Pien Tze Huang Gan Bao ameliorates carbon tetrachloride-induced hepatic injury, oxidative stress and inflammation in rats

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Abstract. Liver damage results from a variety of insults, including hepatitis and chemical toxicity from alcohol, drugs and other toxins. The present study evaluated the hepatoprotective effects and potential mechanisms of action of the Traditional Chinese Medicine Pien Tze Huang Gan Bao (GB) in a rat model of carbon tetrachloride (CCl_4)-induced liver injury. Sixty male Sprague-Dawley rats were randomly divided into six different groups: i) Control, ii) CCl_4 injury model and groups treated with iii) silymarin as a positive drug control, iv) 150 mg/kg GB, v) 300 mg/kg GB and vi) 600 mg/kg GB. Control rats received no treatment, while the remaining ones were intraperitoneally injected with CCl_4 (2 ml/kg) to induce acute liver disease. Silymarin or GB was orally administered prior to CCl_4 treatment in various treatment groups for 7 days. Animals were sacrificed 24 h post-CCl_4 injection. It was revealed that GB significantly reduced serum aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyl transpeptidase and total bilirubin levels in the serum induced by CCl_4. BG also prevented CCl_4-induced changes in liver tissues, as revealed by histopathological analysis. CCl_4-induced reductions in endogenous liver antioxidant enzyme activities of superoxide dismutase, glutathione and glutathione peroxidase as well as increases in malondialdehyde and thiobarbituric acid reactive substances were inhibited by GB treatment. Activated NF-κB in liver tissues was also significantly increased by CCl_4, which was attenuated by GB as indicated by immunohistochemical and PCR analysis. Furthermore, CCl_4-mediated increases in the inflammatory factors tumor necrosis factor-alpha and interleukin-1β secretion into the serum and their expression in liver tissues were reversed following GB treatment, as revealed by ELISA and PCR, respectively. These findings suggested that GB protects against CCl_4-induced hepatic injury, inflammation and oxidative damage in rats and may be useful in future clinical application of liver injury and disease.

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Introduction

The liver is important in metabolism, detoxification and secretory functions in the body, and its disorder and dysfunction may lead to severe liver damage as a result of increasing cellular, tissue and functional disruption. Carbon tetrachloride (CCl_4) is a potent environmental hepatotoxin (1) that has been reported to induce acute and chronic tissue injuries (2-5). Acute administration of CCl_4 is utilized to establish a model of severe liver damage through generation of oxidative stress, recruitment of inflammatory cells and cell death (6-8), and ultimately, liver architectural and functional damage (9,10). The regeneration of damaged liver from CCl_4-induced acute liver injury involves a complex regulated cellular response (11,12).

Oxidative stress and inflammatory responses may be critical in the chemical hepatic injury induced by CCl_4, as CCl_4 exposure induces elevations in reactive and cytotoxic lipoperoxide and free peroxide radicals (13,14). In addition, various factors, including bacterial and viral infections, toxins, dietary factors and alcohol abuse may promote liver inflammation. Pro-inflammatory chemokines and immune cells have an important role in the regulation of the inflammatory response. Nuclear factor-κB (NF-κB) is involved in several inflammatory cytokine responses, including pathways mediated by endotoxins that cause liver damage (15,16). Blocking NF-κB signaling inhibits a positive feedback loop that is mediated by pro-inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α that attenuate inflammation (17).

Natural remedies from medicinal plants are considered safe and effective alternative treatments against hepatotoxicity. The present study investigated the hepatoprotective effects of Pien

Tze Huang Gan Bao (GB), which contains *Calculus bovis*, *Panax notoginseng*, *Artemisia capillaris*, snake gall and Radix Paeoniae alba, against CCl_4-induced hepatotoxicity in rats. GB, a classical Traditional Chinese Medicine formula, has been used for millennia in China and Southeast Asia. GB has demonstrated efficacy in protecting against liver injury due to excessive alcohol consumption. However, no scientific reports are currently available to explain the potential protective effects of GB against chemical injury. The present study was undertaken to evaluate the protective effect of GB on CCl_4-induced hepatotoxicity, and the antioxidant and anti-inflammatory effects of GB in liver-injured rats were investigated.

Materials and methods

Reagents. GB was obtained from and authenticated by the sole manufacturer, Zhangzhou Pien Tze Huang Pharmaceutical Co. Ltd., (Zhangzhou, China; Chinese Food and Drug Administration approval no., HPK-08411). Superoxide dismutase (SOD), malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), glutathione (GSH) and glutathione peroxidase (GSP-PX) assay kits were purchased from Nanjing Jiancheng Biotech Co., Ltd. (Nanjing, China). BCA Protein Assay Kit was purchased from Tiangen Biotech Co., Ltd., (Beijing, China). IL-1β and TNF-α ELISA kits were purchased from Shanghai Xitang Biotech Co., Ltd. (Shanghai, China). TRIzol reagent was obtained from Life Technologies (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A PrimeScript™ RT reagent kit with gDNA Eraser was purchased from Takara Bio Inc., (Tokyo, Japan). CCl_4 was purchased from Shanghai Lingfeng Chemical Co., Ltd. (Shanghai, China).

Animals. A total of 60 male 6-week-old Sprage-Dawley rats (Slike Co. Ltd., Shanghai, China), weighing 180-200 g, were housed at five per cage in an environmentally controlled room at 22±1˚C and relative humidity of 40-60% with a 12-h light/dark cycle. Animals were allowed access to food and water *ad libitum* for one week prior to the start of the study. All experiments involving animals were approved by the Fujian Institute of Traditional Chinese Medicine Animal Ethics Committee (Fuzhou, China). All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Fujian University of Traditional Chinese Medicine (Fuzhou, China).

CCl_4-induced hepatotoxicity and experimental design. The rats were randomly divided into six groups (n=10 in each): Group 1, Control group; group 2, CCl_4 injury model group; group 3, silymarin (Jiangsu ZTE Pharmaceutical Co., Ltd., Jiangsu, China) treatment group [pre-treated with silymarin, 50 mg/kg by oral gavage (per os; p.o.), for 7 days]; group 4, low-dose treatment group (pre-treated with GB, 150 mg/kg p.o., for 7 days); group 5, medium-dose treatment group (pre-treated with GB, 300 mg/kg p.o., for 7 days), and group 6, high-dose treatment group (pre-treated with GB, 600 mg/kg p.o., for 7 days). Rats in groups 1 and 2 were pre-treated with phosphate-buffered saline (0.5 ml/100 g) p.o. for 7 days. On the final day, rats from groups 2-6 received an intraperitoneal (i.p.) injection of CCl_4 at a dose of 2.0 ml/kg in a 50% corn oil solution, while group 1 received 2.0 ml/kg of corn oil only. At 24 h after CCl_4 administration, the animals were anesthetized (40 mg/kg pentobarbital; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany; i.p.), sacrificed by cervical dislocation and subjected to laparotomy. Blood was collected from the aorta abdominalis into non-heparinized tubes and centrifuged (980 x *g* at 4˚C for 10 min) to obtain serum for biochemical tests. The livers were quickly excised, washed, and a portion of the liver was dissected and fixed in 4% formaldehyde saline solution for histological analysis; the remaining tissue was snap frozen in liquid nitrogen and stored at -70˚C until use for determination of oxidative stress markers and for molecular analysis.

Biochemical assays. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (γ-GT) and total bilirubin (TB) levels were evaluated to assess hepatic function using an automatic biochemical analyzer (Bayer ADVIA 2400; Bayer, Siemens, Germany), according to the manufacturer's instructions.

Histopathological analysis. Extracted livers were fixed in 10% buffered formalin for a minimum of 48 h, processed and embedded in paraffin. Paraffin sections were then prepared by an automatic tissue processor (RM2; Leica Microsystems GmbH, Wetzlar, Germany) and cut into 5 μm-thick sections by a rotary microtome. Sections then were stained with haematoxylin-eosin dye and analyzed for histopathological alterations. Photomicrographs of stained tissue sections were obtained using a DMRB/E light microscope (Leica Microsystems GmbH).

Assessment of oxidative stress. Liver tissue was homogenized in cold 50 mM potassium phosphate buffer (pH 7.0) using a Potter-Elvehjem homogenizer (SRH4000-30) to give a 10% (w/v) liver homogenate, which was centrifuged at 980 x *g* for 10 min. The supernatant was used for analysis of TBARS, MDA, SOD, GSH and GSH-PX. Hepatic TBARS were assayed according to previously described methods (18). In brief, TBARS were determined using the thiobarbituric acid reaction with 1.0 mM EDTA added to the reaction medium as a minor modification. The concentration of hepatic TBARS was expressed as MDA equivalents. SOD activity was determined by measurement of the inhibition of cytochrome C reduction via assessment of the absorbance at 550 nm and expressed in U/mg protein (19), whereas GSH-PX activity was determined via assessment of nicotinamide adenine dinucleotide phosphate oxidation of t-butyl peroxide by measuring the absorbance at 412 nm and expressed in U/mg protein. The GSH concentration in homogenates was tested using a previously described method (20). Absorbance was measured at 420 nm and the results are expressed as mg/g protein. Endogenous lipid peroxidation was determined by measuring MDA at 532 nm and the results are expressed as nmol/mg protein.

Immunohistochemistry. NF-κB expression was examined immunohistochemically using rabbit anti-rat polyclonal NF-κB p65 immunoglobulin G antibody (cat. no. sc-372; Santa

Cruz Biotechnologies, Inc., Dallas, TX, USA). In brief, sections were subjected to antigen retrieval, blocking of endogenous peroxidase activity and incubation at 4˚C overnight with rabbit polyclonal anti-NF-κB primary antibody (diluted 1:200). The sections were then incubated at 37˚C for 20 min with appropriate biotinylated secondary antibody (diluted 1:1,000) followed by horseradish peroxidase-conjugated streptavidin and reaction with 3,3-diaminobendizine (Sigma-Aldrich; Merck Millipore) working solution at room temperature for 6 min. Immunolabeled sections were counterstained with Harris hematoxylin (Sigma-Aldrich; Merck Millipore). To rule out any nonspecific labeling, negative controls were used, in which primary antibodies were replaced with PBS. The average proportion of positive cells in each field was counted using a true color multi-functional cell image analysis management system (Image-Pro Plus, Media Cybernetics, Bethesda, MD, USA).

Quantification of mRNA expression by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent was used to isolate total RNA from liver tissues according to the manufacturer's instructions. Following quantification by measuring the absorbance at 260 nm, the RNA was reverse-transcribed into complementary (c)DNA using the PrimeScript™ RT reagent kit with the gDNA Eraser Kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. A total of 1.0 μg total RNA from each sample was added to a mixture of 1.0 μl MultiScribe reverse transcriptase, 2.0 μl 10X reverse transcriptase random primers, 0.8 μl 25X deoxynucleotide triphosphate mix (100 mM), 2.0 μl 10X reverse transcriptase buffer and 3.2 μl nuclease-free water. The reaction conditions were as follows: 25˚C for 10 min, 37˚C for 15 min and 85˚C for 5 sec, followed by cooling to 4˚C. The cDNA was then subjected to PCR amplification using SYBR *Premix Ex Taq* II (Takara) in an ABI 7500 Fast instrument (Applied Biosystems, Thermo Fisher Scientific, Inc.). PCR thermal cycling was performed as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 3 sec and 60˚C for 30 sec, and one cycle of 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec. The PCR mixture contained: 2.0 μl cDNA, 10 μl SYBR *Premix Ex Taq* II (2X), 0.8 μl PCR forword primer (10 μm), 0.8 μl PCR reverse primer (10 μM), 0.4 μl ROX reference dye II and 6 μl dH_2O. mRNA expression values were determined as $\Delta Cq=Cq_{sample}-Cq_{GAPDH}$ and relative quantities between different samples were determined as $\Delta\Delta Cq=\Delta Cq_{sample1}-\Delta Ct_{sample2}$. The values were expressed as $2^{-\Delta\Delta Ct}$. All qPCR reactions were performed in triplicate.

The sequences of the primers used for amplification of NF-κB, IL-1β, TNF-α and β-actin transcripts were as follows: NF-κB forward, 5'-ACGCAAAAGGACCTACGAGACC-3' and reverse, 5'-ATGTTGAAAAGGCATAGGGCTG-3'; IL-1β forward, 5'-TGTTCTTTGAGGCTGAC-3' and reverse, 5'-CTTTGGGATTTGTTTGG-3'; TNF-α forward, 5'-CAGCAGATGGGCTGTACCTT-3' and reverse, 5'-AAGTAGACCTGCCCGGACTC-3'; and β-actin forward, 5'-CGGTCAGGTCATCACTATCGGC-3' and reverse, 5'-GTGTTGGCATAGAGGTCTTTACGG-3'.

ELISA. A commercial ELISA kit was employed to determine TNF-α or IL-1β expression. The method used was a solid-phase sandwich ELISA utilizing a monoclonal antibody specific for rat TNF-α or IL-1β coated on a 96-well plate. Standards and samples were added to the wells and incubated to allow for any present TNF-α or IL-1β to bind to immobilized antibody. The plates were washed and biotinylated polyclonal anti-rat TNF-α or IL-1β antibody was added. Following an additional wash, avidin-horseradish peroxidase was added to form an antibody-antigen-antibody sandwich. Upon repeating the wash, a substrate solution was added to generate a blue color proportional to the amount of rat TNF-α or IL-1β present in the sample. Stop buffer was added to terminate the reaction, leading to a change in color from blue to yellow. The absorbance of the plates at 450 nm was read and the amount of rat TNF-α or IL -1β was expressed as pg/mg protein.

Statistical analysis. Values are expressed as the mean ± standard deviation of three measurements or assessments. Data were analyzed using the SPSS software package for Windows (version 11.5; SPSS, Inc., Chicago, IL, USA). Analysis of differences between groups was performed by one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

GB prevents CCl_4-induced increases in liver parameters. The serum biochemical parameters providing information regarding the hepatoprotective influence of GB in CCl_4-intoxicted rats are shown in Table I. CCl_4-treated rats developed extensive hepatic damage, evidenced by significant increases in the levels of serum AST, ALT, ALP, γ-GT and TB levels compared with those in the control group, which was significantly inhibited by GB at doses of 150, 300 and 600 mg/kg ($P\leq0.05$), particularly in the case of AST, ALT and ALP. The standard control drug silymarin also prevented the elevation of serum enzymes and TB. Treatment with 150, 300 and 600 mg/kg GB and 50 mg/kg silymarin had protective effects as shown by decreases in ALT levels by 32.84, 30.78, 58.38 and 45.68%, AST levels by 56.06, 82.96, 89.68 and 45.35% and ALP levels by 26.41, 34.28, 33.17 and 35.26%, respectively.

GB prevents CCl_4-induced morphological changes in liver tissues. To evaluate the influence of GB therapy on hepatocellular necrosis and inflammation, a histopathologic analysis of liver tissues was performed to support the evidence provided by the biochemical parameters. Liver histology of the control group revealed a lobular architecture and hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus, visible central veins and thin sinusoids (Fig. 1). By contrast, the injury model group showed the most extensive damage among all groups, exhibiting centrilobular necrosis, broad inflammatory cell infiltration, ballooning degeneration and the loss of cellular boundaries. Liver sections of rats pretreated with GB (150, 300 and 600 mg/kg) or silymarin revealed that these treatments reduced or prevented the development of histopathological damage, particularly 600 mg/kg GB (Fig. 1).

GB inhibits CCl_4-induced oxidative stress in the liver. CCl_4 administration induced acute hepatotoxicity and caused

Table I. Effect of GB on ALT, AST, ALP, γ-GT, TB and LDH in serum of rats.

Group	ALT (U/l)	AST (U/l)	ALP (U/l)	γ-GT (U/l)	TB (μmol/l)
Control	48.70±9.38	128.40±20.30	295.22±42.86	1.00±0.67	0.75±0.19
CCl_4	518.67±84.53[a]	1252.83±95.49[a]	359.43±59.18[a]	1.80±0.79[a]	1.71±0.62
Silymarin+CCl_4	281.67±60.47[b]	684.71±85.82[b]	232.71±49.55[b]	1.67±0.99	1.52±0.39
GB (150 mg/kg) + CCl_4	348.33±42.46[b]	550.50±96.11[b]	264.50±45.79[b]	1.11±0.60	1.66±0.46
GB (300 mg/kg) + CCl_4	359.00±47.60[b]	213.38±73.39[b]	236.20±36.17[b]	1.71±0.73	0.61±0.44
GB (600 mg/kg) + CCl_4	215.86±46.57[b]	129.29±36.18[b]	240.20±42.55[b]	1.22±0.83	0.10±0.47

[a]P<0.05 vs. control; [b]P<0.05 vs. CCl_4 group. Animals were pre-treated with GB (150, 300 and 600 mg/kg, i.g.), silymarin (50 mg/kg, i.g.) or vehicle for 7 days. At 24 h following intraperitoneal administration of CCl_4, serum parameters were measured using diagnostic kits. Values are expressed as the mean ± standard deviation (n=10). GB, Pian Zai Huang Gan Bao; AST, Aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GT, gamma glutamyl transpeptidase; TB, total bilirubin; i.g., intragastric.

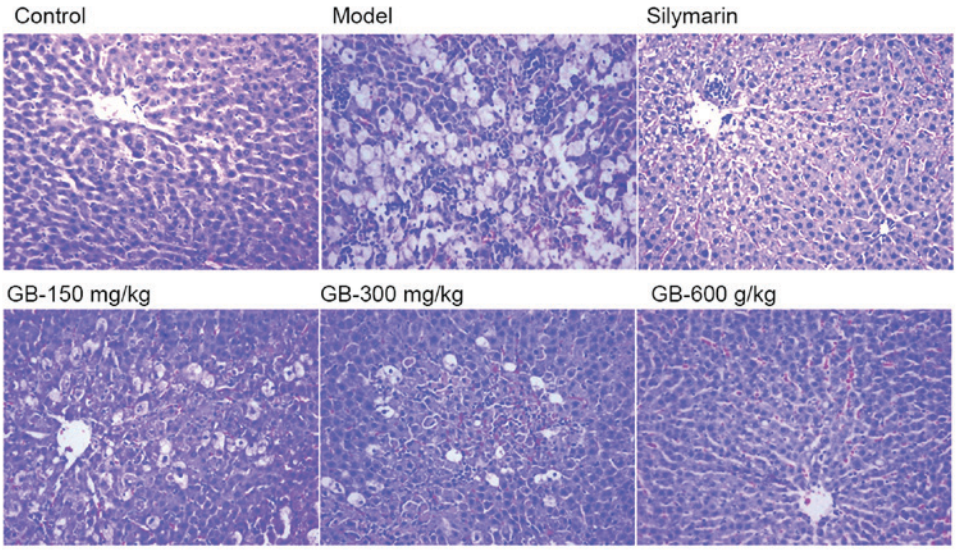

Figure 1. Effects of GB on histopathological changes induced by CCl_4 in rats. Rats were pre-treated with GB (150, 300 and 600 mg/kg, i.g.), silymarin (50 mg/kg, i.g.) or vehicle for 7 days prior to CCl_4 administration (2 ml/kg body weight and 1:1 diluted in corn oil) to induce acute liver injury. Representative images of hematoxylin and eosin-stained liver sections (magnification, x200) are shown. GB, Pian Zai Huang Gan Bao.

severe oxidative damage *in vivo* (Table II). CCl_4 significantly decreased the activity of SOD, GSH, GSH-Px and increased MDA and TBARS in liver samples (P<0.05). This increased lipid peroxidation and the resulting reduced activities of antioxidant enzymes were markedly attenuated by silymarin and GB (150, 300 and 600 mg/kg) (P<0.05).

GB prevents liver inflammation induced by CCl_4. To identify the possible molecular mechanisms responsible for the protective effects of GB against CCl_4-induced hepatotoxicity, NF-κB expression Acute CCl_4 toxicity induced NF-κB expression was assessed by RT-qPCR analysis and immunohistochemistry. As shown in Fig. 2A and B, NF-κB-positive cells in the injury model group were significantly increased compared to those in the control group (P<0.05). Pre-treatment with silymarin or GB (150, 300 or 600 mg/kg) prevented these CCl_4-induced increases in NF-κB expression. The results on mRNA expression of NF-κB were consistent with those on the protein expression (Fig. 2C), as CCl_4-induced increases were prevented by pre-treatment with silymarin or GB (150, 300 and 600 mg/kg). However, no significant difference was observed between the injury model and the 300 mg/kg GB pre-treatment groups.

Certain key genes regulating hepatocellular necrosis and inflammation are known to mediate liver injury. The present study examined the effects of GB on proinflammatory gene expression of acutely liver-injured rats by measuring TNF-α and IL-1β mRNA by RT-qPCR in liver tissues (Fig. 3A). The results showed significant increases in TNF-α and IL-1β mRNA in livers from CCl_4-treated rats. However, pre-treated with GB or silymarin prevented these increases in the proinflammatory cytokines. Inhibition of TNF-α and IL-1β gene expression by GB was confirmed by measuring TNF-α and IL-1β protein using ELISA (Fig. 3B). TNF-α and IL-1β protein was elevated by CCl_4 intoxication but inhibited by pre-treatment with GB or silymarin.

Discussion

For decades, CCl_4-induced liver injury has been utilized as an experimental model, and biochemical and histological

Table II. Effect of GB on MDA and TBARS levels as well as SOD, GSH-Px and GSH activities.

Group	SOD (U/mg prot)	MDA (nmol/mg prot)	GSH (mg/g prot)	GSH-PX (U/mg prot)	TBARS (nmol/mg prot)
Control	502.92±46.61	2.70±0.40	14.36±2.63	122.04±32.90	8.66±0.98
CCl_4	267.73±51.00[a]	5.08±0.48[a]	6.32±0.50[a]	57.73±12.55[a]	28.13±0.98[a]
Silymarin+CCl_4	420.32±43.14[b]	1.48±0.16[b]	6.03±0.36	116.10±24.32[b]	8.36±0.82[b]
GB (150 mg/kg) + CCl_4	379.45±58.08[b]	1.46±0.07[b]	9.08±1.67	114.86±21.18[b]	11.48±1.43[b]
GB (300 mg/kg) + CCl_4	372.98±14.54[b]	1.22±0.08[b]	10.79±1.21[b]	113.02±12.88[b]	7.80±1.28[b]
GB (600 mg/kg) + CCl_4	435.22±44.17[b]	1.37±0.21[b]	12.46±1.64[b]	117.62±24.16[b]	9.38±2.01[b]

[a]P<0.05 vs. Control; [b]P<0.05 vs. CCl_4 group. Animals were pre-treated with GB (150, 300 and 600 mg/kg, i.g.), silymarin (50 mg/kg, i.g.) or vehicle for 7 days, followed by intraperitoneal administration of CCl_4 and assessment of the parameters after 24 h. Values are expressed as the mean ± standard deviation (n=10). GB, Pian Zai Huang Gan Bao; SOD, superoxide dismutase; GSH, glutathione; GSH-PX, glutathione peroxidase; TBARS, thiobarbituric acid reactive substance; MDA, malondialdehyde.

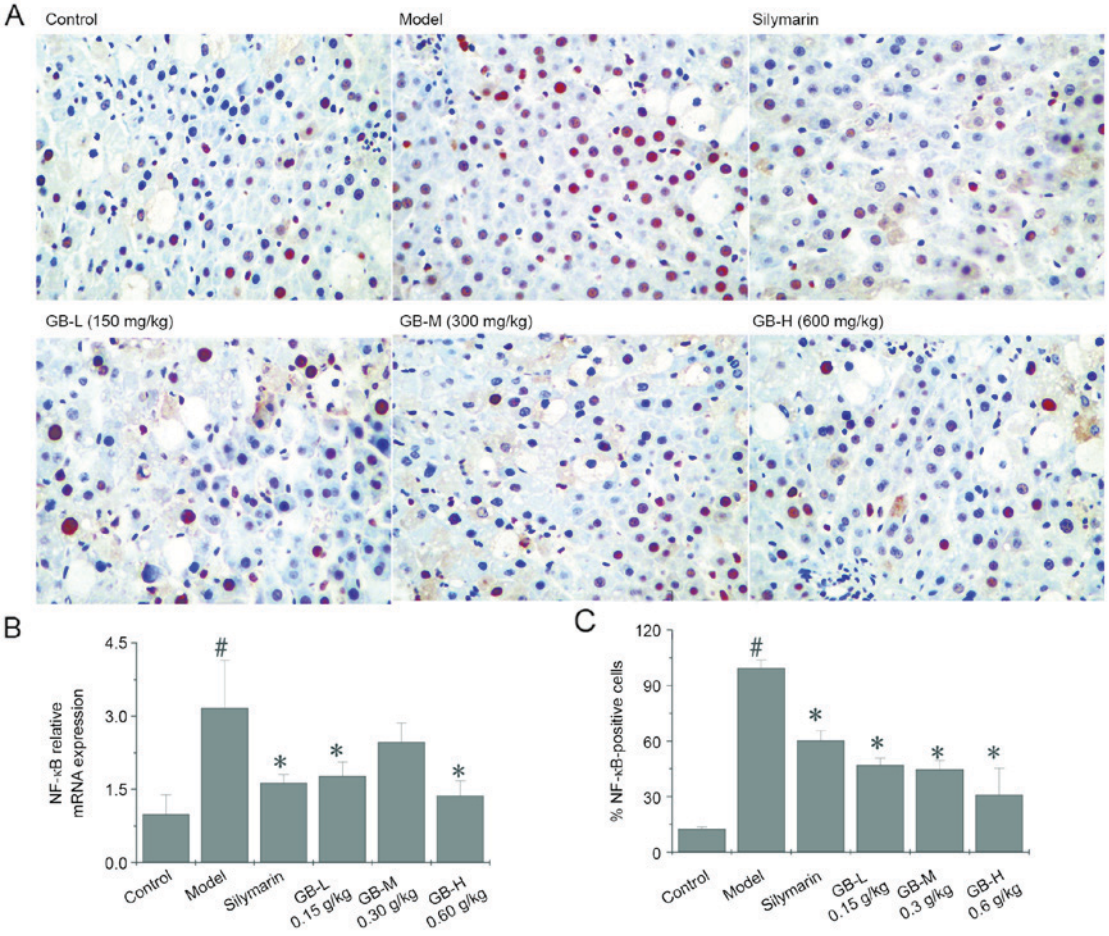

Figure 2. Effects of GB on NF-κB expression in rat liver following CCl_4 administration. (A) At the end of the experiment, liver tissues were processed for IHC staining for NF-κB. Representative images captured at x400 magnification are shown. (B) NF-κB mRNA levels were determined by quantitative polymerase chain reaction with GAPDH used as an internal control. (C) Quantification of IHC labeling presented as the percentage of positively stained cells of total cells. Values are presented as the mean ± standard deviation (n=10/group). #P<0.05 compared with the control group; *P<0.05 compared with the CCl_4-exposed group. GB-L/M/H, low/medium/high dose of Pian Zai Huang Gan Bao; NF, nuclear factor; IHC, immunohistochemical.

changes associated with CCl_4 are similar to those resulting in acute viral hepatitis (21). The present study verified that CCl_4 induced acute liver damage, as demonstrated by increased serum markers of hepatic injury and histology. Specifically, the damage was associated with increased lipid peroxidation products and reduced antioxidative enzymes (22), indicating oxidative stress. Moreover, NF-κB was elevated, and TNF-α and IL-1β, which are pro-inflammatory cytokines regulated by this factor, were also increased. Of note, GB treatment prevented all the alterations induced by CCl_4. It is indicated

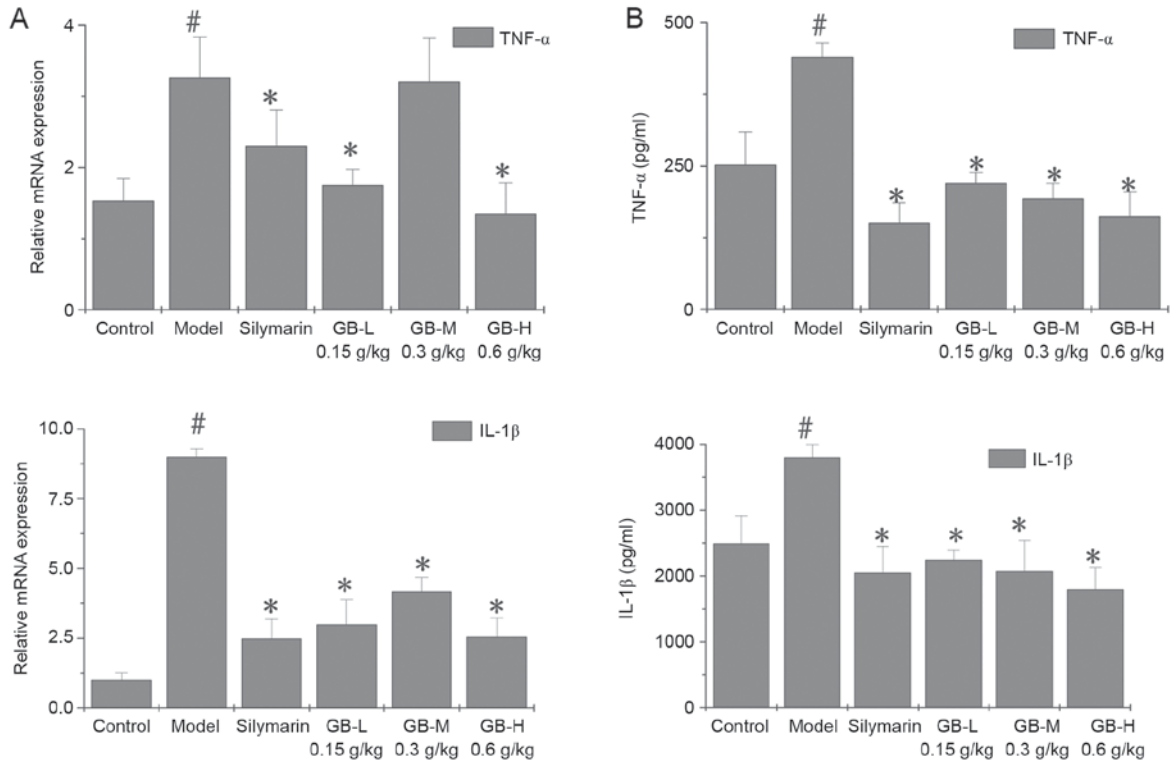

Figure 3. Effects of GB on TNF-α and IL-1β expression. (A) Analysis of TNF-α and IL-1β mRNA expression was determined by quantitative polymerase chain reaction with GAPDH used as an internal control. (B) TNF-α and IL-1β protein in serum was evaluated by ELISA. Values are presented as the mean ± standard deviation (n=10/group). #P<0.05 compared with the control group; *P<0.05 compared with the CCl_4-exposed group. GB-L/M/H, low/medium/high dose of Pian Zai Huang Gan Bao; TNF, tumor necrosis factor; IL, interleukin.

that the antioxidant properties of GB and its ability to inactivate NF-κB, and thus proinflammatory cytokine production (23), are the most likely mechanisms of action of GB.

Under normal conditions, serum hepatobiliary enzymes, including AST, ALT and ALP, are present in high concentrations in the liver. The serum enzyme levels of these enzymes become increased upon onset of hepatocyte membrane damage and necrosis, as they are released into the circulation (24). In CCl_4-induced animals, hepatic cell damage is indicated by increased serum AST, ALT and ALP levels (25), as was observed in the present study. CCl_4-induced increases in serum AST, ALT, ALP and γ-GT were reduced or prevented by pre-treatment with GB or silymarin, indicating hepatoprotective and curative abilities. Bilirubin is a useful clinical indicator of necrotic severity, as it is produced during breakdown of heme in red blood cells, with hyperbilirubinemia indicating liver pathophysiology and bilirubin accumulation being a measure of liver cell conjugation, binding and excretory ability. The results of the present study showed that CCl_4-induced rats produced more total bilirubin, which was inhibited by pre-treatment with GB or silymarin. However, no statistical significance was observed, which may in part be due to liver function restoration.

Lipid peroxidation is an important indicator of oxidative stress. It has been reported that CCl_4-induced hepatic tissue damage causes lipid peroxidation and ultimately triggers MDA production (26). Furthermore, TBARs, the final metabolites of peroxidized polyunsaturated fatty acids, are considered a late biomarker of oxidative stress (27). Increased liver MDA and TBARS levels induced by CCl_4 suggest enhanced lipid peroxidation, leading to hepatic tissue damage and failure of antioxidant defense. In agreement with previous studies of CCl_4-induced oxidative stress, the present study showed that CCl_4 treatment caused an increase of hepatic MDA and TBARS. Of note, pre-treatment with silymarin or GB significantly decreased these markers. GSH is a first line of defense that scavenges free reactive oxygen species (ROS) and exists in its reduced and its oxidized, disulfide state (GSSG). In healthy cells and tissues, >90% of total glutathione is present in the reduced form, while <10% exists in the GSSG form. An elevated GSSG-to-GSH ratio is considered indicative of oxidative stress (28). GSH-dependent enzymes offer a second line of protection by primarily detoxifying noxious byproducts of ROS and preventing free radical dissemination. Peroxides are detoxified by GSH-PX through its reaction with GSH, converting it into GSSG, which is then reduced to GSH by its specific reductase (29). Endogenous antioxidant enzymes such as SOD are also affected by free radicals. The present study revealed that treatment with CCl_4 in rats markedly altered the activity of antioxidant enzymes, while silymarin and GB reversed these effects.

Various studies have shown that hepatocellular injury is not primarily due to the damaging agent itself, but rather a result of inflammatory cells that attack the stressed hepatocytes (30). This inflammatory response is mediated by cytokines, of which TNF-α and IL-1β can induce an acute-phase response (31). NF-κB is an integral inflammatory response regulator, controlling cytokine gene expression. It has been shown that inflammatory molecules are activated or increased following activation of NF-κB by CCl_4-mediated

release of TNF-α and IL-1β (13). Thus, a self-amplifying, detrimental feedback loop is set forth in hepatocytes: TNF-α and IL-1β promote NF-κB activation, and NF-κB enhances production of additional TNF-α and IL-1β. This cycle eventually modifies hepatocyte structures and morphology, and impairs liver cell function (32). As a result, continued NF-κB activation promotes prolonged inflammatory responses, which makes it a key target for various anti-inflammatory drugs used for treating various diseases (33). The present study showed that CCl_4 treatment led to increased NF-κB activation and release of the inflammatory factors TNF-α and IL-1β in the livers of rats, which was prevented by silymarin and GB. The effect of 300 mg/kg of GB was poorer than that of 150 or 600 mg/kg of GB, particularly regarding the gene expression of NF-κB, TNF-α and IL-1β. A possible explanation may be the complexity of mechanisms of action of GB leading to the different effect on gene transcription from that on protein expression.

In conclusion, the findings of the present study indicated that GB was highly effective in preventing CCl_4-induced acute liver damage, oxidative stress, the expression of pro-inflammatory cytokines and the activation of NF-κB. Based on the known effects of GB, the results of the present study suggested that GB prevented transcription factor NF-κB expression and activation to suppress the expression of a series of pro-inflammatory agents, thereby preventing liver injury. Further study is necessary to more broadly assess the therapeutic dose range and mechanism of action of BR in preventing acute hepatotoxicity. However, the present study shed light on the potential clinical efficacy and mode of action of GB in treating liver disorders.

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