

Protective Mechanism of Glycyrrhizin on Acute Liver Injury Induced by Carbon Tetrachloride in Mice

Chan-Ho LEE,^a Sang-Won PARK,^a Yeong Shik KIM,^b Sam Sik KANG,^b Jeong Ah KIM,^c
Seung Ho LEE,^c and Sun-Mee LEE^{*,a}

^a College of Pharmacy, Sungkyunkwan University; 300 Cheoncheon-dong, Jangan-gu, Suwon, Gyeonggi-do 440-746, Korea; ^b College of Pharmacy, Seoul National University; Seoul 110-460, Korea; and ^c College of Pharmacy, Yeungnam University; Gyeongsan 712-749, Korea. Received June 22, 2007; accepted July 11, 2007

Glycyrrhizin is the major active component extracted from licorice (*Glycyrrhiza glabra*) roots, one of the most widely used herbal preparations for the treatment of liver disorders. This study evaluated the potential beneficial effect of glycyrrhizin in a mouse model of carbon tetrachloride (CCl₄)-induced liver injury. The mice were treated intraperitoneally with CCl₄ (0.5 ml/kg). They received glycyrrhizin (50, 100, 200, 400 mg/kg) 24 h and 0.5 h before and 4 h after administering CCl₄. The serum activities of aminotransferase and the hepatic level of malondialdehyde were significantly higher 24 h after the CCl₄ treatment, while the concentration of reduced glutathione was lower. These changes were attenuated by glycyrrhizin. CCl₄ increased the level of circulating tumor necrosis factor- α markedly, which was reduced by glycyrrhizin. The levels of hepatic inducible nitric oxide synthase, cyclooxygenase-2, and heme oxygenase-1 protein expression were markedly higher after the CCl₄ treatment. Glycyrrhizin diminished these alterations for inducible nitric oxide and cyclooxygenase-2 but the protein expression of heme oxygenase-1 was further elevated by the treatment of glycyrrhizin. CCl₄ increased the level of tumor necrosis factor- α , inducible nitric oxide synthase, cyclooxygenase-2, and heme oxygenase-1 mRNA expressions. The mRNA expression of heme oxygenase-1 was augmented by the glycyrrhizin treatment, while glycyrrhizin attenuated the increase in tumor necrosis factor- α , inducible nitric oxide synthase, and cyclooxygenase-2 mRNA expressions. These results suggest that glycyrrhizin alleviates CCl₄-induced liver injury, and this protection is likely due to the induction of heme oxygenase-1 and the downregulation of proinflammatory mediators.

Key words carbon tetrachloride; glycyrrhizin; heme oxygenase-1; hepatoprotective activity; oxidative stress; proinflammatory mediator

Acute and chronic liver diseases constitute a global concern, and the medical treatments for these diseases are often difficult to handle and have limited efficacy. Therefore, there has been considerable interest in the role of complementary and alternative medicines for the treatment of liver diseases.¹⁾ Developing therapeutically effective agents from natural products may reduce the risk of toxicity when the drug is used clinically.

Carbon tetrachloride (CCl₄) is a well-known hepatotoxin that is widely used to induce toxic liver injury in a range of laboratory animals. CCl₄-induced hepatotoxicity is believed to involve two phases. The initial phase involves the metabolism of CCl₄ by cytochrome P450 to the trichloromethyl radical (CCl₃·), which leads to lipid peroxidation.²⁾ Heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme catabolism, is known to be induced by oxidative stress and to confer protection against oxidative stress injuries.³⁾ The second phase of CCl₄-induced hepatotoxicity involves the activation of Kupffer cells, which is accompanied by the production of proinflammatory mediators.⁴⁾ Several microarray studies have been reported describing gene expression changes caused by acute CCl₄ toxicity,⁵⁾ although the significance of these changes has not been fully understood.

Licorice, the root of *Glycyrrhiza glabra*, is one of the oldest and most commonly prescribed herbs in Eastern traditional medicine, and has been used to treat tuberculosis, peptic ulcers, and liver injury in a number of clinical disorders.⁶⁾ Glycyrrhizin is a major active constituent isolated from licorice that scavenges reactive oxygen species (ROS) and has an anti-inflammatory action.^{7,8)} A recent report suggested that glycyrrhizin also inhibits anti-Fas antibody-induced hep-

atitis by acting upstream of the activation of CPP32-like protease.⁹⁾ However, there is limited information available on the *in vivo* hepatoprotective effect of glycyrrhizin.

This study investigated the effect of glycyrrhizin on acute hepatic injury, the specific molecular mechanisms of protection, and the effect of glycyrrhizin on both hepatic oxidative stress and inflammation.

MATERIALS AND METHODS

Isolation and Purification of Glycyrrhizin The dried roots of *Glycyrrhiza glabra* from the Shenyang province of China were purchased from Kwanglim Co. (Daegu, Korea) and were authenticated by Dr. J. H. Lee, an Oriental medicine specialist. A voucher specimen (#06-04-0002) was deposited at the College of Pharmacy, Yeungnam University, Korea. The roots of *Glycyrrhiza glabra* (10 kg) were extracted with methanol (50 l) at room temperature. The methanol extract was evaporated under reduced pressure to obtain a residue (2.6 kg), which was then dissolved in water (3.5 l) and partitioned with methylene chloride (3.5 l \times 3). The methylene chloride soluble fraction (230 g) was chromatographed on silica gel (6.2 kg), with gradient elution using *n*-hexane/ethyl acetate mixtures (100 : 0, 98 : 2, 95 : 5, 90 : 10, 85 : 15, 80 : 20, 5 l for each gradient) to give sixteen fractions (G01—G16). Fraction G04 (3200 ml, *n*-hexane-ethyl acetate, 98 : 2) was purified by crystallization from cold methanol to yield glycyrrhizin (450 mg), which was subjected to analytical HPLC (25—75 μ m, 5 mm i.d. \times 20 cm, Shim-pack ANAL-ODS column; Shimadzu, Japan) with elution by methanol-water-acetic acid (65 : 34 : 1 (v/v), 1 ml/min) to afford its purity

* To whom correspondence should be addressed. e-mail: sunmee@skku.edu

(>95%) and retention time (26 min). The structure of glycyrrhizin was identified with an authentic sample by comparing their NMR and MS spectral data.^{10,11)}

Animals and Treatment Regimens Male ICR mice weighing 25–30 g were fasted overnight but given tap water *ad libitum*. All the animals were treated humanely under the Sungkyunkwan University Animal Care Committee Guidelines. The animals were randomly assigned to 7 groups containing 8 animals per group. The mice in group I (control) received only olive oil (10 ml/kg, i.p.). In groups II to VII, CCl₄ was dissolved in olive oil (1 : 19, v/v) and administered intraperitoneally (final concentration; 0.5 ml/kg). The animal groups I and II (vehicle) were treated intraperitoneally with saline (10 ml/kg). The animals in groups III to VI were treated intraperitoneally with glycyrrhizin (50, 100, 200, 400 mg/kg), and the animals in group VII were treated with silymarin (positive control, 200 mg/kg, i.p.), 24 h and 0.5 h before and 4 h after administering CCl₄. The dose and timing of the glycyrrhizin treatment were selected based on previous reports,¹²⁾ as well as its efficacy in a retrorsine-induced hepatotoxicity model¹³⁾ and an anti-Fas antibody-induced mice hepatitis model.⁹⁾ Blood was collected from the abdominal aorta 24 h after CCl₄ administration. The liver was isolated and used immediately to prepare the mRNA, and was stored at –75 °C for later analysis, except for the part in the left lobe, which was used for histological analysis.

Assessment of Serum Aminotransferase Activities The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined using a Hitachi 747 automatic analyzer (Hitachi, Tokyo, Japan).

Determination of Hepatic Lipid Peroxidation and Glutathione Contents The steady-state level of malondialdehyde (MDA), a lipid peroxidation end product, was analyzed by measuring the level of thiobarbituric acid reactive substances (TBARS) spectrophotometrically at a wavelength of 535 nm, according to the method reported by Buege and Aust¹⁴⁾ using 1,1,3,3-tetraethoxypropane (Sigma, St. Louis, MO, U.S.A.) as the standard. The total glutathione level was measured spectrophotometrically at a wavelength of 412 nm, with yeast glutathione reductase, 5,5'-dithio-bis(2-nitrobenzoic acid), and NADPH, according to the methodology reported by Tietze.¹⁵⁾ The oxidized glutathione (GSSG) level was measured using the same method in the presence of 2-vinylpyridine,¹⁶⁾ and the reduced glutathione (GSH) level was determined by the difference between the total glutathione and the GSSG levels.

Histological Analysis Twenty-four hours after administering CCl₄, a small piece of liver tissue from the anterior portion of the left lateral lobe was removed for histological analysis. The sample was fixed by immersing it in 10% neutral-buffered formalin. The sample was then embedded in paraffin, sliced into 5- μ m sections, and stained with hematoxylin–eosin for a blinded histological assessment. The degree of portal inflammation, hepatocellular necrosis, and inflammatory cell infiltration was evaluated semiquantitatively according to the method reported by Frei *et al.*¹⁷⁾ The stained 5- μ m sections were graded as follows: 0, absent; I, minimal; II, mild; III, modest; and IV, severe. The histological changes were evaluated in nonconsecutive, randomly chosen \times 200 histological fields.

Measurement of Serum Tumor Necrosis Factor- α

(TNF- α) Levels The serum TNF- α level was quantified using enzyme-linked immunosorbent assay (ELISA) with a commercial mouse TNF- α ELISA kit (eBioscience, San Diego, CA, U.S.A.) according to the manufacturer's instructions.

Western Blot Immunoassay Freshly isolated liver tissue was homogenized in a lysis buffer. In order to determine the level of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and heme oxygenase-1 (HO-1) protein expression, 10 μ g of protein samples from the liver homogenates were loaded per lane on 10% polyacrylamide gels. The protein samples were then separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to nitrocellulose membranes using a semi-dry transfer process. After transfer, the membranes were washed with Tris buffered saline (TBS) and blocked for 1 h at room temperature with 5% (w/v) skim milk powder in TBS. The blots were then incubated overnight at 4 °C with the polyclonal antibodies against mouse iNOS (Transduction Laboratories, San Jose, CA, U.S.A.; 1 : 1000 dilution), COX-2 (Cayman, Ann Arbor, MI, U.S.A.; 1 : 1000 dilution), and HO-1 (Transduction Laboratories, San Jose, CA, U.S.A.; 1 : 1000 dilution), and with the monoclonal antibodies against mouse β -actin (Sigma, St. Louis, MO, U.S.A.; 1 : 10000 dilution). On the next day, the primary antibody was removed and the blots were washed thoroughly with T-TBS (0.05% Tween 20 in TBS). The binding of all the antibodies was detected using an ECL detection system (iNtRON Biotechnology Co., Ltd., Korea), according to the manufacturer's instructions. The visualized immunoreactive bands were evaluated densitometrically with ImageQuantTM TL software version 2005 (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Total RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR) The total RNA was extracted using the method reported by Chomczynski and Sacchi.¹⁸⁾ Reverse transcription of the total RNA extracted from the tissue samples was carried out in order to synthesize the first strand cDNA using the oligo(dT)_{12–18} primer and SuperScriptTM II RNase H⁻ Reverse Transcriptase (Invitrogen Tech-LineTM, Carlsbad, CA, U.S.A.). The PCR reaction was carried out with a diluted cDNA sample and was amplified in a 20 μ l reaction volume. The final reaction concentrations are as follows: primers, 10 pmol; dNTP mix, 250 μ M; \times 10 PCR buffer; and Ex *Taq* DNA polymerase, 0.5 U per reaction. RT-PCR was carried out with an initial denaturation step at 94 °C for 5 min and a final extension step at 72 °C for 7 min using GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA, U.S.A.). The amplification cycling conditions are as follows: for *TNF- α* , 28 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s; for *iNOS*, 35 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s; for *COX-2*, 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; for *HO-1*, 30 cycles, and for *β -actin*, 25 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. After RT-PCR, 10 μ l samples of the PCR products were visualized by ultraviolet illumination after electrophoresis through 1.5% agarose gel and ethidium bromide staining. The intensity of each PCR product was analyzed semiquantitatively using a digital camera (DC120, Eastman Kodak, New Haven, CT, U.S.A.) and analyzing software.

Statistical Analysis The overall significance of the results was examined using one-way analysis of variance (ANOVA). The differences between the groups were considered statistically significant at a p value <0.05 with the appropriate Bonferroni correction made for multiple comparisons. The results are presented as a mean \pm S.E.M.

RESULTS

Serum Aminotransferase Activities The serum levels of ALT and AST in the control animals were 66.6 ± 5.5 and 128.0 ± 14.3 U/l, respectively. 24 h after the CCl_4 treatment, the serum ALT and AST levels increased to approximately 174.7 and 94.1 times that in the control animals, respectively. Glycyrrhizin, at the doses of 200 mg/kg and 400 mg/kg, significantly reduced these increases. The ALT and AST activities were also decreased in the silymarin-treated group compared with the vehicle-treated CCl_4 group (Fig. 1).

Lipid Peroxidation and Hepatic Glutathione Contents

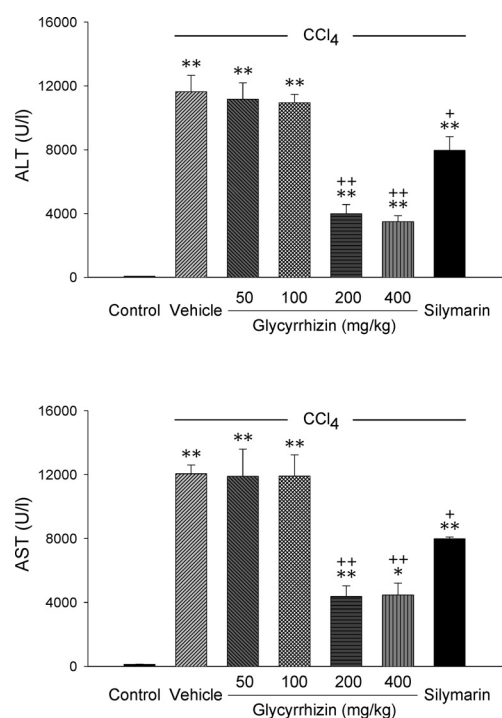


Fig. 1. Effect of Glycyrrhizin on the Serum Aminotransferase Activity after CCl_4 (0.5 ml/kg) Administration

The results are presented as the mean \pm S.E.M. of 8 animals per group. *, ** Denotes significant differences from the control group ($p < 0.05$ and $p < 0.01$); +, ++ denotes significant differences from the vehicle-treated CCl_4 group ($p < 0.05$ and $p < 0.01$).

Table 1. Effect of Glycyrrhizin on Lipid Peroxidation and Glutathione Contents in the Liver after CCl_4 (0.5 ml/kg) Administration

Groups	Dose (mg/kg)	MDA (nmol/g liver)	GSH ($\mu\text{mol/g}$ liver)	GSSG ($\mu\text{mol/g}$ liver)	GSH/GSSG ratio
Control		37.8 ± 1.9	6.3 ± 0.4	0.3 ± 0.1	15.6 ± 0.9
CCl_4					
Vehicle		$49.1 \pm 1.1^{**}$	$3.4 \pm 0.3^{**}$	0.4 ± 0.0	$9.8 \pm 0.7^*$
Glycyrrhizin	50	$49.3 \pm 0.5^{**}$	3.8 ± 1.1	0.3 ± 0.1	10.5 ± 1.1
	100	$50.0 \pm 1.4^{**}$	$3.6 \pm 0.6^*$	0.2 ± 0.0	11.3 ± 2.5
	200	$43.9 \pm 1.1^{* \dagger}$	$5.6 \pm 0.4^{\dagger \dagger}$	0.3 ± 0.0	14.5 ± 3.6
	400	$47.2 \pm 2.0^{**}$	4.3 ± 1.0	0.2 ± 0.0	$15.3 \pm 1.2^{\dagger}$
Silymarin	200	$41.3 \pm 1.9^{\dagger \dagger}$	3.8 ± 1.2	0.4 ± 0.1	9.1 ± 0.9

The results are presented as a mean \pm S.E.M. for 8 animals per group. **, * Denotes significant differences from the control group, $p < 0.05$ and $p < 0.01$, respectively; \dagger , $\dagger \dagger$ denotes significant differences from the vehicle-treated CCl_4 group, $p < 0.05$ and $p < 0.01$, respectively.

The administration of CCl_4 increased the hepatic level of MDA to approximately 1.3 times that of the control animals. This elevation was attenuated by 200 mg/kg of either glycyrrhizin or silymarin. The GSH level in the control animals was $6.3 \pm 0.4 \mu\text{mol/g}$ liver. The GSH content decreased significantly 24 h after CCl_4 administration but was markedly attenuated by 200 mg/kg glycyrrhizin. Hepatic GSSG concentration was unchanged among any of the experimental groups. The ratio of GSH to GSSG, an indicator of the hepatocellular redox state, markedly declined after the CCl_4 treatment. The decrease in the ratio of GSH to GSSG was attenuated by glycyrrhizin (Table 1).

Histological Analysis The histological features shown in Fig. 2 and Table 2 show a normal liver lobular architecture and cell structure of the livers in the control animals. However, the livers exposed to CCl_4 showed multiple and extensive areas of portal inflammation and hepatocellular necrosis, randomly distributed throughout the parenchyma, as well as a moderate increase in inflammatory cell infiltration. These pathological changes were inhibited by glycyrrhizin and silymarin at the doses of 200 mg/kg.

Serum TNF- α Levels The serum levels of TNF- α were low in the control animals. However, in the CCl_4 -treated animals, the serum level increased 2.3-fold 24 h after the CCl_4 treatment. This increase was reduced by glycyrrhizin (200 mg/kg). Glycyrrhizin treatment alone did not affect the serum levels of TNF- α (Fig. 3).

iNOS, COX-2, and HO-1 Protein Expression The amount of iNOS, COX-2, and HO-1 protein in the livers increased markedly 24 h after CCl_4 administration. The increases in iNOS and COX-2 protein levels were significantly attenuated by glycyrrhizin, while the level of HO-1 protein expression was further elevated by the treatment of glycyrrhizin. Glycyrrhizin treatment alone did not alter the protein level of iNOS, COX-2, and HO-1 (Fig. 4).

TNF- α , iNOS, COX-2, and HO-1 mRNA Expression As shown in Fig. 5, the levels of TNF- α , iNOS, COX-2, and HO-1 mRNA in the CCl_4 group were 4.5-, 3.2-, 5.0-, and 1.7-fold higher than the control level, respectively. The increase in TNF- α , iNOS, and COX-2 mRNA levels were significantly suppressed by glycyrrhizin, while the level of HO-1 mRNA expression was augmented by the glycyrrhizin treatment. The mRNA expression of TNF- α , iNOS, COX-2, and HO-1 was unaffected by the glycyrrhizin treatment itself.

DISCUSSION

In this study, the protective effect of glycyrrhizin was ex-

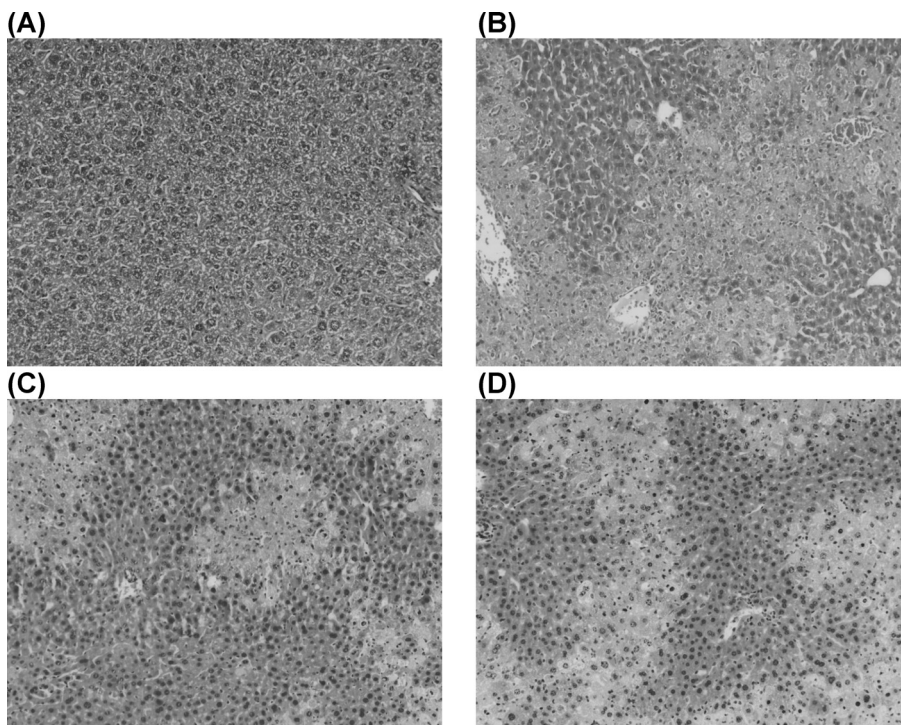


Fig. 2. Histological Analysis of the Livers after CCl₄ (0.5 ml/kg) Administration

Typical images were chosen from the different experimental groups (original magnification ×100). (A) Control group: normal lobular architecture and cell structure; (B) vehicle-treated CCl₄ group: multiple and extensive areas of portal inflammation and hepatocellular necrosis, and a moderate increase in inflammatory cell infiltration; (C) CCl₄ and glycyrrhizin (200 mg/kg)-treated group: minimal hepatocellular necrosis and inflammatory cell infiltration, and mild portal inflammation; and (D) CCl₄ and silymarin (200 mg/kg)-treated group: minimal portal inflammation and inflammatory cell infiltration, and mild hepatocellular necrosis.

Table 2. Quantitative Summary of the Histological Observations on Glycyrrhizin-Dependent Protection of CCl₄-Induced Hepatic Damage

Histopathologic grading	Control	CCl ₄		
		Vehicle	Glycyrrhizin (200 mg/kg)	Silymarin (200 mg/kg)
Portal inflammation				
Grade 0	7	0	0	0
Grade I	1	0	1	5
Grade II	0	1	5	3
Grade III	0	5	2	0
Grade IV	0	2	0	0
Hepatocellular necrosis				
Grade 0	6	0	1	0
Grade I	2	0	6	3
Grade II	0	0	1	3
Grade III	0	6	0	2
Grade IV	0	2	0	0
Inflammatory cell infiltration				
Grade 0	8	0	5	3
Grade I	0	4	3	4
Grade II	0	4	0	1
Grade III	0	0	0	0
Grade IV	0	0	0	0

Liver samples were isolated 24 h after administering the CCl₄ (0.5 ml/kg, i.p.). The samples were fixed in 10% neutral-buffered formalin prior to paraffin-embedding, and stained with hematoxylin and eosin. The histological changes were graded according to the following criteria: 0, absent; I, minimal; II, mild; III, modest; and IV, severe. 8 samples per group.

amined using a model of CCl₄-induced hepatotoxicity. The susceptibility of the liver to chemical injury is as much a function of its anatomical proximity to the bloodstream and

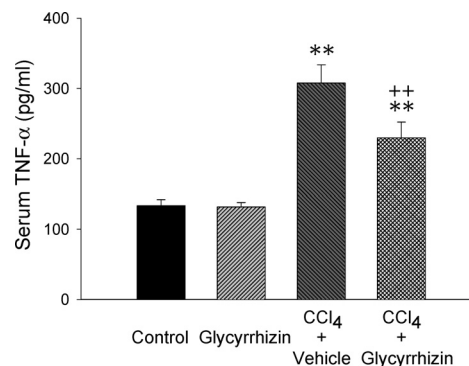


Fig. 3. Effect of Glycyrrhizin (200 mg/kg) on Serum TNF-α Secretion after CCl₄ (0.5 ml/kg) Administration

The results are presented as the mean ± S.E.M. for 8 animals per group. ** Denotes significant differences from the control group ($p < 0.01$); ++ denotes significant differences from the vehicle-treated CCl₄ group ($p < 0.01$).

gastrointestinal tract as to its ability to biotransform and concentrate xenobiotics. CCl₄-induced liver injury in a range of laboratory animals is considered to be an analogue of the liver damage caused by various hepatotoxins in humans.¹⁹⁾

In the vehicle-treated CCl₄ group, the ALT and AST levels increased dramatically compared with the control group, indicating severe hepatocellular damage. In contrast, a treatment with 200 and 400 mg/kg of glycyrrhizin markedly attenuated the release of ALT and AST. Furthermore, the hepatoprotective effect of glycyrrhizin appeared to be higher than that of silymarin, which is used as a potent hepatoprotective agent. The histological observations of the liver samples strongly support the release of aminotransferases by the

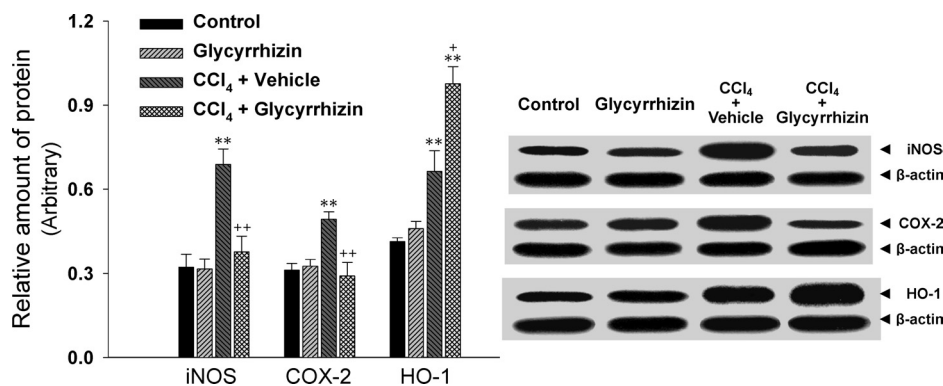


Fig. 4. Effect of Glycyrrhizin (200 mg/kg) on iNOS, COX-2, and HO-1 Protein Expression after CCl₄ (0.5 ml/kg) Administration

The results are presented as a mean \pm S.E.M. for 8 animals per group. ** Denotes significant differences from the control group ($p < 0.01$); +, ++ denotes significant differences from the vehicle-treated CCl₄ group ($p < 0.05$ and $p < 0.01$).

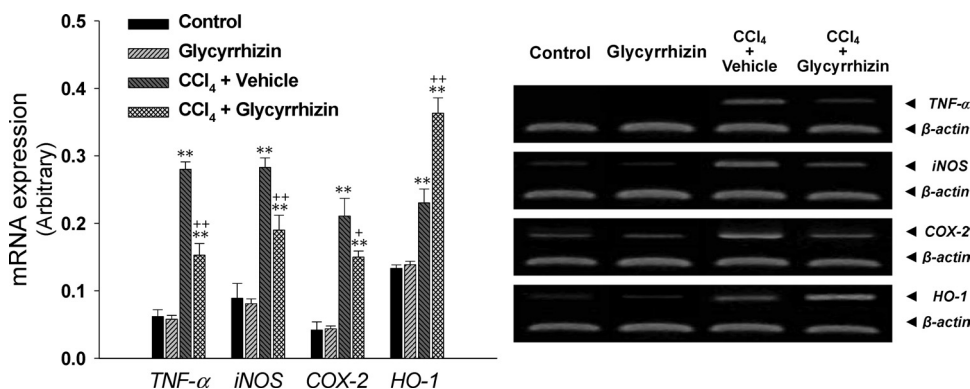


Fig. 5. Effect of Glycyrrhizin (200 mg/kg) on *TNF- α* , *iNOS*, *COX-2*, and *HO-1* mRNA Expression after CCl₄ (0.5 ml/kg) Administration

The results are presented as a mean \pm S.E.M. for 8 animals per group. ** Denotes significant differences from the control group ($p < 0.01$); +, ++ denotes significant differences from the vehicle-treated CCl₄ group ($p < 0.05$ and $p < 0.01$).

damaged hepatocytes as well as the protective effect of glycyrrhizin (Fig. 2, Table 2). CCl₄ caused various histological changes to the liver, including cell necrosis, fatty metamorphosis in the adjacent hepatocyte, ballooning degeneration, cell inflammation, and the infiltration of lymphocytes and Kupffer cells. These alterations were significantly attenuated by glycyrrhizin with the livers showing only minor hepatocellular necrosis and inflammatory cell infiltration, and mild portal inflammation. These results suggest that glycyrrhizin may have potential clinical applications for treating liver disorders.

In contrast to other known hepatotoxins, CCl₄ is not toxic *per se* but is responsible for oxidative stress and lipid peroxidation through the cytochrome P450-mediated generation of the highly reactive CCl₃ \cdot , leading to eventual cellular damage characterized by hepatocellular necrosis.²⁰⁾ The subsequent chloromethylation, saturation, peroxidation, and the progressive destruction of the unsaturated fatty acids of the membrane phospholipids are collectively known as lipid peroxidation, which leads to a functional and structural disruption.²¹⁾ The hepatoprotective effect of glycyrrhizin can also be ascribed to the suppression of lipid peroxidation as well as its propagation in the liver because glycyrrhizin at a dose of 200 mg/kg could attenuate the CCl₄-induced increase in the hepatic MDA content. Another study suggested that glycyrrhizin may provide the maximum conjugation with detrimental free radicals and deprive them of their toxic proper-

ties.²²⁾ A more definitive characterization of CCl₄-induced oxidative stress was evidenced by the decrease in the level of hepatic GSH. The GSH system acts as a major antioxidant defense mechanism against the toxic effects of free radicals.²³⁾ These results suggest that CCl₄ causes direct cellular damage through thiol oxidation and subsequent lipid peroxidation. Moreover, the glycyrrhizin treatment attenuated the lipid peroxidation and decrease in the hepatic GSH content, which suggests that glycyrrhizin increases the hepatic pool of GSH and reduces oxidative stress.

Excessive oxidative stress has been suggested as a reason for the upregulation of HO-1, as this enzyme is known to be readily inducible upon such stressors.²⁴⁾ HO-1 is a rate-limiting enzyme in the catabolism of heme and a heat shock protein (HSP32). By the equimolar production of the antioxidant bilirubin, free iron, and vasodilative carbon monoxide, HO-1 represents a cytoprotective enzyme and, when expressed, produces therapeutic benefits in a number of different conditions and diseases, such as sepsis, inflammation, and ischemia/reperfusion injury.²⁵⁾ In line with this, HO-1 induction has been shown to confer protection in CCl₄-induced hepatotoxicity, as assessed by the measurements of liver transaminase levels and cytological examination of liver histology.³⁾ In our study, the expression of HO-1 protein was significantly increased after the CCl₄ treatment. This is in concordance to the results reported by Nakahira *et al.*³⁾; following CCl₄ treatment, hepatic HO-1 expression was

markedly increased both at transcriptional and protein levels in hepatocytes, especially around the central vein. Additionally, treatment with glycyrrhizin markedly augmented HO-1 protein expression after CCl₄ treatment, which suggests that a strong induction response of HO-1 by glycyrrhizin is to protect liver cells from CCl₄-induced oxidative cellular injuries.

The liver is a major inflammatory organ, and inflammatory processes contribute to a number of pathological events after exposure to various hepatotoxins. Kupffer cells release proinflammatory mediators either in response to necrosis or as a direct action by the hepatotoxin, activated, which are believed to aggravate CCl₄-induced hepatic injury.²⁶⁾ TNF- α , a pleiotropic proinflammatory cytokine, is rapidly produced by macrophages in response to tissue damage.²⁷⁾ While low levels of TNF- α may play a role in cell protection, excessive amounts cause cell impairment. An increase in the TNF- α level has been directly correlated with the histological evidence of hepatic necrosis and the increase in the serum aminotransferase levels.²⁸⁾ DeCicco *et al.*²⁹⁾ have reported the stimulation of TNF- α production in both serum and liver following CCl₄ administration, and it is suggested that CCl₄ activates Kupffer cells to release TNF- α . TNF- α also stimulates the release of cytokines from macrophages and induces the phagocyte oxidative metabolism and nitric oxide production.³⁰⁾ Nitric oxide is a highly reactive oxidant that is produced through the action of iNOS, and plays a role in a number of physiological processes, such as, vasodilation, neurotransmission, and nonspecific host defense.³¹⁾ Nitric oxide can also exacerbate oxidative stress by reacting with reactive oxygen species, particularly with the superoxide anion, and forming peroxynitrite.³²⁾ As nitric oxide has a range of effects on a variety of biological processes, it is unclear if it is beneficial or detrimental in the liver injury induced by hepatotoxins. This study confirmed a significant increase in the serum TNF- α level and iNOS protein expression in the liver after CCl₄ administration. These alterations were attenuated by the glycyrrhizin treatment, which suggests that glycyrrhizin suppresses the TNF- α and iNOS protein secretion and/or enhances the degradation of their protein.

Previous studies reported that the induction of cyclooxygenase in inflammatory response is the secondary effect of CCl₄-induced hepatotoxicity.³³⁾ COX-2 is the mitogen-inducible isoform of cyclooxygenase and is induced in macrophages by several proinflammatory stimuli, such as cytokines and growth factors, leading to COX-2 expression and the subsequent release of prostaglandins.⁴⁾ Arachidonic acid is a well-known substrate of cyclooxygenases or lipoxygenases that is metabolized to produce a variety of proinflammatory substrates called eicosanoids, and COX-2 is the key enzyme in the cascade. Free radical mediated oxidative stress or lipid peroxidation can further activate cyclooxygenases and the subsequent prostaglandin formation from arachidonic acid. The results of this study showed an increase in the expression of COX-2 protein after CCl₄ administration. Glycyrrhizin markedly attenuated this increase, suggesting a suppression of inflammatory responses.

Recent developments in genomic technology have led to new investigations into the changes in gene expression caused by an acute treatment with CCl₄. The acute administration of CCl₄ to rats caused significant changes in the gene

expression profiles.³⁴⁾ The most notable changes in the CCl₄-treated animals were the expression of the genes involved in stress, DNA damage, cell proliferation, and metabolic enzymes.³⁵⁾ These gene expression profiles have catalogued the molecular responses to acute CCl₄ toxicity and revealed the genetic basis of hepatic toxicity. In this study, it was observed that the levels of TNF- α , iNOS, COX-2, and HO-1 mRNA expression were increased significantly by the acute CCl₄ treatment. Glycyrrhizin attenuated the increase in COX-2 mRNA, and the level of HO-1 mRNA expression was augmented by glycyrrhizin treatment. This indicates that although posttranscriptional modifications may occur, COX-2 and HO-1 are controlled primarily at the level of transcription in response to an acute dose of CCl₄. However, glycyrrhizin slightly attenuated the increase in TNF- α and iNOS mRNA expression, which was not well correlated with the levels of their proteins. Therefore, glycyrrhizin may largely regulate the TNF- α and iNOS production by the posttranscriptional level. Additional studies are required to examine this effect in further detail.

These results provide evidence for the pharmacological effect of glycyrrhizin in CCl₄-induced hepatotoxicity. Overall, glycyrrhizin not only provides maximum conjugation with injurious free radicals and diminishes their toxic properties but also suppresses the inflammatory responses of a CCl₄-induced liver injury. Further studies will be needed to fully understand the association between oxidative stress and the inflammatory responses in the hepatoprotective effect of glycyrrhizin against CCl₄-induced hepatotoxicity.

Acknowledgements This work was supported by a grant from the Korea Food and Drug Administration (Studies on the Identification of Efficacy of Biologically Active Components from Oriental Herbal Medicines).

REFERENCES

- 1) Seeff L. B., Lindsay K. L., Bacon B. R., Kresina T. F., Hoofnagle J. H., *Hepatology*, **34**, 595–603 (2001).
- 2) Edwards M. J., Keller B. J., Kauffman F. C., Thurman R. G., *Toxicol. Appl. Pharmacol.*, **119**, 275–279 (1993).
- 3) Nakahira K., Takahashi T., Shimizu H., Maeshima K., Uehara K., Fujii H., Nakatsuka H., Yokoyama M., Akagi R., Morita K., *Biochem. Pharmacol.*, **66**, 1091–1105 (2003).
- 4) Planagumà A., Clària J., Miquel R., López-Parra M., Titos E., Masferri J. L., Arroyo V., Rodés J., *FASEB J.*, **19**, 1120–1122 (2005).
- 5) Harries H. M., Fletcher, S. T., Duggan C. M., Baker V. A., *Toxicol. In Vitro*, **15**, 399–405 (2001).
- 6) Huang K. C., "The Pharmacology of Chinese Herbs," CRC Press, Inc., Boca Raton, FL, 1993, pp. 275–278.
- 7) Gumprecht E., Dahl R., Devereaux M. W., Sokol R. J., *J. Biol. Chem.*, **280**, 10556–10563 (2005).
- 8) Yoshida T., Tsuda Y., Takeuchi D., Kobayashi M., Pollard R. B., Suzuki F., *Cytokine*, **33**, 317–322 (2006).
- 9) Okamoto T., *Eur. J. Pharmacol.*, **387**, 229–232 (2000).
- 10) Kitagawa I., Hori K., Taniyama T., Zhou J. L., Yoshikawa M., *Chem. Pharm. Bull.*, **41**, 43–49 (1993).
- 11) Liu H. M., Sugimoto N., Akiyama T., Maitani T., *J. Agric. Food Chem.*, **48**, 6044–6047 (2000).
- 12) Yamamura Y., Kotaki H., Tanaka N., Aikawa T., Sawada Y., Iga T., *Biopharm. Drug Dispos.*, **18**, 717–725 (1997).
- 13) Lin G., Nnane I. P., Cheng T. Y., *Toxicol.*, **37**, 1259–1270 (1999).
- 14) Buege J. A., Aust S. D., *Methods Enzymol.*, **52**, 302–310 (1978).
- 15) Tietze F., *Anal. Biochem.*, **27**, 502–522 (1969).
- 16) Griffith O. W., *Anal. Biochem.*, **106**, 207–212 (1980).
- 17) Frei A., Zimmermann A., Weigand K., *Hepatology*, **4**, 830–834

- (1984).
- 18) Chomczynski P., Sacchi N., *Anal. Biochem.*, **162**, 156—159 (1987).
 - 19) Muriel P., *Biochem. Pharmacol.*, **56**, 773—779 (1998).
 - 20) Taïeb D., Malicet C., Garcia S., Rocchi P., Arnaud C., Dagorn J. C., Iovanna J. L., Vasseur S., *Hepatology*, **42**, 176—182 (2005).
 - 21) Recknagel R. O., *Life Sci.*, **33**, 401—408 (1983).
 - 22) Hu C. C., Chen W. K., Liao P. H., Yu W. C., Lee Y. J., *Mutat. Res.*, **496**, 117—127 (2001).
 - 23) Griffith O. W., Mulcahy R. T., *Adv. Enzymol. Relat. Areas Mol. Biol.*, **73**, 209—267 (1999).
 - 24) Otterbein L. E., Choi A. M., *Am. J. Physiol.*, **279**, L1029—L1037 (2000).
 - 25) Kyokane T., Norimizu S., Taniai H., Yamaguchi T., Takeoka S., Tsuchida E., Naito M., Nimura Y., Ishimura Y., Suematsu M., *Gastroenterology*, **120**, 1227—1240 (2001).
 - 26) Badger D. A., Sauer J. M., Hoglen N. C., Jolley C. S., Sipes I. G., *Toxicol. Appl. Pharmacol.*, **141**, 507—519 (1996).
 - 27) Brouckaert P., Fiers W., *Curr. Top. Microbiol. Immunol.*, **216**, 167—187 (1996).
 - 28) Bruccoleri A., Gallucci R., Germolec D. R., Blackshear P., Simeonova P., Thurman R. G., Luster, M. I., *Hepatology*, **25**, 133—141 (1997).
 - 29) DeCicco L. A., Rikans L. E., Tutor C. G., Hornbrook K. R., *Toxicol. Lett.*, **98**, 115—121 (1998).
 - 30) Morio L. A., Chiu H., Spowles K. A., Zhou P., Heck D. E., Gordon M. K., Laskin D. L., *Toxicol. Appl. Pharmacol.*, **172**, 44—51 (2001).
 - 31) Lowenstein C. J., Snyder S. H., *Cell*, **70**, 705—707 (1992).
 - 32) Rodenas J., Mitjavila M. T., Carbonell T., *Free Radic. Biol. Med.*, **18**, 869—875 (1995).
 - 33) Basu S., *Biochem. Biophys. Res. Commun.*, **254**, 764—767 (1999).
 - 34) Fountoulakis M., de Vera M. C., Cramer F., Boess F., Gasser R., Albertini S., Suter L., *Toxicol. Appl. Pharmacol.*, **183**, 71—80 (2002).
 - 35) Jiang Y., Liu J., Waalkes M., Kang Y. J., *Toxicol. Sci.*, **79**, 404—410 (2004).