Protective effects of geniposide against *Tripterygium* glycosides (TG)-induced liver injury and its mechanisms

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ABSTRACT — *Tripterygium* glycosides (TG) are commonly used for basic medicine in curing rheumatoid arthritis but with a high incidence of liver injury. Geniposide (GP) has broad and diverse bioactivities, but until now it is still unknown whether GP can protect against TG-induced liver injury. This study, for the first time, observed the possible protection of GP against TG-induced liver injury in mice and its mechanisms underlying. Oral administration of TG (270 mg/kg) induced significant elevation in the levels of serum alanine/aspartate transaminase (ALT/AST), hepatic malondialdehyde (MDA) and pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) (all $P < 0.01$). On the other hand, remarkably decreased biomarkers, including hepatic glutathione (GSH) level, activities of glutathione transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), and anti-inflammatory cytokine interleukin (IL)-10, were observed following TG exposure (all $P < 0.01$). Nevertheless, all of these phenotypes were evidently reversed by pre-administration of GP for 7 continuous days. Further analysis showed that the mRNA expression of hepatic growth factor-beta1 (TGF-β1), one of tissue repair and regeneration cytokines, was enhanced by GP. Taken together, the current research suggests that GP protects against TG-induced liver injury in mice probably involved during attenuating oxidative stress and inflammation, and promoting tissue repair and regeneration.

Key words: *Tripterygium* glycosides, Hepatoprotection, Oxidative stress, Geniposide, Anti-inflammation

INTRODUCTION

Drug-induced liver injury (DILI) as a rare but severe adverse drug reaction represents a major problem in pharmacotherapy, both clinically and economically. At present, DILI has been the major cause of acute liver failure throughout the world, with global incidence as high as about 14 per 100,000 patients per year (Urban et al., 2012). Beyond its cost in terms of patient morbidity and mortality, DILI, among the most frequent reasons for late termination of drug development programs and regulatory actions including withdrawal of drugs from the market, has been resulting in large financial losses to the drug industry that are inevitably passed on to consumers (Urban et al., 2012). Although several hepatoprotective agents have been used in the clinic, some of them have caused adverse reactions underlying especially in chronic or sub-chronic administration (Muriel and Rivera-Espinoza, 2008). On this basis, natural phytochemicals with antioxidant stress and/or anti-inflammation have provided us an effective and safe dietary choice for DILI (Bharrhan et al., 2012; Nabavi et al., 2012).

Although plant extracts constitute potential candidates for DILI, they often contain highly complex mixtures of many compounds, many of which have been incompletely known. Therefore more attention has been focused on bioactive compounds from plant drugs in recent years. *Gardenia jasminoides* Ellis is a medicine and food dual purpose plant, originated in Asia and most commonly found growing wild in Southern China, Taiwan, Vietnam, Japan, Myanmar and India. The fruits of *G. jasminoides* are safe and effective in the treatment of many...
diseases. Many investigations demonstrate that this plant has diverse properties such as hepatoprotection, anti-inflammatory, antioxidant (Chen et al., 2012; Hwang et al., 2010; Uddin et al., 2014).

Extensive chemical studies indicate that geniposide (GP) is one of the main bioactive constituents, which can attenuate oligomeric AB(1-42)-induced inflammatory response by targeting receptor for advanced glycation end products (RAGE)-dependent signaling in BV2 cells (Lv et al., 2014b), attenuate memory deficits through the suppression of mitochondrial oxidative stress (Lv et al., 2014a), exert hepatoprotection in a rat model of nonalcoholic steatohepatitis (NASH) associated with its antioxidant actions or regulation of adipocytokine release and expression of peroxisome proliferator-activated receptor-α (PPARα) (Ma et al., 2011), ameliorate acute alcohol-induced liver injury in mice via up-regulating the expression of the main antioxidant enzymes (Wang et al., 2015), etc. *Tripterygium* glycosides (TG), as the main chemical constituents derived from *Tripterygium wilfordii* (known as lei gong teng), have diverse and broad bioactivities including alleviating Freund's complete adjuvant (FCA)-induced arthritis, ameliorating pulmonary function in adjuvant arthritis rats, treating Behcet's disease and erosive oral lichen planus, etc (Lin and Qi, 2005; Song et al., 2010; Wan et al., 2013), but it may often induce high incidence of liver injury (Li et al., 2011, 2012; Peng et al., 2003; Wan et al., 2012; Wang et al., 2013; Zhang et al., 2012). Nevertheless, there have been no reports on the protective effects of GP against TG-induced liver injury, as far as we are aware.

The present study is designed to investigate the protective effect of GP against the TG-induced acute liver injury and explore the mechanism underlying probably via antioxidant stress and anti-inflammatory, for the first time.

**MATERIALS AND METHODS**

**Animals**

Male Kunming (KM) mice (body weight 20-24 g) were obtained from Experimental Animal Center of Henan Province (Zhengzhou, China) with the license number [SCXK (YU) 2010-0002]. Animals were given rodent laboratory chow and water *ad libitum*, and maintained under controlled conditions with a temperature of 22 ± 1°C, relative humidity 60 ± 10% and a 12/12 hr light/dark cycle (lights on at 8:00 a.m.). All the procedures were in strict accordance with the P.R. China legislation on the use and care of laboratory animals and with the guidelines established by Institute for Experimental Animals of Henan University of Traditional Chinese Medicine and were approved by the university committee for animal experiments.

**Materials**

Geniposide (GP) was provided by Shanghai Jinsui Biotechnology Co., Ltd. (Shanghai, China) with the more than 98% purity as assayed by high-performance liquid chromatography (HPLC). Bifendate was obtained from Zhejiang Medicine Co., Ltd. Xinchang Pharmaceutical Factory (Xinchang, China). *Tripterygium* glycosides tablets were purchased from Shanghai Fudan Forward S&T Co., Ltd. (Shanghai, China). Hot Start Fluorescent PCR Core Reagent Kits (SYBR Green I) were obtained from BBI (Kitchener, Canada). RevertAid™ First Strand cDNA Synthesis Kit was from Thermo Scientific (Shanghai, China). Bradford protein assay kit, Trizol reagent and all primers were purchased from Sangon Biotech (Shanghai, China). Unless indicated, other reagents and consumables were purchased from Sangon Biotech.

**Treatment protocol**

Male mice were divided into several groups (10 mice per group). Mice were administered orally with TG 270 mg/kg at 12 hr after treated with GP (20, 40, 80 mg/kg), or bifendate (150 mg/kg) once a day for seven consecutive days by intragastrical administration (ig) except mice in normal (non-TG treated) group (Peng et al., 2003). The dose selection was based on our pre-experiment results. The normal and control (TG alone) mice were given daily 0.5% CMC-Na (0.2 mL per 10 g) by ig. The blood samples of all groups were collected at 18 hr after TG administration for the measurement of serum biomarkers for liver injury. The livers were immediately removed and cleaned in 0.9% sodium chloride (4°C), and a portion was stored at -80°C for further RNA extraction, for antioxidant and anti-inflammatory parameters.

**Measurement of serum biomarkers for liver injury**

The blood samples were collected from mice in all groups for the measurement of serum biomarkers for liver injury. The serum ALT and AST were analyzed according to the reported method (Kamei et al., 1986).

**Measurement of MDA level**

Liver tissues were homogenized in cold phosphoric acid (PBS). Malondialdehyde (MDA) was assayed by the previous reported method (Högberg et al., 1974). MDA produced as the lipid peroxidation (LPO) end product and served as a sensitive and intensive index of LPO. MDA
Geniposide attenuates TG-induced liver injury

reacts with TBA to form a pink-colored product with an absorbance at 532 nm. The standard curve of MDA was established during the concentration range of 0–40 μM. The LPO level was expressed as micromoles MDA per mg protein on the basis of hepatic protein concentration determined by Bradford protein assay kit.

Measurement of GSH level

The liver GSH level was measured immediately according to the previous research (Oh et al., 2007). The reaction mixture included samples, 150 μL working solution (0.53 U/mL glutathione reductase, 40.7 μg/mL DTNB, 1 mM EDTA in 100 mM sodium phosphate buffer, pH 7.0) and 50 μL 0.16 mg/mL NADPH solution. The change in absorbance was determined at 412 nm against the reagent blank after standing in room temperature for 30 min and the GSH level was determined in comparison with a standard curve. Mouse liver GSH level was calculated on the basis of hepatic protein concentration determined by Bradford protein assay kit.

Enzymatic assays

Liver tissues were homogenized in cold PBS, and then centrifuged at 5,000 g for 5 min and the supernatant was transferred to new tubes for analysis. The hepatic tissue activities of SOD, CAT, GPx and GST were analyzed according to the previous studies (Aebi, 1984; Habig and Jakoby, 1981; Marklund and Marklund, 1974; Rotruck et al., 1973), respectively, and the results were all calculated based on liver protein concentrations determined by Bradford protein assay kit.

Measurement of TNF-α and IL-10

Hepatic tissue was collected as described above and was used to estimate the inflammatory cytokine levels by enzyme-linked immunosorbent assay (ELISA). Tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-10 levels were estimated using commercially available mouse cytokine ELISA kits from R&D System (Minneapolis, MN, USA), according to the manufacturer’s instructions. The results are expressed as picogram (pg) of cytokine per milliliter of protein solution.

Fluorescent quantitative reverse transcription-PCR (FQ-RT-PCR)

Total RNA was extracted from hepatic tissue using Trizol reagent following the manufacturer’s instructions. Reverse transcription (RT) was performed using a cDNA synthesis kit according to the manufacturer’s instructions. The house-keeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Sequences of the PCR primers were as follows: TGF-β1 Forward 5′-AAGGACCTGGTGGAAGTGC-3′, Reverse 5′-TGGGTAGAGGGCAAGGAC-3′ (125 bp product) and GAPDH Forward 5′-GAGGTCATCCATGAACTTTG-3′, Reverse 5′-GGGCCATCCACAGTCTTCTG-3′ (90 bp product). FQ-RT-PCR was performed using Hot Start Fluorescent PCR Core Reagent Kits (SYBR Green I) on a real-time PCR instrument (ABI StepOnePlus, Applied Biosystems). PCR thermal cycling parameters were as follows: the denaturing step at 94°C for 4 min, followed by 40 cycles annealing step at 94°C 30 sec, 60°C 30 sec, and 72°C 30 sec. All amplifications and detections were carried out in a MicroAmp optical 96-well reaction plate with optical adhesive covers. Relative expression of mRNA (%) =2−ΔCT × 100%, where CT represents threshold cycle, ΔCT=CT(TGF-β1)−CT(GAPDH).

Statistical analysis

All data were expressed as mean ± standard error of mean (S.E.M.). The differences among experimental groups were compared by one-way ANOVA (analysis of variance) followed by Student Newman Keuls (SNK) (P < 0.05) using the SPSS (Statistics Package for Social Science) program Version 11.5. A value of P < 0.05 was considered statistically significant for analysis.

RESULTS

Effect of GP on serum biomarkers for TG-induced liver injury

Serum ALT and AST levels are liver injury biomarkers and their significant elevation commonly reflects liver injury (Kamei et al., 1986). In this research, we found dramatically increased levels of serum ALT (137.4 ± 8.2) and AST (185.6 ± 13.3) U/L in only TG-treated mice (Figs. 1A, B) compared with normal (non-TG treated) mice, suggesting that TG induced hepatic injury successfully. After pre-administration with GP (20, 40, 80 mg/kg) and positive drug bifendate (150 mg/kg), respectively, once everyday for 7 d by ig, TG-induced increases of ALT and AST were both significantly reversed (Figs. 1A, B). There were no significant differences in the serum ALT and AST levels between bifendate (150 mg/kg) and GP (80 mg/kg) groups. These results indicate that GP protects against TG-induced mice liver injury in a dose-dependent manner, and has similar action to bifendate.

Effect of GP on liver MDA level

MDA is seen as one of the main end products of LPO (Högberg et al., 1974). As shown in Fig. 2A, liver MDA

Vol. 41 No. 1
level increased in only TG-treated mice while GP (20, 40, 80 mg/kg) and bifendate (150 mg/kg) all inhibited such excessive increase (Fig. 2A), suggesting that GP and bifendate could protect against liver LPO injury induced by TG.

Effect of GP on liver glutathione level

Glutathione, as an antioxidant, helps protect cells against ROS such as free radicals and peroxides (Oh et al., 2007). Its excessive exhaustion can induce oxidative stress injury. In the current research, the liver glutathione level decreased significantly in TG-treated mice, while GP (80 mg/kg) and bifendate (150 mg/kg) all significantly reversed such decrease (Fig. 2B). This result suggests that GP and bifendate can protect TG against destroying the balance between cellular oxidants and antioxidants by inhibiting exhaust of the glutathione level and thus can be likely to protect against liver oxidative stress injury.

Effect of GP on liver glutathione-related enzymes activities

GST and GPx, as intracellular glutathione-related enzymes, cooperate with glutathione in participating during the oxidative stress injury (Habig and Jakoby, 1981; Rottruck et al., 1973). The current study showed that TG significantly reduced hepatic activities (U/mg protein) of GST (175.5 ± 9.6) and GPx (31.2 ± 1.5) in mice while GP (80 mg/kg) and bifendate (150 mg/kg) inhibited such remarkable decrease (Figs. 3A, B), suggesting that the glutathione-related enzymes could participate in the protection of GP and bifendate against liver oxidative stress injury induced by TG. Our results further confirmed that the balance between cellular oxidants and antioxidants was destroyed by TG while GP and bifendate could reverse such damage on this balance.

Effect of GP on liver main antioxidant enzymes activities

SOD and CAT, as intracellular main antioxidant enzymes, participate in the process of the oxidative stress (Aebi, 1984; Marklund and Marklund, 1974). Our results demonstrated that TG decreased the liver activities (U/mg protein) of SOD (68.5 ± 3.3) and CAT (130.2 ± 6.3) in mice while GP (40, 80 mg/kg) and bifendate (150 mg/kg) reversed such remarkable decrease (Figs. 3C, D). These results indicate that the main anti-
Geniposide attenuates TG-induced liver injury

Fig. 2. Effect of GP on the hepatic MDA and glutathione levels in mice. Data are presented as mean ± S.E.M. \((n = 10)\). Significant differences compared with the normal (non-TG treated) group were designated as \(^1P < 0.05\) and \(^2P < 0.01\) and with the control (TG-treated alone) as \(^3P < 0.05\) and \(^4P < 0.01\).

Fig. 3. Effect of GP on hepatic glutathione-related and antioxidant enzymes activities. Data are presented as mean ± S.E.M. \((n = 10)\). Significant differences compared with the normal (non-TG treated) group were designated as \(^1P < 0.05\) and \(^2P < 0.01\) and with the control (TG-treated alone) as \(^3P < 0.05\) and \(^4P < 0.01\).
oxidant enzymes might participate in the protection of GP and bifendate both against TG-induced liver oxidative stress injury.

**GP inhibited TNF-α and IL-10 levels of hepatic inflammatory mediators**

TNF-α and IL-10, as intracellular inflammatory mediators, participate in liver injury (Tilg et al., 2006). Our results demonstrated that TG significantly elevated the hepatic pro-inflammatory cytokine TNF-α and reduced anti-inflammatory cytokine IL-10 levels in mice while GP (40, 80 mg/kg) and bifendate (150 mg/kg) reversed such abnormal levels of inflammatory mediators (Figs. 4A, B). These results indicated that the anti-inflammatory reactions might be involved in the protection of GP and bifendate both against TG-induced liver injury.

**GP enhanced hepatic TGF-β1 mRNA expression**

Cytokine TGF-β1 can promote tissue repair and regeneration (Breitkopf et al., 2006). Our results demonstrated that TG significantly down-regulated the hepatic TGF-β1 mRNA expression in mice while GP (40, 80 mg/kg) and bifendate (150 mg/kg) reversed such remarkable down-regulation (Fig. 5), indicating that GP may probably promote liver tissue repair under TG-induced acute injury conditions.

**DISCUSSION**

ALT and AST are commonly used as sensitive and reliable biomarkers for liver function (Kamei et al., 1986). AST is distributed in the liver, cardiac muscle, skeletal muscle, kidney, pancreas, lung, leukocytes, and erythrocytes, whereas ALT mainly presents in the liver (Rej, 1978). The significant elevation of serum ALT and/or AST indicates increased permeability and damage and/or necrosis of hepatocytes (Goldberg and Watts, 1965). Consistent with previous reports (Peng et al., 2003; Wang et al., 2013), TG markedly caused liver injury in mice with increases in the serum levels of ALT and AST in the present study. Nevertheless, pre-administration with GP and bifendate for a week reversed these changes, suggesting that GP and bifendate protected mice against TG-induced liver injury. Meanwhile, there were no significant
differences in ALT and AST activities between GP (80 mg/kg) and bifendate (150 mg/kg) for 1-week administration, suggesting the equivalent effects between GP and the positive drug bifendate at the corresponding doses.

Hepatic cellular oxidative stress exists in the imbalance of oxidants and antioxidants, and whereas the levels of many antioxidant-related enzymes and non-enzymatic antioxidants may be changed during the pathogenesis of TG-induced liver injury (Peng et al., 2003; Wang et al., 2013). Among them, LPO is a free radical-mediated process (Romero et al., 1998). MDA, as one of the main end products of LPO, has the characterization of cross-linking cellular macromolecules including protein or DNA, and generates widespread cellular damage (Hassan et al., 2005). The results in Fig. 2A show that GP significantly reversed excessive level of MDA induced by TG, suggesting that GP can inhibit TG-induced hepatic LPO injury in mice.

SOD and CAT play important roles in the cellular enzymatic defenses against oxidative stress-mediated injury. SOD, as a metalloenzyme, can convert O$_2$ generated in the process of the oxidative stress to hydrogen peroxide (Marklund and Marklund, 1974). CAT is mainly distributed in all the aerobic cells peroxisomes and helps to protect the cells against the damage of hydrogen peroxide via catalyzing it into molecular oxygen and water without forming toxic free radicals (Aebi, 1984). Our results demonstrated that GP (40 mg/kg and 80 mg/kg) and bifendate (150 mg/kg) remarkably reversed the TG-induced decreased activities of SOD and CAT, suggesting that GP and bifendate could inhibit the TG-induced hepatic oxidative stress injury, while SOD and CAT involved in the protection of GP against TG-induced hepatic oxidative injury. Meanwhile, there were no significant differences in SOD and CAT activities among GP (40 mg/kg), GP (80 mg/kg) and bifendate (150 mg/kg) for 1-week administration, suggesting the equivalent antioxidant effects between GP and the positive drug bifendate.

Glutathione, non-enzymatic antioxidants, is important in protecting hepatocytes against exogenous toxins, and depletion of cellular glutathione is associated with oxidative injury (Carbonell et al., 2000; Han et al., 2006). Our results showed that GP remarkably inhibited the glutathione excessive exhaustion induced by TG, indicating that glutathione could be involved in the protection of GP against TG-induced hepatic oxidative injury.

GST and GPx are cellular glutathione-related antioxidant enzymes. Of them, the cytosolic GSTs exist in almost all aerobic species. It can catalyze the conjugation of electrophilic compounds formed during the oxidative stress with glutathione (Habig and Jakoby, 1981). GPx can catalyze hydrogen peroxide decomposition to the stable form of hydroxides, specifically using reduced glutathione as the electron provider (Rotruck et al., 1973). In this research, GP (80 mg/kg) and bifendate (150 mg/kg) significantly elevated the TG-induced decreased activities of hepatic GST and GPx in mice, which further confirmed that hepatic glutathione-related antioxidant enzymes were
involved in the protection of GP and bifendate against TG-induced hepatic oxidative injury. Meanwhile, there were no significant differences in GST and GPx activities between GP (80 mg/kg) and bifendate (150 mg/kg) for 1-week administration, suggesting the equivalent antioxidant effects between GP and bifendate at the corresponding doses.

Cytokines are pleiotropic, regulatory peptides that can be produced by virtually every nucleated cell in the body, including all types of liver cells (Tilg et al., 2006). The cytokine family consists of several subfamilies such as the interleukins (ILs), the tumor necrosis factor (TNF) family, chemokines such as transforming growth factor-β (TGF-β), colony-stimulating factors (CSFs) and others (Tilg et al., 2006). Among the various cytokines, at least two different cytokines from different cytokine families, namely the pro-inflammatory molecule TNF-α and the anti-inflammatory cytokine IL-10, have emerged as key factors in various aspects of liver diseases (Tilg et al., 2006). In this study, GP significantly reversed the TG-induced increased TNF-α and decreased IL-10 level in mice, indicating that pro- and anti-inflammatory cytokines could be involved in the protection of GP against TG-induced liver injury.

There is increasing evidence supporting a major role for several cytokines in various aspects of inflammatory liver diseases and liver tissue repair (Tilg et al., 2006). Cytokines are proximal mediators of hepatic inflammation, liver-cell death, cholestasis and fibrosis, but paradoxically also mediate regeneration of the liver after injury (Tilg et al., 2006). Among cytokines, TGF-β1 can promote tissue repair and regeneration (Breitkopf et al., 2006). In the current study, GP markedly reversed the TG-induced down-regulated TGF-β1 mRNA expression in mice hepatic tissues while GP (80 mg/kg) and bifendate (150 mg/kg), reversed such remarkable down-regulation, indicating that GP may promote liver tissue repair under TG-induced acute injury conditions.

One limitation to our study was the lack of histopathology of the liver. The data for TGF-β1 mRNA expression changes are deemed insufficient to demonstrate the effect of GP on promotion of repair of liver tissue damage induced by TG. Another limitation to our study was the lack of determination of TGF-β1 level and other gene expression related to tissue repair such as collagen synthesis and degradation in mice liver after treatment with TG and GP. The effects of GP on TG-induced liver changes may be not attributed to the disturbance of TG absorption from gut, because several reports suggest that oral administrations of GP induce diarrhea in mice or rats (Yamano et al., 1988; Yamauchi et al., 1976).

In conclusion, this research indicates that GP has the protective effect against TG-induced liver injury in mice probably via during the antioxidant stress, anti-inflammation, and promoting tissue repair and regeneration. Further studies are in progress in our laboratory to explore the influence of GP on bioactivities of TG, such as anti-inflammation, anti-rheumatoid arthritis, and anti-tumor.

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Conflict of interest—- The authors declare that there is no conflict of interest.

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Geniposide attenuates TG-induced liver injury


