Tropical Journal of Pharmaceutical Research December 2018; 17 (12): 2381-2385
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v17i12.10

Original Research Article

Hepatoprotective effects of ginseng saponins in a mouse model of carbon tetrachloride-induced liver injury

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Sent for review: 22 August 2018 *Revised accepted: 17 November 2018*

Abstract

Purpose: *To investigate the effects of ginsenosides Rg1 and Rb1 on carbon tetrachloride (CCl_4)-induced liver injury in mice.*
Methods: *Thirty-two mice were randomly divided into four groups. In control group, mice were administered sodium carboxymethylcellulose (CMC-Na) by intraperitoneal injection for seven days. In CCl_4 treatment group, mice were treated as control group for the first seven days and then intraperitoneally injected with CCl_4 in olive oil on day 8. In Rb1- and Rg1-treatment group, the mice were intraperitoneally injected with Rb1 or Rg1 (each 30 mg/kg, dissolved in 0.5 % CMC-Na), respectively for seven days, followed by intraperitoneal injection with CCl_4 in olive oil on day 8. Histological damage was examined by haematoxylin and eosin (H&E) staining. Serum levels of alanine aminotransferase (ALT) and aspartate transaminase (AST) were assessed enzymatically. Tissue IL-6 and IL-8 levels were measured by ELISA. Gene and protein levels of transforming growth factor (TGF)-β, Smad2, and Smad3 were analyzed by real-time PCR (RT-PCR) and western blotting, respectively.*
Results: *CCl_4 treatment caused histological damage in mouse liver, and increased the levels of ALT, AST (five-fold), IL-6 (three-fold), and IL-8 (five-fold), and elevated expressions of TGF-β, Smad2, and Smad3. Ginsenosides Rg1 or Rb1 pre-treatment attenuated liver injury by decreasing the serum levels of ALT (from 700 to 200 UI/L) AST (from 550 pg/mL to 250 pg/ml), IL-6 (from 1,100 to 750 pg/mL), and IL-8 (from 600 to 200 pg/mL), and inhibiting the expressions of TGF-β, Smad2, and Smad3.*
Conclusion: *Ginsenosides (Rg1 and Rb1) attenuate CCl_4-induced liver injury and inflammation by regulating TGF-β/Smad signalling pathway.*

Keywords: *Ginseng saponin, Ginsenosides, Rg1, Rb1, Hepatoprotective effect, TGF-β/Smad signalling pathway*

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INTRODUCTION

The liver, an important metabolic organ, plays a key role in many physiological processes, including clearance of drugs and toxins, protein synthesis, and nutrient metabolism [1,2]. Liver injury is a common consequence of various diseases, and acute hepatic failure is known to affect human health worldwide [3]. Liver injury caused by toxin-induced metabolic dysfunction often leads to inflammation, or even hepatic fibrosis [4]. Carbon tetrachloride (CCl_4), a potent chemical hepatotoxin, has been widely used in animal models of liver injury accompanied by

inflammation and fibrosis. The metabolism of CCL_4 can yield reactive free radicals which increase lipid peroxidation and protein oxidation, leading to liver injury [5].

The ginsenosides Rg1 and Rb1, two primary active compounds from *Panax ginseng* C. A. Mey, have been reported to elicit pharmacological effects, such as immunoregulation, neuroprotection, and hepatoprotection [6,7]. Many studies have demonstrated that ginsenosides can function as hepatoprotective agents in multiple models of hepatic injury and other diseases [6,7]. However, the protective mechanism of Rg1 and Rb1 in CCl_4-induced liver injury is still unclear. In this study, the effects of Rg1 and Rb1 in a model of CCl_4-induced acute liver injury was explored, noting changes in tissue histology and cytokine levels. The hepatoprotective mechanism of Rg1 and Rb1 was investigated in detail.

EXPERIMENTAL

Materials

Ginsenosides Rg1 and Rb1 were purchased from Sigma-Aldrich (St Louis, MO, USA). The enzymatic kits for alanine aminotransferase (ALT) and aspartate transaminase (AST) were purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). All primary antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). All primers were purchased from Shanghai Sangon Biological Engineering Co., Ltd. (Shanghai, China). TNF-α and IL-6 ELISA kits were purchased from R&D Systems Inc. (Minneapolis, MN, USA).

Liver injury model

Male ICR mice, purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), were housed at 23 °C, given free access to food and water. All animal experiments were performed in accordance with Guide for the Care and Use of Laboratory Animals [8] and approved by the animal ethics committee of Wuhan Third Hospital (approval no. KY2018—026). Acute liver injury was induced by CCl_4. Thirty-two mice were randomly divided into four groups (control, CCl_4, Rg1, and Rb1). Control mice were administered sodium carboxymethylcellulose (CMC-Na, 0.5 %, the same volume used for the Rg1 and Rb1 groups) by intraperitoneal injection for seven days. CCl_4-treated mice were treated like the controls for the first seven days and then intraperitoneally injected with CCl_4 [10 mg/kg (v/v) in 0.3 % olive oil] on day 8. Rb1- and Rg1-treated mice were intraperitoneally injected with Rb1 and Rg1 (each 30 mg/kg, dissolved in 0.5% CMC-Na), for seven days and then intraperitoneally injected with CCl_4 [10 mg/kg (v/v) in 0.3 % olive oil] on day 8. After 24 h, all mice were sacrificed by cervical vertebra disjointing, liver and blood samples were collected for further analysis.

Haematoxylin and eosin (H&E) staining of mouse liver

The left lobes of mouse liver were washed with phosphate buffer saline then were fixed in formalin (0.4 %) for haematoxylin and eosin (H&E) staining. After dehydration in graded alcohol, the tissue was embedded in paraffin and cut into 3 to 4 μm sections on a Leica SM2010 R Sliding Microtome (Leica, Buffalo Grove, IL, USA). The sections were then deparaffinized, rehydrated, and stained with hematoxylin-eosin for 5 min at 37 °C. Finally, the sections were mounted in neutral Canada balsam after dehydration, observed by optical microscopy, and evaluated by pathologists, blind to the identity of the sections.

Analysis of serum ALT and AST levels

Blood was centrifuged at 3000 rpm/min for 5 min, and the serum was analyzed for ALT (C009-2) and AST (C010-2) levels using commercial ELISA kits according to the manufacturer's protocol.

Determination of liver TNF-α and IL‑6 levels

Mouse livers were rinsed in saline. One lobe of the liver was rinsed in saline [1:9 (m/v)], and homogenized in ice-cold Nonidet P 40 (NP-40) using a glass homogenizer. TNF-α and IL‑6 levels in liver homogenates were measured by ELISA, according to the manufacturer's instructions.

Western blotting

Liver tissues were homogenized in RIPA buffer, and proteins were quantified using the BCA method (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein were loaded and separated on a 10 % SDS-polyacrylamide gel and transferred onto a PVDF membrane (Millipore, MA). After blocking, the membranes were incubated with respective primary antibodies [TGFβ (#79424), Smad2 (#5339), and Smad3 (#9523) at a 1:1,000 dilution] and secondary antibodies (1:10,000 dilution). The proteins were detected by chemiluminescence and quantified using ImageJ software.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total liver RNA was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed into cDNA using the GoScript™ Reverse Transcription System (Promega). Quantitative RT-PCR was performed using the SYBR Green PCR Master Mix reagent kit (Promega). The primers used for RT-PCR were described previously [9].

Statistical analysis

Statistical analysis was performed using one-way ANOVA or Tukey's test by SPSS software (Chicago, IL, USA) Data are presented as mean ± standard deviation (SD), with $p < 0.05$ was considered statistically significant.

RESULTS

Histopathological features

Histological changes in the liver were observed by H&E staining. As shown in Figure 1, Liver from control mice (Figure 1A) had clear lobules and regular cord structures, whereas livers from CCl_4-treated mice showed apparent pathological changes, including vacuoles (arrows) and necrosis, indicative of severe liver damage (Figure 1B). Pre-treatment with Rg1 (Figure 1C) or Rb1 (Figure 1D) attenuated the severity of liver injury, with a corresponding decrease in the number of vacuoles with no apparent necrosis.

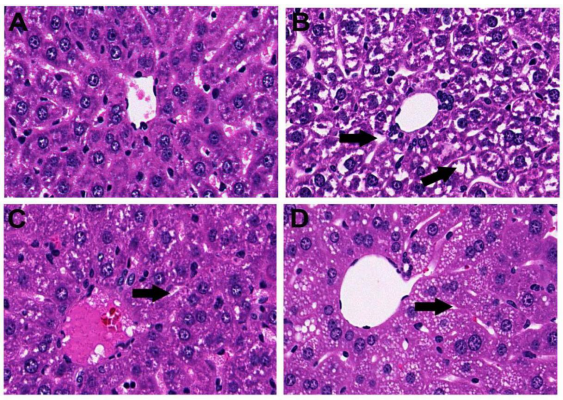

Figure 1: Liver histopathology (H&E staining). A, control group; B, CCl_4 group; C, Rg1 group; D, Rb1 group. Arrows indicated vacuoles areas and necrosis areas

Effects of Rg1 and Rb1 on ALT and AST levels

As shown in Figure 2, ALT levels in the CCl_4 group were about 2.5 times higher than in the control group, and AST levels were present at five-fold higher levels than in the control group. ALT and AST levels in Rg1- or Rb1-treated mice decreased significantly when compared with the CCl_4 group. These results are in accordance with the histopathological results.

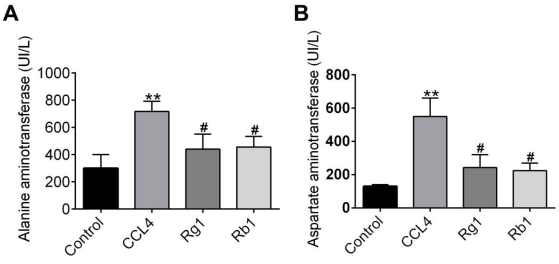

Figure 2. Serum ALT (A) and AST (B) levels in CCl_4-induced mice; n = 8, $^{**}p < 0.01$ vs. control, $^{\#}p < 0.5$ vs. CCl_4

Effects of Rg1 and Rb1 on liver cytokine expression levels

Proinflammatory cytokines, such as IL-6 and IL-8, are crucial mediators contributing to the development of liver inflammation. The effects of Rg1 and Rb1 on IL-6 and IL-8 levels in CCl_4-treated mice were investigated, and the results are shown in Figure 3. IL-6 levels in the CCl_4 group was about three-fold higher than in the control group, and IL-8 levels were five-fold higher than in the control group. IL-6 and IL-8 levels decreased significantly after Rg1 and Rb1 pre-treatment when compared with the CCl_4 group, which indicate that Rg1 and Rb1 attenuate inflammation induced by liver damage.

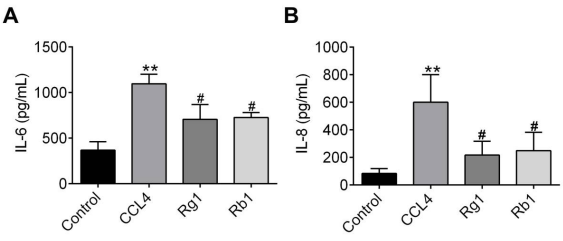

Figure 3: Liver IL-6 (A) and IL-6 (B) levels in CCl_4-induced mice; n = 8, $^{**}p < 0.01$ vs. control, $^{\#}p < 0.5$ vs. CCl_4

Effects of Rg1 and Rb1 on the TGF-β/Smad signalling pathway

As shown in Figure 4, TGF-β, Smad2, and Smad3 levels were increased in CCl_4 group compared with control group. Pre-treatment with Rg1 or Rb1 blocked the high expression of TGF-β, Smad2, and Smad3 induced by CCl_4, which indicates that Rb1 and Rg1 can attenuate liver injury induced by CCl_4 by regulating the TGF-β/Smad signalling pathway.

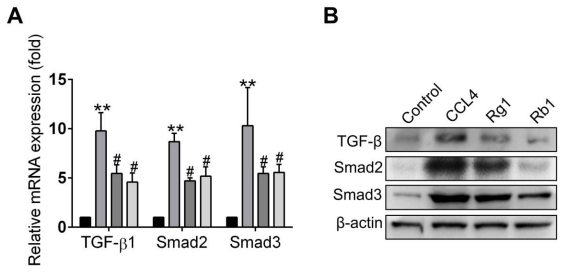

Figure 4: Gene (A) and protein (B) expression levels of TGF-β, Smad2, and Smad3; n = 8, $^{**}p < 0.01$ vs. control, $^{\#}p < 0.5$ vs. CCl_4

DISCUSSION

The liver is an important metabolic organ. Liver injury causes inflammation, which can contribute to various pathological events [10]. As a well-known hepatotoxin that gives rise to free radicals, CCl_4 is widely used in liver injury models. Trichloromethyl radical, the metabolite of CCl_4, causes lipid peroxidation which can activate Küpffer cells and trigger the production of inflammatory cytokines [11,12]. CCl_4-induced liver injury is believed to mimic the liver damage caused by various toxins in humans [13].

Inflammation and oxidative stress are closely related to liver injury and fibrosis. Many studies have demonstrated that anti-inflammatory and oxidative agents such as epigallocatechin-3-gallate, are able to significantly attenuate the severity of CCl_4-induced liver injury and fibrosis [14]. A previous study demonstrated that ginseng essence extracted from four medicinal, edible herbs (*P. ginseng*, *Panax quinquefolius*, *Nelumbo nucifera*, and *Lilium longiflorum*), can attenuate CCl_4-induced liver inflammation and fibrosis by inhibiting oxidative stress [15]. The two compounds in this study, Rg1 and Rb1, are the two primary active compounds in *P. ginseng* C. A. Mey. The current study showed that Rg1 and Rb1 attenuated the severity of CCl_4-induced liver injury and regulate inflammation and fibrosis.

TGF-β, the main fibrogenic cytokine produced by Küpffer and stellate cells in the liver, was overexpressed in CCl_4-induced liver injury in mice. Activation of the TGF receptor leads to the phosphorylation of Smad2 and Smad3 [16,17]. Results of previous studies found that blocking TGF-β signalling significantly protect rats from toxin-induced liver fibrosis [18,19]. The results of this study indicate that Rg1 and Rb1 inhibit the TGF-β/Smad signalling pathway and attenuate liver injury and fibrosis. Moreover, In addition, further studies about the precise mechanism of the association between inflammation and fibrosis are also needed. Such research will allow a better understanding of the hepatoprotective effects of ginsenosides and provide guidance for their clinical use.

CONCLUSION

The results of this study indicate that Rg1 and Rb1 attenuate CCl_4-induced liver injury and inflammation by regulating TGF-β/Smad signalling pathway, suggesting the potential value of ginsenosides in clinical management of liver inflammation.

DECLARATIONS

Acknowledgement

This work was funded by Wuhan Health and Family Planning Commission Project (no. WX18D18).

Disclosure of interest

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Huizhi Lu and Yun Tan designed all the experiments and revised the paper. Luyu Yang, Hui Dong, Youxia Liao and Song Cao performed the experiments, Shouzhi Fu wrote the paper.

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