0.05 and **P < 0.01 vs control, respectively.
liver regeneration is regulated by DHM via the JNK pathway.

**DHM reduces mortality after a lethal dose of CCl₄**

The data demonstrated that oral administration of DHM offered a strong survival benefit for CCl₄-induced ALF mice, and the survival significantly improved probably from the early phase after CCl₄ injection (Figure 5C). However, after JNK inhibitor SP600125 (50 mg/kg) was administered by intraperitoneal injection, DHM could not increase the survival of C57BL/6 mice after a lethal dose of CCl₄ (Figure 5C), which indicated that DHM may protect against CCl₄-induced ALF through the JNK pathway.
DISCUSSION

CCl₄-induced acute liver injury has been used as an ideal model for many years because the mechanism of this hepatotoxin replicates the most cases of human liver disease. Previous studies demonstrated that the pathological roles of CCl₄ are restricted to the liver, while lethality of high-dose CCl₄ is mostly related with ALF. In the present study, we confirmed the protective role of DHM against CCl₄-induced acute liver injury. Serum ALT and AST have been utilized as the biomarkers of liver damage, which were recognized as important indicators to judge the severity of acute liver injury. The results indicated that DHM could markedly attenuate the elevation of serum ALT and AST from an early phase of liver damage. Furthermore, DHM could significantly improve serum albumin, which demonstrated that DHM could promote liver functional recovery. With regard to the role of ROS production in liver disease and pathology, antioxidants might prevent hepatic damage through scavenger activity and increase the activity of intracellular antioxidant enzymes including SOD. Many studies reported that natural antioxidants are efficacious in preventing oxidative stress-related liver injury [15]. In this study, we found that DHM could markedly enhance the activity of SOD both in serum and in the liver, which indicated it as an effective antioxidant. Meanwhile,
DHM-mediated protection on the liver could be significantly reduce serum and liver TNF-α levels, which has close relationship with the regeneration. The results also demonstrated that DHM could significantly suppress CCl4-induced inflammation, necrosis, and destruction of liver architecture.

Liver regeneration is shown as hepatocyte proliferation. In the present study, PCNA results definitely demonstrated the positive role of DHM in hepatocyte proliferation. DHM contributes to faster functional recovery by promoting hepatocyte proliferation during the whole liver damage process.

TUNEL staining results demonstrated that DHM could reduce apoptosis of hepatocytes. Serious hepatocyte apoptosis is also the major cause of the death of ALF mice, and the effect of DHM is mediated partly by inhibiting apoptosis pathways, including the release of cytochrome c from the mitochondria, down-regulating Bax and markedly decreasing the activities of caspases-3, 6, 8 and 9 in the liver tissue. TNF-α is able to induce apoptosis via caspase activation pathways. DHM protects from CCl4-induced ALF by inhibiting activation of caspases via TNF-α mediated pathway.

To investigate the underlying mechanism of the role of DHM in liver regeneration, we used histological methods to reveal the severity of liver necrosis and inflammation. The results also demonstrated that DHM could significantly suppress CCl4-induced inflammation, necrosis, and destruction of liver architecture.

Liver regeneration is shown as hepatocyte proliferation. In the present study, PCNA results definitely demonstrated the positive role of DHM in hepatocyte proliferation. DHM contributes to faster functional recovery by promoting hepatocyte proliferation during the whole liver damage process.

In conclusion, we demonstrated that DHM has strong beneficial effects against CCl4-induced acute liver injury and ALF. The protective effect of DHM makes it a novel therapeutic candidate for acute liver injury and ALF.

**COMMENTS**

**Background**

The liver plays a crucial role in the regulation of body metabolism as well as in detoxification. Due to these essential functions, injuries to this organ need to be rapidly and efficiently remedied.

**Research frontiers**

Dihydromyricetin (DHM) has numerous pharmacological activities, such as anti-inflammatory, antioxidation and anticarcinogenic effects. However, whether DHM could reduce hepatic injury and accelerate hepatocyte proliferation or not is not yet known.

**Innovations and breakthroughs**

This is the first study to demonstrate that DHM can accelerate liver regeneration and protect liver from the death of acute liver failure (ALF) via Jun kinase dependent mechanism.

**Applications**

DHM may serve as a potential effective candidate agent for the therapy of chemical liver injury and ALF.
**Peer-review**

The study is well conceived and performed, and the results are quite interesting and potentially helpful for clinical application.

**REFERENCES**


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