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Water-soluble extract of *Salvia miltiorrhiza* ameliorates carbon tetrachloride-mediated hepatic apoptosis in rats

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Abstract

Apoptosis is one of the events that are involved in liver fibrogenesis. Thus, factors that affect apoptosis may be used to modulate liver fibrosis. We have recently reported that *Salvia miltiorrhiza* plays a protective role in carbon tetrachloride (CCl_4)-induced hepatic fibrosis. In this study, we aimed to evaluate whether *S. miltiorrhiza* modulated CCl_4-induced hepatic apoptosis in rats. Male Wistar rats were given orally either vehicle or water-extract of *S. miltiorrhiza* (50 mg kg^{-1} twice daily) for nine weeks beginning from the start of CCl_4 administration. A group of normal rats was included for comparison. Hepatocyte DNA fragmentation and cytosolic caspase-3 and caspase-8 activity were determined in the experimental animals. Hepatic cytosolic Bax, Bcl-2, cytochrome c, and calpain-μ expressions were measured by Western blot analysis. Hepatic mitochondrial glutathione levels were assessed by colorimetric assay. Compared with normal rats, rats receiving CCl_4 alone showed profound DNA fragmentation associated with an increased cytosolic fraction of cytochrome c and calpain-μ protein expressions and a decreased mitochondrial glutathione level. In contrast, a decreased laddering of DNA fragmentation was noted in rats receiving CCl_4 plus *S. miltiorrhiza* extract. The mitochondrial glutathione level was significantly increased in rats receiving CCl_4 plus *S. miltiorrhiza* extract compared with those receiving CCl_4 alone. Additionally, cytosolic caspase-3 activity and cytosolic fractions of Bax, Bcl-2, cytochrome c, and calpain-μ protein expressions were decreased in rats receiving CCl_4 plus *S. miltiorrhiza* extract compared with those receiving CCl_4 alone. The cytosolic caspase-8 activity in rats receiving CCl_4 alone was no different from those receiving CCl_4 plus *S. miltiorrhiza* extract. These results indicated that chronic administration of *S. miltiorrhiza* ameliorated CCl_4-mediatd hepatic apoptosis in rats. This effect may be related to the antioxidant properties of *S. miltiorrhiza*.

Introduction

Carbon tetrachloride (CCl_4) is widely used in animal models to produce chronic liver injury (Gasso et al 1996; Lee et al 2003). CCl_4 is metabolized by cytochrome P450 to reactive trichloromethyl radical, which is quickly conjugated by hepatic glutathione, reacting with membrane lipids to propagate a chain reaction leading to glutathione depletion and lipid peroxidation that induces cell injury (Basu 2003; Beddowes et al 2003). Based on these results, CCl_4 has been assumed to be a typical substance that produces severe oxidative stress. In addition, formation of apoptotic bodies has been observed in animals receiving CCl_4 (Galle 1997). It is well established that hepatocyte apoptosis plays an important mechanism in hepatobiliary diseases (Guicciardi & Gores 2005). In fact, a number of toxic substances including CCl_4 are known to induce apoptosis (Cain & Freathy 2001). Recent evidence has indicated that the generation of lipid peroxidation (Basu 2003; Beddowes et al 2003), mitochondrial dysfunction (Krahenbuhl et al 2000; Hernandez-Munoz et al 2003), disruption of calcium homeostasis (Hemmings et al 2002), and induction of apoptosis (Cabre et al 1999; Sun et al 2001) are all involved in CCl_4-induced hepatotoxicity. It is conceivable that both hepatocellular necrosis and apoptosis are involved in CCl_4-induced hepatic injury. Regardless of the mechanisms, hepatic apoptosis seems to play a pivotal role in CCl_4-induced acute and chronic liver injury (Shi et al 1998; Cabre et al 1999). Recent studies

have revealed the relationship between oxidative stress and apoptosis (Ronco et al 2002; Ding & Ong 2003). Additionally, a number of studies in animals have shown that administration of Chinese herbs with antioxidative activity leads to a decrease in tissue injury and apoptosis (Wasser et al 1998; Lee et al 2003; Satoh et al 2005).

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 *S. miltiorrhiza* 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 *S. miltiorrhiza* extract reduced hepatic fibrosis caused by CCl_4 administration and bile duct ligation in rats. In addition, our recent study demonstrated that chronic administration of *S. miltiorrhiza* extract in rats reduced the severity of CCl_4-induced hepatic fibrosis, probably through its antioxidant properties (Lee et al 2003). Therefore, this study was undertaken to evaluate whether *S. miltiorrhiza* may exert beneficial effects on CCl_4-induced hepatic apoptosis in rats.

Materials and Methods

Animals

Male Wistar rats (210–230 g; Charles River Laboratories, Cambridge, MA, USA) were housed in a controlled environment and were allowed free access to food and water. Animal studies were approved by the Animal Experiment Committee of National Yang-Ming University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academic Press, USA, 1996). 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 nine weeks as described by Gasso et al (1996). The animals in the vehicle group received CCl_4 only while the experimental groups received *S. miltiorrhiza* extract (50 mg kg^{-1} by oral gavage) twice daily for nine consecutive weeks, beginning at the same time as the injections of CCl_4. The dose of *S. miltiorrhiza* extract was chosen as described by Lee et al (2003). A group of normal rats was included as control.

Preparation of *S. miltiorrhiza* extract

S. miltiorrhiza extract was prepared as described by Lee et al (2003). Briefly, dried root of *S. miltiorrhiza* (400 g) was powdered and extracted with 1000 mL 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. Liu et al (1999) has shown that D(+)β3,4-dihydroxyphenol lactic acid is the major component of the aqueous extract of *S. miltiorrhiza*. To standardize the *S. miltiorrhiza* extract by determining the level of this active component, the aqueous extract of *S. miltiorrhiza* was analysed by reverse-phase high performance liquid chromatography with a phenyl column (250 × 4.6 mm i.d.). Linear gradient elution was conducted with a mobile phase consisting of acetonitrile:methanol:monosodium phosphoric acid (1:3:6) with 0.1 mM octanesulfonic acid (pH 2.8) for 20 min. The flow rate was set at 1.0 mL min^{-1}. The wavelength of the UV-detector was set at 290 nm. The result confirmed the presence of this major active component in the aqueous extract of *S. miltiorrhiza*.

Isolation of mitochondria and measurement of mitochondrial glutathione (GSH) levels

Mitochondria were isolated by differential centrifugation according to the method described by Davis & Lawrence (1986). Liver tissue was homogenized in ice-cold medium containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 0.2 mM EDTA, 0.36 mg mL^{-1} BSA, and adjusted to pH 7.4. Mitochondria were isolated by differential ultracentrifugation at 105 000 *g*. The final pellet containing mitochondria was suspended in the above buffer and diluted to contain approximately 100 mg mitochondrial protein mL^{-1}. The mitochondrial GSH concentrations of liver homogenate were determined with a GSH-400 colorimetric assay kit (Calbiochem Co, San Diego, CA, USA). A 50-μL sample of the centrifuged supernatant was added into the assay buffer provided by the manufacturer to a final volume of 200 μL. The reaction mixtures were incubated at 25°C for 30 min and read by a spectrophotometer at 412 nm. The values of unknown samples were drawn from a standard curve plotted by assaying different known concentrations of GSH. The amounts of GSH were expressed as nmol (mg protein)$^{-1}$.

DNA gel electrophoresis

DNA was extracted from liver tissues as described by Kohli et al (1999). A DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) was used to extract and purify the DNA. Briefly, liver tissue was homogenized and proteinase K was added and incubated overnight at 55°C. The extracts were loaded into the wells of 1.5% agarose gel containing 100 μg mL^{-1} ethidium bromide. Electrophoresis was performed at 90 V for 1 h and imaged with a UV transilluminator.

Determination of hepatic cytosolic caspase-3 and caspase-8 activity

Cytosolic caspase activity was determined with a colorimetric assay kit (BioSource International, Inc. Camarillo, CA, USA). Fresh liver tissues were homogenized with a Teflon homogenizer in lysis buffer containing 25 mmol L^{-1} HEPES (pH 7.4), 5 mmol L^{-1} EDTA and 2 mmol L^{-1} dithiothreitol. The cytosolic fractions were clarified by centrifugation, and supernatants were used for enzyme assays. Caspase activity was carried out using specific

substrates Asp-Glu-Val-Asp-*p*NA for caspase-3, and Ile-Glu-Thr-Asp-*p*NA for caspase-8, according to the protocol provided by the manufacturer. The reaction mixtures were incubated at 37°C for 90 min, and were measured by a spectrophotometer at 405 nm. Specific activity was expressed as U (mg protein)$^{-1}$.

Western blot analysis for cytosolic calpain-μ, cytochrome c, Bax, and Bcl-2 expression

Freshly isolated liver tissue was homogenized in a buffer containing 10 mmol L^{-1} Tris-HCl (pH 7.4) and 1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1 mmol L^{-1} phenylmethylsulfonyl fluoride, and 5 mmol L^{-1} EDTA. The protein concentration of the tissue homogenate was determined by the method of Lowry et al (1951) with BSA as the standard. Protein from liver homogenates (60 or 120 μg) was loaded per lane on 8% or 12% polyacrylamide gels and electrophoresed. Proteins were transferred to nitrocellulose membranes. The membranes were blocked overnight with buffer and then incubated with primary antibodies for 1 h using 1:1000 dilution of mouse monoclonal anti-cytochrome c (Pharmingen, San Diego, CA, USA); 1:500 dilution of mouse monoclonal anti-calpain-μ (Exalpha Biologicals, Inc., Boston, MA, USA); 1:1000 dilution of mouse polyclonal anti-Bax; 1:1000 dilution of mouse monoclonal anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); or 1:1000 dilution of goat polyclonal anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then washed three times in Tris-buffered solution for 15 min each and incubated with 1:5000 dilution of alkaline phosphatase conjugated goat anti-mouse IgG (Calbiochem Com. San Diego, CA, USA) as second antibody for 1 h. The protein was visualized with an enhanced chemiluminescence Western blotting detection kit (Amersham, Arlington Heights, IL, USA). The membranes were finally exposed to X-ray film for 1 min. The relative expression of various proteins was quantified by densitometric scanning with an Image-Analysis system.

Statistics

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

Results

Effect of *S. miltiorrhiza* treatment on DNA fragmentation by gel electrophoresis

Normal rats showed no laddering of DNA in liver tissue. In contrast, profound DNA fragmentation in liver tissue was observed in rats receiving CCl_4 alone. In rats receiving CCl_4 plus *S. miltiorrhiza* extract, marked amelioration in the DNA fragmentation extracted from livers was noted compared with rats receiving CCl_4 alone (Figure 1).

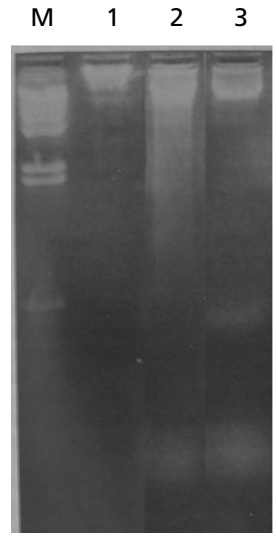

Figure 1 Agarose gel electrophoresis of DNA extracted from rat livers. Sm, *S. miltiorrhiza* extract.

Effect of *S. miltiorrhiza* treatment on hepatic cytosolic caspase-3 and caspase-8 activity

In rats receiving CCl_4 alone, cytosolic caspase-3 activity (68.0 ± 3.7 U (mg protein)$^{-1}$, $P < 0.05$) was significantly higher than in normal rats (51.1 ± 3.2 U (mg protein)$^{-1}$) (Figure 2A). Compared with rats receiving CCl_4 alone, rats receiving CCl_4 plus *S. miltiorrhiza* extract showed a significant decrease in cytosolic caspase-3 activity (58.2 ± 4.3 U (mg protein)$^{-1}$, $P < 0.05$). In contrast, the cytosolic caspase-8 activity was similar among normal rats (23.9 ± 2.1 U (mg protein)$^{-1}$), rats receiving CCl_4 alone (23.1 ± 1.8 U (mg protein)$^{-1}$), and those receiving CCl_4 plus *S. miltiorrhiza* extract (21.4 ± 2.4 U (mg protein)$^{-1}$) (Figure 2B).

S. miltiorrhiza treatment on hepatic cytosolic Bax, Bcl-2 and cytochrome c protein expression after CCl_4 exposure

Compared with normal rats, increased cytosolic Bax and Bcl-2 protein content was found in rats receiving CCl_4 alone. In contrast, the Bax and Bcl-2 protein content decreased in rats receiving CCl_4 plus *S. miltiorrhiza* extract compared with those receiving CCl_4 alone (Figure 3). In addition, cytosolic cytochrome c protein content increased in rats receiving CCl_4 alone compared with normal rats. The cytochrome c protein content also decreased in rats receiving CCl_4 plus *S. miltiorrhiza* compared with those receiving CCl_4 alone (Figure 4).

Effect of *S. miltiorrhiza* treatment on mitochondrial GSH levels

Hepatic mitochondrial GSH levels showed a significant decrease in rats receiving CCl_4 alone (3.5 ± 0.4 nmol (mg protein)$^{-1}$, $P < 0.05$) compared with normal rats

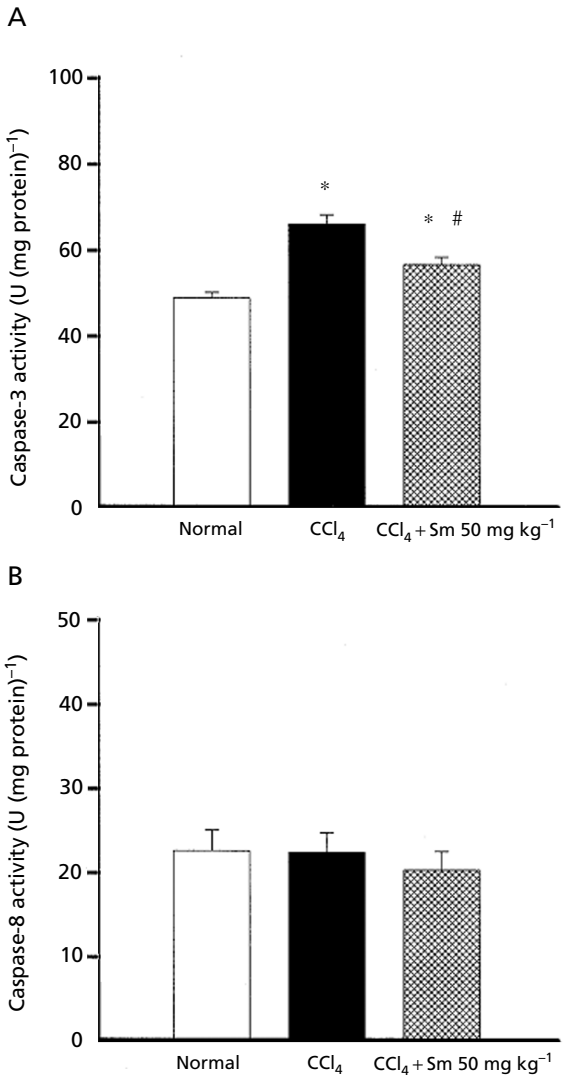

Figure 2 Effect of *S. miltiorrhiza* extract (Sm) on hepatic cytosolic caspase-3 (A) and caspase-8 (B) activity in normal rat, rats receiving CCl_4 alone, and rats receiving CCl_4 plus *S. miltiorrhiza* (n = 8 in each group). $^{*}P < 0.05$ vs normal rats; $^{\#}P < 0.05$ vs rats receiving CCl_4 alone.

$(7.4 \pm 0.6\,\text{nmol (mg protein)}^{-1})$. Hepatic mitochondrial GSH levels significantly increased in rats receiving CCl_4 plus *S. miltiorrhiza* extract $(5.3 \pm 0.4\,\text{nmol (mg protein)}^{-1}$, $P < 0.05)$ compared with those receiving CCl_4 alone (Figure 5).

S. miltiorrhiza treatment on hepatic cytosolic calpain-μ protein expression after CCl_4 exposure

Compared with normal rats, increased hepatic cytosolic calpain-μ protein content was found in rats receiving CCl_4 alone. In contrast, the hepatic calpain-μ protein content decreased in rats receiving CCl_4 plus *S. miltiorrhiza* extract compared with those receiving CCl_4 alone (Figure 6).

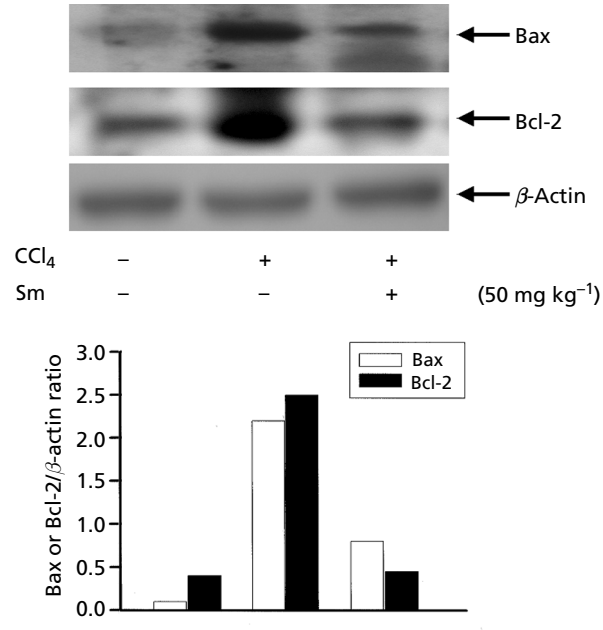

Figure 3 Western blot analysis of hepatic cytosolic Bax and Bcl-2 protein content of normal rats, rats receiving CCl_4 alone, and rats receiving CCl_4 plus *S. miltiorrhiza* extract (Sm). Hepatic cytosolic fractions (60 μg protein per lane) were analysed for immunoreactivity with an antibody recognizing Bax or Bcl-2, as described in Materials and Methods.

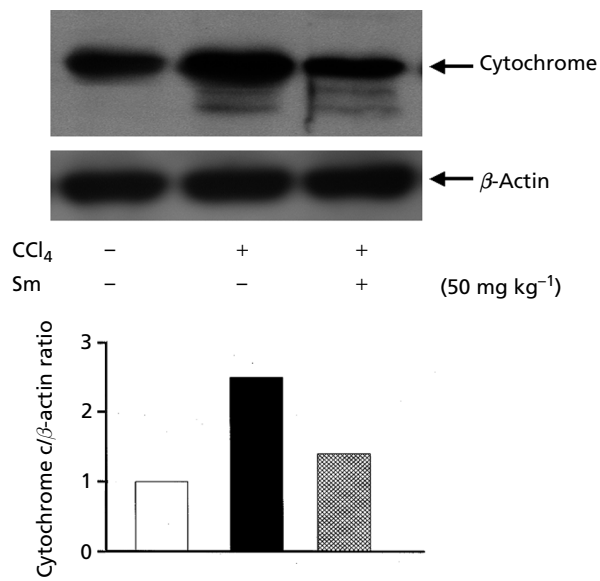

Figure 4 Western blot analysis of hepatic cytosolic cytochrome c content of normal rats, rats receiving CCl_4 alone, and rats receiving CCl_4 plus *S. miltiorrhiza* extract (Sm). The cytosolic fractions (120 μg protein per lane) were analysed for immunoreactivity with an antibody recognizing cytochrome c, as described in Materials and Methods.

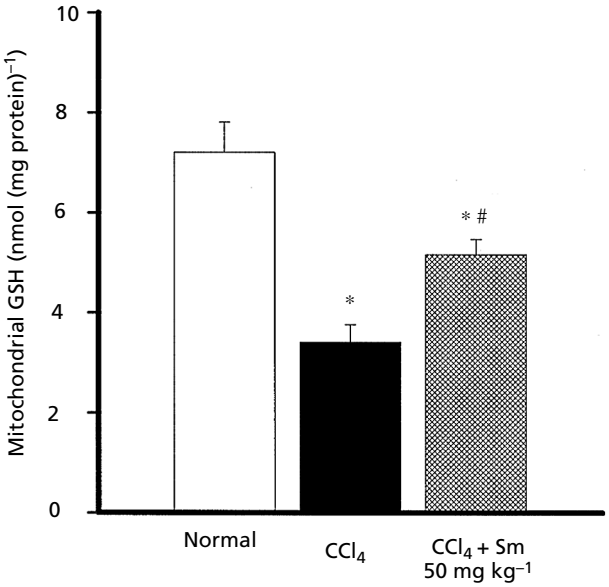

Figure 5 Effect of *S. miltiorrhiza* extract (Sm) on mitochondrial GSH levels in normal rats, rats receiving CCl_4 alone, and rats receiving CCl_4 plus *S. miltiorrhiza* (n = 8 in each group). $*P < 0.05$ vs normal rats; $\#P < 0.05$ vs rats receiving CCl_4 alone.

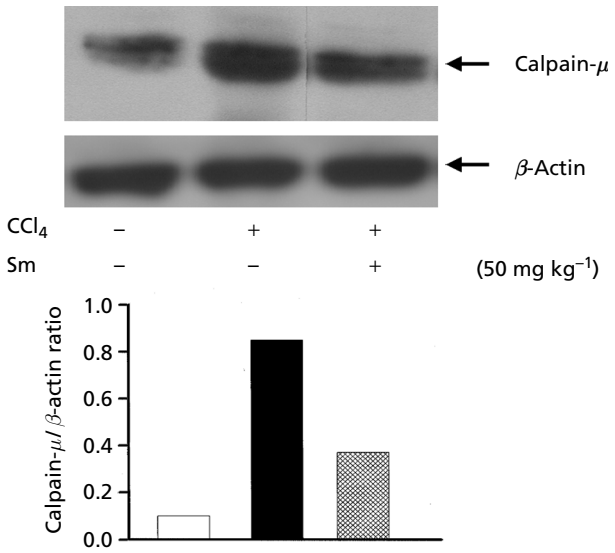

Figure 6 Western blots analysis of hepatic cytosolic calpain-μ in normal rats, rats receiving CCl_4 alone, and rats receiving CCl_4 plus *S. miltiorrhiza* extract (Sm). The cytosolic fractions (120 μg protein per lane) were analysed for immunoreactivity with an antibody recognizing calpain-μ as described in Materials and Methods.

Discussion

Chronic administration of *S. miltiorrhiza* extract significantly attenuated apoptosis induced by CCl_4, evident by the marked amelioration of DNA fragmentation in rats receiving CCl_4 plus *S. miltiorrhiza*. It has been suggested that the apoptotic signalling within the cells is mainly via two pathways: the death receptor pathway (i.e. the extrinsic pathway) and the mitochondrial pathway (i.e. the intrinsic pathway) (Guicciardi & Gores 2005). The extrinsic pathway originates at the cell surface, mainly involving death-mediated receptors such as Fas receptors. Ligand/receptor binding leads to activation of caspase-8 and initiates the apoptotic pathway. The intrinsic pathway is triggered by different extra- and intra-cellular signals such as γ-irradiation, oxidative stress, toxins, etc., which cause mitochondrial dysfunction. These factors induce alteration of organelle architecture and membrane permeability with subsequent release of cytochrome c, which contributes to protease activation such as caspase-3. On the other hand, the protein Bid may provide crosstalk between the two pathways, because Bid is activated by caspase-8 following death receptor engagement and translocates to the mitochondria where it contributes to mitochondrial dysfunction.

In this study, the cytosolic caspase-8 activity was not altered in rats receiving CCl_4 plus *S. miltiorrhiza* extract compared with those receiving CCl_4 alone. In contrast, we found that cytosolic cytochrome c protein content and caspase-3 activity increased in rats receiving CCl_4 alone, while these values reduced in rats receiving CCl_4 plus *S. miltiorrhiza*. It is well established that CCl_4 is assumed to be a typical substance that produces severe oxidative stress. We have reported also that chronic administration of *S. miltiorrhiza* extract in rats reduced the severity of CCl_4-induced hepatic fibrosis, probably through its antioxidant properties (Lee et al 2003). Moreover, in this study a replenishing of mitochondrial GSH levels in rats receiving CCl_4 with *S. miltiorrhiza* was observed. Taken together, our results indicated that the effects of *S. miltiorrhiza* extract in the amelioration of CCl_4-induced apoptosis in rats mainly involved the mitochondrial pathway through the antioxidant properties of *S. miltiorrhiza*.

The mitochondria are known to be a vulnerable target of various toxins and oxidative stress. The mitochondrial apoptotic pathway is regulated by the Bcl-2 family of proteins. These consist of both anti-apoptosis (such as Bcl-2) and pro-apoptosis (such as Bax) proteins (Green & Reed 1998). In the oxidative stress-induced apoptotic pathway, activation of the pro-apoptotic Bcl-2 family of proteins such as Bax is known to play an important role (Ding & Ong 2003). It has been shown that Bax can translocate from cytosol to the mitochondria and exhibit conformational change under the apoptotic process (Gilmore et al 2000). In this study, we found that Bax protein content markedly increased in rats receiving CCl_4 alone, suggesting that oxidative stress caused by CCl_4 administration might have activated Bax. On the other hand, we also found that Bcl-2 protein content markedly increased in rats receiving CCl_4 alone. In other words, the Bcl-2 protein content increased during the apoptotic process. A similar observation regarding an increase in Bcl-2 protein content during the course of apoptosis of hepatic stellate cells was reported by Cales (1998). Bcl-2 is a protein found on the intracellular membrane with a potent inhibition effect on apoptosis. Theoretically, increased Bcl-2 protein may lead to less hepatic apoptosis

in rats receiving CCl_4 alone. However, it has been shown that Bax can dimerize with Bcl-2, inhibiting their function and thereby promoting apoptosis (Reed 1994). In addition, Miyashita et al (1995) demonstrated that the half-life of Bax was increased by the over-expression of Bcl-2. Accordingly, these effects may probably mask the beneficial effect of increased anti-apoptotic protein (Bcl-2) content during the course of apoptosis in rats receiving CCl_4 alone. In this study, both Bax and Bcl-2 protein contents markedly decreased in rats receiving CCl_4 plus *S. miltiorrhiza* extract. We demonstrated previously (Lee et al 2003) that chronic administration of *S. miltiorrhiza* extract in rats exposed to CCl_4 caused a reduction in hepatic transforming growth factor-β1 levels. It is known that transforming growth factor-β1 is a potent hepatic apoptogen (Lin & Chou 1992; Oberhammer et al 1993). Therefore, the production of less transforming growth factor-β1 in rats receiving CCl_4 plus *S. miltiorrhiza* may result in a lesser degree of hepatic apoptosis and a lower level of Bax protein content. Thereafter, the Bcl-2 protein content decreased also because of a lesser degree of hepatic apoptosis. However, whether *S. miltiorrhiza* exerted a direct effect on the Bcl-2 family of proteins needs to be elucidated further.

Calpain-μ is considered as participating in various intracellular signalling pathways mediated by Ca^{2+} (Sorimachi et al 1997). In addition, calpain-μ is a major Ca^{2+}-dependent cytosolic protease that has been implicated as an intracellular mediator leading to apoptosis (Squier et al 1994) as well as necrosis (Mezey et al 2001). It has been suggested that changes in mitochondrial permeability lead to the release of mitochondrial Ca^{2+} (Ding & Ong 2003), and oxidative stress may be one of the important factors that cause alteration in the mitochondrial Ca^{2+} concentration. In rats receiving CCl_4 alone, the metabolites of CCl_4 might have stimulated hepatic reactive oxygen species generation and enhanced mitochondrial Ca^{2+} release, thereafter activating calpain. This study observed an increase in calpain-μ protein content during the course of apoptosis in rats receiving CCl_4 alone. Similar observations regarding over-expression of calpain have been seen in human diseases marked by overt apoptosis (Saito et al 1993). In contrast, in rats receiving CCl_4 plus *S. miltiorrhiza* extract, a decrease in calpain-μ protein content was found. Our results suggested that in rats receiving CCl_4 plus *S. miltiorrhiza*, the decrease in oxidative stress following *S. miltiorrhiza* extract administration may have decreased mitochondrial Ca^{2+} release. Accordingly, the calpain-μ content decreased in rats receiving CCl_4 plus *S. miltiorrhiza*. However, we cannot exclude the possibility that *S. miltiorrhiza* administration caused hepatocyte necrosis to a lesser degree, and therefore a lower expression of calpain-μ content. Further studies are needed to clarify this phenomenon.

Conclusions

Chronic administration of *S. miltiorrhiza* extract ameliorated CCl_4-mediatd hepatic apoptosis in rats. The beneficial effect of *S. miltiorrhiza* extract in the amelioration of CCl_4-induced apoptosis in rats may have involved mainly the mitochondrial pathway, which probably acted through the antioxidant properties of *S. miltiorrhiza*.

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