Protective Effects of *Cuscutae semen* against Dimethylnitrosamine-Induced Acute Liver Injury in Sprague-Dawley Rats

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We investigated the protective effect of *Cuscutae semen* (CS) on acute liver injury induced by dimethylnitrosamine (DMN) in Sprague-Dawley rats. CS is an important traditional herbal medicine widely used as a tonic and aphrodisiac to nourish the liver and kidney and to treat impotence and seminal emission. Rats were given a single intraperitoneal injection of DMN (40 mg/kg), and were then treated with CS daily by oral gavage for 4 d. Immunohistochemical studies for alpha-smooth muscle actin (α-SMA) and proliferating cell nuclear antigen (PCNA) were performed, along with hydroxyproline and biological assay. Liver injury caused by DMN-injection was significantly inhibited in the CS-treated group compared to the silymarin-treated group. The results of blood biological assay were significantly protected by CS in serum total protein (T-protein), T-bilirubin (T-bili), D-bilirubin (D-bili), GOT, GPT, and ALP. The hydroxyproline content and amount of active α-SMA and PCNA were significantly decreased in the CS-treated group than in the silymarin-treated group. CS exhibited an in vivo hepatoprotective effect and anti-fibrogenic effects against DMN-induced acute liver injury and inhibited the formation of hydroxyproline, which suggests that CS may be useful in preventing fibrogenesis after liver injury.

**Key words** acute liver injury; dimethylnitrosamine; alpha-smooth muscle actin; proliferating cell nuclear antigen; hydroxyproline

Today, many chemicals that are inhaled or swallowed can damage the liver. Drug-induced liver injury is the most frequent cause of acute liver failure.

Dimethylnitrosamine (DMN) is a potent hepatotoxin, carcinogen and mutagen. A high dose of DMN is administrated by one injection to the experimental animal, which causes submassive necrosis that resembles human fulminant hepatitis. Therefore this compound has been widely used as a model agent to study the mechanisms of liver injury.

Silymarin, a known standardized extract obtained from seeds of milk thistle (*Silybum marianum*) is widely used in treatment of liver diseases of varying origin. It is composed of a mixture of four isomeric flavonolignans: silibinin, isosilibinin, silydianin and silychristin. Silymarin is widely used for protection against various liver diseases in Europe. It is also reported to offer protection against chemical hepatotoxins such as CCl₄, DMN, and alcoholic liver. Due to its proven hepatoprotective, silymarin is being used as a standard agent for comparison in the evaluation of hepatoprotective effects of CS.

*Cuscutae semen* (CS), the ripe seed of Cuscuta chinensis Lam, belongs to an annual voluble parasitic herb of the family Convolvulaceae. CS is used in traditional herbal medicine to invigorate the kidneys and consolidate kidney essence, nourish the liver, improve eyesight, arrest diarrhea and soothe an unborn fetus. It has been reported to have positive effects on chronic prostatitis, sperm abnormality, gynecological disease, chronic bronchitis, and cancer growth. However, the effects of CS on acute liver injury and fibrosis have not yet been reported.

Thus, we investigated the effects of CS on acute liver injury by biochemical analyses of serum, immunohistochemical analysis of alpha-smooth muscle actin (α-SMA) and proliferating cell nuclear antigen (PCNA) and hydroxyproline assay, using the DMN-induced liver injury model.

**MATERIAL AND METHODS**

**Animals and Reagents** Animal care and all experimental procedures were conducted in accordance with the Guide for Animal Experiments edited by the Korean Academy of Medical Sciences. Male 6-week-old Sprague-Dawley rats were purchased from Samtako (Seoul, Korea). The rats were housed at 22±2°C in 50±10% humidity with 12 h–12 h light–dark cycle. The rats were given autoclaved diets and water. Prior to the experiments, the rats were allowed to adapt to the laboratory environment for 1 week. DMN and quercetin was purchased from Sigma (St. Louis, MO, U.S.A.). Silymarin, named Legalon 70 (Bukwang Param, Seoul, Korea), was purchased from a drugstore. The mature seeds of CS were purchased from Omni herb (Youngcheon, Korea) and it was powdered.

**Treatment of Rats** The 32 rats were divided into four groups of 8 rats each. The normal group was not treated. The control (−) group was treated with an intraperitoneal injection of DMN (diluted with 0.15 m NaCl) at a dose of 40 mg/kg. The silymarin group was treated with an intraperitoneal injection of DMN at a dose of 40 mg/kg, followed by daily treatment with silymarin (suspended in D.W.) at a dose of 100 mg/kg by oral gavage for 4 d. The CS group was treated with an intraperitoneal injection of DMN (diluted with saline) at a dose of 40 mg/kg, followed by daily treatment CS (suspended in D.W.) at a dose of 1000 mg/kg by oral gavage for 4 d. On the fourth day, all rats in each group were sacrificed under anesthesia with diethyl ether. Blood samples for biochemical analyses were obtained from the right ventricle. The liver and kidney were removed immediately and weighed. The liver specimens were fixed in 10% formaldehyde.
neutral buffered formalin and then dehydrated and paraffin-embedded for histochemical studies.

**The HPLC Analysis of Standard Material for Sample Testing** The extraction of quercetin from CS hot water extract was carried according to pre-treatment method of Woo.\(^\text{(3)}\) About 10.0 g of finely powdered CS sample was accurately weighed, put in a 500 ml round plate bottom flask, and then dissolved in 50 ml of methanol–2 M HCl (HPLC grade, 41, Millinckrodt Baker Inc., Phillipsburg, NJ, U.S.A.) by refluxing for 1 h at 95 °C and then centrifuged for 10 min at 3000 rpm at room temperature (RT). A 10.0 mg of standard material (marker substance), quercetin was accurately weighed and dissolved in absolute alcohol (GR grade, 11, Ducksan Pure Chemicals, Ansan, Korea). The dissolved standard solution was diluted to 0.025, 0.05, and 1.0 mg/ml, after which the standard HPLC chromatogram was obtained.

The relationship between the concentration and the peak-area was measured by minimum square method \((R^2)\) value.

The quantity of standard material, quercetin contained in CS was calculated by following formula:

\[
\text{the amount (mg) of standard material, quercetin} = \left(\frac{\text{the quantitative amount (mg) of standard material, quercetin} \times \text{AT/AS}}{n}\right), (n=3)
\]

Where AT is the peak-area of the test sample containing the standard material, and AS is the peak-area of the standard material. HPLC analysis was carried out with a Waters 2695 Alliance System with a 2996 PDAD detector, and a Waters Empower System (Ver. 5.00, Waters Co., Milford, U.S.A.). The test sample was analyzed by reverse-phased HPLC on a C18 column (XTerra\(^\text{TM}\), 5 µm, ODS, 150 mm×3.9 mm (I.D.), Waters, U.S.A.) with a linear gradient condition of 5% to 95% methanol–acetonitrile \((1:1)\)/H\(_2\)O (containing with 0.1% formic acid) \((v/v)\) at 1 ml/min, with detection performed at UV 215 nm and with injection performed in 10 µl portions.

**Biochemical Analyses of Serum** Serum T-protein, albumin, T-bili, D-bili, GOT, GPT, and ALP were entrusted to SCL (Seoul Clinical Laboratories, Seoul, Korea) for analyses.

**Hydroxyproline Assay** Hydroxyproline content was determined by the Jamall methods as previously reported.\(^\text{(6)}\) Liver tissue (0.3 g) was homogenized in 6 M HCl and hydrolyzed at 110 °C for 18 h. Twenty-five microliter aliquots were dried at 60 °C. The sediment was dissolved in 1.2 ml of 50% isopropanol and incubated with 200 µl of 0.56% chloramine T Solution (Sigma, St. Louis, MO, U.S.A.) in acetate citrate buffer pH 6.0. After incubation for 10 min at RT, 1 ml of Ehrlich’s reagent was added and the mixture was incubated at 50 °C for 90 min. After cooling, the absorbance was measured at 558 nm (BECMAN, DUS30, U.S.A.).

**Immunohistochemistry** Four-micrometer liver sections were deparaffinized. For the immunohistochemical analysis, sections for PCNA were immersed in 0.1 M citric acid buffer (pH 6.0) and boiled for 10 min, and then the slides were rinsed gently with D.W. Endogenous peroxidases of the samples for PCNA and α-SMA were blocked by incubation in 3% hydrogen peroxide in methanol for 30 min. The samples were washed three times with TBS and blocked for 10 min at RT with 10% normal horse serum (VECTOR, Burlingame, CA, U.S.A.) in TBS. After washing three times with TBS, the samples were incubated with 18 h at 4 °C with the appropriate dilution of primary Ab. We used antibodies against mouse α-SMA (diluted 1:200, Neomarker, Fermont, CA, U.S.A.) and mouse PCNA (diluted 1:50, Zymed, San Francisco, CA, U.S.A.). The samples were rinsed three times with TBS and incubated for 30 min at RT with the appropriate dilution of secondary Ab, biotinylated anti-mouse IgG (diluted 1:200, Zymed, San Francisco, CA, U.S.A.). The slides were rinsed with TBS, incubated for 30 min with ABC solution (VECTOR, Burlingame, CA, U.S.A.), and washed with TBS, after which the signal was detected using 3,3-diaminobenzidine (DAB; Sigma, St. Louis, MO, U.S.A.). PCNA and α-SMA were counterstained with hematoxylin and eosin, respectively, and then mounted in Canada balsam. The stained sections were examined using an optical microscope (Olympus, BX51, Japan).

**Imaging Analysis** For the morphometric analysis, we assessed mean value of a smooth muscle actin positive cells and the number of PCNA positive cells in three ocular fields per one specimen as percent area at 40× magnification using an image-analysis system (NIH image 1.62, U.S.A.). Alpha-SMA positive cells were expressed as percentage of total area of the specimen, and PCNA positive cells as labeling index in specimen.

**Western Blot** Frozen rat livers were homogenized in a lysis buffer containing 20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 10% glycerol, 1% Trixon X-100, 1 mM NaVO\(_4\), and 1 mM PMSF. After centrifugation at 9000×g for 30 min, supernatants were used as whole cell extracts. Protein concentration was measured by Bradford method. Equal amounts of proteins were separated on 10% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were blocked with 5% nonfat skim milk in Tris-buffered saline (TBS) containing 0.5% Tween-20 (TBS) at room temperature for 1 h and then incubated for 16 h at 4 °C with rabbit anti-α SMA (1:250, Neomarker, Fermont, CA, U.S.A.), or anti-PCNA (1:500, Zymed, San Francisco, CA, U.S.A.) antibodies diluted in 5% nonfat milk in TTBS. Horseradish peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was used as a secondary antibody (1:5000–1:10000 dilution in 5% nonfat skim milk in TTBS, for 1 h incubation at room temperature) and the antigen–antibody complexes were visualized by using an ECL Plus kit (Amersham Biosciences, Piscataway, NJ, U.S.A.). Western blot experiments were repeated at least three times with different cell preparations.

**Statistical Analysis** All values were expressed as the mean±standard error. Raw data were subjected to one-way ANOVA followed by Dunnett’s post-hoc comparisons. \(p\) value of <0.05 was considered statistically significant.

**RESULTS**

**The HPLC Analysis of Standard Material for Sample Testing** Quercetins are flavonoids rarely distributed in the plant kingdom, characteristic constituents of CS.\(^\text{(17)}\) From the results of the standard calibration curve, the \(R^2\) value and linear progressive equation of the standard material, quercetin was \(Y=115817800.0X+308839.4\) \((R^2: 0.999991)\), which shows a high linear relationship with that passing through the zero point. The amount (mg) of standard material, quercetin, for quantitative analysis of CS was qualified as 0.546±
0.006 mg/raw material 1 g (0.055% (0.055)/H11006 0.001%) (Fig. 1).

**Gross Manifestation of Liver**  Body and liver weights were decreased, but spleen weights were increased in all groups compared to the normal group. Liver colors of the control (−) group were very dark brown. On the other hand, silymarin- and CS-treated groups were light brown (data not shown).

**Biochemical Analyses of Serum**  The effects of CS on serum parameters in the liver cirrhosis model are shown in Table 1. T-protein, T-bili, D-bili, GOT, GPT and ALP were significantly suppressed by CS treatment, whereas only ALP was decreased in the silymarin-treated group (Table 1).

**Hydroxyproline**  The total collagen in the liver, measured in terms of hydroxyproline content, was significantly decreased in the silymarin-treated (222.70±8.69, p<0.01) and CS-treated (219.67±14.02, p<0.05) groups compared to the control (252.90±6.88) (Fig. 2).

**Immunohistochemistry**  Alpha-SMA expression, an indicator of activated hepatic stellate cells (HSC), was detected by the immunohistochemistry method.18,19) The means of α-SMA positive regions were reduced in CS treated group (0.91±0.11, p<0.001) and silymarin-treated groups (1.65±0.22) compared to control group (1.89±0.21) (Fig. 3).

The number of PCNA immunopositive hepatocytes was reduced in the CS group (144.58±32.09). However, the silymarin-treated group (195.69±54.07) showed no difference compared to the control group (220.21±20.69) (Fig. 4).

**Western Blot** There was no significant difference in the expression of α-SMA between CS-treated group and control

![Fig. 1. HPLC Chromatogram of Standard Material, Quercetin (0.025 mg/ml, Up) and CS (0.1 g/ml, Down)](image1)

![Fig. 2. Effect of CS on the Hydroxyproline (μg/g Liver) Levels in the Liver of DMN-Treated Rats](image2)

![Fig. 3. Immunohistochemistry Alpha-SMA expression](image3)

![Fig. 4. Number of PCNA Immunopositive Hepatocytes](image4)

**Table 1. Effects of CS on Serum Parameters of DMN-Treated Rats**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Control (−)</th>
<th>Silymarin</th>
<th>CS</th>
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<tr>
<td>T-protein (g/dl)</td>
<td>5.71±0.03</td>
<td>3.99±0.05&lt;sup&gt;***&lt;/sup&gt;</td>
<td>4.02±0.07</td>
<td>4.51±0.24*</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.59±0.02</td>
<td>2.52±0.03&lt;sup&gt;***&lt;/sup&gt;</td>
<td>2.53±0.04</td>
<td>2.81±0.16</td>
</tr>
<tr>
<td>T-bilirubin (mg/dl)</td>
<td>0.23±0.02</td>
<td>0.47±0.02&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.54±0.04</td>
<td>0.32±0.04**</td>
</tr>
<tr>
<td>D-bilirubin (mg/dl)</td>
<td>0.19±0.03</td>
<td>0.28±0.02&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.34±0.03</td>
<td>0.16±0.02***</td>
</tr>
<tr>
<td>GOT (IU/l)</td>
<td>156.43±5.11</td>
<td>518.50±22.15&lt;sup&gt;***&lt;/sup&gt;</td>
<td>550.86±39.36</td>
<td>386.43±44.86**</td>
</tr>
<tr>
<td>GPT (IU/l)</td>
<td>54.62±1.51</td>
<td>191.18±8.94&lt;sup&gt;***&lt;/sup&gt;</td>
<td>187.24±11.98</td>
<td>132.00±21.56*</td>
</tr>
<tr>
<td>ALP (IU/l)</td>
<td>461.70±9.94</td>
<td>636.67±12.02&lt;sup&gt;***&lt;/sup&gt;</td>
<td>566.92±20.11***</td>
<td>550.25±36.02*</td>
</tr>
</tbody>
</table>

The values are expressed as means±standard error. <sup>***</sup>p<0.001 vs. normal group, <sup>*</sup>p<0.05, <sup>**</sup>p<0.01, <sup>***</sup>p<0.001 vs. control (−) group.
groups (Fig. 5). PCNA expression was increased significantly in control group and silymarin-treated groups compared with normal group, whereas it was markedly decreased in CS treatment group (Fig. 5).

DISCUSSION

DMN administration causes severe necrosis and the deposition of extracellular matrix macromolecules, particularly collagen, the hallmark of the fibrous deposition in the liver associated with cirrhosis. In our study, high-dosage DMN treatment causes acute liver injury, leading to shrunken livers, accumulation of hydroxyproline and biological change.

This suggests that a high dosage DMN-induced liver injury in rats can be used as a research model for acute liver injury and the early stage of liver fibrosis.

Among the many drugs for liver injury, silymarin is the most clinically popular for patients and is known to have hepatoprotective and anti-fibrotic properties. Therefore we used silymarin as the positive control in this experiment, and observed that CS exhibited greater liver protective effects than silymarin.

CS administration showed restorative effects on serum T-protein, T-bili, D-bili, GOT, GPT and ALT, which were related to the improvement of inflammation and the suppressed concentration of hydroxyproline.

HSCs are regarded as the primary target cells for inflammatory stimuli in the injured liver. HSCs and the derived myofibroblasts play a central role in experimental models of hepatocellular damage and liver fibrosis, as well as in human chronic liver disease. HSCs are activated in early hepatic fibrosis. Activated HSCs express $\alpha$-SMA and procollagen-1 and are the major source of collagens and other matrix proteins that are deposited in fibrosis.
Cell proliferation is thought to play an important role in several steps of the carcinogenic process. The detection of PCNA using immunohistochemical methods is a common way to study the proliferating activity of transformed cells.\textsuperscript{30} Therefore we used the hydroxyproline assay as a tool to examine the change in expression of collagen in liver tissues. CS administration inhibited \(\alpha\)-SMA and PCNA compared to DMN-induced acute liver injury. In particular, \(\alpha\)-SMA was markedly inhibited by CS administration.

Hydroxyproline, which is a major component of the protein collagen, is produced by hydroxylation of the amino acid proline, and helps provide stability to the triple-helical structure of collagen by forming hydrogen bonds.\textsuperscript{32} The alteration of hydroxyproline levels in the liver is considered to be an index of collagen metabolism and provides valuable information about the biochemical and pathologic events of hepatic fibrosis.\textsuperscript{31} Therefore we used the hydroxyproline assay as a method for collagen measure. Our results showed that CS prevented acute liver injury and improved liver function by inhibiting the formation of hydroxyproline. We proposed CS as a possible anti-fibrogenic agent due to its effective suppression of \(\alpha\)-SMA and PCNA.

Considering the above all results, the present study provided evidence that CS inhibits acute liver injury by suppression of biological change, hydroxyproline, \(\alpha\)-SMA and PCNA. In conclusion, CS shows potential as a hepatic agent against acute liver injury and fibrosis.

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REFERENCES