Ameliorative effect of *Ganoderma lucidum* on carbon tetrachloride-induced liver fibrosis in rats

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Abstract

AIM: To investigate the effects of *Reishi mushroom*, *Ganoderma lucidum* extract (GLE), on liver fibrosis induced by carbon tetrachloride (CCl₄) in rats.

METHODS: Rat hepatic fibrosis was induced by CCl₄. Forty Wistar rats were divided randomly into 4 groups: control, CCl₄, and two GLE groups. Except for rats in control group, all rats were administrated orally with CCl₄ (20%, 0.2 mL/100 g body weight) twice a week for 8 weeks. Rats in GLE groups were treated daily with GLE (1 600 or 600 mg/kg) via gastrogavage throughout the whole experimental period. Liver function parameters, such as ALT, AST, albumin, and albumin/globulin (A/G) ratio, spleen weight and hepatic amounts of protein, malondialdehyde (MDA) and hydroxyproline (HP) were determined. Histochemical staining of Sirius red was performed. Expression of transforming growth factor β1 (TGF-β1), methionine adenosyltransferase (MAT1) 1A and MAT2A mRNA were detected by using RT-PCR.

RESULTS: CCl₄ caused liver fibrosis, featuring increase in plasma transaminases, hepatic MDA and HP contents, and spleen weight; and decrease in plasma albumin, A/G ratio and hepatic protein level. Compared with CCl₄ group, GLE (600, 1 600 mg/kg) treatment significantly increased plasma albumin level and A/G ratio (P<0.05) and reduced the hepatic HP content (P<0.01). GLE (1 600 mg/kg) treatment markedly decreased the activities of transaminases (P<0.05), spleen weight (P<0.05) and hepatic MDA content (P<0.05); but increased hepatic protein level (P<0.05). Liver histology in the GLE (1 600 mg/kg)-treated rats was also improved (P<0.01). RT-PCR analysis showed that GLE treatment decreased the expression of TGF-β1 (P<0.05-0.001) and changed the expression of MAT1A (P<0.05-0.01) and MAT2A (P<0.05-0.001).

CONCLUSION: Oral administration of GLE significantly reduces CCl₄-induced hepatic fibrosis in rats, probably by exerting a protective effect against hepatocellular necrosis by its free-radical scavenging ability.

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Key words: Ganoderma lucidum; Carbon tetrachloride; Liver fibrosis


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INTRODUCTION

Reishi mushroom, *Ganoderma lucidum* (Fr.) Krast (Polyporaceae), is a well-known Chinese crude drug used clinically in East Asia. The fruit bodies are used for the treatment of neurasthenia, deficiency fatigue, insomnia, bronchial cough in elderly people and carcinoma[9].

A number of animal studies have indicated that water or ethanol extracts of *G. lucidum* showed protective actions against acute hepatitis in rats or mice[2-4]. Other reports had previously indicated that triterpenoids isolated from *G. lucidum* possessed the protective effect against acute hepatitis caused by CCl₄[5,6]. Furthermore, Park et al.[7] demonstrated that, in rats, polysaccharides extracted from *G. lucidum* could antagonize liver fibrosis caused by biliary obstruction. These results demonstrate that *G. lucidum* possesses a protective effect in the liver.

Liver fibrosis is the common end-stage of most chronic liver disease, regardless of etiology, and its progression leads to cirrhosis and liver cancer[8]. Although the exact mechanisms of pathogenesis in liver cirrhosis are still obscure, the role of free radicals and lipid peroxides has attracted considerable attention[9]. It has been found that metabolism of CCl₄ involves the production of free radicals through its activation by drug-metabolizing enzymes located in the endoplasmic reticulum[10]. CCl₄ is capable of causing liver lipid peroxidation, resulting in liver fibrosis[10].

Data from *in vitro* and *in vivo* studies have indicated that *G. lucidum* has potent antioxidative and radical-scavenging effects[3,12-18], which contribute to hepatoprotection[15-18]. Nevertheless, to our knowledge, no reports have recorded the effect of *G. lucidum* on chronic hepatitis. In the present study, we therefore investigated the effect of extracts...
of G. lucidum on chronic CCl₄-induced liver fibrosis.

**MATERIALS AND METHODS**

**Preparation of test substance**
Crude G. lucidum extract (GLE), which also contains cracked spores of G. lucidum, was obtained from the Taiwan branch of the American company NuSkin Pharmanex. GLE was suspended in distilled water and administered orally to each rat at a volume of 1 mL/100 g body weight. To guarantee reproducibility of pharmacological experiments, we assayed the total triterpene content of GLE.

**Determination of total triterpenes in GLE by HPLC**
GLE (100 mg) was extracted with ethyl acetate and then evaporated to dryness under vacuum. The residue was dissolved in methanol and diluted to 2 mL. The sample solutions were filtered through a 0.45-µm filter before HPLC analysis as follows. HPLC instrument: Waters 2690 separation unit plus Waters 996 PDA; column: Phenomenex Luna C18(2); flow rate: 1.0 mL/min; detection: absorption at 252 nm; gradient solvent system: CH₂CN+0.1% trifluoroacetic acid. The total peak area for a retention time of 8.0-38.0 min was used to calculate total triterpenes. The peak area of ganodermic acid A (Shanghai R&D, Pharmanex) was used as standard. This method showed that the total triterpene content of GLE was over 6%.

**Animals**
Male Wistar rats were obtained from the National Laboratory Animal Breeding and Research Center, National Science Council, and fed with a standard laboratory diet and tap water ad libitum. Experimental animals were housed in an air-conditioned room at 22-25 ºC and a 12 h light/dark cycle. Rats were allowed free access to powdered feed and mains water that was supplied through an automatic watering system. When they reached 250-300 g, forty rats were divided randomly into 4 groups, such as control, model and two GLE treatment groups, according to body weight 1 d before administration of the test substance. All animals received humane care and the study protocols were in compliance with Institutional Guidelines for the use of laboratory animals.

**CCl₄-induced liver fibrosis**
Liver fibrosis was induced by oral administration of 0.2 mL/100 g body weight of CCl₄ (200 mL/L; diluted in olive oil) twice a week for 8 wk. Animals received CCl₄ only (model group), CCl₄ with GLE (600 or 1 600 mg/kg per day) throughout whole experimental period. During CCl₄ administration, the time interval between CCl₄ and GLE administration was at least 5 h to avoid disturbance in absorption of each substance. At the end of the experimental period, rats were sacrificed under ether anesthesia and blood was withdrawn from the abdominal artery. Liver and spleen were quickly removed, weighed after washing with cold normal saline and removing excess moisture. The largest lobe of liver was divided into four parts, which were then used as follows: (1) submerged in 100 mL/L neutral formalin for the preparation of pathological sections; (2) frozen directly in liquid nitrogen for transcript analysis; (3) after weighing, the liver was completely dried at 100 ºC for the determination of collagen content; and (4) remaining samples were stored at -80 ºC as reserves.

**Assessment of liver functions**
Whole blood was centrifuged at 4 700 r/min at 4 ºC for 10 min to separate the plasma. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT), albumin and total protein were determined spectrophotometrically with an automatic analyzer (Cobas Mira; Roche, Rotkreuz, Switzerland) using commercially available kits (Roche Diagnostics).

**Assays for hepatic protein, lipid peroxidation and hydroxyproline**
Livers were homogenized in 9 vols ice-cold 0.15 mol/L KCl and 1.9 mmol/L ethylenediaminetetraacetic acid. Liver protein concentration was measured according to Lowry et al[16] using bovine serum albumin as standard. Lipid peroxidation was measured by the methods of Ohkawa et al[17] using 2-thiobarbituric acid. Lipid peroxidation was expressed as the amount of malondialdehyde/mg protein.

Hydroxyproline determination followed a method designed by Neuman and Logan[18]. After hydrolysis, dried liver tissue was oxidized by H₂O₂ and colored by p-dimethylaminobenzaldehyde; and absorbance was determined at 540 nm. The amount of hydroxyproline was expressed as µg/g tissue.

**RNA extraction and RT-PCR analysis**
Total RNA was isolated from rat livers using the acid guanidium thiocyanate-phenol-chloroform extraction methods, as described by Chomczynski and Sacchi[19]. A total of 5 µg RNA from each liver sample was subjected to reverse transcription (RT) by using MMuLV reverse transcriptase in a 50 µL reaction volume. Aliquots of the reverse transcription mixture were used for amplification by polymerase chain reaction (PCR) of fragments specific to transforming growth factor-β1 (TGF-β1), methionine adenosyltransferase 1A (MAT1A) and MAT2A using the primer pairs listed in (Table 1). The levels of expression of all transcripts were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the same tissue sample. The primer pairs for TGF-β1 and GAPDH were designed by Wolf et al[20]. In addition, the primer pairs for MAT1A and MAT2A were designed using the Primer select program[21]. The identities of the resultant PCR products were confirmed by sequence analysis. The cycling parameters were 30 min at 55 ºC for cDNA first strand synthesis, and 5 min at 95 ºC, 1 min at 55 ºC and 1 min at 72 ºC for 32 cycles in a Perkin Elmer 9700 Gene Amp PCR system. The PCR product was electrophoresed on a 20 g/L agarose gel recorded by Polaroid film, and the bands were quantitated by using densitometry.

**Pathological examinations**
After formalin fixation, tissue samples were sliced, embedded in a standard manner and stained with Sirius red.
red. Fibrosis was graded according to the method of Ruward et al.\(^{[22]}\) as follows: Grade 0 = normal liver; grade 1 = increase of collagen without formation of septa; grade 2 = formation of incomplete septa from portal tract to central vein (septa that do not interconnect with each other); grade 3 = complete but thin septa interconnecting with each other, so as to divide the parenchyma into separate fragments; and grade 4 = as grade 3, except with thick septa (complete cirrhosis). To avoid sampling error, all biopsies were obtained from the same lobe and these semi-quantitative grades were performed by the observer without knowledge of sample treatment.

### Statistical analysis

Data were presented as mean \(\pm\) SD. All other experimental data, except the pathological findings, were analyzed by one-way analysis of variance using the Dunnett’s test. Liver histopathological examination data were analyzed by the Kruskall-Wallis non-parametric test, followed by a Mann-Whitney U-test. A \(P\) value \(< 0.05\) was considered statistically significant.

### RESULTS

#### Body weight and weights of liver and spleen

Treatment with \(\text{CCl}_4\) caused a significant decrease in the body weight of rats as compared with control rats. There were no differences in the body weight of rats in the \(\text{CCl}_4\) alone and \(\text{CCl}_4+\text{GLE}\) groups. The final body weights for the control and \(\text{CCl}_4\)-treated groups were 474.5 ± 26.7 and 290.4 ± 126.3 g, respectively.

#### Effects of \(\text{GLE}\) on plasma AST and ALT activity in \(\text{CCl}_4\)-treated rats

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Dose (mg/kg)</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>71.5 ± 15.6</td>
<td>41.9 ± 1.9</td>
</tr>
<tr>
<td>(\text{CCl}_4+\text{H}_2\text{O})</td>
<td>–</td>
<td>610.8 ± 149.9</td>
<td>464.7 ± 126.7</td>
</tr>
<tr>
<td>(\text{CCl}_4+\text{GLE})</td>
<td>600</td>
<td>648.9 ± 153.8</td>
<td>499.9 ± 112.1</td>
</tr>
<tr>
<td></td>
<td>1600</td>
<td>459.3 ± 105.3</td>
<td>324.7 ± 41.3</td>
</tr>
</tbody>
</table>

\(^aP<0.05\ vs \ \text{CCl}_4+\text{H}_2\text{O}\ \text{group}; \ ^bP<0.001\ vs \text{control group.}\)

### Effects of \(\text{GLE}\) on biochemical parameters

As shown in (Table 3), \(\text{CCl}_4\) treatment resulted in a significant increase in plasma AST and ALT activities as compared to the control group. Oral administration of \(\text{GLE}\) (1 600 mg/kg) significantly reduced the \(\text{CCl}_4\)-induced increase in AST and ALT activities.

The plasma albumin content and A/G ratio in \(\text{CCl}_4\)-treated groups were significantly lower than that in the control group. The \(\text{CCl}_4\)-induced decrease in plasma albumin concentration and A/G ratio were significantly increased following the administration of \(\text{GLE}\) (600 and 1 600 mg/kg; Table 4).

### Hepatic protein, malondialdehyde and hydroxyproline concentrations

\(\text{CCl}_4\)-induced liver fibrosis in rats resulted in a significant decrease in hepatic protein content compared to the control group. \(\text{GLE}\) (1 600 mg/kg) attenuated the decrease of hepatic protein level induced by \(\text{CCl}_4\) (Table 5).

\(\text{CCl}_4\)-induced liver fibrosis in the rats, accompanied by a marked elevation of malondialdehyde and hydroxyproline concentrations. \(\text{GLE}\) (600 and/or 1 600 mg/kg) could lower the increase in hepatic malondialdehyde and hydroxyproline content (Table 5).

### TGF-β1, MAT1A and MAT2A mRNA expression

Fragments specific to TGF-β1, MAT1A and MAT2A were amplified by using RT-PCR (Figure 1). Values from densitometric analysis, after normalization against the corresponding GAPDH transcript, were expressed as the TGF-β1:GAPDH, MAT1A:GAPDH and MAT2A:GAPDH ratios (Table 6). \(\text{CCl}_4\)-treatment could significantly increase the levels of both TGF-β1 and MAT2A. The administration of \(\text{GLE}\) (600 or 1 600 mg/kg) significantly decreased the expression of TGF-β1 and MAT2A mRNA.
MAT2A mRNA. In contrast, the level of MAT1A mRNA was significantly decreased by CCl4 treatment. However, treatment with GLE significantly increased the level of MAT1A mRNA.

Pathological changes
As shown in Figure 2, CCl4-induced liver lesions in rats. Sirius red stain showed clear nodular fibrosis (Figure 2B). Treatment with GLE (1 600 mg/kg) showed a marked improvement in the pathological changes to these tissues (Figure 2C and Table 7).

DISCUSSION
The present study revealed the beneficial effect of GLE in prevention of liver fibrosis induced by CCl4 treatment. An improvement brought about by GLE was also seen in plasma biochemical parameters.

CCl4 treatment caused hepatocellular damage in rats, as indicated by a drastic increase in both plasma ALT and AST levels after CCl4 administration. Rats treated with GLE showed a protection against CCl4-induced hepatotoxicity, with the levels of both plasma AST and ALT being reduced.

It is well known that adenosylmethionine-dependent methylation is central to many biological processes [23]. Methionine adenosyltransferase (MAT) is a key enzyme for liver methionine metabolism, which catalyzes the synthesis of S-adenosylmethionine [24]. In mammalian tissue, three different forms of MAT (MAT I/III and MAT II) have been identified, which are the product of two different genes (MAT1A and MAT2A). MAT1A is primarily restricted to adult liver [25]. MAT2A is high in fetal liver, decays at birth to negligible levels and, in the adult liver, increases during regeneration after partial hepatectomy [26-28]. Thus, in response to liver injury, MAT1A expression is switched off and MAT2A expression is switched on. Consistent with this, the expression of MAT1A was found to be reduced in the livers of rats with chronic CCl4 injury, whereas the expression of MAT2A increased. In this study, we also found that the changes in MAT expression in chronic CCl4-injured rats were reduced by GLE treatment. These results further support the fact that GLE possesses a hepatoprotective effect.

The liver synthesizes not only the protein it needs, but also produces numerous export proteins. Among the latter, plasma albumin is the most important [29]. Export proteins are synthesized on polyribosomes bound to the rough endoplasmic reticulum of the hepatocytes. In contrast, protein destined for intracellular use is synthesized on free polyribosomes rather than bound polyribosomes [29]. In this experiment, CCl4 induced liver fibrosis in rats and it appeared to cause a decrease in both hepatic protein and plasma albumin contents. GLE clearly reduced the decrease in protein content in the liver and albumin content in the plasma; thus it was shown to ameliorate the decline in liver synthesis function caused by CCl4-induced fibrosis.

Immunoglobulin is synthesized by immunocytes and hyperglobulinemia is found in hepatocellular disorders, appearing as an inflammatory reaction of liver [30]. In the present experiments, we observed CCl4-induced chronic liver lesions in rats and also a decrease in A/G ratio. GLE could clearly lessen the decrease in the A/G ratio caused by CCl4, thereby exhibiting suppressive actions on liver

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Table 5 Effect of GLE on hepatic protein, malondialdehyde and hydroxyproline content in CCl4-treated rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Protein (mg/g tissue)</th>
<th>Malondialdehyde (nmol/mg protein)</th>
<th>Hydroxyproline (μg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>186.3 ± 26.4</td>
<td>2.6 ± 0.5</td>
<td>590.0 ± 42.5</td>
</tr>
<tr>
<td>CCl4 + H2O</td>
<td>–</td>
<td>108.7 ± 4.8</td>
<td>4.4 ± 1.1</td>
<td>1201.2 ± 151.7</td>
</tr>
<tr>
<td>CCl4 + GLE 600</td>
<td>600</td>
<td>124.4 ± 10.2</td>
<td>3.8 ± 0.4</td>
<td>1050.4 ± 187.2</td>
</tr>
<tr>
<td>CCl4 + GLE 1600</td>
<td>1600</td>
<td>128.2 ± 13.5</td>
<td>3.4 ± 0.5</td>
<td>877.5 ± 137.9</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01 vs CCl4 + H2O group; ***P<0.001 vs control group.

Table 6 Effect of GLE on the mRNA expression of hepatic TGF-β1, MAT1A and MAT2A

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>TGF-β1/GAPDH DH ratio</th>
<th>MAT1A/GAPDH DH ratio</th>
<th>MAT2A/GAPDH DH ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>0.18 ± 0.03</td>
<td>4.34 ± 0.81</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>CCl4 + H2O</td>
<td>–</td>
<td>22.17 ± 7.20</td>
<td>1.75 ± 0.46</td>
<td>4.95 ± 0.21</td>
</tr>
<tr>
<td>CCl4 + GLE 600</td>
<td>600</td>
<td>16.99 ± 3.26</td>
<td>3.07 ± 1.15</td>
<td>1.68 ± 0.14</td>
</tr>
<tr>
<td>CCl4 + GLE 1600</td>
<td>1600</td>
<td>4.83 ± 0.48</td>
<td>3.83 ± 1.35</td>
<td>0.56 ± 0.03</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01, ***P<0.001 vs CCl4 + H2O group; ****P<0.001 vs control group.

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Figure 1 Effect of GLE on mRNA expression of TGF-β1, MAT1A and MAT2A in hepatic tissue. M: DNA marker

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had been proposed as a major cellular mechanism involved in CCl4 hepatotoxicity[10]. Furthermore, a close relationship has been reported between lipid peroxidation and fibrogenesis in rats, in which fibrosis was induced by CCl4 administration[11]. Our results also confirmed these findings that hepatic lipid peroxidation increases during hepatic fibrogenesis. Moreover, we observed that GLE inhibited CCl4-induced hepatic lipid peroxidation. These results indicated that GLE might inhibit lipid peroxidation and consequently attenuate the development of liver fibrosis. Numerous studies have indicated that G. lucidum extracts are good free-radical scavengers[12–14], suggesting that the ameliorative effects of GLE on liver fibrosis induced by CCl4 are due, at least in part, to its free-radical scavenging ability.

TGF-β1 is a profibrogenic cytokine as it directly stimulates extracellular matrix production by both endothelial and stellate cells[15,16]. Increased levels of TGF-β1 mRNA expression have been found in patients with liver fibrosis as well as in experimental models of liver fibrosis[17,18]. Blockade of TGF-β1 synthesis or signaling is a primary target for the development of antifibrotic approaches, and modern hepatology has facilitated the design of drugs removing this causative agent[19]. In this study, CCl4 treatment increased while GLE treatment significantly reduced TGF-β1 mRNA expression, suggesting that GLE might ameliorate liver fibrosis via reducing TGF-β1 secretion.

In conclusion, oral administration of GLE is effective in the reduction of chronic liver injury, probably via a protective effect against hepatocellular necrosis by its free-radical scavenging ability.

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10. Slater TF, Sawyer BC. The stimulatory effects of carbon tetrachloride and other halogenoalkanes on peroxidative

| Table 7 Effect of GLE on CCl4-induced liver fibrosis in rats |
|---------------------|---------------------|-------|-------|-------|
| Group               | Dose (mg/kg)       | 0    | 1    | 2    |
| Control             | –                  | 0    | 0    | 0    |
| CCl4 + H2O          | –                  | 0    | 0    | 1    |
| CCl4 + GLE          | 600                | 0    | 0    | 3    |
|                     | 1600               | 0    | 0    | 6    |

Figure 2 Sirius red staining of rat liver sections. A: Control; B: CCl4 + H2O, showing micronodular formation and complete septa interconnection with each other; C: CCl4 + GLE (600 mg/kg); D: CCl4 + GLE (1 600 mg/kg), showing a marked reduction in fiber deposition.

It is well known that liver fibrosis is a result of increased collagen synthesis[30]: hydroxyproline is the characteristic component in collagen[31]. The amount of collagen can be reflected by determining hydroxyproline concentration and can be used to express the extent of fibrosis[32]. In this experiment, when CCl4 was administered to induce liver fibrosis, the hydroxyproline contents in liver obviously increased. Interestingly, GLE could reduce hydroxyproline concentration, indicating that it could lessen the actions of hepatic fibrosis caused by CCl4, which was further proved by histopathological inspection.

When the liver is damaged, it can initiate regenerative actions[33], thus increasing the weight of liver. If it was heavily damaged, however, liver fibrosis and cirrhosis appear resulting in liver atrophy[34]. Therefore, the change in weight of liver can not directly predict the pathological process in chronic liver injuries.

Liver fibrosis leads to blockage of blood flow into the liver and causes portal hypertension. It also influences blood flow to the spleen and gives rise to splenomegaly[35]. In this study, CCl4 induced chronic hepatic fibrosis and splenomegaly, but GLE could improve splenomegaly, indicating that GLE possesses actions in ameliorating fibrosis.

Increased free radical production and lipid peroxidation

inflammation caused by CCl4.

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