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Original Article

Hepatoprotective effects of Ger-Gen-Chyn-Lian-Tang in thioacetamide-induced fibrosis in mice

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Abstract

Background: Many researchers have focused on developing traditional herbal medicines as pharmacological medicines to treat hepatic fibrosis. In this study, we evaluated the possible mechanism of Ger-Gen-Chyn-Lian-Tang (GGCLT) on thioacetamide (TAA)-induced hepatic injury in mice.
Methods: Hepatic fibrosis mice were established by intraperitoneal injection with TAA (100 mg/kg, 3 times/week), and treated with daily oral administration of 30 mg/kg, 100 mg/kg, and 300 mg/kg of GGCLT for 6 weeks. There were 40 mice randomly assigned to control, TAA and TAA+GGCLT groups. When the experiment was completed, Masson's trichrome staining was used to measure the degree of liver fibrosis. Hepatic fibrosis molecules were assessed by Western blot and real-time polymerase chain reaction. Hepatic glutathione levels, matrix metalloproteinase (MMP-2 and MMP-9), and hydroxyproline were also measured.
Results: Treatment with GGCLT significantly reduced the toxicity of TAA and exhibited effective hepatoprotective activity. The mechanism of the hepatoprotective effect of GGCLT is proposed to be by normalizing oxidative stress. Additionally, the data of fibrotic areas, expression of procollagen III, and MMP2 and 9 mRNA levels in the TAA+GGCLT group were much lower than those in the TAA group ($p < 0.05$). Furthermore, the upregulation of hepatic protein levels of nuclear factor-κB, transforming growth factor (TGF)-β receptor-1, and smooth muscle α-actin induced by TAA was significantly inhibited after GGCLT treatment.
Conclusion: GGCLT can efficiently ameliorate hepatic fibrosis by its inhibitory effects on the intrahepatic oxidative stress in TAA mice model. The antioxidant properties afforded by GGCLT may be attributed to its modulation on TGF-β/TGFβ receptor signaling through the down-regulation of integrated signal pathways involving smooth muscle α-actin and lipid peroxidation.

Keywords: fibrosis; herbal medicines; oxidative stress

1. Introduction

Liver fibrosis represents the consequences of a sustained wound healing response to various chronic liver injuries including viral, autoimmune, drug-induced, cholestatic, and metabolic diseases.[1] This process is associated with the increased expression of contractile filaments such as smooth muscle α-actin (α-SMA) and production of extracellular

Conflicts of interest: The authors declare that there are no conflicts of interest related to the subject matter or materials discussed in this article.

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matrix, and a substantial production of profibrogenic factors such as cytokines and reactive oxygen species.[2] During hepatic fibrogenesis, transforming growth factor (TGF)-β1 plays a pivotal role in the progression of liver fibrosis by promoting the transdifferentiation and migration of hepatic stellate cells (HSCs).[3,4]

Although the modern medicinal system has undergone phenomenal development, discovering a new drug for treating liver diseases remains a distant reality. Therefore, a number of therapeutic plants are used in the traditional system of medicine for the management of liver disorders. However, many of them have not been formally investigated for their efficacy and possible side effects. Ger-Gen-Chyn-Lian-Tang (GGCLT) is a traditional extract mixture of four Chinese medicine herbs, consisting of Puerariae Radix, Coptidis Rhizoma, Scutellariae Radix, and Glycyrrhizae Radix. GGCLT is used to control inflammatory response,[5] and the actions partly by the major constituents of GGCLT. Puerarin, derived from *Pueraria lobata,*[6–8] baicalin, an active component of *Scutellaria baicalensis*,[9–11] berberine, the major alkaloid of the *Coptis chinensis*,[12,13] and glycyrrhizin from *Glycyrrhiza uralensis*,[14,15] all exhibit antioxidant properties and have been shown to ameliorate hepatic injury in earlier studies.[8–11,13–15]

Alternative medicines for chronic liver disease, mostly herbal preparations from single or multiple plants, can be traced back through ancient China and hold promise for the development of cheap and potentially low-risk natural drugs. Compared to many studies currently focusing on the discovery of therapeutic pure compounds, the application of mixed-type Chinese medicine in treating liver disease is still limited and requires more extensive investigation. In this study, we address whether mix product GGCLT could have a beneficial effect in hepatic fibrosis. GGCLT is an officially approved standardized remedy available in Taiwan and widely used as a health medicine in Chinese society. Thus, identifying new therapeutic uses and effects of GGCLT would accelerate its beneficial use in the larger society. To address this issue, the antioxidant property of GGCLT was evaluated in TAA-induced hepatic injury in mice.

2. Methods

2.1. Preparation of GGCLT

GGCLT powder consists of crude ingredients extracted from the following four medicinal herbs mixed in the ratio in parenthesis: Puerariae Radix (roots of *Pueraria lobata*, Ge Gen), Scutellariae Radix (roots of *Scullellaria baicalensis,* Huang Qin), Coptidis Rhizoma (rhizomes of *Coptis chinensis*, Huang Lian), and Glycyrrhizae Radix (roots of *Glycyrrhiza uralensis*, Gan Cao) with a ratio of 8:3:3:2 in weight. The GGCLT was prepared by boiling the dried powder with distilled water for 2 hours at 80°C. The resulting extract was filtered and lyophilized (VirTis Freezemobile, Gardiner, NY, USA) to a light-brownish residue with an approximate yield of 12.5% (w/w). These substances were freeze-dried, and kept at −20°C. The dried extract was dissolved in distilled water before use. The high-performance liquid chromatography (HPLC) chromatogram of three reference standards was shown in HPLC profiles of GGCLT injection (Fig. 1). The four main index components of the GGCLT decoction were recognized by HPLC. The analytic column was Cosmosil C18

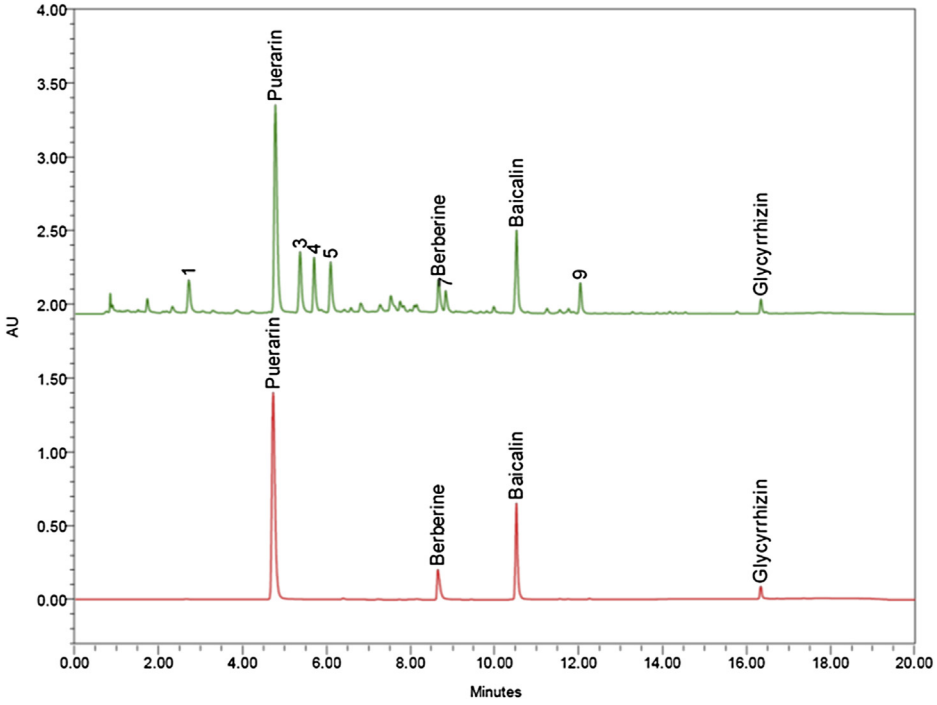

Fig. 1. The chromatogram of four reference standards was shown in high-performance liquid chromatography-UV profiles of raw material of Ger-Gen-Chyn-Lian-Tang injection. Four main index components were recognized by comparing the retention times and UV spectra with standards of compound (lower). The peaks are puerarin, berberine, baicalin, and glycyrrhizin, which represented Puerariae Radix, Scutellariae Radix, Coptidis Rhizoma, and Glycyrrhizae Radix, respectively.

(250 mm × 4.6 mm i.d.). The HPLC mobile phase was a mixture of 0.03% phosphoric acid-water-acetonitrile. The flow rate was 1.0 mL/minute, 20 μL of solution was injected into HPLC system for analysis. Puerarin, berberine, baicalin, and glycyrrhizin are represented by Puerariae Radix, Scutellariae Radix, Coptidis Rhizoma, and Glycyrrhizae Radix, respectively (Fig. 1).

2.2. Animals and experimental designs

Peritoneal injection of TAA (Sigma Chemical Co., St. Louis, MO, USA) was employed to induce liver injury (100 mg/kg every 2 days for 6 weeks). Male C57BL/6 mice were purchased from the *National Laboratory Animal Center (Taipei, Taiwan)*. All animals were housed in a controlled environment and allowed free access to food and water. The animal studies were approved by the Animal Experiment Committee of Chang Gung University, Taoyuan, Taiwan (IACUC Approval No.: CGU12-135) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Mice were randomized into three groups: control, TAA, and TAA+GGCLT groups (8 animals in each group). The TAA+GGCLT group received TAA plus GGCLT (30 mg/kg/day, 100 mg/kg/day, or 300 mg/kg/day) by gastric gavage from the initiation of TAA administration. The TAA group received TAA plus normal saline administration. The mice in each group were sacrificed under anesthesia at the end of the experiments. Portions of liver tissues were fixed in 10% neutral buffered paraformaldehyde for histopathologic and immunohistochemical examinations, or immediately frozen in liquid nitrogen and stored at −80°C for further RNA and protein analysis. Serum was collected and stored at −20°C until analysis.

2.3. Histopathology assay and biochemical measurement

The liver tissue was fixed in 10% formalin and then embedded in paraffin, cut into 5-μm thick sections, and stained with Masson's trichrome, and then examined under light microscopy by an experienced pathologist. Then, blood was obtained for serum biochemical analysis, and the activities of alanine aminotransferase (ALT) were measured using an Auto Dry Chemistry Analyzer (Hitachi 736-60, Tokyo, Japan).

The liver homogenate for lipid peroxidation was prepared with 2 mL of 50mM potassium phosphate buffer, pH 7.4, and thiobarbituric acid-reactive substances (TBARS) were determined.[16] The fluorescence of the samples was detected at an excitation wavelength of 515 nm and an emission wavelength of 555 nm in a F4500 fluorescence spectrophotometer (Hitachi, Japan). 1,1,3,3-Tetramethoxypropane was used as the TBARS standard. Results were expressed in nmol/mg protein, and protein concentrations were determined by the method of Lowry et al.[17]

Hepatic hydroxyproline content was measured using a modified version of the method of Jamall et al.[18] Briefly, liver samples were homogenized and hydrolyzed in 6N HCl at 110°C for 18 hours. After filtration of the hydrolysate through a 0.45-mm Millipore Filter (Millipore, Bedford, MA, USA), chloramine T was added to a final concentration of 2.5mM. The mixture was then treated with 410mM paradimethylamino-benzaldehyde and incubated at 60°C for 30 minutes. After cooling to room temperature, the samples were read at 560 nm against a reagent blank which contained the complete system without added tissue. The concentration of hydroxyproline in each sample was determined from a standard curve generated from known quantities of hydroxyproline.

Hepatic levels of glutathione were determined using a glutathione-400 colorimetric assay kit (Calbiochem Co., San Diego, CA, USA) and performed by using a spectrophotometer as in our previous study.[19] Liver tissue was homogenized with 2 mL of 10% (w/v) metaphosphoric acid solution at 4°C. Each sample was then centrifuged at 3000*g* for 10 minutes at 4°C. After each sample was vortexed, 50 μL aliquot of the centrifuged supernatants were added to the assay buffer provided by the manufacturer until the volume was the equivalent of 200 μL. The reaction mixtures were incubated at 25°C for 30 minutes, and were measured by a spectrophotometer at 412 nm. The values of unknown samples were drawn from a standard curve plotted by assaying different known concentrations of glutathione (GSH). The amounts of GSH were expressed as nmol/mg protein.

2.4. Western blotting

The liver tissue was lysed with distilled water containing protease inhibitors (BD Pharmingen, San Jose, CA, USA), and a Bio-Rad (Hercules, CA, USA) rapid Coomassie kit was used to determine the total protein concentration. Samples of 60 μg protein were run on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel followed by Western blotting with various mouse or rabbit monoclonal antibodies TGFβ-R1, TGFβ-R2, and α-SMA from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA) or Histone H1 and antinuclear factor(NF)-κB antibody (Chemicon, Temecula, CA, USA). Chemiluminescence (ECL; Amersham, Piscataway, NJ, USA) in conjunction with video densitometry was used to quantify protein expression.

2.5. Real-time polymerase chain reaction analysis

Total RNA was extracted from the hepatic tissue using the guanidinium-phenol- chloroform method. Total RNA (5 μg) was reverse-transcribed using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The cDNA was amplified using the TaqDNA polymerase kit (Fermentas, Vilnius, Lithuania). Real-time polymerase chain reaction was performed on a LightCycler 1.5 apparatus (Roche Diagnostics GmbH, Basel, Switzerland) using the LightCycler FastStart DNA MasterPLUS SYBR-Green I kit according to the manufacturer's protocol. cDNA synthesis was achieved with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Commercially available primers were purchased from

Purigo Biotech, Inc. (Taipei, Taiwan R.O.C.) The primer pairs used were are as follows. TGF-β: forward—TGCCC TCTACAACCAACACAACCCG, reversed—AACTGCTCC ACCTTGGGCTTGCGAC; MMP-2: forward—GCTGATAC TGACACTGGTACTG, reverse—CAATCTTTTCTGGGAGC TC; MMP-9: forward—CGTCGTGATCCCCACTTACT, reverse—AGAGTACTGCTTGCCCAGGA; procollagen-3: forward—GGTGGTTTTCAGTTCAGCTATGG, reverse—CT GGAAAGAAGTCTGAGGAATGC.

2.6. Statistical analysis

The results are expressed as mean ± standard error of the mean. Quantitative variables were tested with analysis of variance rank Kruskal–Wallis tests. A p value of < 0.05 was considered to be statistically significant (Statsoft Statisica 3.1, Tulsa, OK, USA).

3. Results

3.1. GGCLT improved liver fibrosis

As shown in Fig. 2, TAA treatment mice had abundant fibrosis showing a characteristic pattern of perivenular and periportal deposition of collagen fiber with the development of portal-to-portal septa. However, mice that received GGCLT displayed thinner septa of collagen and more preserved hepatic parenchyma than the mice with untreated TAA. This result was confirmed by the Western blot analysis of α-SMA (Fig. 3B) in which samples from the mice receiving chronic GGCLT treatment showed a significant reduction in the percentage of fibrosis area as compared to samples from TAA mice. The percentage of liver tissue that showed a reduction in the TGFβ-R1 level after GGCLT treatment when compared to the untreated TAA mice is shown in Fig. 3C and D. The fibrosis was also evaluated histologically by visualizing fibers of collagen in sections of liver samples stained with Masson's trichrome (Fig. 2). This histopathological analysis is consistent with the results for α-SMA (Fig. 3B), TGF-βR1 (Fig. 3C), and TGF-βR2 (Fig. 3D) which were evaluated using Western blot analysis. α-SMA and TGF-βR1 were elevated significantly after the TAA treatment but decreased partially but significantly by treatment with GGCLT.

Additionally, we examined the effect of GGCLT on hepatic mRNA expression of TGF-β, a marker of HSC activation. There is procollagen III, a fibril-forming collagen that predominates in chronic liver disease, and MMP-2, a matrix metalloproteinase that degrades collagen and MMP-9. As shown in Fig. 4, TGF-β, procollagen III, MMP-2, and MMP-9 levels were significantly upregulated in TAA mice receiving GGCLT treatment as compared to untreated TAA animals.

3.2. Induction of intracellular glutathione in GGCLT-treated TAA mice

To evaluate the effect of GGCLT on the underlying mechanism of the TAA-induced hepatic damage, the activity of the NF-κB proteins was examined. The expression of NF-κB was significantly decreased by GGCLT treatment in TAA mice (Fig. 3A). Additionally, the induction of lipid peroxidation in TAA mice was dramatically reduced by treatment with GGCLT, whereas the GSH levels also changed. These data indicate that at least the antioxidant effects of GGCLT are involved in the TAA-mediated oxidative stress. As expected, a significantly lower GSH concentration was found in the liver tissue from TAA mice as compared to the normal group (Fig. 5D). GGCLT treatment led to much higher concentrations of total GSH in the liver than those found in TAA mice. Notably, the significant increase in GSH concentration was associated with a decrease in the level of the lipid peroxidation marker TBARS (Fig. 5C). The inhibition of oxidative stress exhibited by GGCLT has been associated with lipid peroxidation inhibition and the subsequent decrease

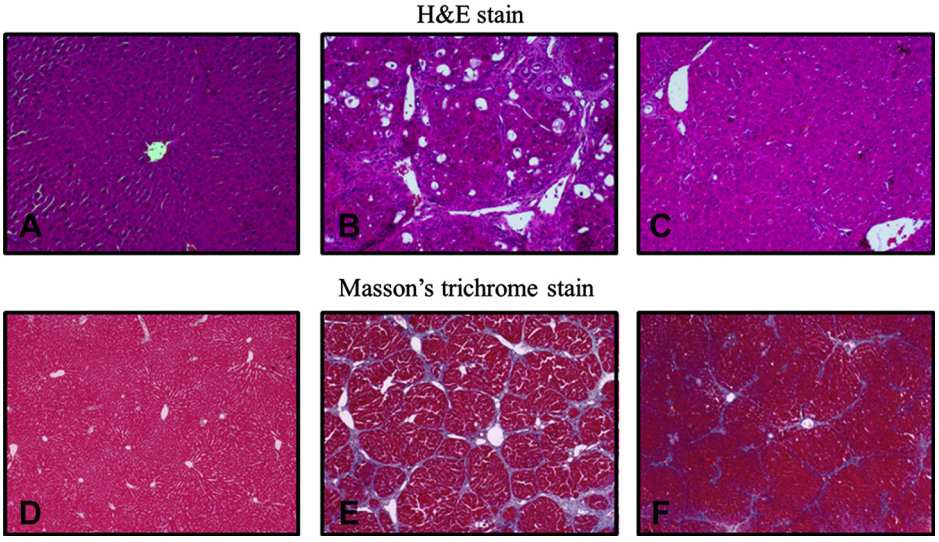

Fig. 2. Light microscopic analysis of mice liver sections of normal mice, and after thioacetamide (TAA) treatment with or without Ger-Gen-Chyn-Lian-Tang administration. (A, D) Normal mouse liver; (B, E) TAA alone; and (C, F) TAA concomitantly treated with Ger-Gen-Chyn-Lian-Tang (300 mg/kg) for 6 weeks. Paraffin embedded sections were stained with Masson's trichrome. The original magnification was 200×. H&E stain = hematoxylin and eosin stain.

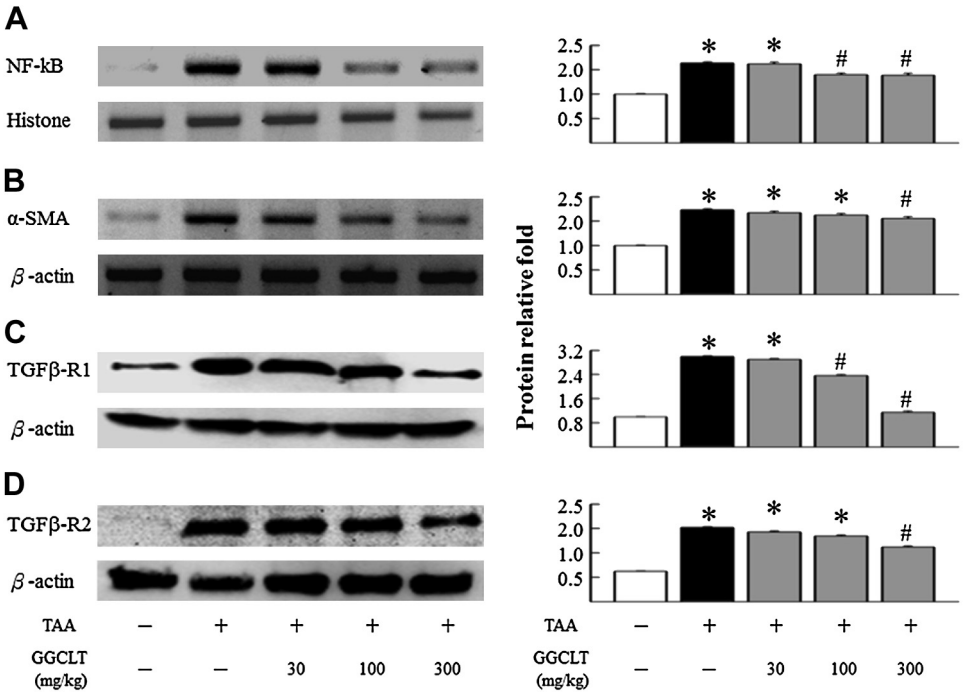

Fig. 3. Western blot analysis of (A) hepatic nuclear factor (NF)-κB, (B) smooth muscle α-actin (α-SMA), (C) transforming growth factor β receptor (TGF-βR)1, and (D) TGF-βR2 protein contents of normal mice, in mice after TAA treatment, with thioacetamide and Ger-Gen-Chyn-Lian-Tang (30 mg/kg, 100 mg/kg, 300 mg/kg) administration after thioacetamide injection for 6 weeks. Liver homogenate fractions (60 μg protein/lane) were analyzed for immunoreactivity with an antibody-recognizing specific antibody. The membranes were also probed with an antibody-recognizing β-actin to assure equal protein loading in the respective lines. The gels are representative of three experiments from separate animals.

of TBARS levels. Finally, In the GGCLT treatment animals, the levels of ALT (Fig. 5A) and hydroxyproline (Fig. 5B) were significantly lower than those of the TAA group ($p < 0.05$).

4. Discussion

Natural Chinese medicine has an extensive history of use in Taiwan and comprises mixed-type ingredients. Compared to many studies focusing on the discovery of therapeutic pure compounds, application of mixed-type Chinese medicine in treating liver injury is still limited and needs more extensive investigation. GGCLT mix product is an officially approved standardized remedy available in Taiwan and widely used as a health medicine in Chinese society. GGCLT is used to control inflammatory response,[5] and this benefit is primarily ascribed to the actions of some major constituent flavonoids in GGCLT. Puerarin,[6–8] baicalin,[9–11] berberine,[12] and glycyrrhizin[14] all exhibit anti-inflammatory activity and were shown to ameliorate tissue damage following inflammation. This includes the prevention of brain infarction, inhibition of ischemic brain injury, alcoholism, and liver steatosis. Moreover, the hepatoprotective properties of puerarin, baicalin, and glycyrrhizin have also been demonstrated.[8–10,14]

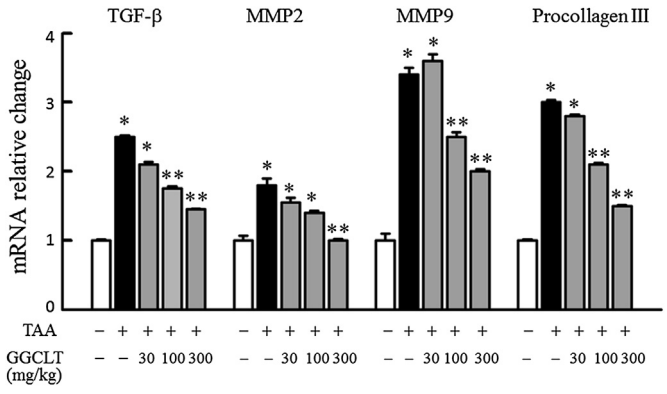

Fig. 4. Ger-Gen-Chyn-Lian-Tang (GGCLT) attenuated hepatic fibrogenesis factors of thioacetamide treatment mice. The effects of GGCLT on (A) transforming growth factor (TGF)-β1, (B) matrix metalloproteinase (MMP)-2, (C) MMP-9, and (D) procollagen III transcription levels. The mRNA levels were analyzed by real-time polymerase chain reaction. * $p < 0.05$ compared with lean control group; ** $p < 0.05$ compared with thioacetamide mice without GGCLT treatment.

The principle findings of this study relate to a potential mechanism of GGCLT that is linked with hepatic fibrogenesis. These data suggest that the mechanistic inhibition of hepatic oxidative stress would attenuate fibrosis in chronic liver disease. The bases of these theories are discussed in the following paragraphs.

4.1. Antifibrotic mechanisms of GGCLT include stimulation of the hepatic antioxidant defenses

The induction of fibrosis by TAA occurs *in vivo* and is believed to involve the generation of oxidative stress,[20–22] and an imbalance between fibrosis and antifibrosis signaling pathways.

Several reports have shown that the oxidative stress associated with lipid peroxidation is involved in the development

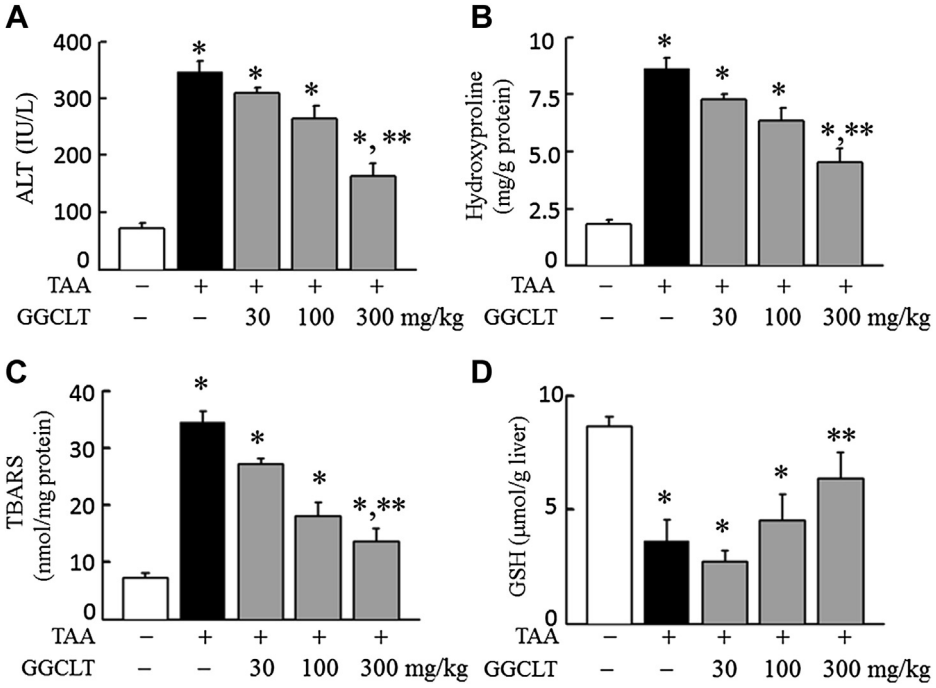

Fig. 5. Ger-Gen-Chyn-Lian-Tang (GGCLT) attenuated oxidative stress and restored glutathione (GSH) in the liver of thioacetamide treated mice. The effects of GGCLT on (A) alanine aminotransferase levels (ALT), (B) hydroxyproline concentrations, (C) thiobarbituric acid reactive substances (TBARS) content, and (D) GSH levels. * $p < 0.05$ compared with control group; ** $p < 0.05$ compared with thioacetamide treatment without GGCLT treatment.

of liver injury in TAA rats.[23–25] The present study indicates that administration of GGCLT reduced the increased levels of plasma ALT after TAA challenge. Additionally, GGCLT decreased the oxidative stress and the decreased levels of TBARS, as compared to the TAA treated mice. In the current study, the levels of TBARS and hydroxyproline were significantly elevated due to TAA, whereas GGCLT treatment markedly reduced these marker levels, which tended to decrease with GGCLT treatment. This also supports the hypothesis that GGCLT ameliorates liver injury through its antioxidant effect. GSH is an essential component of the cellular defense mechanism against oxidative stress that is induced by reactive oxygen species in rats with TAA.[22,26–28] The restoration of hepatic GSH levels by GGCLT treatment could in part be related to its antioxidant and free-radical scavenging effect. Another explanation for this statistically significant increase in the GSH levels seen in the GGCLT treated mice compared with the TAA group is the effect of GGCLT on the enzymes involved in GSH synthesis, whereby GGCLT may help to maintain the levels of glutathione during oxidative stress. Additionally, GGCLT has the ability to elicit hepatoprotective effects by enhanced tissue GSH expression and lower ALT levels, which is consistent with the data showing decreased TBARS levels. Our results suggest that the upregulation of TBARS in the liver may increase hepatic tissue susceptibility to harmful stimuli and may cause the deterioration of liver functions. The change in TBARS expression after GGCLT treatment may account for the attenuation of the cellular functions by ameliorating oxidative stress stimuli in TAA-treated mice.

4.2. Possible mechanism revealed that TGFβ signaling might be involved in the anti-fibrotic action of GGCLT

The aspect of liver injury brought on by TAA is mediated through oxidative stress, which can cause dysfunction of hepatocytes and the discharge of inflammation-related cytokines as NF-κB and IL-6 and fibrogenic mediators as TGF-β1. Intracellular oxidative stress may be a relevant contributor to fibrogenesis, as well as TGF-β expression. However, NF-κB may play an essential role in the activation of HSCs, which bind to the specific regulating sequence of the α-SMA gene. The present study revealed that GGCLT treatment attenuated the level of prominent profibrogenic gene TGF-β1 expression. This indicates the inhibitory activity of GGCLT to the proliferative activity of HSCs, which might be confirmed by reduced collagen deposition in the liver tissues of animals treated with GGCLT. Similarly, the high levels of NF-κB observed in the fibrosis group mice were reversed in the mice treated with GGCLT indicating the anti-inflammatory activity of GGCLT. For the first time, GGCLT has also been shown to inhibit TGF-β levels, probably by its inhibitory action on the NF-κB pathway. Moreover, GGCLT may attenuate HSC activation via the downregulation of TAA-induced α-SMA, and TGF-β signaling activation. The enhancement by GGCLT of GSH production, and the inhibition of MMP-2 and MMP-9 production by HSCs seem to be additional mechanisms of its antifibrotic activity.

Indeed, our results demonstrate that hepatocellular injuries are reduced by the GGCLT treatment of TAA mice, are associated with TGFβ and α-SMA activities, and involve hepatic GSH levels. Our current observations suggest that GGCLT plays

a role in attenuating hepatic fibrosis in the initiation and perpetuation of HSC activation. The expression of two well-characterized and validated markers of the initiation phase of HSC activation, α-SMA and TGF-β, were decreased in GGCLT-treated TAA mice. Likewise, the markers for the perpetuation phase of stellate cell activation, procollagen III and TIMP transcripts, were also diminished in the presence of GGCLT.

The studies have implicated dysregulation of the TGF-β signaling in various liver diseases and their complications (e.g., hepatitis, fibrosis, cirrhosis, and ischemia−reperfusion). This observation raised the possibility that the pharmacological modulation of the hepatic TGF-β signaling could be a valuable therapy in cases of hepatic injury. In the current study, we alternatively investigated whether GGCLT attenuated the activation of hepatic TGF-β signaling receptors in mice with pre-existing fibrosis decreased hepatic collagen abundance and, therefore, reversed fibrosis. Our study reveals a potential, crucial antifibrogenic role of the administration of GGCLT efficiently reducing fibrogenesis, and may open new therapeutic avenues for the treatment of liver fibrosis.

In conclusion, we have demonstrated the antifibrosis effect of GGCLT and its molecular mechanism. GGCLT protects hepatic cells from TAA-induced damage via the amelioration of oxidative stress status. This molecular action suggests that GGCLT may prove useful as a research tool or in clinical applications.

Acknowledgments

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