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

Tzung-Yan Lee[1], Lee-Ming Mai[2], Guei-Jane Wang[3], Jen-Hwey Chiu[1], Yun-Lian Lin[3] and Han-Chieh Lin[4,5,*]

[1]*Institute of Traditional Medicine, National Yang-Ming University School of Medicine, Taipei 112, Taiwan*
[2]*Institute of Anatomy, National Yang-Ming University, Taipei 112, Taiwan*
[3]*National Research Institute of Chinese Medicine, Taipei, Taiwan*
[4]*Division of Gastroenterology, Department of Medicine, Taipei Veterans General Hospital, No. 201, Sec. 2, Shih-Pai Rd, Taipei 11217, Taiwan*
[5]*National Yang-Ming University School of Medicine, Taipei 112, Taiwan*

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Abstract. The purpose of this study was to investigate the possible mechanisms of *Salvia miltiorrhiza* (Sm) in carbon tetrachloride (CCl_4)-induced acute hepatotoxicity in rats. Male Wistar rats received a single dose of CCl_4 (2 ml/kg in corn oil, intraperitoneally). Three hours after CCl_4 intoxication, rats received either Sm (100 mg/kg) or silymarin (100 mg/kg) by gastrogavage twice a day for 2 consecutive days. CCl_4-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 CCl_4 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 CCl_4 intoxication. We concluded that administration of Sm is effective in amelioration of CCl_4-induced hepatotoxicity. This effect may be due to its ability to decrease the metabolic activation of CCl_4 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 (CCl_4) is widely used in animal models to induce acute liver injury (1 – 3). It is generally believed that the toxicity of CCl_4 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 CCl_4-induced hepatotoxicity. In addition, it has been shown that CCl_4-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 CCl_4-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

*Corresponding author (affiliation #4). FAX: +886-2-2873-9318
E-mail: hclin@vghtpe.gov.tw

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 CCl_4 administration due to the reaction of glutathione with CCl_4-derived free radicals in the hepatocytes (11, 12). Accordingly, CCl_4-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 CCl_4- 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 CCl_4 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 mechanism with respect to acute liver injury caused by CCl_4 are lacking. The current study was undertaken to evaluate the protective effects of Sm on CCl_4-induced hepatotoxicity and to elucidate the possible mechanisms underlying these protective effects in rats. The effect of Sm on CCl_4-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 CCl_4-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*

Groups	Serum ALT (U/L)	Serum AST (U/L)
Normal	45 ± 8	124 ± 15
Normal + Sm (100 mg/kg)	54 ± 6	130 ± 9
CCl_4	644 ± 53	1677 ± 108
CCl_4 + Sm (10 mg/kg)	638 ± 40*	1603 ± 119*
CCl_4 + Sm (50 mg/kg)	468 ± 44*	1579 ± 142*
CCl_4 + Sm (100 mg/kg)	375 ± 72*,#	911 ± 47*,#
CCl_4 + Sm (200 mg/kg)	394 ± 51*,#	879 ± 69*,#
CCl_4 + Sily (100 mg/kg)	355 ± 66*,#	881 ± 52*,#

Rats were pretreated with carbon tetrachloride (CCl_4, 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 CCl_4 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

CCl_4 (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 CCl_4, respectively. Among these 3 groups, one group (the vehicle group) was given CCl_4 alone and the other 2 groups of rats received gastrogavage 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 gastrogavage of saline and served as the normal control. Drugs or saline were given starting 3 h after CCl_4 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 CCl_4 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 CCl_4 administration for various assays. In addition, a section of liver tissues was obtained from each group of rats receiving CCl_4 at 48 h after CCl_4 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 $10{,}000 \times g$ for 15 min in a refrigerated centrifuge at 4°C (30RF; Universal, Hettich, Germany). The supernatant was then centrifuged at $105{,}000 \times g$ 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).

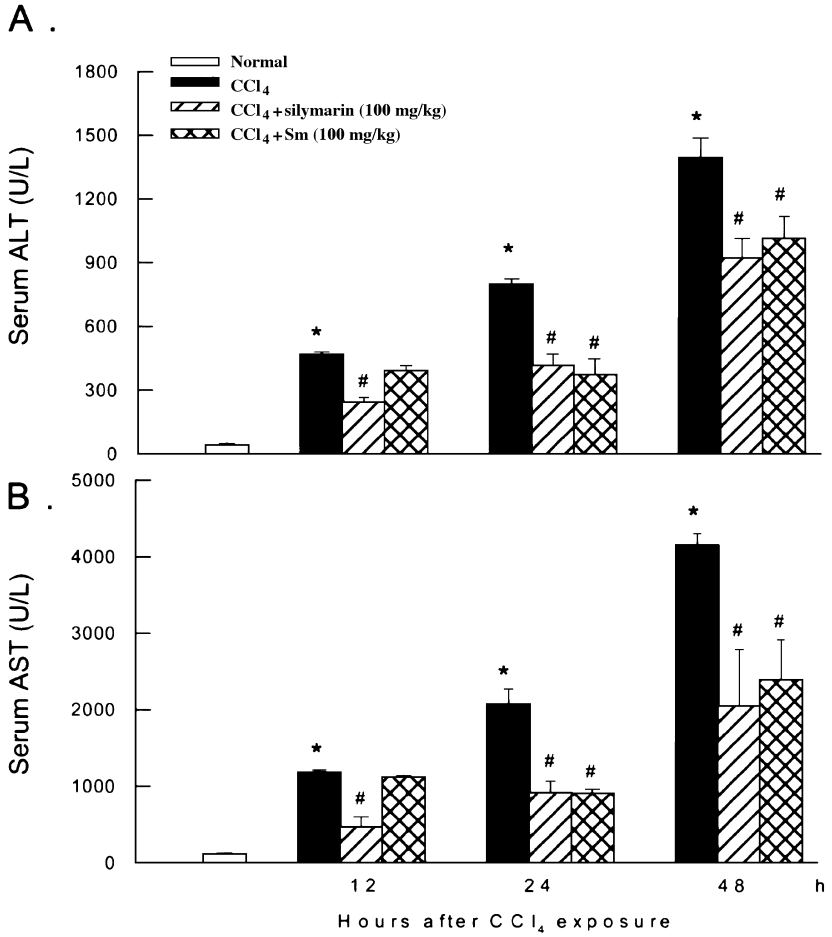

Fig. 1. Serum values of AST and ALT in normal rats were found to be 124 ± 15 U/L and 45 ± 8 U/L, respectively. Serum ALT and AST levels in normal rats, in rats receiving CCl_4 alone, in rats with CCl_4 and silymarin, and with CCl_4 and Sm at 12, 24, and 48 h after CCl_4 administration. Values are means ± S.E.M. for eight rats in each group. *P<0.05 vs normal control. $^{\#}P$<0.05 vs CCl_4 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 1h. 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 $3000 \times g$ 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 H_2O_2 at 240 nm and was expressed as units per milligram of protein as described by Aebi (26).

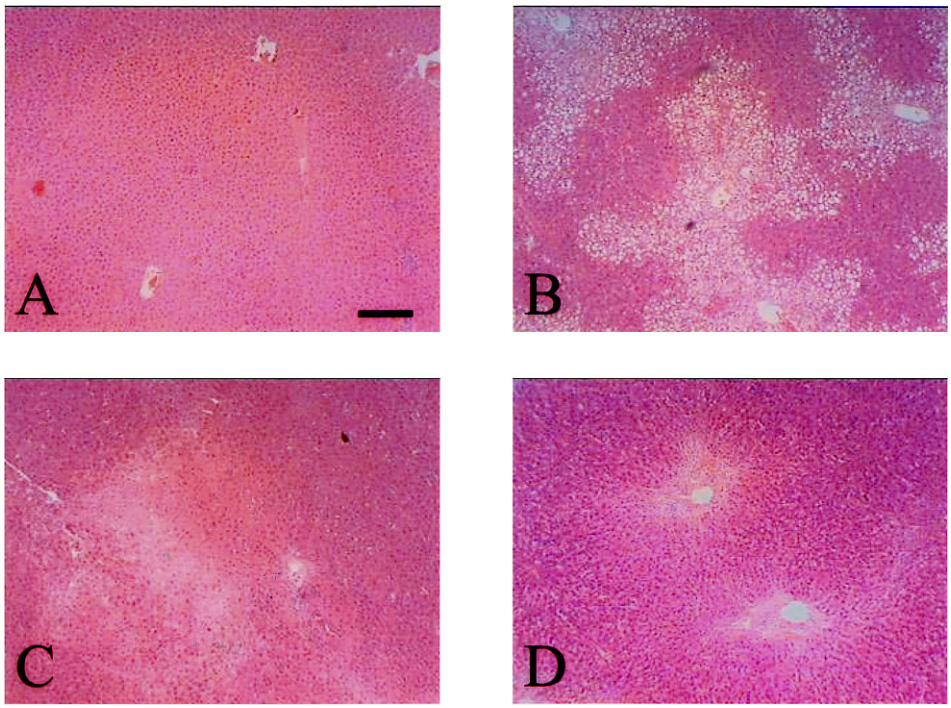

Fig. 2. Liver tissue sections from a normal rat (A), a rat receiving CCl_4 only (B), a rat with CCl_4 and silymarin (C), and a rat with CCl_4 and Sm (D). Histological sections were prepared from liver 48 h after CCl_4 exposure and stained with hematoxylin and eosin (Original magnification×100, calibration bar = 120 μm).

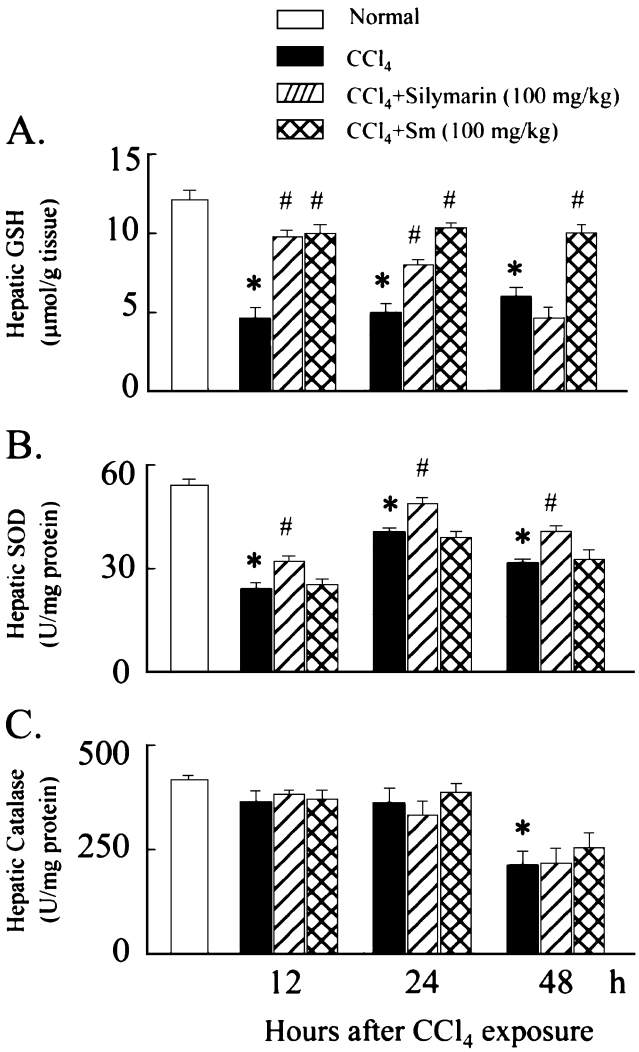

Fig. 3. Hepatic glutathione (A), SOD (B), and catalase (C) levels at different time points after CCl_4 (2 ml/kg) or drug administration. Values are means ± S.E.M. for eight rats in each group. *P<0.05 vs normal rats. #P<0.05 vs CCl_4 alone.

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,

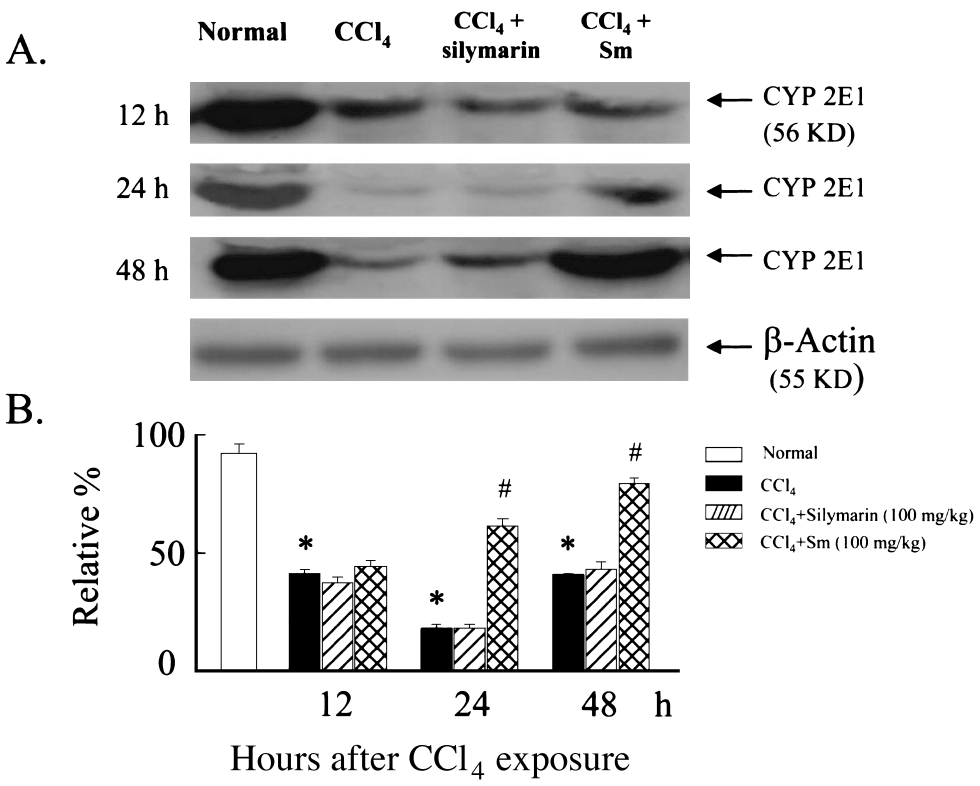

Fig. 4. Changes of hepatic P450 2E1 content in different groups of rats. A: Western blot analysis of hepatic microsomal cytochrome P450 2E1 content (CYP 2E1) in normal rats, in rats receiving CCl_4 alone, in rats with CCl_4 and silymarin, and in rats with CCl_4 and Sm at 12, 24, and 48 h after CCl_4 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 CCl_4 alone.

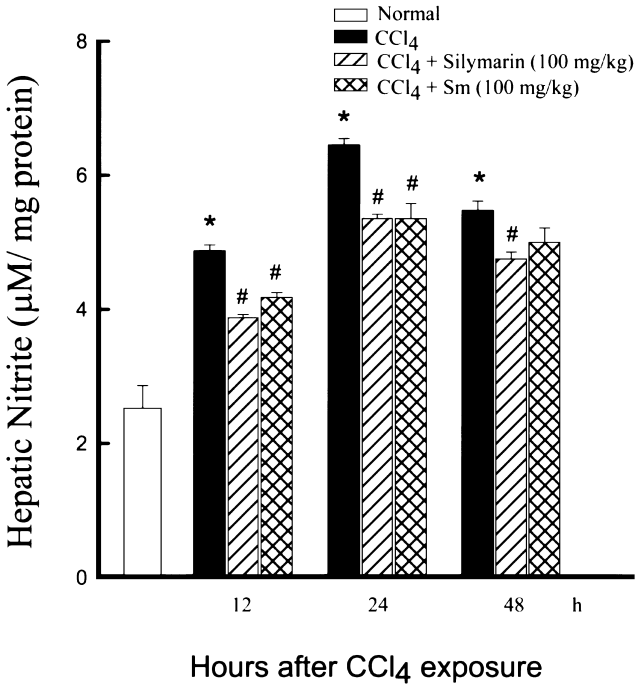

Fig. 5. Hepatic nitrite concentrations in normal rats, in rats receiving CCl_4 alone, in rats with CCl_4 and silymarin, and in rats with CCl_4 and Sm at 12, 24, and 48 h after CCl_4 administration. Values are means ± S.E.M. for eight rats in each group. *P<0.05 vs normal control. $^{\#}P$<0.05 vs CCl_4 alone.

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 CCl_4 administration. The hepatic iNOS protein content was significantly increased in the 3 groups of rats at 12, 24, and 48 h after administration of CCl_4 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 CCl_4 alone.

Discussion

Liver injuries induced by CCl_4 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 CCl_4-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

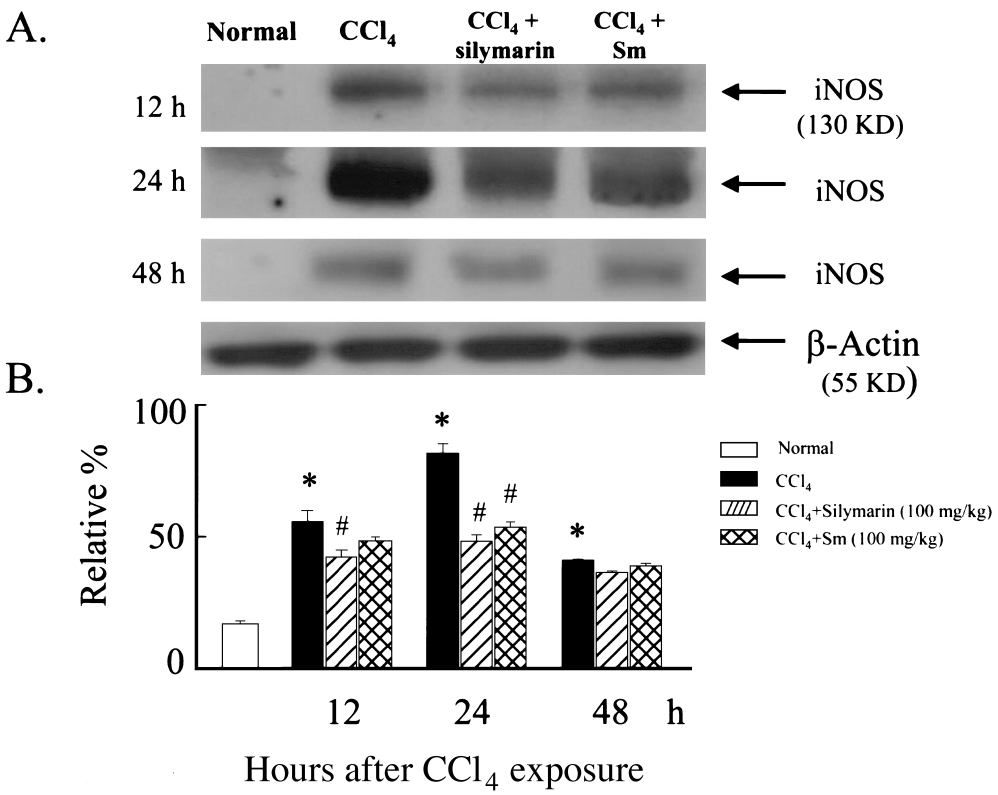

Fig. 6. Changes of hepatic iNOS content in different groups of rats. A: Western blot analysis of hepatic iNOS content in normal rats, in rats receiving CCl_4 alone, in rats with CCl_4 and silymarin, and in rats with CCl_4 and Sm at 12, 24, and 48 h after CCl_4 administration. B: Densitometric analysis 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 CCl_4 alone.

in the protection against trichloromethyl radical-induced liver damage (4, 27). It has been suggested that the lipid peroxidates generated after CCl_4 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 CCl_4 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 CCl_4 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 CCl_4-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 CCl_4 alone. It is possible that administration of Sm or silymarin reduced CCl_4-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 CCl_4 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 P450 pathways (28). In the present study, a significant decrease in hepatic P450 2E1 protein content was found in rats treated with CCl_4 alone. In contrast, in rats that received Sm, the hepatic P450 2E1 protein content was less suppressed at 24 h after CCl_4 treatment. Furthermore, the P450 2E1 protein level almost returned to the normal state at the time point of 48 h following CCl_4 exposure. This result suggests that Sm exerted 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 CCl_4-induced liver injury. Similar observations regarding a decrease in P450 2E1

protein content was found in rats treated with acetaminophen (10) or CCl_4 (29) and also was found in human liver microsomes following CCl_4 administration (30). In their studies, an increase in P450 2E1 protein content following pharmacological manipulations ameliorated the hepatotoxicity after acetaminophen (10) or CCl_4 (29) administration. However, it is also possible that administration of Sm caused a reduction of CCl_4-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 CCl_4 and treated with Sm or silymarin than in rats receiving CCl_4 alone. Accordingly, the other possible mechanism of action in the protection against CCl_4-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 CCl_4 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 protein and enzyme activity (32, 33). Moreover, the endotoxin-induced increase in nitric oxide production and the decrease in cytochrome P450 levels in rats were ameliorated by administration of a nitric oxide synthase inhibitor (32). It is possible that the reduced cytochrome P450 protein content is the result of an increased generation of nitric oxide following CCl_4 intoxication. The role of nitric oxide in liver damage remains controversial (34, 7). Although several studies have found that nitric oxide protected against CCl_4-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 CCl_4 that reached its peak level at 24 h after CCl_4 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 CCl_4 alone. The present results suggest that both Sm and silymarin ameliorate CCl_4-induced acute liver injury that probably acts, in part, through the down-regulation of iNOS content. A similar observation has found that CCl_4-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 CCl_4-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 CCl_4 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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