A traditional formula, Chunggan extract, attenuates thioacetamide-induced hepatofibrosis via GSH system in rats

Kyeong-Gue Kwak¹, Jing-Hua Wang¹, Jang-Woo Shin¹, Dong-Soo Lee² and Chang-Gue Son¹

Abstract
Chunggan extract (CGX) is a hepatotherapeutic herbal formula which has been traditionally used for patients suffering from various hepatic disorders. This study aimed to elucidate antifibrotic effect and mechanisms of CGX in thioacetamide (TAA) model. Hepatic fibrosis was induced in 45 Sprague-Dawley rats by TAA (200 mg kg⁻¹, intraperitoneally [ip]) on twice per week for 12 weeks. CGX (100 or 200 mg kg⁻¹, per oral [po]) was administrated once a day throughout the experiment. CGX treatment ameliorated serum biomarkers. CGX administration significantly attenuated distortion of histopathologic finding, and accumulation of hydroxyproline and malondialdehyde (MDA). CGX treatment significantly decreased transforming growth factor-beta (TGF-β) concentrations and inactivated hepatic stellate cells (HSCs). CGX treatment drastically restored glutathione (GSH) system, while inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF-α) significantly down-regulated in liver tissue. CGX showed antifibrotic effect in thioacetamide-induced chronic liver injury model. Its corresponding mechanisms may be mediated via anti-oxidative stress property sustaining GSH system and inhibition of ROS production.

Keywords
liver fibrosis, glutathione system, oxidative stress, hepatic satellite cells, thioacetamide

Introduction
The process of hepatic fibrosis is a critical step deciding clinical outcome of chronic liver diseases. Liver cirrhosis, end-stage of fibrosis, contribute to leading cause of deaths worldwide, 1.3% of total deaths in 2004 and predicted to be 1.2% in 2015.¹ Any chronic liver injuries including alcoholic disorder, viral hepatitis, biliary obstruction, autoimmune-related biliary disorders, and hemochromatosis consequently can lead to fibrosis.²³ The progress of liver fibrosis and cirrhosis is evidently accompanied with abnormal liver architecture resulting in severe alternation of intra-/extra-hepatic hemodynamic and finally failure of liver function.⁴

It has been commonly accepted that liver cirrhosis is an irreversible disease and none of therapeutic drug has been established to date.⁵ So, realistic strategies are the eradication of the underlying etiology and inhibition of fibrotic progress.⁶ Recently, many interests in herbal medicine have been noticed on hepatoprotective or antifibrotic.⁷⁻⁹ Chunggan extract (CGX) is a herbal drug with 13 medicinal plants, originated from a traditional formula, Chunggan meaning ‘cleaning liver.’ It is prescribed for patients with various chronic liver diseases such as alcoholic liver disorders, chronic viral hepatitis, and liver cirrhosis.¹⁰ Its hepatoprotective effect has been demonstrated in carbon tetrachloride

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DMN-induced hepatic fibrosis is a well-accepted pre-clinical model, owing to quick development of rigorous fibrosis, but failed to mimic the pathogenesis in human body. On the contrary, thioacetamide (TAA) model is believed to closely follow human characteristic in morphological and biochemical aspects.

Therefore, herein, a long-term TAA-induced fibrosis model (12 weeks) was chosen to confirm the antifibrotic effects of CGX and its potential mechanisms.

Materials and methods

Reagents and chemicals

Thioacetamide (TAA) and other reagents, including silymarin, hydroxyproline, \( p \)-dimethylaminobenzaldehyde, 1,1,3,3-tetraethoxypropane (TEP), chloramines-T, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione, glutathione reductase (GSH-red), glutathione peroxidase (GSH-px), and \( \beta \)-nicotinamide adenine dinucleotide phosphate, reduced form (\( \beta \)-NADPH), were obtained from Sigma (St. Louis, Missouri, USA); perchloric acid was obtained from GFS Chemical Co. (Columbus, Ohio, USA); and thio-barbituric acid (TBA) was purchased from Lancaster Co. (Lancashire, England, UK).

Preparation of CGX

The CGX consists of 13 herbs including 5 g each of Artemisiae Capillaris Herba, Trionycis Carapax, Raphani Semen; 3 g each of Atractylodis Macrocephalae Rhizoma, Poria, Alismatis Rhizoma, Atractylodis Rhizoma, Salviae Miltiorrhizae Radix; 2 g each of Polyporus, Amomi Fructus, Aurantii Fructus, and 1 g of Glycyrrhizae Radix and Helenii Radix. All the herbs used in this formulation are fulfilled with the Korean Pharmacopoeia standards. CGX was manufactured by Samik Pharmacy (Seoul, Korea) using the approved Good Manufacturing Practice (GMP) of the Korea Food and Drug Administration (KFDA) according to over-the-counter Korean monographs. Briefly, 120 kg of CGX was boiled in 1200 L of distilled water for 4 hours at 100\(^\circ\)C, and then filtered using a 300-mesh filter. Some part was filtered through filter paper (Advantec, Toyo Roshi Kaisha, Tokyo, Japan) and lyophilized in our laboratory for this study. The final extraction gave a yield of 10.71\%, and the batch used in this experiment was stored for future use (voucher specimen No: CGX-2007-09-WE-SI). The final CGX extract satisfied the criteria of the Korean Pharmaceutical Codex, including the quantity of each herb, contamination by heavy metals, general bacteria, fungi and specific pathogens, and the quantity of ingredients.

Fingerprint of CGX

Fingerprint for CGX was developed using three-dimensional high-performance liquid chromatography (3D-HPLC) profile of five major compositional herbs and their major compounds: Glycyrrhizae Radix, Artemisiae Capillaris Herba, Atractylodis Macrocephalae Rhizoma, Salviae Miltiorrhizae Radix and Ponciri Fructus vs liquiritin and glycyrrhizin, 6,7-dimethoxycoumarin, atractylenolide III, rosmarinic acid, naringin, and poncirin, respectively. Briefly, after dissolution (20 mg of CGX and 0.01 mg of seven standards in 1 mL of water or 50% methanol) and filtration, these drugs were subjected to HPLC analysis. The HPLC system consisted of SCL-10A system controller, LC-10AD pump, SPD-10MVP diode array detector, and CTO-10AS column temperature controller (Shimadzu, Kyoto, Japan). A phenomenon prodigy C18 (2.0 \( \times \) 150 mm) column was eluted with solvents A (10% acetonitrile in water containing 0.05% formic acid) and B (90% acetonitrile in water) at flow rate of 0.4 mL min\(^{-1}\). Solutions 100% A and 0% B changing over 30 min to 25% B, 60 min to 75% B were used. The chromatogram were obtained using wavelength of 220 to 300 nm. Standard components of five herbs were detected and three main peaks and several minor peaks were observed (Figure 1).

Animals and experimental schedule

Forty-five, 6-weeks-old Sprague Dawley (SD) male rats were procured from a commercial animal breeder (Orient Bio, Gyeonggi-do, Korea). After 1 week of acclimation in an environmentally controlled room at 22 \( \pm \) 2\(^\circ\)C with a 12/12-hour light/dark cycle with commercial pellets (Orient Bio) and tap water ad libitum, the rats were divided randomly into five groups of nine animals each: Normal, TAA (TAA only), CGX 100 (TAA with 100 mg kg\(^{-1}\) CGX), CGX 200 (TAA with 200 mg kg\(^{-1}\) CGX), and positive control (TAA with 50 mg kg\(^{-1}\) silymarin).

To induce liver fibrosis, TAA (200 mg kg\(^{-1}\)) was intraperitoneally (ip) injected twice a week for 12 weeks, to four groups except normal group (normal saline, ip). CGX (100 or 200 mg kg\(^{-1}\)), silymarin...
(50 mg kg⁻¹), or distilled water was given by gastric gavage six times per week throughout the experimental period. Body weight was recorded once a week. After last drug administration, animals were fasted for 18 hours, and then blood was collected from abdominal aorta under ether anesthesia. Liver and spleen were removed to determine absolute and relative weight. A portion of liver and spleen tissue stored at −70°C separately were used for hydroxyproline, lipid peroxidation, and antioxidant enzymes determination. Liver tissue fixed in Bouin’s solution was processed for histomorphological finding and immunohistochemistry. A small portion of liver tissue fixed in RNAlater solution was stored at −20°C for gene expression studies.

This animal experiment was approved by the Institutional Animal Care and Use Committee of Daejeon University (DJUARB2009-010) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH).¹⁶

**Serum biochemical analysis**

Serum was prepared following blood clotting. The serum levels of total protein, albumin, total bilirubin, alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) were determined using an Autoanalyzer (Chiron, Emeryville, California, USA).

**Histomorphology and immunohistochemistry for α-smooth muscle actin**

After general processing of tissue treatment, paraplast-embedded liver tissues were sectioned (4 μm thick) and stained with hematoxylin and eosin.
(H&E) or Masson’s trichrome dye for histopathological evaluation.

Immunohistochemistry for α-smooth muscle actin (α-SMA) was also performed to examine the activation of hepatic stellate cells (HSCs). Briefly, liver tissue sections were deparaffinized, hydrated and heated in citrate buffer at 100°C for 15 min, and then treated with normal serum for 30 min. Next, the slides were treated with an anti-α-SMA mouse monoclonal antibody (1:200; Abcam, Cambridge, UK) overnight. After washing three times with PBS, samples were stained with the secondary antibody, N-Histofine Simple Stain MAX PO (Nichirei Biosciences, Tokyo, Japan), and its substrate, diaminobenzidine (BioGenex, San Ramon, California, USA). After counterstaining with Mayer’s hematoxylin (Wako Pure Chemical Industries), the slides were examined under an optical microscope (Leica Microsystems, Wetzlar, Germany).

**Determination of hydroxyproline in liver tissues**

Hydroxyproline determination was performed with a slight modification of a method described previously. Briefly, liver tissues (200 mg) stored at −70°C were homogenized in 2 mL of 6 N HCl and incubated overnight at 110°C. After filtering the acid hydrolyzates using filter paper (Toyo Roshi Kaisha, Tokyo, Japan), 50 μL samples or hydroxyproline standards in 6 N HCl were incubated at 60°C to dry. The dried samples were dissolved with methanol (50 μL); then 1.2 mL of 50% isopropanol and 200 μL of chloramine-T solution were added to each sample, followed by incubation at room temperature for 10 min. Ehrlich’s solution (1.3 mL) was added, and the samples were incubated at 50°C for 90 min. The optical density of the reaction product was read at 558 nm using a spectrophotometer. A standard curve was constructed using serial twofold dilutions of 1 mg solution of hydroxyproline.

**Determination of malondialdehyde in liver tissues**

Lipid peroxidation levels in the liver tissue were determined using the method of TBA reactive substances (TBARS), as described previously. The concentration of TBARS was expressed as μmol per gram tissue using TEP as a standard. Briefly, 200 mg of liver tissue was homogenized in 2 mL of ice-cold 1.15% KCl, and 0.13 mL of homogenate was mixed with 0.08 mL of 1% phosphoric acid and 0.26 mL of 0.67% TBA. After heating the mixture for 45 min at 100°C, 1.03 mL of n-butanol was added, followed by vigorous vortexing and centrifugation at 3000 rpm for 15 min. The absorbance of the upper organic layer was measured at 535 and 520 nm with a spectrophotometer (Cary 50; Varian, Palo Alto, California, USA) and compared to the value from freshly prepared TEP as a standard.

**Determination of catalase, superoxide dismutase, GSH-reductase and GSH-px activity, and total glutathione (GSH) content in liver tissues**

Briefly, 100 mg liver tissue was homogenized with RIPA buffer and centrifuged at 10,000 × g for 15 min at 4°C. The supernatant fraction was transferred into a clean tube, and used to determine the antioxidant enzymes and protein.

Catalase activity in the liver tissue was determined using the method of Beers and Siezer. Bovine erythrocyte SOD (Sigma) was diluted serially from 100 to 0.001 U mL–1 and used as a standard. GSH-red and GSH-px activity in liver tissues were determined with GSH-red assay kit (Sigma), and GSH-px cellular activity assay kit (Sigma), respectively. Total GSH content was determined according to the method of Ellman. Briefly, duplicate 50 μL aliquots of the supernatant (or GSH standard) were combined with 80 μL of a previously prepared DTNB/NADPH mixture (10 μL 4 mM DTNB and 70 μL 0.3 mM NADPH) in a 96-well plate. Finally, 20 μL (0.06 U) of GSH-red solution was added to each well and the absorbance was measured at 405 nm after 5 min.

**Determination the level of transforming growth factor-beta (TGF-β), platelet-derived growth factor-beta, connective tissue growth factor, tissue inhibitor of matrix metalloprotease-1 in liver tissues**

One hundred milligram liver tissue was homogenized with RIPA buffer and centrifuged at 10,000 × g for 15
min at 4°C. The supernatant fraction was used to determine the level of fibrosis-related cytokines. Transforming growth factor-beta (TGF-β) level was measured using ELISA assay kit (Biosource, Camarillo, California), and platelet-derived growth factor-beta (PDGF-β) and tissue inhibitor of matrix metalloprotease-1 (TIMP-1) level were also determined by ELISA assay kit (R&D system, Minneapolis, Minnesota).

Quantitation of tissue connective tissue growth factor (CTGF) was performed using a modification of sandwich ELISA method described previously. Briefly, a 96-well ELISA plate was coated with 100 μL of goat polyclonal anti-rat antibody (Santa Cruz Biotechnology, Germany) at a concentration of 10 μg/mL in PBS and 0.02% sodium azide overnight. After incubation with blocking buffer (PBS, 0.02% sodium azide and 1% bovine serum albumin) and washing (four times), 50 μL of sample or recombinant human CTGF standard was added for 1 hour. Then, 100 μL of rabbit polyclonal anti-goat antibody (2 μg/mL, Santa Cruz Biotechnology, Germany) as first antibody and 50 μL of donkey anti-rabbit IgG-HRP antibody (as 1:2000 dilution, Santa Cruz Biotechnology, Germany) as second antibody were attached. CTGF was quantified by measuring the absorbance at 405 nm after mix substrate solution and stop solution (2 N H2SO4).

Reverse transcription-polymerase chain reaction (RT-PCR) for analyzing gene expression in liver tissue
Liver tissue samples in RNAlater (Ambion, Austin, Texas, USA) were homogenized in TRI reagent (Molecular Research Center, Cincinnati, Ohio, USA), and total RNA was extracted using an RNA Easy column (QIAGEN, Valencia, California). The same amounts of RNA from each sample were mixed and used for complementary DNA (cDNA) synthesis. PCR was performed for 14 genes including the β-actin. The primers sequences were used as follows (forward and reverse, respectively): for β-actin, GTGGGGCGCCCCAGGACCA and CTCCTTAATGTCACGCACGATTTC; for α-SMA, CATCAGGAACCTCGAGAAGC and TCGGATACTTCAGGTCAG; for cytochrome P450 2E1 (CYP2E1), CATGGCTACAAGGCTGTCAA, and TGGCCTTTTGTCTTTTTGAG; for hepatocyte growth factor (HGF), ACACATCTGTGGGGATCAT and TGGTGCTGACTGCATTTCTC; for inducible nitric oxide synthase (iNOS), TGGTGGTGAACATTT and TGTTGCGTTGGAAGTGTA; for PDGF-β, CTGCCTCTCTGCTGCTACCT and GATGAGCTTTCCGACTCGAC; for transforming growth factor beta (TGF-β), CTCCCAGTTCTTCTTCGAGG and TGGGAAGACTTCCCTCAGGTA; for connective tissue growth factor (CTGF), ATGGAGCATGGCCGAAGGC and TTGCATGACAATGACACAGC; for tumor necrosis factor alpha (TNF-α), CTCCCAGTTCTTCTTCGAAGG and TGGGAAGACTTCCCTCAGGTA; for glutathione peroxidase type 1 (GSH-px 1), GAGGCACACTGTCATCCAT and AGCCGAGCAGCAGACATACT; for TIMP-1, CAGGACAGATTACAGGATTAACTGGAAGC and GACACATTGGCCAGGTCTC; for SOD-2, GGCCAAGGGAGATGTTACAA and GAACCTTGGACTCCCACAGA; for SOD-3, CTGTTTGGGAGAGCTTGTC and CCGTTGTTTTCTAGCTCCA.

Statistical analysis
The results are expressed as the mean ± standard deviation (n = 9). Statistical analysis was carried out using Student’s t-test. Difference at the level of p < 0.05 and p < 0.01 was regarded as statistically significant.

Results
Body and organ weights
TAA-treatment considerably inhibited gain of body weight by 50% comparing to normal group. However, CGX treatment significantly attenuated this weight loss when compared with TAA group (p < 0.01 for both 100 and 200 mg of CGX). The relative (but not absolute) weight of liver and spleen was increased by TAA treatment, and 100 mg of CGX treatment significantly reduced the change of relative weight of liver (P < 0.05, Table 1). The above-mentioned observations were similar in silymarin group.

Serum biochemistry
TAA treatment elevated serum levels of AST, ALT, ALP, and total bilirubin by two or three folds than normal group. These elevations were significantly attenuated by CGX administration: serum ALT (p < 0.05, CGX 200), ALP (p < 0.05, CGX 100 and 200), bilirubin (p < 0.05, CGX 200). For AST, the statistical significance was not observed. Total protein was lowered by TAA treatment, but this abnormality was significantly recovered in CGX 200 group (p <
Table 1. Organ weights and serum biochemistry

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normal</th>
<th>TAA only</th>
<th>TAA + CGX100</th>
<th>TAA + CGX200</th>
<th>TAA + silymarin</th>
</tr>
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<tbody>
<tr>
<td>Body mass (g)</td>
<td>545.7 ± 64.8</td>
<td>364.7 ± 16.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>403.2 ± 24.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>396.1 ± 19.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>391.8 ± 30.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver mass (g)</td>
<td>14.58 ± 2.92</td>
<td>14.02 ± 1.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.25 ± 1.51</td>
<td>13.98 ± 1.88</td>
<td>13.97 ± 1.65&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver relative weight (%)</td>
<td>2.7 ± 0.25</td>
<td>3.8 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5 ± 0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.5 ± 0.47</td>
<td>3.5 ± 0.29&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Spleen mass (g)</td>
<td>0.92 ± 0.15</td>
<td>0.94 ± 0.28</td>
<td>0.96 ± 0.11</td>
<td>0.95 ± 0.21</td>
<td>1.04 ± 0.14</td>
</tr>
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<td>Spleen relative weight (%)</td>
<td>0.17 ± 0.03</td>
<td>0.26 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.24 ± 0.02</td>
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<td>0.23 ± 0.10</td>
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<tr>
<td>AST (IU L&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>131.2 ± 17.6</td>
<td>299.0 ± 150.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>242.0 ± 114.4</td>
<td>219.1 ± 64.9</td>
<td>247.4 ± 74.1</td>
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<tr>
<td>ALT (IU L&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>49.1 ± 8.7</td>
<td>114.5 ± 45.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93.3 ± 55.6</td>
<td>72.0 ± 24.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>83.0 ± 40.3</td>
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<td>ALP (IU L&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>217.9 ± 35.5</td>
<td>638.0 ± 202.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>419.4 ± 119.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>421.4 ± 119.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>445.9 ± 146.5&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Bilirubin (g dL&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>0.11 ± 0.02</td>
<td>0.38 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23 ± 0.12</td>
<td>0.20 ± 0.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.21 ± 0.11&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Total protein (g dL&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>6.32 ± 0.17</td>
<td>5.47 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.73 ± 0.34</td>
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<td>Albumin (g dL&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>2.36 ± 0.11</td>
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<td>2.46 ± 0.17</td>
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<td>A/G ratio</td>
<td>0.59 ± 0.03</td>
<td>0.78 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.76 ± 0.05</td>
<td>0.69 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.73 ± 0.05</td>
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Abbreviations: TAA: thioacetamide, AST: aspartate transaminase, ALP: alkaline phosphatase, ALT: alanine transaminase.

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0.01, Table 1). Those improvement patterns were observed partially in silymarin group.

**Histopathological findings**

By visual observation, a severe nodule feature on liver surface was observed in TAA group, whereas it was mild in CGX and silymarin group (data not shown). In TAA group, liver showed features of moderate inflammation, whereas the CGX treatment significantly ameliorated these changes (Figure 2A). In addition, severe hepatic collagen deposition and large septa was observed in TAA group by Masson’s trichrome stain, and then the CGX treatment reduced it significantly (Figure 2B). Immunostain revealed positive signal for α-SMA antibody around collagen septa region in TAA group; however, it was markedly weak in CGX-treated group (Figure 2C). Silymarin treatment showed a similar pattern with CGX group.

**Hydroxyproline and malondialdehyde content in liver tissue**

The concentration of hydroxyproline, as a constituent of collagen, was markedly higher, approximately threefold in TAA group than normal group, CGX treatment significantly reduced it in a dose-dependent manner (p < 0.01; Figure 3A). In addition, CGX treatment significantly reduced malondialdehyde (MDA) that was increased in TAA group (p < 0.05, Figure 3B). Silymarin treatment showed similar effects with 100 mg of CGX treatment.

**Antioxidant enzymes and GSH content in liver tissue**

TAA treatment notably depleted activity of catalase, SOD, GSH-red, and GSH-px in liver tissue, CGX administration ameliorated especially GSH-red and GSH-px with statistical significance (p < 0.05 for CGX 200, Figure 3D–F). Compared with TAA group, GSH content was also significantly normalized by CGX treatment (but not by Silymarin; p < 0.05). Silymarin treatment improved only GSH-px activity.

**TGF-β, PDGF-BB, CTGF and TIMP-1 levels of liver tissue**

In analysis for three main profibrotic cytokines, TAA treatment increased protein level of TGF-β and PDGF-BB. CGX treatment significantly reduced TGF-β comparing to TAA group (p < 0.05, Figure 4A), while no change was observed for PDGF-BB (Figure 4B). CTGF was not changed by either TAA or CGX treatment (Figure 4C). TIMP-1 level in tissue was increased by threefold, and this alteration was significantly reduced by CGX treatment (p < 0.01, Figure 4D). Silymarin treatment showed very similar pattern of effects with CGX treatment.

**Gene expression associated with liver fibrosis**

The pattern of gene expression associated with liver fibrosis was analyzed. TAA treatment remarkably up-regulated gene expressions of α-SMA, iNOS, PDGF-β, TGF-β, CTGF, TNF-α and TIMP-1 while
it significantly down-regulated the gene expression of GSH-px 1. Then, CGX treatment almost completely normalized gene expression of iNOS. CGX treatment also moderately suppressed gene expression of PDGF-β, TGF-β, CTGF and TNF-α. The gene expression of GSH-px 1 was down-regulated by TAA treatment, while it was slightly recovered by administration of CGX. No change was observed for the gene expression of CYP2E1, HGF, SOD-1, SOD-2 and SOD-3 by TAA, or CGX treatment (Figure 5).

**Discussion**

In this study, 12 weeks repeated injection with TAA induced severe histopathologic deterioration including accumulation of extracellular matrix (ECM) in liver tissues, while CGX treatment significantly ameliorated those histopathologic findings (Figure 2A and B). These results were in accordance with serum parameters (Table 1) and hydroxyproline level in liver tissues (Figure 3A). In particular, immunohistochemistry for α-SMA showed a significant inactivation of hepatic stellate cells (HSCs; Figure 2C). It is well known that activated HSCs are corresponding cell to secrete ECM including collagen types I and III.21

To explain the mechanisms of CGX effect on antifibrosis in liver tissues, we investigated the fibrogenesis-specific cytokines and oxidative stress-associated proteins in liver tissues. CGX treatment suppressed the protein levels of TGF-β and TIMP-1 (Figure 4A and D). TGF-β is a major key cytokine leading to activation of HSCs to secrete ECM.22 TIMP-1 is an endogenous inhibitor of metallo-matrix proteinase degrading ECM. Previous reports showed that high expression of TGF-β and TIMP-1 led to substantial accumulation of ECM and liver fibrosis.23,24 Other two cytokines, PDGF-β and CTGF, were not affected by CGX administration, and these findings were also correlated with gene expression pattern (Figure 5).

Most of pathologic condition, for example alcohol over-consumption or virus infection, and imbalance of prooxidant-antioxidant homeostasis due to administration of toxic agents, including TAA and DMN, are well known.14,15 Oxidative stress causes alteration of DNA, protein, and lipids and activates HSCs directly or indirectly; nevertheless, it can be overwhelmed by activation of antioxidant enzymes.25,26 Lipid peroxide represents holistic cellular oxidative stress, antioxidant effect of CGX was confirmed by quantification of the end products of lipid peroxidation, MDA (Figure 3B). Liver tissue has extensive antioxidant enzymes such as SOD and catalase as well as GSH system.27 TAA treatment drastically exhausted
Figure 3. Hydroxyproline, malondialdehyde (MDA) and antioxidant enzyme activities in liver tissues. Nine male Sprague Dawley (SD) rats per group were treated with TAA together with CGX (100, 200 mg kg⁻¹), silymarin (50 mg kg⁻¹), or distilled water. At the end of the experiment, hydroxyproline, MDA, superoxide dismutase (SOD), catalase, glutathione reductase (GSH-red), glutathione peroxidase (GSH-px) and GSH were determined in the liver tissues. #p < 0.05, ##p < 0.01, compared with the normal group, *p < 0.05, **p < 0.01, compared with the thioacetamide (TAA) group.
activities of those enzymes. In particular, CGX administration significantly restored GSH system including GSH-red and GSH-px enzymes and total GSH content in this experiment (Figure 3D/C0).

Interestingly, the first defense of antioxidant enzymes SOD and catalase activity was not affected by CGX (Figure 3C). The pharmacological capacities of CGX in reducing oxidative stress were supported by dramatic downregulation of iNOS and TNF-α gene expression representing inhibition of ROS production and inflammation, respectively (Figure 5).

Chronic hepatic injury commonly leads to liver fibrosis, resulting in development of liver cirrhosis and consequently failure of hepatic function. In spite of poor clinical evidence or conflicting data, many plant-derived drugs such as phyllanthus, silybum marianum (milk thistle), glycyrrhizin (licorice root extract), Sho-saiko-to and LIV-52 are reported to have antifibrotic property. CGX is a traditional herbal remedy that has been used for patients with chronic hepatic disorders since 1991. Our previous studies have demonstrated the safety of CGX and its hepatoprotective and antifibrotic properties in different animal models.

Previously, we have shown that ip administration of DMN resulted in a drastically shrunken liver, decreased liver weight, and spleen enlargement. However, 12 weeks repeated ip injection with TAA

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**Figure 4.** Profibrotic cytokines in liver tissue. Fibrosis-related cytokines (platelet-derived growth factor-beta [PDGF-β], transforming growth factor-beta [TGF-β], connective tissue growth factor [CTGF], and tissue inhibitor of matrix metalloprotease-1 [TIMP-1]) were determined in the liver lysates using ELISA method. ##p < 0.01, compared with the normal group, *p < 0.05, **p < 0.01, compared with the thioacetamide (TAA) group.
drug targeting hepatofibrosis is thought to be TAA model.\textsuperscript{15} Our results strongly emphasize the antifibrotic effect of CGX and its probable mechanism of action.

In summary, CGX has hepatoprotective effects evidenced by inhibition of hepatic inflammation, and exhibits antifibrotic property through inactivation of HSCs in TAA-induced chronic liver fibrosis model. The responsible mechanism may involve reduction of oxidative stress via inhibition of ROS production and maintaining GSH system.

**Conflict of Interest**

The authors declare that there are no conflicts of interest.

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**Reference**


