Protective Mechanism of *Salvia miltiorrhiza* on Carbon Tetrachloride-Induced Acute Hepatotoxicity in Rats

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Abstract. The purpose of this study was to investigate the possible mechanisms of *Salvia miltiorrhiza* (Sm) in carbon tetrachloride (CCl₄)-induced acute hepatotoxicity in rats. Male Wistar rats received a single dose of CCl₄ (2 ml/kg in corn oil, intraperitoneally). Three hours after CCl₄ intoxication, rats received either Sm (100 mg/kg) or silymarin (100 mg/kg) by gastrogavage twice a day for 2 consecutive days. CCl₄-induced liver damage was shown by significant elevation of serum aminotransferase levels. Additionally, a significant decrease was observed in hepatic microsomal P450 2E1 protein content and hepatic concentrations of antioxidant enzymes. In contrast, rats given both Sm and silymarin supplement had less elevation of serum aminotransferase concentrations associated with less severe lobular damage of hepatocytes than rats receiving CCl₄ alone. Sm administration restored the reduction of hepatic microsomal P450 2E1 protein content as well as inducing an increase in hepatic glutathione concentration. On the other hand, administration of silymarin resulted in an elevation of hepatic superoxide dismutase levels. Moreover, both Sm and silymarin treatment inhibited the elevation of hepatic inducible nitric oxide (iNOS) protein content and nitrite concentration in liver homogenate 24 h after CCl₄ intoxication. We concluded that administration of Sm is effective in amelioration of CCl₄-induced hepatotoxicity. This effect may be due to its ability to decrease the metabolic activation of CCl₄ by an increase in P450 2E1 protein content and its antioxidant activity associated with less increase in hepatic iNOS protein content.

Keywords: *Salvia miltiorrhiza*, carbon tetrachloride, cytochrome P450, glutathione, nitric oxide

Introduction

Carbon tetrachloride (CCl₄) is widely used in animal models to induce acute liver injury (1 – 3). It is generally believed that the toxicity of CCl₄ results from its reductive dehalogenation by the cytochrome P450 enzyme system into the highly reactive free radical trichloromethyl radical (4). Wong et al. (5) suggested that P450 2E1 is the major factor involved in CCl₄-induced hepatotoxicity. In addition, it has been shown that CCl₄-induced toxicity may stimulate endogenous reactive oxygen and nitrogen species that have also been suggested to play an important role in the pathogenesis of hepatotoxicity (6). Hierholzer et al. (7) suggested that inducible nitric oxide synthase (iNOS)-generated nitric oxide not only directly contributes to tissue damage but that it also upregulates the inflammatory response through specific signaling mechanisms. Recent reports have demonstrated that induced nitric oxide overproduction occurs in the liver of rats with CCl₄-induced acute liver injury (8, 9) and suggested that iNOS may act as a mediator in the pathogenesis of hepatotoxicity in rats (9, 10). On the other hand, trichloromethyl free radicals...
can react with sulfhydryl groups such as glutathione and protein thiols. The covalent binding of trichloromethyl free radicals to cell protein is considered the initial step in a chain of events that eventually leads to membrane lipid peroxidation and finally cell necrosis (4). Moreover, hepatic glutathione level was decreased after CCl₄ administration due to the reaction of glutathione with CCl₄-derived free radicals in the hepatocytes (11, 12). Accordingly, CCl₄-induced hepatotoxicity in rodents was reported to be partially protected by the pretreatment of antioxidants (13).

_Salvia miltiorrhiza_ Bunge (Labiatae) is officially listed in the Chinese Pharmacopoeia and is used for the treatment of cardiovascular disorders as well as inflammation (14, 15). The water-soluble extract of _Salvia miltiorrhiza_ (Sm) contains phenolic compounds that are effective in protecting liver microsomes, hepatocytes, and erythrocytes against oxidative damage (16). Wasser et al. (17) and Nan et al. (18) have demonstrated that chronic administration of water-soluble of Sm reduced both CCl₄- and bile duct ligated-induced hepatic fibrosis. Our previous report (19) also showed that long-term administration of Sm ameliorated the portal hypertensive state in bile duct ligated rats. On the other hand, silymarin, a flavonoid isolated from milk thistle, is used clinically in Europe and Asia for the treatment of liver diseases. In a rat model of liver damage induced by CCl₄ or paracetamol, silymarin exerted a radical scavenger effect that prevented lipid peroxidation and preventing subsequent hepatic damage (20). Although Sm has been previously reported to reduce fibrosis and portal hypertension (17–19), scientific studies on its underlying these protective effects in rats. The effect of Sm on CCl₄-induced acute liver injury was also compared to the effect of silymarin.

**Materials and Methods**

**Animals**

Male Wistar rats (220–250 g) were used in the experiments. All rats were caged at 24°C, with a 12-h light-dark cycle, and allowed free access to food and water. Animal studies were approved by the Animal Experiment Committee of the University and conducted humanely.

_Salvia miltiorrhiza_ preparation

Sm was prepared by the methods described in our previous study (19). Briefly, Sm (400 g) of the dried root was powdered and extracted with 1000 ml of boiled distilled water for 2 h at 80°C. The resulting extract was filtered and lyophilized (freeze-mobile; Virtis, New York, NY USA) to a light brownish residue with an approximate yield of 25%. These substances were stored at −20°C until used. A separate study showed that treatment with 100 mg/kg of Sm extract and 100 mg/kg of silymarin significantly decreased the CCl₄-induced elevation of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in rats as shown in Table 1.

**Table 1. Changes of liver enzymes in normal rats and in rats receiving carbon tetrachloride and treated with silymarin or different doses of _Salvia miltiorrhiza_**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum ALT (U/L)</th>
<th>Serum AST (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>45 ± 8</td>
<td>124 ± 15</td>
</tr>
<tr>
<td>Normal + Sm (100 mg/kg)</td>
<td>54 ± 6</td>
<td>130 ± 9</td>
</tr>
<tr>
<td>CCl₄</td>
<td>644 ± 53</td>
<td>1677 ± 108</td>
</tr>
<tr>
<td>CCl₄ + Sm (10 mg/kg)</td>
<td>638 ± 40*</td>
<td>1603 ± 119*</td>
</tr>
<tr>
<td>CCl₄ + Sm (50 mg/kg)</td>
<td>468 ± 44*</td>
<td>1579 ± 142*</td>
</tr>
<tr>
<td>CCl₄ + Sm (100 mg/kg)</td>
<td>375 ± 72*/#</td>
<td>911 ± 47*/#</td>
</tr>
<tr>
<td>CCl₄ + Sm (200 mg/kg)</td>
<td>394 ± 51*/#</td>
<td>879 ± 69*/#</td>
</tr>
<tr>
<td>CCl₄ + Sily (100 mg/kg)</td>
<td>355 ± 66*/#</td>
<td>881 ± 52*/#</td>
</tr>
</tbody>
</table>
| Rats were pretreated with carbon tetrachloride (CCl₄, 2 ml/kg, i.p.). Three hours later, rats were treated with _Salvia miltiorrhiza_ (Sm; 10, 50, 100, or 200 mg/kg, p.o.) or silymarin (sily; 100 mg/kg, p.o.) twice daily. Normal rats were given in saline. Hepatotoxicity was determined following CCl₄ exposure 24 h by measuring serum ALT and AST activities. Each value represents the mean ± S.E.M. of five rats. *Significantly different from normal rats. **Significantly different from rats receiving carbon tetrachloride alone.

**Study protocol**

CCl₄ (2 ml/kg) was dissolved in corn oil (1:1, v/v%) and administered intraperitoneally to induce acute liver injury as previously described (2). Three groups of rats (eight rats in each group) each received a single intraperitoneal injection of CCl₄, respectively. Among these 3 groups, one group (the vehicle group) was given CCl₄ alone and the other 2 groups of rats received gastro-gavage of either Sm (100 mg/kg) or silymarin (100 mg/kg) (Sigma Chemical Co., St. Louis, MO, USA), respectively. Another one group of rats received gastro-gavage of saline and served as the normal control. Drugs or saline were given starting 3 h after CCl₄ administration and continued every 12 h for 2 consecutive days. The frequency of Sm administration was selected according to our previous study (19). Under diethyl ether anesthesia, blood samples obtained from the femoral artery were collected from each rat at different time points (12, 24, and 48 h) after CCl₄ administration and then centrifuged. Thereafter, rats were killed by an
overdose of diethyl ether. Similarly, a wedge resection of the liver was obtained from each rat at different time points (12, 24, and 48 h) after CCl₄ administration for various assays. In addition, a section of liver tissues was obtained from each group of rats receiving CCl₄ at 48 h after CCl₄ administration and stored in formalin for histological examination.

**Histopathology and biochemistry assay**

Liver tissue samples were fixed in 10% formalin and then embedded in paraffin, cut into 5-μm sections, stained with hematoxylin-eosin (HE), and examined under light microscopy by a pathologist. Serum AST and ALT levels were measured by an auto-analyzer (model 736-60, Hitachi, Tokyo).

**Hepatic microsomal preparation**

Microsomes were prepared by differential centrifugation as previously described with minor modification (22). Briefly, livers were quickly removed from rats and homogenized in 0.15 M KCl solution containing 10 mM EDTA using a Potter-type Teflon glass homogenizer. The homogenate was centrifuged at 10,000 × g for 15 min in a refrigerated centrifuge at 4°C (30RF; Universal, Hettich, Germany). The supernatant was then centrifuged at 105,000 × g for 60 min at 4°C in a preparative ultracentrifuge (XL-90; Beckman, Palo Alto, CA, USA). The resulting pellet was suspended in a lysis buffer containing 10 mmol/L Tris-HCl (pH 7.4), 1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 5 mmol/L EDTA (pH 7.4). The final products were stored at −80°C until use.

**Measurement of hepatic nitrite levels**

Hepatic nitrite levels were estimated by the Greiss reaction with sodium nitrite as a standard (23).

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**Fig. 1.** Serum values of AST and ALT in normal rats were found to be 124 ± 15 U/L and 45 ± 8 U/L, respectively. Serum ALT and AST levels in normal rats, in rats receiving CCl₄ alone, in rats with CCl₄ and silymarin, and with CCl₄ and Sm at 12, 24, and 48 h after CCl₄ administration. Values are means ± S.E.M. for eight rats in each group. *P<0.05 vs normal control. **P<0.05 vs CCl₄ alone.
Western blot analysis for hepatic iNOS and cytochrome P450 2E1

Freshly isolated liver tissue was homogenized in a lysis buffer. The protein concentration of the tissue homogenate and the cytosolic and microsomal fractions was determined according to the method of Lowry et al. (24). One hundred µg of protein from liver homogenates or 50 µg of protein from purified microsome was loaded per lane on 8% or 12% polyacrylamide gels and electrophoresis was performed. Proteins were then transferred onto nitrocellulose membranes. The membrane was blocked overnight with buffer and then incubated with primary antibodies for 1 h using 1:1000 dilution of rabbit anti-mouse iNOS antibody (Transduction Laboratory, Lexington, KY, USA) and 1:750 dilution of goat polyclonal anti-rat CYP 2E1 antibody (Chemicon Co., Temecula, CA, USA). The membranes were then washed 3 times in TTBS solution containing Tris buffer solution (TBS) with 0.1% Tween-20 for 15 min, incubated with 1:5000 dilution of horseradish peroxidase-conjugated anti-mouse IgG, and then 1:2000 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG as the second antibody for 1 h. The protein was visualized with an enhanced chemiluminescence Western blotting detection kit (Amersham, Arlington Heights, IL, USA), and exposed to X-ray film for 3 min. The relative content of iNOS and cytochrome P450 2E1 protein in each tissue was quantified by densitometric scanning using an image-analysis system (Kodak Co., Rochester, NY, USA).

Hepatic glutathione and antioxidant enzymes — superoxide dismutase (SOD) and catalase determinations

Hepatic levels of glutathione were determined using a glutathione-400 colorimetric assay kit (Calbiochem Co., San Diego, CA, USA) and performed by using a spectrophotometer. Liver tissue was homogenized with 2 ml of 10% (w/v) metaphosphoric acid (MPA) solution at 4°C. Then each sample was centrifuged at 3000 × g for 10 min at 4°C. After each sample was vortexed, 200 µl aliquot of the centrifuged supernatants was read at 412 nm in a spectrophotometer. The values of unknown samples were drawn from a standard curve plotted by assaying different known concentrations of GSH. The amounts of hepatic GSH were expressed as µmol/g liver wt. Hepatic SOD activity was determined as described by Marklund and Marklund (25). One unit of SOD activity was defined as the amount of protein that inhibits the rate of pyrogallol reduction by 50%. Catalase activity was determined by measuring the exponential disappearance of H₂O₂ at 240 nm and was expressed as units per milligram of protein as described by Aebi (26).

Fig. 2. Liver tissue sections from a normal rat (A), a rat receiving CCl₄ only (B), a rat with CCl₄ and silymarin (C), and a rat with CCl₄ and Sm (D). Histological sections were prepared from liver 48 h after CCl₄ exposure and stained with hematoxylin and eosin (Original magnification×100, calibration bar = 120 µm).
Both enzyme activities were expressed as units per mg of protein.

**Statistics**

The results are expressed as means ± S.E.M. Statistical analyses were performed with one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls’ multiple range test or Student’s t-test when appropriate. Significance was determined at \( P < 0.05 \).

**Results**

**Serum ALT and AST levels**

The changes of serum AST and ALT levels are shown in Table 1. Normal rats receiving Sm (100 mg/kg) resulted in no significant changes in serum AST and ALT levels compared to those of the normal control. Serum AST and ALT levels were markedly elevated in rats receiving CCl\(_4\) alone. In contrast, in rats receiving either Sm (100 mg/kg) or silymarin (100 mg/kg), there were significantly less elevations of serum AST and ALT levels than in rats receiving CCl\(_4\) alone.

In rats receiving CCl\(_4\) only, AST and ALT were significantly increased at 12, 24, and 48 h after CCl\(_4\) administration (Fig. 1). In contrast, rats receiving either Sm or silymarin had less elevation of both AST and ALT than rats receiving CCl\(_4\) alone (Fig. 1).

**Histological assessment**

A liver tissue section from normal rats is shown in Fig. 2A. In rats receiving CCl\(_4\) alone, the liver histology at 48 h after CCl\(_4\) exposure showed extensive necrosis of hepatocytes in centrilobular regions of the liver (Fig. 2B). In contrast, less severe lobular damage of hepatocytes was seen in rats receiving either silymarin treatment (Fig. 2C) or Sm treatment (Fig. 2D).

**Hepatic glutathione, SOD, and catalase levels**

Compared to normal rats, rats receiving CCl\(_4\) alone showed a decreased hepatic level of glutathione at each time point. In rats receiving silymarin, the decrease in hepatic glutathione levels was delayed after CCl\(_4\) exposure compared to rats receiving CCl\(_4\) alone. In contrast, Sm administration preserved glutathione levels (Fig. 3A). Hepatic SOD levels decreased in rats receiving CCl\(_4\) alone compared to normal rats. However, in rats receiving silymarin, the reduction of hepatic SOD levels was less than in rats receiving CCl\(_4\) alone (Fig. 3B). The changes of hepatic catalase levels were similar among the 3 groups of rats receiving CCl\(_4\) (Fig. 3C).

**Hepatic microsomal P450 2E1 protein content**

Compared to normal rats, rats receiving CCl\(_4\) alone showed significant decreases in P450 2E1 levels at each time point examined. In rats receiving silymarin, P450 2E1 levels were similar to those observed in CCl\(_4\)-treated rats at each time-point. In contrast, in rats receiving Sm, higher levels of P450 2E1 were found starting at 24 h after CCl\(_4\) administration compared to rats receiving CCl\(_4\) alone. In addition, the P450 2E1 level was similar to that of normal rats at 48 h after CCl\(_4\) administration (Fig. 4).

**Hepatic nitrite levels and iNOS protein content**

Hepatic nitrite levels were significantly elevated in all groups of rats receiving CCl\(_4\) at each time point examined compared to normal rats (Fig. 5). However,
Salvia miltiorrhiza ameliorates hepatotoxicity

A smaller increase in hepatic nitrite levels was found in the Sm group at 12 and 24 h as well as in the silymarin group at 12, 24, and 48 h after CCl₄ administration. The hepatic iNOS protein content was significantly increased in the 3 groups of rats at 12, 24, and 48 h after administration of CCl₄ compared to normal rats (Fig. 6). However, a lower level of hepatic iNOS protein content was found in rats receiving Sm or silymarin than in rats receiving CCl₄ alone.

Discussion

Liver injuries induced by CCl₄ are the best characterized system of xenobiotic-induced hepatotoxicity and commonly used models for the screening of anti-hepatotoxic and/or hepatoprotective activities of drugs (4). To our knowledge, this is the first study to demonstrate that administration of Sm ameliorated CCl₄-induced acute liver injury in rats as evidenced by both histological findings and serum AST and ALT levels. Similar protective effects were also observed in rats receiving silymarin, although the mechanism of action for these effects may not be the same.

Glutathione content in the liver plays a primary role...
in the protection against trichloromethyl radical-induced liver damage (4, 27). It has been suggested that the lipid peroxides generated after CCl₄ intoxication is eliminated by glutathione peroxidase in the presence of glutathione, thus curbing the propagation of lipid peroxidation (4). The present study found a significant decrease in hepatic glutathione and SOD levels following CCl₄ exposure. This is consistent with the results of Recknagel et al. and Nishida et al. (4, 12). However, our results found that the hepatic glutathione and SOD levels were higher in rats receiving either Sm or silymarin than in rats exposed to CCl₄ alone. In addition, hepatic glutathione level is replenished to a greater extent in rats receiving Sm than in rats receiving silymarin. In contrast, hepatic SOD activity was increased more profoundly in rats receiving silymarin than in rats receiving Sm. Therefore, although the mechanism of action may not be identical, both Sm and silymarin appear to exert their protective effect against CCl₄-induced hepatic injury, at least in part, through their effect on hepatic antioxidant levels. On the other hand, we observed less hepatotoxicity in rats receiving Sm or silymarin than rats receiving CCl₄ alone. It is possible that administration of Sm or silymarin reduced CCl₄-induced hepatotoxicity that lead to less depletion of hepatic glutathione or SOD levels. Further studies may be needed to elucidate this phenomenon.

It has been established that CCl₄ is metabolized by the cytochrome P450 system yielding trichloromethyl radical, which causes direct damage to hepatocytes. The trichloromethyl radical may bind either at the heme group of cytochrome P450 or at the active site of the enzyme near the heme group, leading to the inactivation of P450 pathways (28). In the present study, a significant decrease in hepatic P450 2E1 protein content was found in rats treated with CCl₄ alone. In contrast, in rats that received Sm, the hepatic P450 2E1 protein content was less suppressed at 24 h after CCl₄ treatment. Furthermore, the P450 2E1 protein level almost returned to the normal state at the time point of 48 h following CCl₄ exposure. This result suggests that Sm exerts a more beneficial effect on hepatic P450 2E1 content restoration than silymarin. Therefore, it is possible that Sm may restore the hepatic P450 2E1 function and lead to a rapid recovery from CCl₄-induced liver injury. Similar observations regarding a decrease in P450 2E1

![Fig. 6. Changes of hepatic iNOS content in different groups of rats. A: Western blot analysis of hepatic iNOS content in normal rats, in rats receiving CCl₄ alone, in rats with CCl₄ and silymarin, and in rats with CCl₄ and Sm at 12, 24, and 48 h after CCl₄ administration. B: Densitometric analysis of Western blot in different groups of rats. Values are means ± S.E.M. for eight rats in each group. *P<0.05 vs normal rats. †P<0.05 vs CCl₄ alone.](image-url)
protein content was found in rats treated with acetaminophen (10) or CCl₄ (29) and also was found in human liver microsomes following CCl₄ administration (30). In their studies, an increase in P450 2E1 protein content following pharmacological manipulations ameliorated the hepatotoxicity after acetaminophen (10) or CCl₄ (29) administration. However, it is also possible that administration of Sm caused a reduction of CCl₄-related hepatotoxicity that lead to an early recovery of P450 2E1 protein content in rats receiving Sm. Therefore, decreased hepatotoxicity following Sm administration would lead to a less elevation of liver enzyme activity.

The mechanism of action regarding the hepatoprotective effects of Sm may be multi-factorial. In the current study, we found a smaller increase in hepatic nitrites level and a lower level of hepatic iNOS protein content in rats receiving CCl₄ and treated with Sm or silymarin than in rats receiving CCl₄ alone. Accordingly, the other possible mechanism of action in the protection against CCl₄-induced hepatotoxicity appears to be, at least in part, due to the decreased hepatic nitric oxide production. It is known that certain cytokines (such as tumor necrosis factor-α) are induced following CCl₄ and lipopolysaccharide exposure that may lead to an up-regulation of hepatic iNOS activity (31). In addition, previous studies have demonstrated that administration of cytokines and cytokine inducers to animals reduces cytochrome P450 450 protein and enzyme activity (32, 33). Moreover, the endotoxin-induced increase in nitric oxide production and the decrease in cytochrome P450 450 levels in rats were ameliorated by administration of a nitric oxide synthase inhibitor (32). It is possible that the reduced cytochrome P450 450 protein content is the result of an increased generation of nitric oxide following CCl₄ intoxication. The role of nitric oxide in liver damage remains controversial (34, 7). Although several studies have found that nitric oxide protected against CCl₄-induced liver injury using a NOS knockout mice or a NOS inhibitor (35, 8), certain evidences have found that excessive nitric oxide production by iNOS may lead to hepatic injury (36–40). In the present study, we found an increase in iNOS protein content in rats receiving CCl₄ that reached its peak level at 24 h after CCl₄ exposure. This result corresponded with the decreased hepatic microsome 2E1 protein content. In contrast, in rats that received either Sm or silymarin, the hepatic iNOS protein content and nitrite levels were increased less than in rats receiving CCl₄ alone. The present results suggest that both Sm and silymarin ameliorate CCl₄-induced acute liver injury that probably acts, in part, through the down-regulation of iNOS content. A similar observation has found that CCl₄-induced hepatic injury in mice can be prevented by the administration of aminoguanidine, a specific iNOS inhibitor (9).

In conclusion, the results of this study demonstrate that Sm has a hepatoprotective action on CCl₄-induced acute hepatic injury in rats. These results show that the protective effect of Sm may be due to its ability to decrease the metabolic activation of CCl₄ by the increase in P450 2E1 protein content and the antioxidant activity, in combination with a less elevation of hepatic iNOS protein content, all of which are capable of exerting hepatocellular protection.

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