Original Article
Hepatoprotective activity of Pien Tze Huang Gan Bao in CCl$_4$-induced chronic liver injury models

Jinyan Zhao$^{1,2}$, Daxin Chen$^{1,2}$, Shan Lin$^{1,2}$, Yuchen Zhang$^{1,2}$, Yun Wan$^{1,2}$, Zhenfeng Hong$^1$

$^1$Academy of Integrative Medicine, $^2$Fujian Key Laboratory of Integrative Medicine on Geriatric, Fujian University of Traditional Chinese Medicine, Quyang Road, Shangjie Minhou, Fuzhou 350122, Fujian, China

Received December 20, 2018; Accepted April 9, 2019; Epub September 15, 2019; Published September 30, 2019

Abstract: The aim of the current study was to evaluate the effects and underlying molecular mechanisms of Pien Tze Huang Gan Bao (GB) on chronic liver injuries induced by carbon tetrachloride (CCl$_4$) in rat models. Chronic liver injuries in Sprague-Dawley male rats were induced by intraperitoneal injections with CCl$_4$ (1.0 mL/kg body weight, twice weekly), dissolved in olive oil (1:1, v/v). After four weeks, experimental and control rats were orally administered physiological saline (PS), silymarin (50 mg/kg), GB-Low (150 mg/kg), GB-moderate (300 mg/kg), or GB-high (600 mg/kg) for 4 weeks. Twenty-four hours after the last treatment, the animals were sacrificed. Biochemical assays were used to analyze indicators of hepatotoxicity. H&E staining was employed to observe histopathological changes in the liver and Masson’s staining was used to assess liver fibrosis. Gene and protein expression levels of TGF-β1, α-SMA, IL-1β, and TNF-α in the liver were determined by qPCR and ELISA or Western blotting, respectively. Results showed that both silymarin and GB normalized elevated levels of ALT, AST, ALP, and LDH and attenuated hepatic steatosis and fibrosis induced by CCl$_4$. GB treatment also decreased secretion of LN and HA and downregulated expression of TGF-β1, α-SMA, IL-1β, and TNF-α. GB demonstrated protective effects against chronic liver injuries induced by CCl$_4$, likely through the alleviation of inflammation-induced hepatic fibrosis.

Keywords: Pien Tze Huang Gan Bao, carbon tetrachloride, liver injury, liver fibrosis, inflammation

Introduction

The liver is an important metabolic organ in the body, though it is susceptible to injury by various factors, including many chemical agents. Chronic liver disease represents a major public health concern, worldwide. Hepatocellular apoptosis, hepatic inflammation, oxidative stress, and fibrosis are prominent features of chronic liver disease [1, 2]. Of these, progressive hepatocellular fibrosis is the common pathway of most chronic liver injuries, leading to liver cirrhosis and hepatocellular carcinoma [3]. Carbon tetrachloride (CCl$_4$)-induced liver injury is a classic model of chemical liver injury in rodents [4-6], popular for its symptoms resembling those of chronic liver injury in humans [7]. Despite advances in the understanding of the molecular pathology of liver damage, there are only a limited number of hepatoprotective interventions. In view of this, many have posited the potential of natural compounds, which have demonstrated worldwide importance and effectiveness [8].

Natural remedies from medicinal plants are considered safe and effective alternatives or complementary treatments against hepatotoxicity. Pien Tze Huang Gan Bao (GB), a Traditional Chinese Medicine containing Calculus bovis, Panax notoginseng, Artemisia capillaris, snake gall, and Radix Paeoniae alba, has been used for millennia in China and Southeast Asia. GB has been demonstrated to be an effective protectant against liver injuries caused by excessive alcohol consumption. However, there remains a lack of information concerning the molecular mechanisms by which GB confers protective actions. The present study established a chronic liver injury model via CCl$_4$ in rats, aiming to investigate the underlying mechanisms of the protective roles of GB in chronic liver injuries. This study was conducted with an emphasis on attenuation of inflammation and fibrosis.
Methods and materials

GB was obtained and authenticated by the sole manufacturer, Zhangzhou Pien Tze Huang Pharmaceutical Co. Ltd. (Zhangzhou, China; Chinese FDA approval no. HPK-08411). Silymarin (cat no: 02000585) was obtained from Sigma-Aldrich Company (Sigma-Aldrich; Merck KGaA). TRIzol Reagent was purchased from Life Technologies (Thermo Fisher Scientific, Inc., Waltham, MA, USA). PrimeScript™ RT reagent kit with gDNA Eraser and SYBR Premix Ex Taq II (Tli RNaseH plus) was purchased from Takara Bio Inc., (Tokyo, Japan). BCA Protein Assay Kit was purchased from Tiangen Biotech Co., Ltd., (Beijing, China). Laminin (LN), Hyaluronic acid (HA), Interleukin (IL)-1β, and Tumor necrosis factor (TNF)-α enzyme linked immunosorbent (ELISA) assay kits were purchased from Shanghai Xitang Biotech Co., Ltd. (Shanghai, China). GAPDH (5174S; 1:1,000 dilution) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit monoclonal antibodies for alpha smooth muscle actin (α-SMA, cat no. ab32575) and rabbit polyclonal to transforming growth factor beta 1 (TGF-β1, cat no. ab92486) were obtained from Abcam (Abcam, Cambridge, MA, USA; 1:1,000 dilution). Horseradish peroxidase-conjugated secondary antibodies (cat. no. ab205718) were obtained from Abcam (1:2,000 dilution). CCl₄ was purchased from Shanghai Lingfeng Chemical Co., Ltd. (Shanghai, China).

Animals

Sixty male Sprague-Dawley rats (6 weeks; 180-200 g; Slike Co., Ltd., Shanghai, China) were grouped with five in one cage (ventilated) in an environmentally controlled facility. Temperature was maintained at 22 ± 1°C and 40-60% humidity. The housing area was kept under a 12-hour light/dark cycle, with a light intensity of around 150-300 lux. Food and water were provided ad libitum for one week prior to the beginning of experimentation. All animal studies were approved by the Fujian Institute of Traditional Chinese Medicine Animal Ethics Committee (Fuzhou, China). Experimental procedures were carried out in accordance with Guidelines for Animal Experimentation of Fujian University of Traditional Chinese Medicine (Fuzhou, China).

Chronic liver injuries were induced by intraperitoneal injections of CCl₄ (1.0 mL/kg body weight, twice weekly), dissolved in olive oil (1:1, v/v), for 8 weeks. At the beginning of treatment, the animals were randomly assigned into six groups (n = 10, each), according to the following conditions: Group 1: Control group; Group 2: CCl₄ model group; Group 3: Silymarin treated CCl₄ group (treated with silymarin, 50 mg/kg), a positive control; Group 4: Low-dose GB treatment CCl₄ group (treated with GB, 150 mg/kg); Group 5: Medium-dose GB treatment CCl₄ group (treated with GB, 300 mg/kg); and Group 6: High-dose GB treatment CCl₄ group (treated with GB, 600 mg/kg). On the fifth week, Groups 3 to 6 were administered oral doses of silymarin or GB for an additional four weeks. Instead, control and CCl₄ model groups were given oral doses of PS for 4 weeks. Twenty-four hours after the final treatment, the rats were anesthetized by 40 mg/kg intraperitoneal injections of pentobarbital (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Once anesthesia confirmed, the animals were terminated by cervical dislocation. They were immediately subjected to laparotomy procedures, in which the abdominal cavity was opened to expose the abdominal aorta. Blood was collected from the aorta abdominis into non-heparinized tubes. These tubes were centrifuged at 3,000 rpm at 4°C for 10 minutes, obtaining serum for biochemical testing and ELISA assays. Liver tissues were also quickly excised into multiple portions and washed. This was followed by fixation in formaldehyl saline (4%) solution for histological analysis. The remaining portion was snap-frozen in liquid nitrogen and stored at -80°C for molecular analysis.

Serum biochemical assays

Various markers of hepatic function, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH), were evaluated using an automatic biochemical analyzer (Bayer ADVIA 2400; Bayer, Siemens, Germany), according to manufacturer instructions.

Histopathological analysis

Liver tissues allocated for histopathological analysis were fixed in 10% buffered formalin for ≥ 48 hours. This was followed by processing and paraffin-embedding. Paraffin sections were prepared by an automatic tissue proces-
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sor (RM2; Leica Microsystems GmbH, Wetzlar, Germany) and sectioned into 5 μm-thick serial slices using a rotary microtome. Sections were subsequently stained by hematoxylin-eosin dye and histopathologically analyzed. Photomicrographs of the stained tissues were captured using a DMRB/E light microscope (Leica Microsystems GmbH).

Masson’s trichrome staining

The hepatic tissue sections were deparaffinized, dehydrated, washed, and stained in Weigert’s iron hematoxylin for 5 minutes. Next, they were washed and stained in Biebrich scarlet acid fuchsin solution for 5 minutes. The sections were then washed and differentiated in 1% phosphomolybdic-phosphotungstic acid solution for 5 minutes. This was followed by aniline blue counterstain for 5 minutes. Finally, the sections were differentiated in 1% acetic acid solution for 1 minute, followed by washing, dehydration, and treatment with resin mounting medium.

ELISA

Commercial enzyme-linked immunosorbent assays (ELISA) were used for evaluation of various markers, including LN, HA, TNF-α, and IL-1β. The kits operated on a solid-phase sandwich principle, beginning with the addition of a monoclonal antibody specific for rat LN, HA, TNF-α, or IL-1β coated on 96-well plates. Standards and samples were incubated in antibody-containing solution, allowing for the binding of any present LN, HA, TNF-α, or IL-1β to the immobilized antibody. Plates were washed and a secondary biotinylated polyclonal anti-rat LN, HA, TNF-α, or IL-1β antibody was added. Finally, avidin-horseradish peroxidase tertiary antibodies were added as the last sequence in the antibody-antigen-antibody sandwich formation. To visualize positive signals, a substrate solution was added, generating a blue color proportional to the amount of rat LN, HA, TNF-α, or IL-1β present in the sample. A stop buffer was next added to terminate the reaction. Absorbance values of each reaction were then measured at 450 nm on a plate reader. Optical density levels were converted to pg/mg of protein for each amount of rat LN, HA, TNF-α, or IL-1β.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total liver RNA was isolated using TRIzol Reagent, according to manufacturer instructions. Purified RNA was quantified by measuring the absorbance of the preparation at 260 nm. Moreover, cDNA synthesis was performed using the PrimeScript™ RT reagent kit, according to manufacturer instructions. One microgram of total RNA was incubated with 4.0 μl 5X PrimeScript buffer, 1.0 μl PrimeScript RT enzyme mix I, 1.0 μl Oligo dT primer (50 μM), 1.0 μl random 6 mers (100 μM), and RNase-free water to a final volume of 20 μl. The reaction mixture was processed at 25°C for 10 minutes. It was heated to 37°C for 15 minutes, heated to 85°C for 5 seconds, and cooled to 4°C.

Next, cDNA was subjected to PCR amplification using SYBR Premix Ex Taq II in an ABI 7500 instrument. Expression of mRNA was determined using the 2−ΔΔCq method [9], based on the expression of the internal control gene for β-actin. All qPCR reactions were conducted in triplicate. Primer sequences were as follows: IL-1β forward, 5’-TGT TCT TTG AGG CTG AC-3’ and reverse, 5’-CTT TGG GAT TTG TTT GG-3’; TNF-α forward, 5’-CAG CAG ATG GGC TGT ACC TT-3’ and reverse, 5’-AAG TAG ACC TGC CCG GAC TC-3’; TGF-β1 forward, 5’-TGA ACC AAG GAG ACG GAA TAC AGG-3’ and reverse, 5’-GCA GTA GTT GGT ATC CAG GGC TCT-3’; α-SMA forward, 5’-ATC ACC ATC GGG AAT GAA CGC TT-3’ and reverse, 5’-ATC CTG TCA TGA ATG CCT GGG TA-3’; and β-actin forward, 5’-CGG GTA GTT GGT ATC CAG GCC TCT-3’ and reverse, 5’-GTG TTG GCA TAG AGG TCT TTA CG G-3’.

Western blot analysis

Livers tissues were mechanically homogenized and total protein was extracted by radioimmunoprecipitation using a commercial preparation (Thermo Fisher Scientific, Inc.), containing protease and phosphatase inhibitor cocktails. After homogenization, protein lysate was centrifuged at 12,000 × g for 15 minutes at 4°C. Protein concentrations were determined by the BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Fifty microgram samples were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The mem-

branes were incubated overnight at 4°C with blocking solution in TBS, followed by primary antibody incubation (rabbit monoclonal antibodies against TGF-β1, α-MSA, and GAPDH) for 2 hours at room temperature. Subsequently, the membranes were washed and incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2,000) for 1 hour at room temperature. To visualize proteins, the membranes were washed a final time in TBST solution. This was followed by the addition of SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) for enhanced chemiluminescence detection.

**High performance liquid chromatography (HPLC)**

The samples were analyzed on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) using a Welch Ultimate XB-C18 (250 mm × 4.60 mm; 5 μm). Absorbance was measured at 274 nm. The mobile phase consisted of methanol and 0.1% phosphoric acid (45:55) at a flow rate of 0.9 mL/min, with an injection volume of 5 μL. Column temperature was maintained at 20°C. Baicalin (Sigma-Aldrich; Merck KGaA) served as a positive control [10].

**Statistical analysis**

Data were analyzed using SPSS version 11.5 (SPSS, Inc., Chicago, IL, USA) and are expressed as mean ± standard deviation of three independent experiments. Statistical analysis was performed with one-way analysis of variance, followed by post-hoc Fisher’s least significant difference testing. P < 0.05 indicates statistically significant differences.

**Results**

*GB prevents CCl₄-induced increases in hepatic enzymes*

Administration of CCl₄ markedly increased (P < 0.01) the activity of hepatic enzymes AST, ALT, and ALP, compared to the control group. Elevation in secretion levels of these enzymes was significantly decreased (P < 0.01) by silymarin and 600 mg/kg of GB, compared to the CCl₄ group, as shown in Figure 1. Similarly, serum LDH was increased in CCl₄-treated animals, an effect reversed by silymarin- or GB-treatment. Treatment with 150, 300, and 600 mg/kg PZH-GB, as well as silymarin at a dose of 50 mg/kg, exhibited a reduction of in serum LDH by 44.30%, 31.90%, 45.17%, and 59.53%, respectively.

*GB ameliorates hepatic gross morphology and histopathological changes*

Hepatic gross morphology was captured using a single lens reflex camera (Nikon, Japan). While control rats exhibited a normal morphology (capsule smooth, complete, red-brown red, and a soft texture), livers in the CCl₄ group showed typical hepatic fibrosis morphology, with a yellowed rough surface, blunt edge, fiber exudation, and adhesion with surrounding tissues. In contrast, after treatment with silymarin and GB (especially 600 mg/kg), the livers showed obvious signs of recovery (Figure 2A). Furthermore, liver histopathology was analyzed with H&E staining, revealing a lobular architecture and hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus, visible central veins, and thin sinusoids in the con-
GB prevented liver injuries via attenuation of fibrosis

Liver fibrosis was evaluated histologically via Masson's staining. Results demonstrated that normal hepatic lobules in the \( \text{CCl}_4 \) group were diminished and collagen deposits were evident. Treatment with silymarin and GB markedly alleviated collagen deposition and lowered levels of fibrosis, compared to the \( \text{CCl}_4 \) group (Figure 3A). To confirm the anti-fibrosis effects of silymarin and GB, expression levels of common liver fibrosis biomarkers HA and LN were determined in the serum. HA in the injury group was elevated by 39.76\%, while LN was increased 39.02\%, compared with the control group. Treatment with silymarin and different doses of GB reduced serum levels of HA and LN. Compared with the induced injury model, silymarin and GB (150, 300, and 600 mg/kg) decreased levels of LN or HA by 60.67, 36.89, 30.39, and 49.68\% or 35.34, 24.10, 36.55, and 36.55\%, respectively (Figure 3B). Evaluation of signaling molecules TGF-\( \beta \)1 and \( \alpha \)-SMA revealed that GB (especially 600 mg/kg group) inhibited TGF-\( \beta \)1 and \( \alpha \)-SMA (Figure 4). Taken together, present data demonstrates that silymarin and GB ameliorated liver fibrosis, indicated histologically by serum biomarkers.
and expression of TGF-β1 and α-SMA.

GB downregulated expression of pro-inflammatory factors

Pro-inflammatory cytokines are known to play a crucial role in liver injuries and fibrosis [11-14]. The present study examined the effects of GB on pro-inflammatory gene expression of chronic liver-injury rats, specifically by measuring TNF-α and IL-1β, using RT-qPCR, in liver tissues (Figure 5A). Results showed that significant increases in TNF-α and IL-1β mRNA occurred in the livers of CCl₄-treated rats. As shown in Figure 5A, CCl₄ intoxication increased the mRNA of TNF-α and IL-1β by 42.29% or 29.08%, respectively. However, treatment with GB and silymarin prevented these expression level
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Inhibition of TNF-α and IL-1β gene expression by silymarin and GB was further confirmed by measurement of TNF-α and IL-1β protein using ELISA assays (Figure 5B). TNF-α and IL-1β protein was elevated by CCl₄ but inhibited by either GB or silymarin.

Discussion

Liver injury models induced by CCl₄ are most commonly used for screening the hepatoprotective activity of natural substances [15-17]. The current study induced chronic liver injuries in rats via intraperitoneal injections of CCl₄ for 8 weeks. This was based on previous data showing that CCl₄ at a dose of 1 mL/kg of body weight, twice per week for 8 weeks, was enough for development of hepatic injuries and fibrosis in rats. Present results are in accord with previous findings [18, 19].

In the present study, rats treated with CCl₄ for 8 weeks exhibited inflammation and fibrosis. Furthermore, serum ALT, AST, ALP, and LDH were significantly increased in CCl₄-induced rats. Morphological data further showed the infiltration of inflammatory cells and collagen deposition in the livers after CCl₄ treatment. However, every single damage parameter was demonstrably reversed by silymarin and different doses of GB.

Progression of liver fibrosis is characterized by the accumulation of extracellular matrix (ECM) proteins in injured tissues. Upon liver chronic injury, hepatic stellate cells (HSCs) are activated by TGF-β1 secreted via Kupfer cells [20, 21], allowing trans-differentiation into myofibroblast-like cells, which propagates ECM deposition [22, 23]. FN and HA are key components of the ECM. Their expression levels are closely associated with hepatic fibrosis, cellular damage, differentiation, and repair. In the present study, FN and HA expression increased after CCl₄ treatment. It subsequently decreased with silymarin and GB treatment. Activated HSCs can upregulate expression of cytoskeletal proteins, such as α-SMA, a crucial step for cellular differentiation. Thus, α-SMA has been considered a marker of HSC activation, taken to reflect the degree of liver fibrosis in other studies [24]. In the present study, α-SMA expression increased in response to CCl₄ injections. However, after treatment with silymarin or GB, α-SMA protein expression returned to baseline levels, suggesting the reversibility of liver fibrosis.

Inflammatory response plays an important role in the progression of liver fibrosis. Pro-inflammatory cytokines, such as TNF-α and IL-1β, have been shown to increase in livers with CCl₄-induced hepatitis [25, 26]. Pro-inflammatory cytokines and chemokines stimulate HSCs transformation into myofibroblasts, playing a role in activating the production of ECM. Thus, liver fibrosis is closely related to liver inflammation. Results of this study demonstrated the increased expression of both TNF-α and IL-1β in the liver injury group. Conversely, current findings demonstrated that liver fibrosis and inflammation markers elicited by CCl₄ treatment were ameliorated by the protective effects of silymarin and GB. This further indicated the anti-fibrotic and anti-inflammatory properties of GB. Taken together, results sug-
suggest that the hepatoprotective capacity of GB, including inhibition of ECM deposition and the resulting histopathology, is probably linked to modulated expression of inflammatory factors.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant No. 81303125), Nature Science Foundation of the Fujian Province of China (No. 2017J01845), and Developmental Fund of Chen Keji Integrative Medicine (CKJ2013015).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhenfeng Hong, Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, Qiuyang Road, Shangjie Minhou, Fuzhou 350122, Fujian, China. Tel: +86-591-22861012; Fax: +86-591-22861012; E-mail: zhenfeng.hong@yahoo.com

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