Hepatoprotective effect of galangin on carbon tetrachloride-induced hepatotoxicity via the LKB1/AMPK pathway

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Abstract
To investigate the protective effects of galangin on liver toxicity induced by carbon tetrachloride (CCl4) in mice. Mouse hepatotoxicity model was established by intraperitoneal injection (i.p.) of 10 ml/kg body weight CCl4 that diluted with corn oil to a proportion of 1:500 on Kunming mice. The mice were randomly divided into five groups named control group, model group, and 1, 5, and 10 mg/kg galangin group. The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed by ELISA. Liver histopathological examination was observed via optical microscopy. The levels of superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), and glutathion (GSSG) were analyzed to assess oxidative stress. Finally, western blot assay was carried out to analyse the expression levels of total AMP-activated protein kinase (AMPK), phospho-AMPK (p-AMPK), total liver kinase B1 (LKB1), and phospho-LKB1 (p-LKB1). Compared with the control group, in the model group, the levels of AST, ALT, MDA, and GSSG increased significantly (p < 0.01); the activity of SOD and GSH decreased significantly (p < 0.01); and the histopathological examination revealed liver necrosis. However, treatment with galangin (5 and 10 mg/kg) significantly reversed these CCl4-induced liver damage indicators. Furthermore, treatment with galangin (10 mg/kg) significantly increased the p-AMPK and p-LKB1 expression levels (p < 0.01). This study supports the hepatoprotective effect of galangin against hepatotoxicity, perhaps occurring mainly through the LKB1/AMPK-mediated pathway.

Keywords
antioxidant, carbon tetrachloride, galangin, hepatotoxicity, LKB1/AMPK

Introduction
Liver, one of the body’s vital organs, plays a central role in the clearance of metabolites and exogenous compounds. Therefore, liver disease seriously affects health. One of the major reported causes of mortality and morbidity worldwide are liver diseases, and drug-induced liver toxicity is a major cause of hepatic dysfunction (e.g. CCl4 toxicity).1,2 Thus, it is extremely necessary to find a treatment to prevent drug-induced liver toxicity.

The concentration of reactive oxygen species (ROS) can be normally reduced by antioxidant systems,
including antioxidant enzymes and antioxidant molecules, such as SOD and GSH, respectively. However, oxidative stress and oxidative damage will occur if the amount of ROS is way above what the aforementioned enzymes can diminish. Several studies have demonstrated that ROS-induced oxidative stress holds an important position in drug-induced liver toxicity. In the CCl₄-induced acute liver injury model, CCl₄ induced increased ROS production. The ROS attack DNA and proteins, resulting in damage to liver tissues. Studies have found that improving oxidative stress can indeed reduce CCl₄-induced liver damage, such as phosphatidylserine liposomes. Therefore, an effective strategy may be to identify natural antioxidants to prevent acute liver injury.

Flavonoids exhibited unique antioxidant properties and other pharmacological activities that may be associated with the protection of the liver from CCl₄ injury. Many flavonoids were also reported to protect against liver injury induced by CCl₄. Galangin, a flavonoid in Zingiberaceae, is isolated from the roots of Alpinia officinarum Hance. Some studies have suggested that galangin possesses some effective pharmacological properties, including free radical scavenging and anti-inflammatory activities. Studies have found that galangin exerts preventive effect on acute hepatorenal toxicity in novel propacetamol-induced acetaminophen-overdosed mice. However, as a therapeutic agent to prevent drug-induced liver toxicity, the hepatoprotective effect and mechanism of action of galangin is still little known. Therefore, in the present study, we evaluated whether the hepatoprotective effects of galangin are related to antioxidation.

Materials and methods

Compounds, chemicals, and reagents

Galangin (purity ≥ 98%) was purchased from Sichuan Victor Bio-Technol Co., Ltd. (Sichuan, China). The mouse alanine aminotransferase (ALT) ELISA kit and mouse aspartate aminotransferase (AST) ELISA kit were purchased from Shanghai JiNing Industrial Co., Ltd. (Shanghai, China). The mouse Superoxide dismutase (SOD) ELISA kit, mouse malondialdehyde (MDA) ELISA kit, mouse glutathione (GSH) ELISA kit and mouse glutathion (GSSG) ELISA kit were purchased from Shanghai JiNing Industrial Co., Ltd. (Shanghai, China).

Other chemicals and reagents used in this study were of analytical grade.

Animals

Kunming mice, weighting between 20 and 30 g, were obtained from Jinan Pengyue Experimental Animal Breeding Co., Ltd. (No. SCXK (lu) 2014-0007). All animal experimentation was followed the recommendations of the ethics committee of Yantai Yuhuangding Hospital of Laishan branch (ethics approval number: YHD.No20190607c102)

Experimental groups

An acute liver injury model was established as previously described. Each group of more than six experimental animals can basically eliminate individual differences. In order to eliminate individual differences, this study selects 10 experimental animals per group. A total of 50 clean grade male Kunming mice were randomly divided into five groups of 10 each: the control group, the model group, the 1 mg/kg galangin group, the 5 mg/kg galangin group, and the 10 mg/kg galangin group. The mice of the control group received distilled water for 7 days. The mice of the model group also received distilled water, while CCl₄, diluted with corn oil at a 1:500 ratio, was intraperitoneally administered 10 mL/kg body weight once on day 8. In the three different doses of galangin (1, 5, and 10 mg/kg/day) groups, after galangin were administered intragastrically once daily for 7 days, a single i.p. dose of CCl₄ (10 mg/kg body weight) on day 8 was followed.

Estimation of biochemical parameters

Determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum were carried out spectrophotometrically following the manufacturer’s protocol of the ELISA kit from Nanjing Jiancheng Bio-Engineering Institute Co., Ltd. (Nanjing, China).

Assay of oxidative stress

The serum was used for the oxidative stress assay. After the serum was separated, superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), and glutathion (GSSG) activity were spectrophotometrically analyzed using an ELISA kit.
from Shanghai JiNing Industrial Co., Ltd (Shanghai, China).

**General histological survey of livers**

The mice were sacrificed by cervical dislocation. Afterward, the livers were excised and irrigated with phosphate buffer, and dried using tissue paper. Hepatic tissue was immersed in 10% formalin and embedded in paraffin. The paraffin-embedded tissue was sectioned 5 μm thick, placed on slides, deparaffinized in xylene, hydrated in decreasing concentrations of ethanol, and washed in water. Conventional haematoxylin and eosin staining were performed. After HE staining, the sections were observed under a light microscope.

**Western blot analysis**

Protein levels of total AMPK, phospho-AMPK, total LKB1 and phospho-LKB1 were measured by western blot. Total protein in the liver tissue was extracted with lysis buffer (50 mM Tris-HCl, pH 7.6, 0.5% Triton X-100, and 20% glycerol). Equal amount of the resulting whole-cell protein extracts were separated by 12% SDS polyacrylamide gel and subsequently transferred to nylon membrane. According to the protocol, the samples were incubated first with the primary antibodies at 4°C overnight, including AMPK, P-AMPK, LKB1, p-LKB1, and β-actin polyclonal antibodies (Cell Signaling, Beverly, MA, USA). After incubated for 12 h at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibody (Cell Signaling, Beverly, MA, USA), the hybridization signal of protein bands were visualized using ECL-plus reagent and imaged using the Bio-Rad Gel Doc 2000 imaging system. Following normalization to β-actin levels, the expressions of p-AMPK and p-LKB1 were expressed as the ratios of the levels of them and their relative inactivated AMPK, and LKB1, respectively.

**Statistical analysis**

In the present study, all the experiments were carried out and analyzed at least six independent experiments. The data are expressed as the mean ± standard deviation. Statistical significance was determined by the one-way analysis of variance (ANOVA), followed by Bonferroni correction. In all cases, $p < 0.05$ was considered significant differences. The statistical Program for Social Sciences Software (IBM SPSS, International Business Machines Corporation, Armonk City, New York, USA) was used to create the artwork.

**Results**

**Hepatoprotective effect of galangin on carbon tetrachloride-induced liver toxicity in mice**

As shown in Figure 1, liver sections of control group showed normal cell morphology, with a well-preserved cytoplasm, a prominent nucleus, and compact arrangement of hepatocytes. However, the liver cells of model group were observed significantly abnormal. The liver sections showed incomplete cytoplasm, loose cell arrangement, and necrosis, which is similar to the liver sections of the mice administered with 1 mg/kg galangin. However, after administered with 5 mg/kg galangin, liver sections of the mice showed a reduced degree of liver cell necrosis. Furthermore, treatment with 10 mg/kg galangin exhibited significant protective effect against CCl4-induced liver damage in mice, as evidenced by the presence of hepatic cords and the absence of necrosis. To investigate the hepatoprotective activities of galangin, the liver function serum markers, ALT and AST, were measured. As depicted in Table 1, compared with the control group, the intoxication of mice with CCl4 notably increased the levels of ALT and AST. In contrast, the increased levels of ALT and AST decreased to a nearly normal level because of the ameliorative effect of galangin (5 and 10 mg/kg). A dose of 1 mg/kg galangin did not significantly improve the pathological damage caused by CCl4.

**Alleviation effect of galangin on oxidative stress of hepatotoxic injury induced by CCl4**

The oxidative stress markers in serum were shown in Table 2. The intoxication of mice with CCl4 significantly decreased the activities of SOD and GSH in comparison with the control group. In addition, a significant increase in the levels of MDA and GSSG was observed in the model group. Compared with the model group, groups of the 5 and 10 mg/kg galangin-pretreated showed a notable ameliorative effect via the elevation of the reduced activity of SOD and GSH and reduction of the increased levels of the MDA and GSSG. However, the 1 mg/kg galangin-pretreated group did not exhibit a...
significant reversal in the oxidative parameter changes caused by CCl₄.

Effects of galangin on phosphorylation of LKB1 and AMPK in the liver tissue

Given that the significant hepatoprotective effects observed in the treatment of 10 mg/kg galangin, this concentration, 10 mg/kg, was selected for the subsequent assays. Expression levels of AMPK, LKB1, p-AMPK, and p-LKB1, detected using western blot, were shown in Figure 2. The result showed that galangin administration enhanced LKB1 phosphorylation and AMPK phosphorylation.

Discussion

The protective effect of galangin on CCl₄-induced liver toxicity in a mouse model, through decreases in MDA and GSSG levels and increases in SOD and GSH activity, was revealed in this study. Thus, the hepatoprotective effect of galangin may be attributed to its antioxidant activity, which the LKB1/AMPK signaling pathway may perform a major role in it.

CCl₄, known as a hepatotoxin, has been widely adopted to induce liver injury and to research the cellular mechanisms behind oxidative damage in laboratory animals. Studies on CCl₄-induced liver damage in mice and rats showed a significant elevation of the serum aminotransferase (e.g. AST and ALT) levels. With it, in this study, there observed a significant elevation in the levels of serum marker enzymes, AST and ALT, in the model group. The administration of galangin reduced the toxic effect of CCl₄ by restoring the levels of AST and ALT to normalcy. CCl₄-induced hepatic lesions arrest with features of coagulation necrosis and hepatocyte vacuolation, which is mainly situated in the central to middle portion of the hepatic lobules. The HE staining assays demonstrated that intra-peritoneal injection of CCl₄ can cause incomplete

Figure 1. Different concentrations of galangin protected against CCl₄-induced histopathological damage. Haematoxylin and eosin staining (Original magnification, ×200) showed that livers in the model group exhibited massive inflammatory cells and cellular necrosis compared to the findings of those in the control group, and symptoms of the histopathological damage were significantly alleviated by galangin (5 and 10 mg/kg) treatment (n = 10).

Table 1. Prophylactic effect of galangin on the restoration of liver function markers in CCl₄-intoxicated mice.

| Group   | ALT (U/L)       | AST (U/L)       |
|---------|-----------------|-----------------|
| Control | 33.29 ± 3.37    | 39.91 ± 5.57    |
| Model   | 231.07 ± 16.03##| 170.70 ± 11.02##|
| 1 mg/kg | 219.76 ± 5.68   | 149.57 ± 15.07  |
| 5 mg/kg | 171.54 ± 10.87##| 102.35 ± 7.89## |
| 10 mg/kg| 109.13 ± 9.54** | 76.75 ± 9.23**  |

Values are expressed as the mean ± SD (n = 10/group).

##p < 0.01 compared to the control group. **p < 0.01 compared to the model group.
cytoplasm, loose cell arrangement, and necrosis, reflecting liver damage, while galangin preconditioning alleviated abovementioned liver damage. All of these results bore out the protective effects of galangin on the livers of mice administered CCl4.

Lipid peroxidation, regarded as one of the principal causes of CCl4-induced liver injury, is mediated by the free-radical derivatives of CCl4.17,18 When CCl4 enters the body, cytochrome P450 is metabolized to produce the trichloromethyl radicals (•CCl3 and/or CCl3OO•), which result in membrane lipid peroxidation and eventually cell necrosis.19,20 Once the damage to hepatic antioxidant defense system, an increase in MDA and GSSG and/or alteration in enzymatic and non-enzymatic antioxidants, including SOD and GSH, occurred. SOD converted the superoxide radicals into H2O2 and O2, which will be further detoxified to water by CAT or GPx. GSH functioned in initiating the detoxification of alkylolation of lipid, protein, and nucleic acid as well as providing cellular resistance to lipid peroxidation by elevating the conjugation of toxic electrophiles with GSH. Hence, the activities of these enzymes were used for the oxidative stress evaluation in liver tissue.21,22 ROS induced by CCl4 tips the balance between ROS production and the antioxidant defense system. In this work, down-regulation of the hepatic SOD activity and GSH content and up-regulation of the content of MDA and GSSG notably induced CCl4 were all markedly restored by galangin. This suggests that the hepatoprotective effect of galangin may be attributed to its antioxidant activity.

The LKB1/AMPK pathway is essential for maintaining cellular homeostasis, especially for oxidation-reduction reactions. It is reported that activating the LKB1/AMPK signaling pathway can reduce the excessive production of ROS.23,24 Studies have also found that the LKB1/AMPK signaling pathway plays an important role in liver diseases, such as non-alcoholic fatty liver disease (NAFLD), and activation of the pathway can improve lipid metabolism disorders in individuals with NAFLD.25 However, it is unclear whether the LKB1/AMPK signaling pathway plays a hepatoprotective role in acute liver injury. In the present study, our results revealed that the phosphorylated levels of LKB1 and AMPK in the galangin-pretreated mice were higher than those in model group mice. Taken together, these results

### Table 2. The effect of galangin pretreatment on oxidative stress parameters of mice in CCl4-induced hepatotoxicity.

| Group       | SOD (U/mL) ± SD | MDA (nmol/mL) ± SD | GSH (ng/L) ± SD | GSSG (nmol/mL) ± SD |
|-------------|-----------------|--------------------|----------------|---------------------|
| Control     | 59.52 ± 6.01    | 6.59 ± 0.14        | 756.51 ± 36.71 | 7.74 ± 0.60        |
| Model       | 35.74 ± 2.87###| 10.31 ± 0.45###   | 395.47 ± 17.56###| 10.09 ± 0.51###    |
| 1 mg/kg     | 39.96 ± 3.12    | 9.66 ± 0.32        | 377.70 ± 11.86 | 9.52 ± 0.50        |
| 5 mg/kg     | 44.45 ± 3.59###| 8.49 ± 0.35###    | 452.58 ± 21.47###| 8.89 ± 0.23###    |
| 10 mg/kg    | 53.49 ± 2.78**  | 7.70 ± 0.44**     | 559.10 ± 25.21**| 8.15 ± 0.50**     |

Values are expressed as the mean ± SD (n = 10/group). **p < 0.01 compared to the model group.
suggest that galangin’s protective effect against CCl4-induced liver injury is likely mediated via activation of the LKB1/AMPK pathway.

Limitations of our present study should be explored in further study. The first key limitation of our study is that it did not use techniques such as gene knockout or gene silencing to further study the relationship between the hepatoprotective effect of galangin and the LKB1/AMPK pathway. The second major limitation is that we did not study other factors such as inflammation and apoptosis. The lack of power calculations to estimate the sample size selected for the study is a limitation of this study. Therefore, further studies should be directed toward these objectives.

Conclusion

In conclusion, we found that galangin can significantly reduce CCl4-induced liver damage and oxidative stress, and this hepatoprotective effect may be related to the LKB1/AMPK signaling pathway.

Animal welfare

The present study involved client-owned animals; it demonstrated a high standard (best practice) of veterinary care. Experimental animals were killed and their bodies were disposed of: the cervical vertebra was dislocated after anesthesia with 3% pentobarbital sodium intraperitoneal injection. The animal carcasses were delivered to the animal center of Yantai Yuhuangding Hospital of Laishan branch for innocuous treatment.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval

Ethical approval for this study was obtained from the ethics committee of Yantai Yuhuangding Hospital of Laishan branch (no. 20160617).

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