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Long-term administration of *Salvia miltiorrhiza* ameliorates carbon tetrachloride-induced hepatic fibrosis in rats

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Abstract

Carbon tetrachloride (CCl_4) is metabolized by cytochrome P450 to form a reactive trichloromethyl radical that triggers a chain of lipid peroxidation. These changes lead to cell injury, and chronic liver injury leads to excessive deposition of collagen in liver, resulting in liver fibrosis. The aim of this study was to evaluate the effects of long-term *Salvia miltiorrhiza* administration in CCl_4-induced hepatic injury in rats. *Salvia miltiorrhiza* (10, 25 or 50 mg kg^{-1} twice a day) was given for 9 weeks, beginning at the same time as the injections of CCl_4. Rats receiving CCl_4 alone showed a decreased hepatic glutathione level and an increased glutathione-S-transferase content. The hepatic thiobarbituratic acid-reactive substance levels were increased. CCl_4 also caused a prominent collagen deposition in liver histology that was further supported by the increased hepatic mRNA expression of transforming growth factor-β1, tissue inhibitor of metallproteinase-1 and procollagen I. *Salvia miltiorrhiza* administration led to a dose-dependent increase in hepatic glutathione levels and a decrease in peroxidation products. Additionally, it reduced the mRNA expression of markers for hepatic fibrogenesis. In conclusion, long-term administration of *Salvia miltiorrhiza* in rats ameliorated the CCl_4-induced hepatic injury that probably related to a reduced oxidant stress and degree of hepatic fibrosis.

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Introduction

Carbon tetrachloride (CCl_4) is widely used in animal models to produce chronic liver injury and liver fibrosis. It has been demonstrated that CCl_4 is metabolized by cytochrome P450 to reactive trichloromethyl radical and further converted to reactive oxygen species. These substances react with membrane lipids to propagate a chain reaction, leading to lipid peroxidation in cellular membranes that induces cell injury (Recknagel et al 1989). Following hepatic injury, activation of hepatic stellate cells (the key fibrogenic cells) is the dominant event in liver fibrogenesis, causing an excessive accumulation of extracellular matrix (Friedman 2000; Friedman et al 2000). Additionally, oxidative stress is a direct fibrogenic stimulus (Pratico et al 1998; Yamamoto et al 1998). During the fibrogenic process, matrix production by stellate cells is markedly increased through the activation of transforming growth factor-β1 (TGF-β1) (Friedman 2000; Friedman et al 2000). Other substances such as tissue inhibitor of metalloproteinase (TIMP-1) also play a role in collagen synthesis (Pinzani & Gentilini 1999; Friedman 2000; Friedman et al 2000). A growing body of evidence has shown that administration of antioxidants or inhibition of cytochrome P450 decreases CCl_4-induced liver injury with decreased lipid peroxidation (elSisi et al 1993; Gasso et al 1996; Pastor et al 1997; Kawada et al 1998). Accordingly, the degree of fibrosis may be ameliorated.

Salvia miltiorrhiza Bunge (Labiatae) is officially listed in the Chinese Pharmacopoeia, and is used for the treatment of cardiovascular disorders and inflammation (Chen 1993; Li 1998). The water-soluble extract of *Salvia miltiorrhiza* (Sm) contains phenolic compounds that are effective in protecting liver microsomes, hepatocytes and erythrocytes against oxidative damage (Liu et al 1992). Wasser et al (1998) and Nan et al (2001) have demonstrated that chronic administration of water-soluble

Sm reduced hepatic fibrosis caused by CCl_4 administration and bile duct ligation. Our previous study suggested that long-term administration of Sm improved the portal hypertensive state in rats with bile duct ligation (Huang et al 2001). These studies have resulted in considerable interest in Sm as a therapeutic agent in chronic liver disease. The aim of the current study was to investigate the beneficial effects of long-term Sm administration on oxidative stress and liver fibrosis in rats with CCl_4-mediated hepatic injury. The hepatic glutathione (GSH) and glutathione-S-transferase (GST) status, and the peroxidation products estimated mainly as thiobarbituratic acid-reactive substances (TBARS) were measured in CCl_4-exposed rats receiving different doses of Sm. In addition, molecular markers of liver fibrogenesis, including TGF-β1, TIMP-1 and procollagen I, were also measured.

Materials and Methods

Animals

Male Wistar rats (Charles River Laboratories, Cambridge, MA) weighing 210–230 g were used in the current study. All rats were housed in a controlled environment and allowed free access to food and water. Animal studies were approved by the Animal Experiment Committee of the National Yang-Ming University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academic Press, 1996). In order to accelerate the speed of liver fibrosis, rats received 0.35 g L^{-1} pentobarbital diluted in the drinking water beginning 1 week before initial exposure to CCl_4 injection. Hepatic injury was produced in rats by intraperitoneal injection of 0.5 mL CCl_4 diluted 1:1 (v/v) in olive oil twice per week for 9 weeks as previously described (Gasso et al 1996). The animals in the vehicle group received CCl_4 only, and the experimental groups received increasing doses of Sm (10, 25 or 50 mg kg^{-1} body weight by oral gavage) twice daily for 9 consecutive weeks, beginning at the same time as the injections of CCl_4. A group of normal rats was also included as controls.

Preparation of Sm

Sm was prepared by the methods described in our previous study (Huang et al 2001). 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 (Virtis, freeze-mobile, NY) to a light brownish residue with an approximate yield of 25%. These substances were stored at −20 °C until used. The active ingredients of the aqueous extract of Sm were confirmed by high performance liquid chromatography (HPLC). Similar to the study reported by Liu et al (1999), the analytical results showed that D-(+)-β-3,4-dihydroxyphenol lactic acid is the major component of the aqueous extract of Sm.

Liver histopathology and biochemistry

The liver tissue was fixed in 10% formalin and then embedded in paraffin, cut into 5-μm thick sections, stained with Masson's trichrome and reticulum silver, and examined under light microscopy by an experienced pathologist. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured by an auto-analyzer (Hitachi 736-60, Tokyo, Japan).

Hepatic GSH and TBARS measurements

The GSH concentrations in the liver homogenate were determined with a GSH-400 colorimetric assay kit (Calbiochem Co., San Diego, CA). Liver tissue was homogenized with 2 mL of 10% (w/v) metaphosphoric acid solution at 4 °C. Each sample was then centrifuged at 3000 *g* for 10 min at 4 °C. After vortexing, a 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 in μmol g^{-1} liver wt. The liver homogenate for lipid peroxidation was prepared with 2 mL of 50 mM potassium phosphate buffer, pH 7.4, and TBARS were determined (Fraga et al 1988). The fluorescence of the samples was detected at an excitation wavelength of 515 nm and an emission wavelength of 555 nm in a F4500 fluorescence spectrophotometer (Hitachi, Japan). 1,1,3,3-Tetramethoxypropane was used as the TBARS standard. Results were expressed in nmol mg^{-1} protein, and protein concentrations were determined by the method of Lowry et al (1951).

Microsome preparation and Western blot analysis for GST

Microsomes were prepared by differential centrifugation as described previously (Johansson et al 1988). In brief, one part of the liver was cut with scissors and then homogenized in 15 mM 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 (Universal 30RF, Hettich, Germany). The supernatant was then centrifuged at 105 000 *g* for 60 min in a preparative ultracentrifuge (XL-90; Beckman, USA). The pellet of microsomes was suspended in the homogenization solution and stored at −70 °C until used. The protein concentration of the microsome fraction was determined according to Lowry et al (1951). The purified microsome protein (120 μg) was loaded into lanes on 12% polyacrylamide gels and electrophoresed. Proteins were transferred to nitrocellulose membranes, blocked with buffer overnight, and then incubated with primary antibodies for 1 h using a 1:1000 dilution of mouse monoclonal anti-GST (Exalpha Biologicals Inc., Boston, MA) or a 1:1000 dilution of goat polyclonal anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then washed three times for 15 min in Tris-buffered solution and incubated with a 1:5000 dilution of alkaline

phosphatase conjugated goat anti-mouse IgG (Calbiochem Co., San Diego, CA) as the second antibody for 1 h. The protein was visualized using an enhanced chemiluminescence Western blotting detection kit (Amersham, Arlington Heights, IL), and then exposed to X-ray film for 1 min. The relative expression of protein was semi-quantified by densitometric scanning using an Image-analysis system (Kodak Co., Rochester, NY).

Reverse transcription-polymerase chain reaction (RT-PCR) amplification

Approximately 300 mg of liver was snap-frozen in liquid nitrogen and homogenized in 3 mL of Triazol solution (BRL, Gaithersburg, MD). Total cellular RNA was extracted according to the manufacturer's instructions. One microgram of total RNA from each sample was resuspended in 20 μL of the reaction buffer. After the reaction mixture reached 42 °C, 400 U of reverse transcriptase was added to each tube, and the samples were incubated for 30 min at 42 °C. Reverse transcription was stopped by denaturing the enzyme at 99 °C. The reaction mixture was diluted with distilled water to a final volume of 50 μL. Commercially available PCR primer for TGF-β1, TIMP-1, procollagen I and G3PDH was used (Purigo Biotech, Inc. Taipei, Taiwan) and contained the following sequences: TGF-β1, sense 5′-TATAGCAA CAATTCCTGGCG-3′, antisense 5′-TGCTGTCACAG-GAGCAGTG-3′; TIMP-1, sense 5′-CCACAGATATC-CGGTTCGCCTACA-3′, antisense 5′-GCACACCC-CACAGCCAGCACTAT-3′; procollagen I, sense 5′-TACTACCGGGCCGATGATGC-3′, antisense 5′-TC-CTTGGGGTTCGGGCTGATGTA-3′; G3PDH, sense 5′-CCCTTCATTGACCTCAACTACATGG-3′, antisense 5′-CATGGTGGTGAAGACGCCAG-3′. The sizes of amplified PCR products were 162 bp for TGF-β1, 218 bp for TIMP-1, 319 bp for procollagen I and 209 bp for G3PDH. The primer pairs used for the amplification of TGF-β1, TIMP-1, procollagen I and G3PDH were described as previously (Wasser et al 1998). Five microlitre aliquots of the synthesized cDNA were added to 45 μL of the PCR mixture containing 5 μL of 10 × PCR buffer, 1 μL deoxynucleotides, 0.5 μL of sense and antisense primers, and 0.25 μL of DNA polymerase (Gene Amp PCR kit, Perkin Elmer Co., Foster, CA). The reaction mixture and amplification was initiated by 1 min of denaturation at 94 °C for 1 cycle, followed by multiple cycles (range, 25–35) at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min using a GeneAmp PCR system 2400 DNA thermal cycler (Perkin Elmer Co., Foster, CA). The amplified PCR products were electrophoresed at 75 V through a 2% agarose gel for 1 h. The PCR products were size-fractionated on agarose gels and visualized by ethidium bromide staining, and the relative amount of messenger RNA transcripts was photographed using the Pharmacia Biotech System (D&R, Israel) and scanned by an Imaging Master. Densitometric analysis of the captured image was standardized for G3PDH content.

Statistics

The results are expressed as mean ± s.e.m. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls' multiple range test. Significance was determined at $P < 0.05$.

Results

Liver and body weight, and serum aminotransferase levels

Initial body weight was not significantly different between groups of experimental animals. Despite comparable body weight in the groups, the liver weight was significantly heavier in the group of animals that received CCl_4 alone compared with normal rats. By contrast, when Sm was given in addition to CCl_4, the relative liver weight was significantly decreased in the groups of animals that received 25 and 50 mg kg^{-1} Sm compared with the rats that received CCl_4 alone (Table 1). In rats receiving CCl_4 alone, the serum AST and ALT levels were significantly increased compared to those in normal rats (Table 1). In rats receiving CCl_4 plus 10 mg kg^{-1} Sm, the serum AST and ALT levels were similar to those receiving CCl_4 alone. In contrast, the serum AST and ALT levels were lower in

Table 1 Effects of *Salvia miltiorrhiza* on liver weights and serum aminotransferase levels in CCl_4-exposed rats.

Group	N	CCl_4	CCl_4 + Sm (10 mg kg^{-1})	CCl_4 + Sm (25 mg kg^{-1})	CCl_4 + Sm (50 mg kg^{-1})
n	6	12	12	12	12
Final body weight (g)	415 ± 7	410 ± 8	399 ± 11	405 ± 9	400 ± 5
Liver weight (g)	11.8 ± 0.7	16.7 ± 0.4*	16.5 ± 0.9*	14.5 ± 0.4*,**	13.5 ± 0.8*,**
Liver weight index (liver weight/100 g body weight)	2.7 ± 0.4	4.2 ± 0.2*	4.1 ± 0.6*	3.5 ± 0.1*,**	3.2 ± 0.3*,**
AST (IU/L)	120 ± 4	268 ± 43*	260 ± 50*	206 ± 43*	188 ± 55*
ALT (IU/L)	34 ± 2	213 ± 53*	210 ± 43*	177 ± 45*	180 ± 55*

Values are mean ± s.e.m. One-way ANOVA (Student–Newman–Keuls). *$P < 0.05$ compared to normal group, **$P < 0.05$ compared to rats receiving CCl_4 only. N, normal; Sm, *Salvia miltiorrhiza*; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

rats receiving CCl_4 plus 25 mg kg^{-1} and 50 mg kg^{-1} Sm than in those receiving CCl_4 alone, respectively. However, the differences did not reach statistical significance.

Sm improved liver histopathology of CCl_4-exposed rats

Histological examination also showed a significant change in the profile of collagen fiber deposition in the liver sections of rats treated with Sm compared to rats that received CCl_4 alone (Figure 1). Liver sections from the normal rats showed no fibrosis (Figure 1A, E). CCl_4 injection produced typical histological changes in the liver, characterized by central–central architecture disruption and bridge fibrosis formation (Figure 1B, F). The CCl_4-exposed rats that were also treated with Sm, either 25 (Figure 1C, G) or 50 (Figure 1D, H) mg kg^{-1}, had significantly less histological collagen accumulation compared to the CCl_4 alone group.

Effect of Sm on hepatic GSH and TBARS levels in CCl_4-exposed rats

CCl_4 exposure for 9 weeks significantly decreased GSH levels (Figure 2A) and increased TBARS concentrations (Figure 2B) in the hepatic homogenate of rats. Administration of Sm caused a dose-dependent effect on GSH and TBARS levels.

Sm administration in hepatic microsomal GST protein content after CCl_4 exposure

The group of normal rats showed basal levels of GST protein in hepatic microsomes, as demonstrated by Western blot analysis (Figure 3). GST protein content was markedly expressed in liver microsomes after CCl_4 exposure. Long-term treatment with Sm resulted in a dose-dependent decrease in GST protein content.

Sm reduced levels of TGF-β1, TIMP-1 and procollagen I transcript in CCl_4-exposed rats' livers

TGF-β1, TIMP-1 and procollagen I mRNA transcripts, isolated from the livers of normal rats and rats exposed to CCl_4 alone or CCl_4 concomitant with Sm, were examined by RT-PCR to determine the relative changes in gene expression. Livers from normal rats contained low levels of TGF-β1 (Figure 4), while increased levels were observed within 9 weeks after CCl_4 exposure. Treatment with Sm caused a marked reduction in the level of expression of the TGF-β1 transcript.

Nine weeks after CCl_4 exposure, the levels of TIMP-1 expression (Figure 5) were significantly increased compared to normal rats. Treatment with Sm resulted in a marked reduction in the level of expression of the TIMP-1 transcript. As with procollagen I transcript (Figure 6), livers from normal rats expressed constitutive levels of procollagen I mRNA that were significantly increased following exposure to CCl_4 alone. Concomitant treatment with Sm and CCl_4 reduced the level of procollagen I mRNA expression.

Discussion

Glutathione is a tripeptide that is found in the liver tissue at high concentration (Kaplowitz et al 1985). It plays

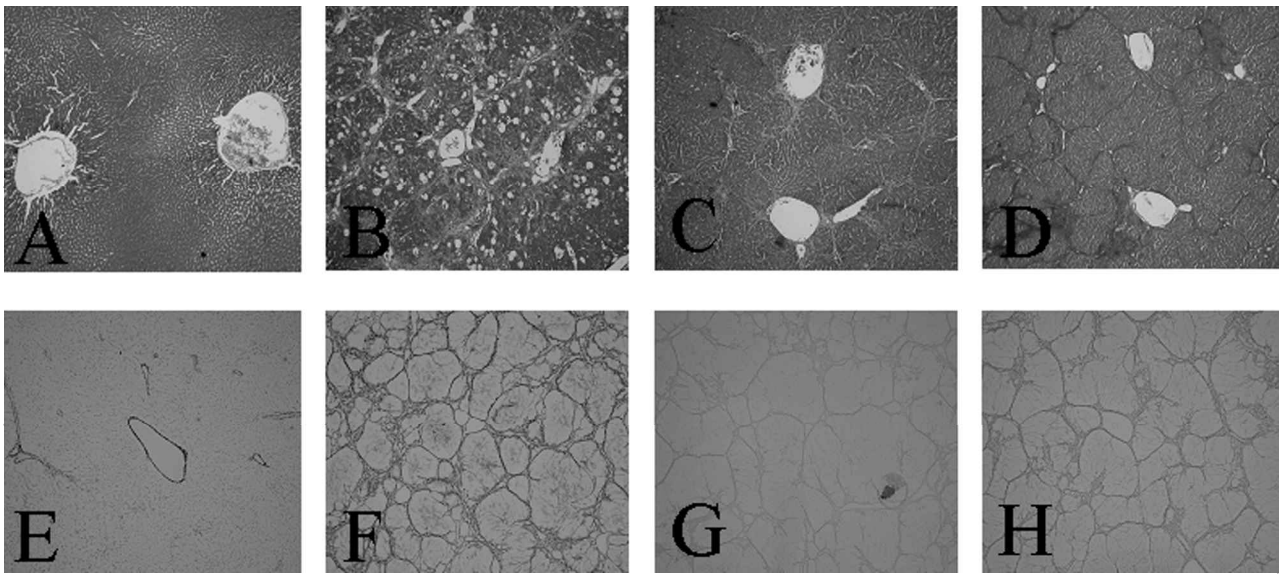

Figure 1 Light microscopic analysis of rat liver sections of normal rats and after CCl_4 treatment with or without Sm administration. Normal rat liver (A, E); CCl_4-treated (B, F), CCl_4-treated with concomitant Sm 25 mg kg^{-1} (C, G) and 50 mg kg^{-1} (D, H) administration after 9 weeks. Paraffin-embedded sections were stained with Masson's trichrome (A–D) and reticulum silver (E–H). Original magnification ×40.

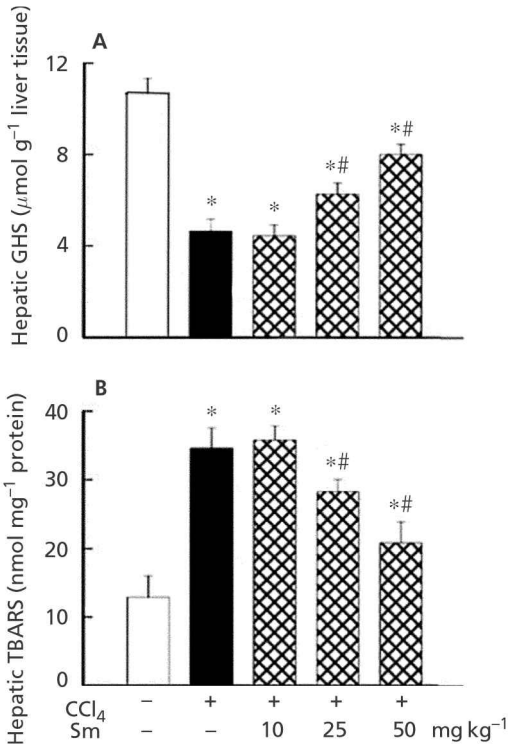

Figure 2 Effect of increasing concentrations of Sm administration on hepatic homogenate GSH (A) and TBARS (B) levels 9 weeks after CCl_4 exposure. All values were expressed as mean ± s.e.m. from at least eight rats. $^*P < 0.05$ vs normal group; $^\#P < 0.05$ vs CCl_4 alone group.

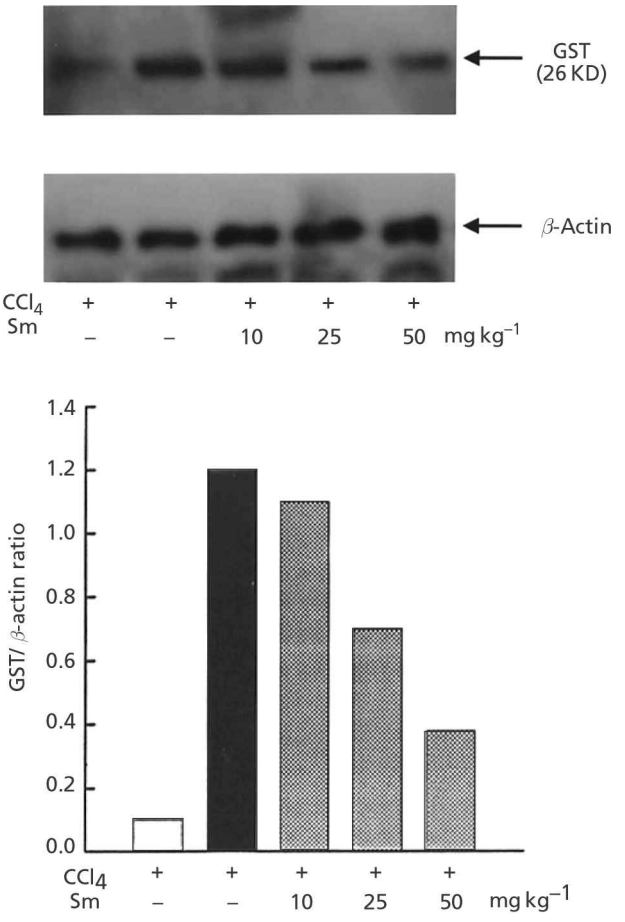

Figure 3 Western blot analysis of hepatic microsome GST of normal rats and after CCl_4 administration, with or without increasing concentrations of Sm. Microsome fractions (120 μg protein per lane) were analyzed for immunoreactivity with an antibody recognizing GST, as described in Materials and Methods.

a pivotal role in protection against trichloromethyl radical-induced liver injury (Connor et al 1990; Nishida et al 1998). It has been suggested that the lipid peroxidates generated after intoxification are eliminated by glutathione peroxidase in the presence of GSH (Meister & Anderson 1983). In the current study, we demonstrated that there is a significant decrease in hepatic GSH levels in rats receiving CCl_4 alone, whereas long-term administration of Sm caused a dose-dependent restoration of hepatic GSH levels. Our results are in line with previous studies showing a depletion of GSH levels following CCl_4 intoxification (Hsiao et al 2001; Luckey & Petersen 2001). As GSH plays a pivotal role in the detoxification of CCl_4 as well as in the defence against lipid peroxidation, it is conceivable that the increase of intracellular GSH concentrations induced by long-term Sm administration may partially account for its protective effect against CCl_4 toxicity. The current study suggests that Sm may neutralize the oxidative stress induced by CCl_4. This was further supported by the changes in hepatic TBARS levels (an estimation of peroxidation products). Additionally, this study demonstrates that long-term Sm administration significantly suppresses the increase of TBARS formation induced by CCl_4 in rats. We also demonstrated that hepatic microsomal GST protein content increased after 9 weeks of CCl_4 exposure. GST catalyzes the reaction of GSH with metabolites of xenobiotics to form non-toxic conjugates (Meister & Anderson 1983). The cellular GST level is probably involved in oxidative stress-induced cellular damage, such as genotoxic damage and lipid peroxidation (Traverso et al 2002; Tsuneyama et al 2002; Veal et al 2002). Long-term administration of Sm caused a dose-dependent reduction in cellular GST protein content. This result suggests that Sm reduces oxidative stress in rats with CCl_4 exposure. Taken together, the current study indicates that administration of Sm enhances the protection against hepatic damage caused by lipid peroxidation and strengthens the detoxification of CCl_4. However, only a modest decrease in serum AST and ALT levels was found in rats receiving 25 and 50 mg kg^{-1} of Sm. A similar observation has shown that Sm treatment ameliorated the hyperdynamic circulation in cirrhotic rats with portal hypertension but did not affect serum biochemical profiles (Huang et al 2001). It is possible that serum AST and ALT levels reflect the extent of hepatocellular damage but not the extent of fibrosis.

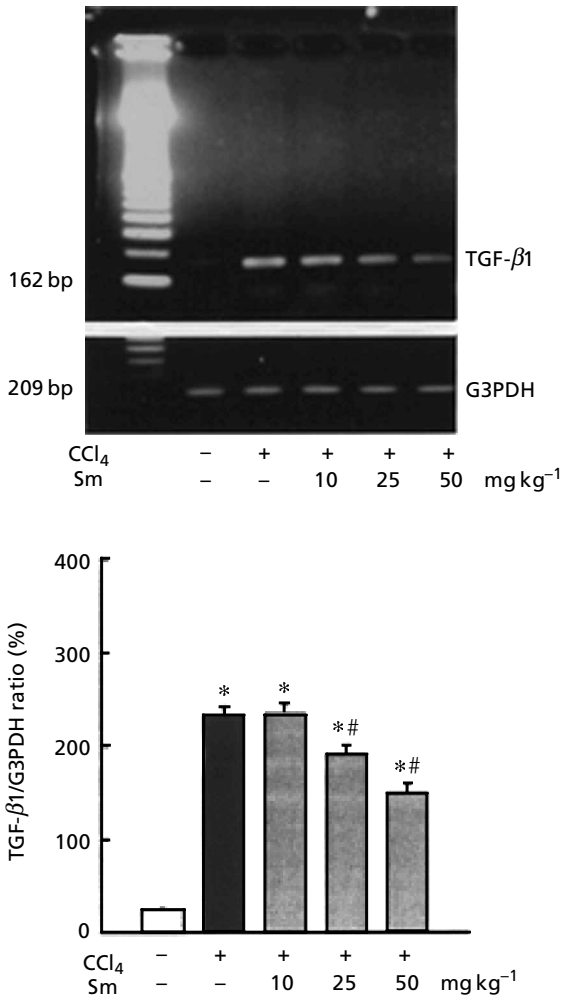

Figure 4 Amplification of hepatic TGF-β1 mRNA by RT-PCR from livers of rats: normal, CCl_4-treated and CCl_4-treated with concomitant administration of increasing concentrations of Sm. Hepatic samples were obtained at the same time-points. Using cDNA equivalents of 10 ng RNA, samples were amplified for 35 cycles using specific TGF-β1 primers.

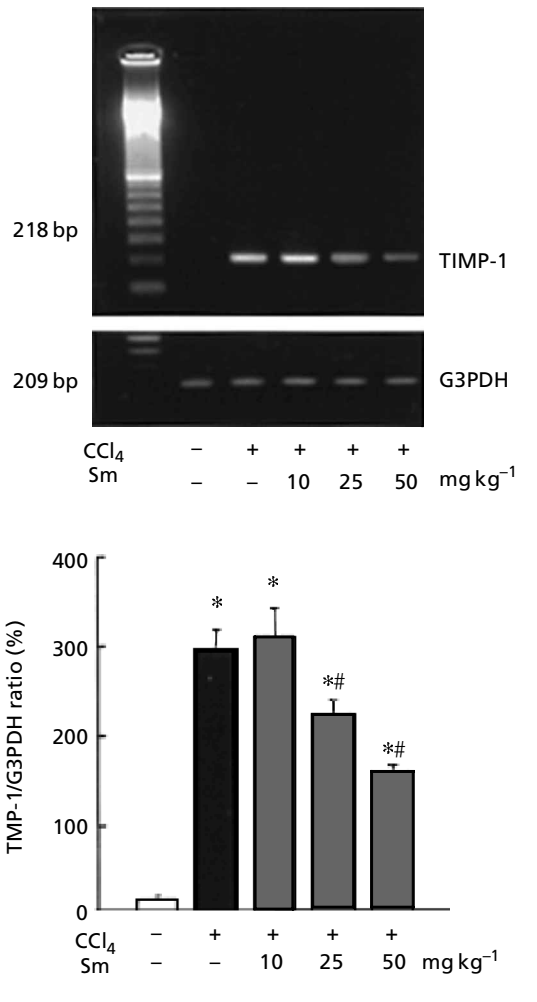

Figure 5 Amplification of hepatic TIMP-1 mRNA by RT-PCR from livers of rats: normal, CCl_4-treated and CCl_4-treated with concomitant administration of increasing concentrations of Sm. Hepatic samples were obtained at the same time-points. Using cDNA equivalents of 10 ng RNA, samples were amplified for 30 cycles using specific TIMP-1 primers.

However, the current study cannot exclude the possibility that long-term administration of Sm directly reduces CCl_4-induced hepatocellular injury. It is also possible that the long-term administration of Sm directly stimulates GSH synthesis. These possibilities may all lead to a higher hepatic GSH level and lower peroxidation products in CCl_4-exposed rats with chronic Sm treatment than in rats receiving CCl_4 alone. Further study is necessary to elucidate this phenomenon.

In the current study, we found that less collagen deposition is seen in liver histopathology associated with a lower liver weight in rats receiving CCl_4 and long-term Sm treatment than in rats receiving CCl_4 alone. This was further supported by the lower expression of TGF-β1 content in rats receiving CCl_4 and long-term Sm than in rats receiving CCl_4 alone. Accordingly, the mRNA expression of procollagen I was reduced in rats receiving long-term Sm. TGF-β1 is responsible not only for induction of hepatic fibrosis (Wasser et al 1998; Tahashi et al 2002), but also for suppression of GSH synthesis in hepatic stellate cells or primary hepatocytes (Sanchez et al 1997; De Bleser et al 1999). CCl_4-induced hepatic damage not only depletes hepatic GSH storage, but also appears to induce an activation of TGF-β1, which leads to hepatic fibrosis (Wasser et al 1998). In addition, it has been shown that increased expression of TIMP-1 occurs soon after liver injury and then persists throughout the time-course of the development of liver fibrosis (Iredale et al 1996; Herbst et al 1997) in the CCl_4 model. The current study found that long-term administration of Sm inhibited the over-expression of TIMP-1 in CCl_4-treated rats, which may lead to less collagen synthesis. Therefore, long-term

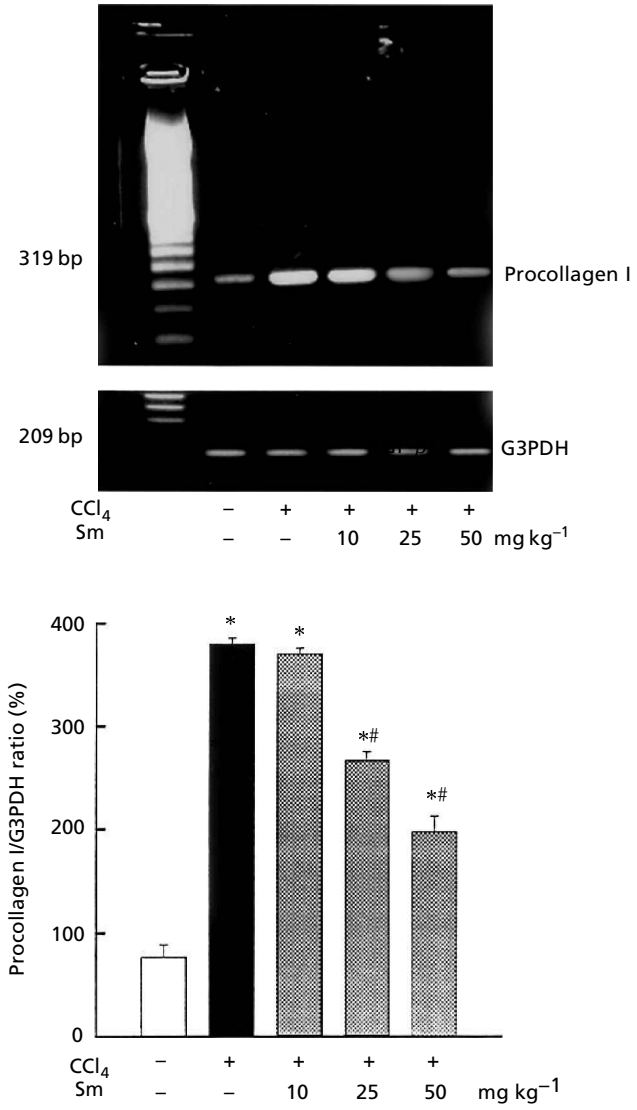

Figure 6 Amplification of hepatic procollagen I mRNA by RT-PCR from livers of rats: normal, CCl_4-treated and CCl_4-treated with concomitant administration of increasing concentrations of Sm. Hepatic samples were obtained at same time-point. Using cDNA equivalents of 10 ng RNA, samples were amplified for 28 cycles using specific procollagen I primers.

administration of Sm may reduce the severity of liver fibrosis induced by chronic CCl_4 administration, which mostly acts through the antioxidant effect of Sm. It is known that oxidant stress per se is a direct fibrogenic stimulus, and administration of antioxidants may have potential beneficial effects for liver fibrosis, elucidated in experimental models and in-vitro studies (Gasso et al 1996; Pastor et al 1997; Kawada et al 1998). However, another possible mechanism by which Sm ameliorates the severity of hepatic fibrosis induced by CCl_4 administration may be through the direct inhibition of TGF-β1 expression. Further studies are needed to examine this possibility.

Conclusions

This study indicates that long-term Sm administration ameliorates hepatic injury in rats under chronic CCl_4 exposure. The beneficial effect of Sm was mostly attributed to the increased hepatic GSH concentrations with decreased lipid peroxidation associated with an inhibition of TGF-β1 mRNA expression.

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