

# Protective effect and mechanism of ginsenoside Rg1 on carbon tetrachloride-induced acute liver injury

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**Abstract.** Liver injury is a common pathological state in various types of liver disease; severe or persistent liver damage is the basis of hepatic failure. Ginsenoside Rg1 (Rg1), one of the primary active ingredients of ginseng, has been reported to reduce concanalin A-induced hepatitis and protect against lipopolysaccharide- and galactosamine-induced liver injury. However, the underlying protective mechanism of Rg1 in acute liver injury remains unclear. In the present study, a carbon tetrachloride (CCl<sub>4</sub>)-induced acute liver injury model was established, and the protective effect of Rg1 on CCl<sub>4</sub>-induced acute liver injury was demonstrated in cell culture and animal experimental systems. Further investigation of the mechanisms demonstrated that pretreatment with Rg1 reduced elevated levels of alanine aminotransferase and aspartate aminotransferase, enhanced the antioxidant activity of superoxide dismutase (SOD) and decreased malondialdehyde (MDA) content. Experiments *in vitro* demonstrated that Rg1 decreased p65 expression and inhibited nuclear factor (NF)-κB activity. In addition to the effect of Rg1, an NF-κB inhibitor promoted cell survival, enhanced SOD activity and reduced MDA level. It was observed through *in vivo* experiments that pretreatment with Rg1 inhibited NF-κB expression and activity in Kupffer cells and reduced the serum levels of tumor necrosis factor-α and interleukin-6. In conclusion, the results of the present study indicated that pretreatment with Rg1 may rescue CCl<sub>4</sub>-induced

acute liver injury *in vivo* and *in vitro* through inhibition of NF-κB activity, to restore the anti-oxidative defense system and down-regulate pro-inflammatory signaling pathways. The present observations provide a theoretical foundation for the clinical application of Rg1 therapy in acute liver injury.

## Introduction

The liver, one of the largest organs in the human body, serves a vital role in the metabolism of carbohydrates, lipids and proteins, the regulation of immune responses, and the clearance of toxins and pathogens (1,2). The liver is frequently exposed to various insults, which may cause cell swelling, degeneration, necrosis, apoptosis, hepatic fibrosis and dysfunction. As the fifth most common cause of mortality following heart disease, stroke, lung disease and cancer (3), the rates of liver disease, unlike other major causes of mortality, are increasing (4). However, the available synthetic drugs, including interferon and corticosteroids, are expensive and may present the risk of adverse effects (5). Therefore, treating liver disease with alternative medicine seems attractive, as a number of medicinal plants, which have been traditionally used for centuries, are accessible and appear to exhibit decreased toxicity (6). Ginsenoside Rg1 (Rg1) is one of the primary active ingredients in ginseng, and possesses the potential to protect cardiovascular and nervous system activity, as well as anti-tumor, anti-fatigue and anti-aging capabilities (7-9). Komatsu *et al* (10) demonstrated that Rg1 protected against lipopolysaccharide- and galactosamine-induced hepatic damage. Additionally, Cao *et al* (11) reported that Rg1 reduced concanavalin A-induced hepatitis in mice through inhibition of cytokine secretion and lymphocyte infiltration. Pretreatment with Rg1 protected mice from IR-induced liver injury by reducing hepatocellular apoptosis and inhibiting inflammatory responses (12). The present study aimed to investigate the effect and underlying mechanism of Rg1 on carbon tetrachloride (CCl<sub>4</sub>)-induced acute liver injury *in vitro* and *in vivo*, and to provide the theoretical foundation of the clinical application of Rg1 in acute liver injury.

Liver injury is a common pathological state in various types of liver disease; severe or persistent liver damage is the basis of hepatic failure (13). CCl<sub>4</sub> is a commonly-applied

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chemical substance which may induce acute and chronic liver injury in animal models. It is well known that following activation by cytochrome P450 metabolism in the liver, CCl<sub>4</sub> generates numerous free radicals and reactive oxygen species which cause oxidative stress. These species cause membrane lipid peroxidation and bind protein macromolecules through covalent linkage, ultimately leading to interference with protein function, the destruction of cell membrane structure, increased permeability of the cell membrane, leakage of liver enzymes and liver cell death (14,15).

Nuclear factor (NF)- $\kappa$ B, a transcription factor known to regulate inflammatory responses in a number of cell types (16), was demonstrated to be involved in a variety of types of liver injuries (17-21). Liu *et al* (9) observed that Rg1 inhibited NF- $\kappa$ B activity to protect against H<sub>2</sub>O<sub>2</sub>-induced cell death in rat adrenal pheochromocytoma PC12 cells; additionally, the study of Tao *et al* (12) demonstrated that pretreatment with Rg1 protected mice from ionizing radiation-induced liver injury by reducing hepatocellular apoptosis and inhibiting inflammatory responses, in part via the NF- $\kappa$ B signaling pathway. However, the effect of Rg1 on CCl<sub>4</sub>-induced acute liver injury and its mechanism, including the association between Rg1 and NF- $\kappa$ B, have not been systematically studied. The present study demonstrated that pretreatment with Rg1 may protect against CCl<sub>4</sub>-induced acute liver injury *in vivo* and *in vitro*, and the protective effect was associated with inhibition of the NF- $\kappa$ B signaling pathway to restore the anti-oxidative defense system. A reduction in elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels was observed, as well as the inhibition of lipid peroxidation expressed as enhancement in superoxide dismutase (SOD) activity and a decrease in malondialdehyde (MDA) expression. Attenuation of inflammatory responses expressed as a decrease in serum tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-6 levels was also observed.

## Materials and methods

**Reagents.** Rg1 (C<sub>42</sub>H<sub>72</sub>O<sub>14</sub>) was obtained from Shanghai YaJi Biotechnology Co., Ltd. (Shanghai, China; cat. no. MUST-11041201); CCl<sub>4</sub> was purchased from Shanghai ShenXiang Chemical Reagent Co., Ltd. (Shanghai, China; cat. no. 20090510); minimum essential medium with Earle's balanced salts (MEM-EBSS) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA); fetal bovine serum (FBS) was from Hangzhou Evergreen Biological Material Co., Ltd. (Hangzhou, China); penicillin and streptomycin were from North China Pharmaceutical Co., Ltd. (Shijiazhuang, China); dimethyl sulfoxide (DMSO) was from Beijing YiLi Fine Chemicals Co., Ltd. (Beijing, China); NF- $\kappa$ B p65 antibody (rabbit anti-mouse) was from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany; cat. no. SAB4502609); bicinchoninic acid protein concentration assay kit was from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China); NF- $\kappa$ B electrophoretic mobility shift assay (EMSA) kit (cat. no. 20148) was from Thermo Fisher Scientific, Inc.; biotin-labeled NF- $\kappa$ B probes (oligonucleotide sequences: 3'-TCAACTCCCCTGAAAGGGTCCG-5' and 5'-AGTTGAGGGGACTTTCCCAGGC-3') were purchased from Shanghai Sangon Biological Engineering Co., Ltd.

(Shanghai, China); TNF- $\alpha$  (cat. no. MTA000B) and IL-6 (cat. no. M6000B) ELISA kits were from R&D Systems, Inc. (Minneapolis, MN, USA); and ALT (cat. no. C009-3), AST (cat. no. C010-3), SOD (cat. no. A001-2), and MDA (cat. no. A003-1) kits were from Nanjing JianCheng Bioengineering Institute (Nanjing, China).

**CCl<sub>4</sub> injury mouse model.** A CCl<sub>4</sub>-induced acute liver injury model of mice was established as described by Li *et al* (22). Male Kunming mice (20 $\pm$ 2 g; n=60) were purchased from the Experimental Animal Center of Anhui Medical University [animal license no. SCXK (Anhui) 2011-002; Hefei, China]. The facilities and the protocol for these experiments were consistent with the regulations of animal use for biomedical experiments as issued by the Ministry of Science and Technology of China and were also approved by the animal ethics committee of Bengbu Medical College (Bengbu, China). The mice were kept in the dark and had free access to standard chow and water. Following 3 days of adaptive feedings, the 60 mice were randomly divided into five groups: i) Control group [olive oil (Hong Rui Men; Jiangxi Hongrui Oil Food Co. Ltd., Nanchang, China), 0.1 ml/10 g body weight]; ii) model group (CCl<sub>4</sub>, 0.1 ml/10 g body weight), iii) low Rg1 group (Rg1, 10 mg/kg body weight; CCl<sub>4</sub> 0.1 ml/10 g body weight), iv) middle Rg1 group (Rg1, 20 mg/kg; CCl<sub>4</sub> 0.1 ml/10 g body weight), v) high Rg1 group (Rg1, 40 mg/kg; CCl<sub>4</sub> 0.1 ml/10 g body weight). Oral doses of Rg1 were administered once a day for 7 days and all groups, except the control group, were intraperitoneally injected with 0.1% CCl<sub>4</sub> in olive oil (v/v) 2 h subsequent to the last dose of Rg1 in order to induce acute liver injury, while the mice in control group were injected with an equal volume of olive oil. All mice were fasted and had free access to water overnight. The blood was obtained from the orbital sinus, centrifuged at 268.3 x g for 5 min at 37°C and prepared for ALT and AST content detection. The liver was rapidly removed subsequent to the mice being sacrificed, and was rinsed three times with saline to remove blood. The left lobe of the liver was prepared for hematoxylin and eosin (H&E) staining and the right lobe of the liver for SOD, MDA, TNF- $\alpha$  and IL-6 content detection.

**Isolation and purification of Kupffer cells.** Following intraperitoneal injection with CCl<sub>4</sub> olive oil solution into mice for 1 h, Kupffer cells from each group of animals were collected (n=4). Hepatic perfusion and digestion was performed according to the method described by Fukada *et al* (23). Following intraperitoneal injection with CCl<sub>4</sub> olive oil solution into the mouse for 1 h, the mouse was anesthetized with chloral hydrate (5% chloral hydrate, 0.1 ml/10 g body weight) and the abdomen opened to confirm the location of the portal vein. The portal vein was cannulated with a 22 G catheter, ~1 cm away from the hepatic portal with 0.8-cm insertion and an arterial clamp. The liver was perfused with the first infusion, composed of NaCl (137 mmol/l), KCl (2.68 mmol/l), Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O (0.7 mmol/l), HEPES (10 mmol/l), glucose (10 mmol/l), EDTA-Na<sub>2</sub> (0.5 mmol/l) and heparin (2,000 U/l), pH 7.4, until the liver volume had expanded to ~2X the original volume and a slight white color was observed. The inferior vena cava was subsequently cut. Finger pressure was applied to the segments, and they were intermittently opened to enlarge and retract the

liver alternately following liver retraction. When the liver had turned white, with 15 min reperfusion, the liver was removed to a sterile petri dish (reserving the portal vein catheterization). Perfusion of the liver was continued with the second infusion, composed of NaCl (137 mmol/l), KCl (2.68 mmol/l),  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  (0.7 mmol/l), HEPES (10 mmol/l), glucose (10 mmol/l),  $\text{CaCl}_2$  (5 mmol/l) and collagenase IV (0.05%), pH 7.4, for ~15 min, until the liver became soft and structural collapse was observed. Liver cells were scattered to form a cell suspension using stirring and percussion with a dropper.

For the isolation of Kupffer cells, the method described by Fukada *et al* (23) and Smedsrød and Pertoft (24) in 1985 was employed and improved. The cell suspension was subjected to low-speed centrifugation ( $16.7 \times g$  for 3 min at  $4^\circ\text{C}$ ) and the supernatant was removed prior to the precipitate being resuspended in 10 ml PBS. Subsequently, 15 ml 50% Percoll liquid (Shanghai Xinyu Biotechnology Co., Ltd., Shanghai, China) was added into a new tube and 20 ml 25% Percoll liquid was carefully added along the tube wall, followed by 10 ml cell suspension. Following centrifugation at  $268.3 \times g$  for 15 min at  $4^\circ\text{C}$ , four apparent partitions were observed: From top to bottom these were cell debris, non-parenchymal cells enriched in endothelial cells, Kupffer cells and red cells, which precipitated at the bottom of the tube. The Kupffer cells were collected, ~15 ml, and were diluted with 15 ml PBS. Following centrifugation at  $268.3 \times g$  for 10 min at  $4^\circ\text{C}$ , the precipitate was resuspended in culture medium.

Subsequently, purification of Kupffer cells was performed; the survival rate of the cells was >90% as observed with Trypan blue staining (3 min at  $4^\circ\text{C}$ ). The cells were counted and the concentration adjusted to  $2 \times 10^6/\text{ml}$ , and 1 ml cell suspension/well was seeded on 12-well culture plates. Following incubation for 2 h at  $37^\circ\text{C}$  in an atmosphere of 5%  $\text{CO}_2$  in air, the cells were gently washed with fresh culture medium, and the adherent cells were considered to be Kupffer cells. Cell culture was continued, with the medium being changed every 2-3 days.

**H&E staining of liver tissue.** The left lobe of the removed liver was fixed in neutral formalin, paraffin-embedded and sliced. A  $2.0 \times 2.0 \times 0.3$  cm block of tissue was selected from the same site of each mouse, washed with saline and placed in 10% neutral formalin buffer at  $37^\circ\text{C}$ . Following dehydration, the blocks were wax embedded, sliced into  $5\text{-}\mu\text{m}$  sections, dewaxed, H&E stained for 5 min at  $37^\circ\text{C}$ , dehydrated, and mounted in neutral balsam. The tissues were observed using an optical microscope in the Department of Pathology at the First Affiliated Hospital of Bengbu Medical College and images of morphological changes were captured using a light microscope (magnification,  $\times 100$ ).

**Determination of serum ALT and AST content.** The blood obtained from the orbital sinus was centrifuged at  $1,341.6 \times g$  at  $4^\circ\text{C}$  for 10 min and the serum content of ALT and AST was measured in accordance with the manufacturer's protocol of the ALT and AST activity kits.

**Determination of liver SOD and MDA content.** The right liver lobes of the sacrificed mice were cut and weighed and 400 mg liver tissue was combined with saline (1:9 v/v) and

crushed on ice using a glass homogenizer. The homogenized solutions were centrifuged at  $1,341.6 \times g$  at  $4^\circ\text{C}$  for 15 min, and the resulting 10% liver homogenate supernatants were measured for SOD and MDA content in accordance with the manufacturer's protocol for the kits.

**Determination of liver TNF- $\alpha$ , IL-6 levels using ELISA.** The remainder of the right lobes of the livers were cut and weighed, and 400 mg was combined with saline (1:9 v/v) and crushed on ice using a glass homogenizer. The homogenates were centrifuged at  $1,341.6 \times g$  at  $4^\circ\text{C}$  for 15 min, and the optical density of the supernatant was detected at 450 nm and compared to the TNF- $\alpha$  or IL-6 standard curve in accordance with the manufacturer's protocol for the kit.

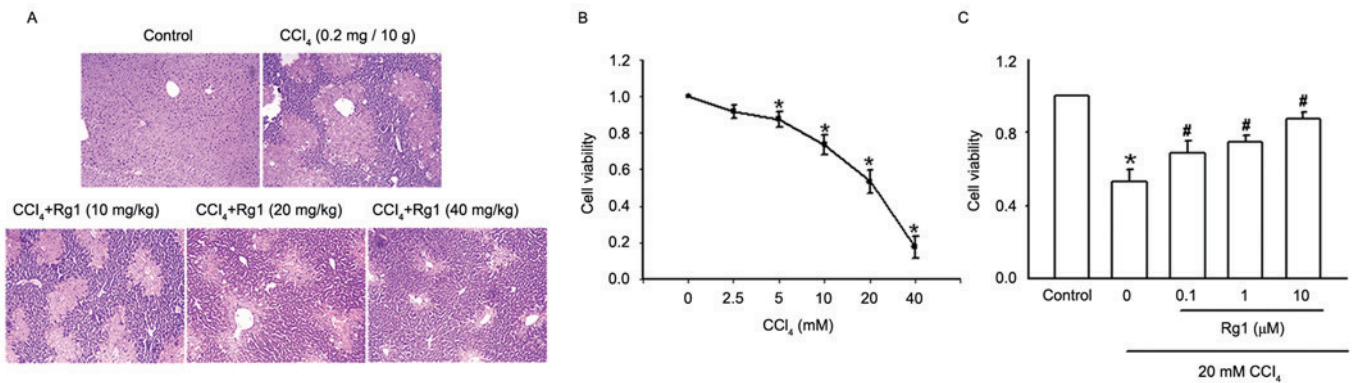
**$\text{LO}_2$  cell culture.**  $\text{LO}_2$  human normal liver cells, donated by the College of Pharmacy, Sun Yat-sen University, were maintained in MEM-EBSS containing 10% FBS and 1% penicillin/streptomycin at  $37^\circ\text{C}$  in an atmosphere of 5%  $\text{CO}_2$  in air. The cells were trypsinized and diluted to  $5 \times 10^4$  cells/ml in the logarithmic phase prior to the experiment.

**$\text{CCl}_4$  injury cell model.** The viability of  $\text{LO}_2$  cells incubated with  $\text{CCl}_4$  or Rg1 was measured by MTT assay. A total of  $5 \times 10^3$  cells/well were seeded into 96-well plates for 24 h and then increasing concentrations of  $\text{CCl}_4$  in normal saline (2.5, 5, 10, 20 and 40 mM) were added for 3 h. Each group was plated in triplicate and normal saline was used as control group. For Rg1 treatment, 0.1, 1 and 10  $\mu\text{M}$  Rg1 was added to  $\text{LO}_2$  cells. After 21 h, 20 mM  $\text{CCl}_4$  (the most appropriate concentration from the liver cell injury model) was added and incubated for a further 3 h. An NF- $\kappa\text{B}$  inhibitor [caffeic acid phenethyl ester (CAPE); 25  $\mu\text{M}$  for 2 h] was additionally assessed. Subsequently, 20  $\mu\text{l}$  MTT (Shanghai Gefan Biotechnology Co., Ltd., Shanghai, China; 5 mg/ml) was added to each well for a further 4 h, the supernatants were carefully discarded and 150  $\mu\text{l}$  DMSO was added to each well in order to dissolve the crystals. The absorbance at 570 nm was measured and the cell viability was calculated as follows: Survival rate=(absorbance of treated group-absorbance of blank group)/(absorbance of control group-absorbance of blank group).

**Determination of cell AST, ALT levels, SOD activity and MDA content.** A total of  $5 \times 10^4$   $\text{LO}_2$  cells/well were seeded into 24-well plates and incubated at  $37^\circ\text{C}$  for 24 h. Treatment with Rg1 at 0.1, 1 and 10  $\mu\text{M}$  was performed for 21 h, and 20 mM  $\text{CCl}_4$  was added for a further 3 h. The supernatant was collected, and AST and ALT expression levels, SOD activity and MDA, content were measured according to the manufacturer's protocols.

**Determination of cell NF- $\kappa\text{B}$  activity using EMSA.** The NF- $\kappa\text{B}$  activity in  $\text{LO}_2$  and Kupffer cells was detected by EMSA as described by Li *et al* (25). The nuclear proteins were incubated in 1X binding buffer (50 ng/ $\mu\text{l}$  poly (deoxyinosinic-deoxycytidylic), 0.05% NP-40, 5 mM  $\text{MgCl}_2$ , 50 mM KCl, 2.5% glycerol, and ultra-pure  $\text{H}_2\text{O}$  at room temperature for 10 min, and the biotin-labeled NF- $\kappa\text{B}$  probe was added for a further 20 min. All reactions were electrophoresed on a 6% polyacrylamide gel, transferred to a positively charged





**Figure 1.** Protective effect of Rg1 on liver histopathological alterations in CCl<sub>4</sub>-induced mice and the viability of CCl<sub>4</sub>-treated LO<sub>2</sub> cells. (A) Representative liver histopathology with hematoxylin/eosin staining of CCl<sub>4</sub> induced liver injury in mice, assessed by light microscope image capture of liver sections (magnification, x100; n=8). (B) The viability of LO<sub>2</sub> cells was assessed by MTT following treatment with CCl<sub>4</sub> (2.5–40 mM) for 3 h. Data are expressed as the mean ± standard error of the mean (n=3). (C) Rg1 (0.1–10 μM) was added to LO<sub>2</sub> cells for 21 h prior to induction with 20 mM CCl<sub>4</sub> for 3 h. Treatment with 0 μM Rg1 represents CCl<sub>4</sub> group. Data are expressed as the mean ± standard error of the mean (n=3). \*P<0.05 vs. control group, #P<0.05 vs. CCl<sub>4</sub> group. Rg1, ginsenoside Rg1; CCl<sub>4</sub>, carbon tetrachloride.

nylon membrane and UV cross-linked to fix the DNA. The bands were visualized by Amersham ECL™ Plus Detection kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

**Western blotting.** Cells were harvested using lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM β-glycerophosphate and 1:1,000 protease inhibitors]. Protein concentration was determined using the BCA kit then 25 μg total protein from each sample was analyzed by SDS-PAGE and transferred onto a nitrocellulose membrane followed by immunoblotting. The membranes were probed with antibodies against p65 (1:1,000) and β-actin (A1978; 1:8,000; Sigma-Aldrich; Merck KGaA) and the antibodies were blocked with the primary antibody dilution (Bi Yun Tian Biotechnology Co., Ltd., Shanghai, China), at 4°C overnight. Then the membranes were probed by goat anti-rabbit or anti-mouse IgG antibody HRP conjugate secondary antibodies for p65 (1:2,000) and β-actin (BL003A; 1:10,000; Biosharp, Hefei, China) at room temperature for 1 h. Immunopositive bands were visualized by Amersham ECL™ Plus Western Blotting Detection kit (RPN2232; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The expression of p65 and β-actin as detected by western blotting were analyzed by Image J software version 1.48 (National Institutes of Health, Bethesda, MD, USA) as was the density of western blotting.

**Statistical analysis.** Data were processed using SPSS software (version 16; SPSS, Inc., Chicago, IL, USA). The results are expressed as the mean ± standard error of the mean. Two-group comparisons were analyzed using an unpaired Student's t test. P<0.05 was considered to indicate a statistically significant difference. All analyses were plotted using SigmaPlot version 10.0 (Jandel Scientific, San Rafael, CA, USA).

## Results

**Protective effect of Rg1 on acute CCl<sub>4</sub>-induced liver injury models.** In order to characterize the function of Rg1 in acute CCl<sub>4</sub>-induced liver injury in mice, histopathology of liver

tissues was performed using H&E staining (Fig. 1A). The control group exhibited normal liver morphology and intact lobular architecture, neat hepatic cords, clear boundaries of liver cells and round and well-defined nuclei in the absence of inflammatory cell infiltration. In the liver of the CCl<sub>4</sub>-treated mouse presented in the second panel, normal liver tissue structure disappeared and lobular structure was disrupted, inflammatory cell infiltration was observed at lobular and portal areas, including disordered hepatic cords, and significant swelling of liver cells suggested high levels of necrosis. However, with the addition of Rg1, particularly for the higher dose group, liver cell degeneration and necrosis was markedly reduced, a clearer lobular structure and neatly arranged liver cells were observed, and the inflammatory cell infiltration was also reduced.

The effect of Rg1 on acute CCl<sub>4</sub>-induced liver injury in LO<sub>2</sub> cells was also investigated (Fig. 1B and C). Increasing concentrations of CCl<sub>4</sub> (2.5–40 mM) were added to LO<sub>2</sub> cells for 3 h, and the effect of CCl<sub>4</sub> cytotoxicity on LO<sub>2</sub> cells was detected by MTT assay. As presented in Fig. 1B, CCl<sub>4</sub> led to an apparent concentration-dependent decrease in cell viability. The cell survival rates with 2.5 and 20 mM CCl<sub>4</sub> were 91.23 and 53.39%, respectively. When the 40 mM CCl<sub>4</sub> cells were observed under the microscope, the survival rate was 20.12%. A concentration of 20 mM CCl<sub>4</sub> was used for the following CCl<sub>4</sub>-induced LO<sub>2</sub> cell injury model.

The addition of Rg1 at a range of concentrations (0.1–10 μM) to LO<sub>2</sub> cells for 21 h prior to exposure to 20 mM CCl<sub>4</sub>, and detection of cell viability by MTT assay, demonstrated that incubation with CCl<sub>4</sub> alone for 3 h decreased cell viability to 53.08%; the addition of Rg1 markedly improved cell viability in a concentration-dependent manner. The cell survival rate of the 0.1 μM Rg1 group was 66.67% and the 10 μM group reached 84.69% (Fig. 1C).

**Effect of Rg1 on ALT and AST levels in acute CCl<sub>4</sub>-induced liver injury models.** The serum contents of ALT and AST presented in Table I demonstrated that, compared with levels of ALT and AST in the control group (34.54±5.63 and 75.76±7.64 U/l, respectively), the expression levels of ALT and

Table I. Effects of Rg1 on the serum levels of ALT and AST in mice with acute liver injury induced by CCl<sub>4</sub> (mean ± standard error of the mean; n=8).

Groups	Rg1 dose, mg/kg	ALT, U/l	AST, U/l
Control	0	34.54±5.63	75.76±7.64
CCl <sub>4</sub> model	0	89.90±8.18 <sup>a</sup>	182.20±8.33 <sup>a</sup>
Rg1 (low)	10	80.81±5.46	169.02±7.60 <sup>b</sup>
Rg1 (middle)	20	67.25±9.21 <sup>b</sup>	148.77±5.45 <sup>b</sup>
Rg1 (high)	40	50.88±10.85 <sup>b</sup>	100.64±8.53 <sup>b</sup>

<sup>a</sup>P<0.05 vs. control; <sup>b</sup>P<0.05, vs. CCl<sub>4</sub> model group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; Rg1, ginsenoside Rg1; CCl<sub>4</sub>, carbon tetrachloride.

Table II. Effects of Rg1 on ALT and AST levels in supernatants of CCl<sub>4</sub>-treated LO<sub>2</sub> cells (mean ± standard error of the mean; n=8).

Groups	Rg1 dose, mg/kg	ALT, U/l	AST, U/l
Control	0	19.35±4.82	25.88±6.54
CCl <sub>4</sub> model	0	85.88±7.55 <sup>a</sup>	128.36±9.03 <sup>a</sup>
Rg1 (low)	0.1	78.14±6.60	116.04±7.91 <sup>b</sup>
Rg1 (middle)	1	52.33±8.37 <sup>b</sup>	70.07±4.96 <sup>b</sup>
Rg1 (high)	10	32.49±7.15 <sup>b</sup>	36.50±6.8 <sup>b</sup>

<sup>a</sup>P<0.05 vs. control; <sup>b</sup>P<0.05, vs. CCl<sub>4</sub> model group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; Rg1, ginsenoside Rg1; CCl<sub>4</sub>, carbon tetrachloride.

AST in CCl<sub>4</sub>-treated mice significantly increased (89.90±8.18 and 182.20±8.33 U/l, respectively; P<0.05). However, oral administration of Rg1 gradually reduced the serum levels of ALT and AST; particularly in the high-dose group (40 mg/kg), the serum ALT expression level reduced to 50.88±10.85 U/l and the AST expression level reduced to 100.64±8.53 U/l.

ALT and AST levels in the cell culture supernatant of CCl<sub>4</sub>-treated LO<sub>2</sub> cells were assessed following pretreatment with increasing concentrations of Rg1 (0.1-10 μM). Consistent with the results of ALT and AST expression levels *in vivo*, 20 mM CCl<sub>4</sub> caused a significant increase in the levels of ALT and AST, while pretreatment with Rg1 decreased ALT and AST expression levels in a concentration-dependent manner (Table II).

**Effect of Rg1 on SOD activity and MDA content in acute CCl<sub>4</sub>-induced liver injury models.** In order to investigate the effect of Rg1 on SOD activity and MDA content in acute CCl<sub>4</sub>-induced liver injury in mice, 10% liver homogenate supernatants of the different treatment groups were prepared, and the results presented in Table III demonstrated that SOD activity in mice that were intraperitoneally injected with CCl<sub>4</sub> was decreased (113.21±11.31 U/mg) compared with control

Table III. Effects of Rg1 on the activity of SOD and concentration of MDA in liver homogenates of CCl<sub>4</sub>-treated mice (mean ± standard error of the mean; n=8).

Groups	Rg1 dose, mg/kg	SOD, U/mgprot	MDA, nmol/mgprot
Control	0	212.31±5.65	3.42±0.88
CCl <sub>4</sub> model	0	113.21±11.31 <sup>a</sup>	8.78±0.62 <sup>a</sup>
Rg1 (low)	10	128.14±11.50 <sup>b</sup>	8.35±0.73
Rg1 (middle)	20	145.61±8.27 <sup>b</sup>	7.18±0.94 <sup>b</sup>
Rg1 (high)	40	185.20±11.25 <sup>b</sup>	4.86±0.92 <sup>b</sup>

<sup>a</sup>P<0.05 vs. control; <sup>b</sup>P<0.05, vs. CCl<sub>4</sub> model group. SOD, superoxide dismutase; MDA, malondialdehyde; Rg1, ginsenoside Rg1; CCl<sub>4</sub>, carbon tetrachloride.

group (212.31±5.65 U/mg); however, pretreatment with Rg1 caused a dose-dependent enhancement of SOD activity. By contrast, the MDA expression level was increased in CCl<sub>4</sub>-treated mice (8.78±0.62 nmol/mg) compared with the control group (3.42±0.88 nmol/mg), while pretreatment with Rg1 caused a dose-dependent reduction in the MDA expression level.

SOD activity and MDA content in the cell culture supernatants of the CCl<sub>4</sub>-induced LO<sub>2</sub> cell injury and Rg1 treatment groups were also examined (Table IV). CCl<sub>4</sub> inhibited SOD activity and increased the MDA expression levels of LO<sub>2</sub> cells; however, Rg1 alleviated the inhibited SOD activity and reduced the increased MDA level, which was consistent with the results of SOD activity and MDA content experiments *in vivo*.

**Effect of Rg1 on NF-κB activity in acute CCl<sub>4</sub>-induced liver injury models.** Liver Kupffer cells serve a role as a barrier to liver injury. In order to investigate whether NF-κB is involved in the protective effect of Rg1 in acute CCl<sub>4</sub>-induced liver injury in mice, liver Kupffer cells were isolated from the different treatment groups. NF-κB p65 protein expression was determined using western blotting and NF-κB binding activity using EMSA analysis. The results of the western blotting demonstrated that NF-κB p65 protein expression was significantly increased in the CCl<sub>4</sub> model group, although pretreatment with Rg1 (10-40 mg/kg) reduced the protein expression of p65 in a dose-dependent manner (Fig. 2A). Additionally, the results of the EMSA analysis demonstrated that CCl<sub>4</sub> (0.2 mg/10 g) used alone enhanced NF-κB binding activity, however, pretreatment with Rg1 alleviated the enhanced NF-κB binding in a dose-dependent manner (Fig. 2B).

NF-κB function in the protective effect of Rg1 on acute CCl<sub>4</sub>-induced liver injury was further confirmed *in vitro*. CAPE, an inhibitor of NF-κB, was used and its role observed on CCl<sub>4</sub>-induced LO<sub>2</sub> cell injury. The results of the MTT assay presented in Fig. 2C demonstrated that exposure of LO<sub>2</sub> cells to 20 mM CCl<sub>4</sub> for 3 h inhibited cell growth, and CAPE (25 μM, 2 h) attenuated this cell growth inhibition. P65 expression in LO<sub>2</sub> cells treated with CCl<sub>4</sub> alone or in combination with Rg1

Table IV. Effects of Rg1 on SOD activity and MDA concentration in supernatants from LO<sub>2</sub> cells treated with CCl<sub>4</sub> (mean ± standard error of the mean; n=8).

Groups	Rg1 concentration, $\mu$ M	SOD, U/mgprot	MDA, nmol/mgprot
Control	0	5.24±0.353	0.71±0.12
CCl <sub>4</sub> model	0	2.01±0.246 <sup>a</sup>	1.85±0.24 <sup>a</sup>
Rg1 (low)	0.1	2.21±0.26	1.66±0.16
Rg1 (middle)	1	4.17±0.33 <sup>b</sup>	1.24±0.19 <sup>b</sup>
Rg1 (high)	10	4.85±0.27 <sup>b</sup>	0.88±0.12 <sup>b</sup>

<sup>a</sup>P<0.05 vs. control; <sup>b</sup>P<0.05, vs. CCl<sub>4</sub> model group. SOD, superoxide dismutase; MDA, malondialdehyde; Rg1, ginsenoside Rg1; CCl<sub>4</sub>, carbon tetrachloride.

Table V. Effects of CAPE on SOD activity and MDA concentration in supernatants from LO<sub>2</sub> cells treated with CCl<sub>4</sub> (mean ± standard error of the mean; n=8).

Groups	CAPE concentration, $\mu$ M	SOD, U/mgprot	MDA, nmol/mgprot
Control	0	5.24±0.353	0.71±0.12
CCl <sub>4</sub> model	0	2.01±0.246 <sup>a</sup>	1.85±0.24 <sup>a</sup>
CAPE	25	4.96±0.31 <sup>b</sup>	0.95±0.27 <sup>b</sup>

<sup>a</sup>P<0.05 vs. control; <sup>b</sup>P<0.05, vs. CCl<sub>4</sub> model group. CAPE, caffeic acid phenethyl ester; SOD, superoxide dismutase; MDA, malondialdehyde; Rg1, ginsenoside Rg1; CCl<sub>4</sub>, carbon tetrachloride.

was observed by western blotting. When treated with 20 mM CCl<sub>4</sub> for 15 min, there was no significant alteration in p65 expression; however, when the incubation time was prolonged to 30 or 60 min, the expression level increased, and 10  $\mu$ M Rg1 alleviated the increased p65 expression (Fig. 2D and E). The present results were also confirmed by EMSA assay. Rg1 (0.1-10  $\mu$ M) used alone did not affect NF- $\kappa$ B binding activity; however, it significantly alleviated the enhanced binding of NF- $\kappa$ B when used in combination with CCl<sub>4</sub>; the NF- $\kappa$ B inhibitor CAPE served a comparable role and inhibited CCl<sub>4</sub>-induced NF- $\kappa$ B binding (Fig. 2F).

**Mechanism of NF- $\kappa$ B in Rg1 protective effect on acute CCl<sub>4</sub>-induced liver injury models.** The results presented in Table IV demonstrated that pretreatment with Rg1 alleviated the inhibited SOD activity and reduced MDA expression level in CCl<sub>4</sub>-induced LO<sub>2</sub> cell injury. Inhibition of NF- $\kappa$ B activity by CAPE demonstrated comparable results: CCl<sub>4</sub> inhibited SOD activity and increased the MDA expression level compared with the control group; however, using 25  $\mu$ M CAPE for 2 h enhanced the inhibited SOD activity and reduced the MDA expression level (Table V). The expression levels of TNF- $\alpha$  and IL-6 in 10% liver homogenate supernatants of the different treatment groups of mice were measured by ELISA, and it was observed that CCl<sub>4</sub> injection

Table VI. Effects of Rg1 on serum levels of TNF- $\alpha$  and IL-6 in CCl<sub>4</sub>-induced liver injury in mice.

Groups	Rg1 concentration, $\mu$ M	TNF- $\alpha$ , pg/ml	IL-6, pg/ml
Control	0	18.42±3.57	23.66±5.32
CCl <sub>4</sub> model	0	823.45±7.43 <sup>a</sup>	97.30±13.09 <sup>a</sup>
Rg1 (low)	10	81.39±14.75	96.20±10.53
Rg1 (middle)	20	68.56±11.17 <sup>b</sup>	78.75±7.17 <sup>b</sup>
Rg1 (high)	40	39.22±7.06 <sup>b</sup>	48.75±9.54 <sup>b</sup>

<sup>a</sup>P<0.05 vs. control; <sup>b</sup>P<0.05, vs. CCl<sub>4</sub> model group. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-6, interleukin-6; Rg1, ginsenoside Rg1; CCl<sub>4</sub>, carbon tetrachloride.

led to an increase in the serum levels of TNF- $\alpha$  and IL-6; however, pretreatment with Rg1 reduced this increase in a dose-dependent manner (Table VI).

It is well known that NF- $\kappa$ B expression and activity in liver Kupffer cells may be modulated by TNF- $\alpha$ , IL-6 and other cytokines, and NF- $\kappa$ B may, in turn, activate these inflammatory regulators (26,27). The results of the present study, displayed in Fig. 2A and B, demonstrated that CCl<sub>4</sub> increased NF- $\kappa$ B p65 protein expression and enhanced NF- $\kappa$ B binding activity, while pretreatment with Rg1 reduced p65 protein expression and alleviated the enhanced NF- $\kappa$ B binding. Therefore, Rg1 protected CCl<sub>4</sub>-induced liver injury, by directly alleviating the inhibition of SOD activity and reducing MDA, and possibly by inhibiting NF- $\kappa$ B activity, thereby further alleviating the inhibited SOD activity and reducing MDA, TNF- $\alpha$  and IL-6 expression levels.

## Discussion

The present study demonstrated that pretreatment with Rg1 protected the liver from CCl<sub>4</sub>-induced acute injury in cell culture and animal experimental systems. It was observed that pretreatment with Rg1 attenuated the pathological damage to liver tissues *in vivo* and LO<sub>2</sub> cell death *in vitro*. The indicative enzymes of liver cell damage, serum transaminases ALT and AST, were markedly increased in the CCl<sub>4</sub> treatment model group and decreased in the Rg1 pretreatment group. Further investigations into the antioxidant properties of Rg1 in acute CCl<sub>4</sub>-induced liver injury demonstrated that SOD activity was inhibited in the CCl<sub>4</sub> treatment group, and recovered in the Rg1 pretreatment group; MDA level was increased by treatment with CCl<sub>4</sub> and attenuated by pretreatment with Rg1. Inflammatory cytokines TNF- $\alpha$  and IL-6, which were enhanced by CCl<sub>4</sub> treatment, were attenuated in the Rg1 pretreatment group. Rg1 inhibited p65 expression and NF- $\kappa$ B activity *in vivo* and *in vitro*, and NF- $\kappa$ B inhibitor CAPE, which was observed to exhibit comparable effects, enhanced SOD activity, reduced MDA level and promoted LO<sub>2</sub> cell survival. As NF- $\kappa$ B serves a role in the activation of inflammatory cytokines, it was hypothesized that Rg1 protected CCl<sub>4</sub>-induced liver injury by directly alleviating the inhibition of SOD activity and reducing MDA, and additionally by inhibiting

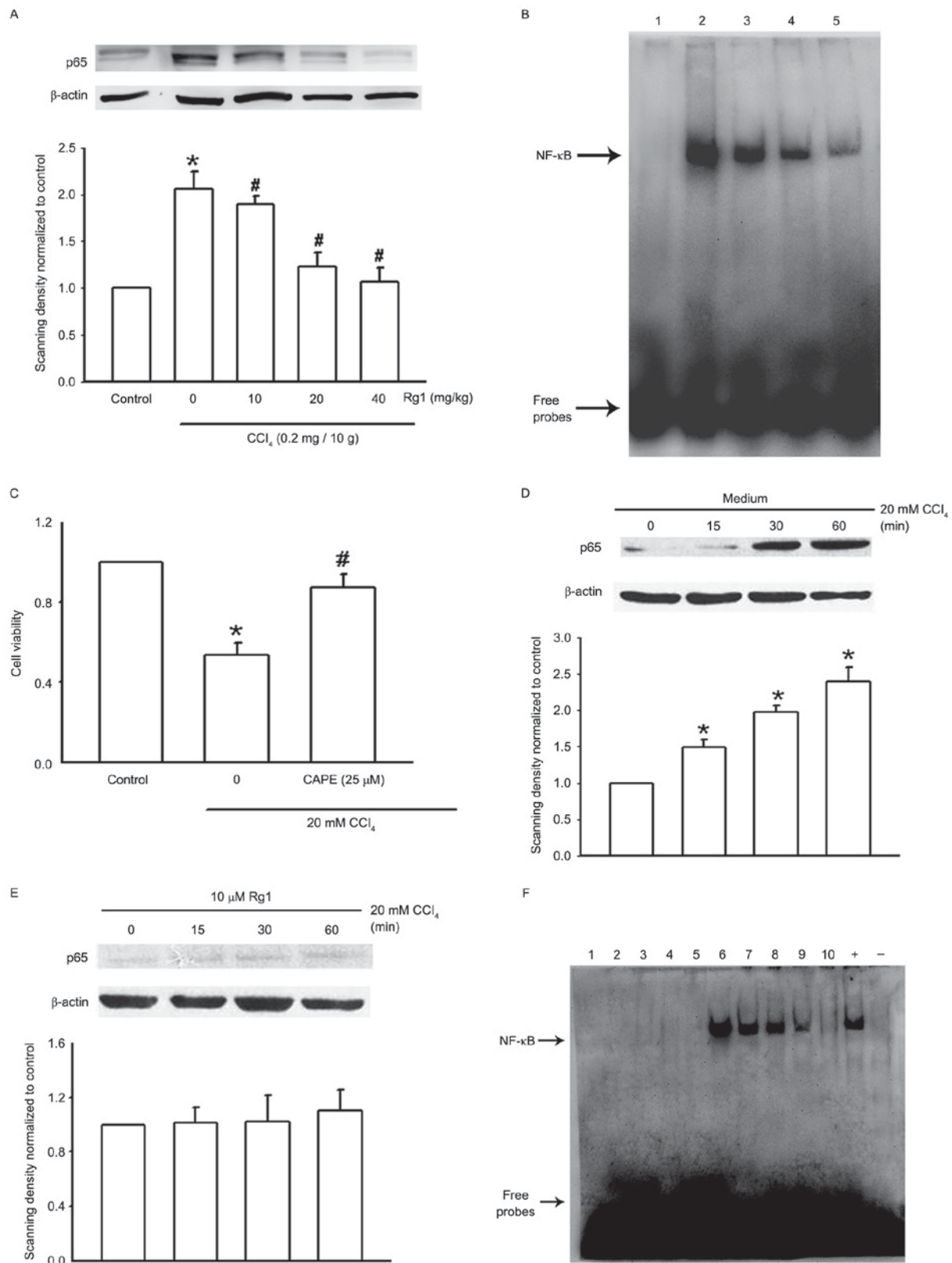


Figure 2. Expression and binding activity of NF- $\kappa$ B in the Kupffer cells of mice with  $\text{CCl}_4$ -induced liver injury and  $\text{CCl}_4$ -treated LO<sub>2</sub> cells. (A) Representative images of NF- $\kappa$ B P65 protein expression assessed by western blotting in Kupffer cells isolated from mice treated with  $\text{CCl}_4$  and/or Rg1 (n=4). (B) Representative image of NF- $\kappa$ B binding activity assessed by EMSA in the Kupffer cells of acute liver injury mice pretreated with Rg1 (n=4). Lane 1, control group; lane 2,  $\text{CCl}_4$  group; lane 3,  $\text{CCl}_4$  + Rg1 (10 mg/kg); lane 4,  $\text{CCl}_4$  + Rg1 (20 mg/kg); lane 5,  $\text{CCl}_4$  + Rg1 (40 mg/kg). (C) The function of the NF- $\kappa$ B inhibitor CAPE (25  $\mu$ M, 2 h) in  $\text{CCl}_4$ -treated LO<sub>2</sub> cells was assessed by MTT assay. The data are expressed as the mean  $\pm$  standard error of the mean (n=3). (D) Representative images of NF- $\kappa$ B P65 protein expression assessed by western blotting in lysates from LO<sub>2</sub> cells treatment with  $\text{CCl}_4$  (n=3). (E) Representative images of NF- $\kappa$ B P65 protein expression assessed by western blotting in lysates from LO<sub>2</sub> cells treatment with 10  $\mu$ M Rg1 (n=3). (F) Representative image of NF- $\kappa$ B binding activity assessed by EMSA in  $\text{CCl}_4$ -treated LO<sub>2</sub> cells (n=3). Lane 1, probe without nucleoprotein; lane 2 control group; lane 3, Rg1 (0.1  $\mu$ M); lane 4, Rg1 (1  $\mu$ M); lane 5, Rg1 (10  $\mu$ M); lane 6,  $\text{CCl}_4$  group; lane 7,  $\text{CCl}_4$  + Rg1 (0.1  $\mu$ M); lane 8,  $\text{CCl}_4$  + Rg1 (1  $\mu$ M); lane 9,  $\text{CCl}_4$  + Rg1 (10  $\mu$ M); lane 10, CAPE (25  $\mu$ M); +, positive control (20 ng/ml tumor necrosis factor- $\alpha$  stimulation for 30 min); -, negative control (untreated LO<sub>2</sub> cells). \* $P < 0.05$  vs. control group, # $P < 0.05$  vs.  $\text{CCl}_4$  group. CAPE, caffeic acid phenethyl ester; Rg1, ginsenoside Rg1;  $\text{CCl}_4$ , carbon tetrachloride; NF- $\kappa$ B, nuclear factor- $\kappa$ B; EMSA, electrophoretic mobility shift assay.



NF- $\kappa$ B activity, thereby further alleviating the inhibited SOD activity and reducing MDA, TNF- $\alpha$  and IL-6 expression levels.

As a common pathological state of various types of liver disease, liver injury may be caused by ischemia, viral infection, autoimmune disorders and various xenobiotic substances, including alcohol, drugs and toxins (28). The present study used a CCL<sub>4</sub>-induced acute liver injury model, and this type of liver injury model is widely used in hepatoprotective drug screening (29,30). The characteristics of the acute liver injury induced by CCL<sub>4</sub> are liver dysfunction and cell morphology deterioration (31); therefore, alterations in histopathology and liver function were investigated, and it was observed that pretreatment with Rg1 attenuated the pathological damage to liver tissues and LO<sub>2</sub> cell death. In addition, increased levels of ALT and AST expression caused by CCL<sub>4</sub> in animal experimental systems and cell culture, which are direct hepatic functional indicators and have been demonstrated to correlate with the severity of liver injury (32), were markedly reduced by pretreatment with Rg1. The results of the present study demonstrated that pretreatment with Rg1 decreased hepatic dysfunction and cell morphology deterioration induced by CCL<sub>4</sub>.

Oxidative stress is considered to serve an important role in the development of CCL<sub>4</sub>-induced acute liver injury (33,34). When CCL<sub>4</sub> enters the liver and is activated by cytochrome P450 metabolism, it generates various free radicals and reactive oxygen species for oxidative stress and causes membrane lipid peroxidation (14,15). The activity of SOD, an effective metalloenzyme which catalyzes the dismutation of superoxide anions into H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub> (35-37), was markedly increased compared with the injury group. The studies of Zhang *et al* (31) and Li C (22), demonstrated that the expression level of MDA was associated with CCL<sub>4</sub>-induced acute liver injury in mice; increased expression of MDA, a lipid peroxidative product of cell membranes (38), was prevented by pretreatment with Rg1 in the present study. The hepatoprotective effect of Rg1 may be partly due to attenuation of oxidative stress and inhibition of lipid peroxidation.

Kupffer cells, as tissue macrophages, reside within the liver sinusoid and serve a role in homeostatic liver regeneration and protection (39-41). Activated Kupffer cells mediate the hepatic inflammation process by releasing a wide range of cytokines, including TNF- $\alpha$  and IL-6 (42). A previous study reported that NF- $\kappa$ B is sensitive to redox status in abnormal physiological conditions, including CCL<sub>4</sub>-induced acute liver injury (43). Additionally, the study of Tao *et al* (12) demonstrated that pretreatment with Rg1 inhibited the inflammatory response and protected the mouse liver against ischemia-reperfusion injury, partly through the NF- $\kappa$ B signaling pathway. The present study demonstrated that pretreatment with Rg1 inhibited NF- $\kappa$ B activity and p65 expression in Kupffer cells and reduced the serum levels of TNF- $\alpha$  and IL-6 in acute CCL<sub>4</sub>-induced liver injury in mice. As NF- $\kappa$ B expression and activity in liver Kupffer cells may be modulated by TNF- $\alpha$ , IL-6 and other cytokines, and NF- $\kappa$ B may, in turn, activate these inflammatory regulators, it is hypothesized that the protective effect of Rg1 on CCL<sub>4</sub>-induced liver injury was partly involved in the attenuation of inflammatory responses expressed as a reduction of serum TNF- $\alpha$  and IL-6 levels via the NF- $\kappa$ B signaling pathway.

NF- $\kappa$ B was first identified as a transcription factor in 1986 by Sen and Baltimore (44). In an inactive state, NF- $\kappa$ B is

sequestered in the cytoplasm as a heterotrimer consisting of p50, p65, and inhibitor of NF- $\kappa$ B (I $\kappa$ B) subunits. On activation, I $\kappa$ B $\alpha$  undergoes phosphorylation and ubiquitination-dependent degradation leading to p65 nuclear translocation and binding to a specific consensus sequence in the DNA, which results in gene transcription. The nuclear translocation of NF- $\kappa$ B leads to gene expression of prostaglandin G/H synthase 2, inducible nitric oxide synthase, chemokines, adhesion molecules, matrix metalloproteinases and various pro-inflammatory cytokines (45,46). These released inflammatory factors subsequently activate further amplification of NF- $\kappa$ B activity in a regulatory cycle. As a result, the oxidative stress in injury liver activates NF- $\kappa$ B, triggering expression of oxidative stress-responsive genes, which ultimately leads to liver cell necrosis and apoptosis (47-49). The present study demonstrated that Rg1 inhibited p65 expression and NF- $\kappa$ B activity, *in vivo* and *in vitro*. The NF- $\kappa$ B inhibitor CAPE, which was confirmed to exhibit a similar effect compared with Rg1, enhanced SOD activity and reduced the MDA expression level in addition to promoting LO<sub>2</sub> cell survival. Therefore, the possible underlying mechanisms of the beneficial effect of Rg1 may be attributed to an attenuation of the inflammatory response and oxidative stress in CCL<sub>4</sub>-induced acute liver injury via inhibition of NF- $\kappa$ B.

In conclusion, the present study reveals the potential clinical value of Rg1, and demonstrates that pretreatment with Rg1 may protect against CCL<sub>4</sub>-induced acute hepatotoxicity *in vivo* and *in vitro*, via inhibition of NF- $\kappa$ B activity to restore the anti-oxidative defense system and downregulate pro-inflammatory signaling pathways.

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