Filtrate of fermented mycelia from *Antrodia camphorata* reduces liver fibrosis induced by carbon tetrachloride in rats

Wen-Chuan Lin, Shu-Ching Kuo, Wei-Lii Lin, Hsun-Lang Fang, Bor-Chen Wang

AIM: To investigate the effects of filtrate of fermented mycelia from *Antrodia camphorata* (FMAC) on liver fibrosis induced by carbon tetrachloride (CCl₄) in rats.

METHODS: Forty Wistar rats were divided randomly into control group and model group. All model rats were given 200 mL/L CCl₄ (2 mL/Kg, po) twice a week for 8 wk. Four weeks after CCl₄ treatment, thirty model rats were further divided randomly into 3 subgroups: CCl₄ and two FMAC subgroups. Rats in CCl₄ and 2 FMAC subgroups were treated with FMAC 0, 0.5 and 1.0 g/kg, daily via gastrogavage beginning at the eighth week. Spleen weight, blood synthetic markers (albumin and prothrombin time) and hepatic malondialdehyde (MDA) and hydroxyproline (HP) concentrations were determined. Expression of collagen I, tissue inhibitor of metalloproteinases (TIMP)-1 and transforming growth factor β1 (TGF-β1) mRNA were detected by RT-PCR. Histochemical staining of Masson’s trichrome was performed.

RESULTS: CCl₄ caused liver fibrosis, featuring increased prothrombin time, hepatic MDA and HP contents, and spleen weight and decreased plasma albumin level. Compared with CCl₄ subgroup, FMAC subgroup (1 g/kg) significantly decreased the prothrombin time (36.7 ± 7.2 and 25.1 ± 10.2 in CCl₄ and FMAC groups, respectively, P < 0.05) and increased plasma albumin concentration (22.7 ± 1.0 and 30.7 ± 2.5 in CCl₄ and FMAC groups, respectively, P < 0.05). Spleen weight was significantly lower in rats treated with CCl₄ and FMAC (1 g/kg) compared to CCl₄ treated rats only (2.7 ± 0.1 and 2.4 ± 0.2 in CCl₄ and FMAC groups, respectively, P < 0.05). The amounts of hepatic MDA and HP in CCl₄ ± FMAC (1 g/kg) subgroup were also lower than those in CCl₄ subgroup (MDA: 3.9 ± 0.1 and 2.4 ± 0.6 in CCl₄ and CCl₄ + FMAC groups, respectively, P < 0.01; HP: 1730.7 ± 258.0 and 1311.5 ± 238.8 in CCl₄ and CCl₄ + FMAC groups, respectively, P < 0.01). Histologic examinations showed that CCl₄ + FMAC subgroups had thinner or less fibrotic septa than CCl₄ group. RT-PCR analysis indicated that FMAC (1 g/kg) reduced mRNA levels of collagen I, TIMP-1 and TGF-β1 (collagen I: 5.63 ± 2.08 and 1.78 ± 0.48 in CCl₄ and CCl₄ + FMAC groups, respectively, P < 0.01; TIMP-1: 1.70 ± 0.82 and 0.34 ± 0.02 in CCl₄ and CCl₄ + FMAC groups, respectively, P < 0.01; TGF-β1: 38.03 ± 11.9 and 4.26 ± 2.17 in CCl₄ and CCl₄ + FMAC groups, respectively, P < 0.01) in the CCl₄-treated liver.

CONCLUSION: It demonstrates that FMAC can retard the progression of liver fibrosis induced by CCl₄ in rats.

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Key words: *Antrodia camphorata*; Liver fibrosis; Carbon tetrachloride


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INTRODUCTION

*Antrodia camphorata* is a new species of the genus *Antrodia* (Polyporaceae) parasitic in the inner cavity of the endemic species *Cinnamomum kanehirai* Hay. Traditionally, it has been used as a remedy for food-, alcohol-, drug-intoxication, diarrhea, abdominal pain, hypertension, skin itching, and liver cancer among Chinese. The growth rate of natural *A. camphorata* in the wild is very slow, and it is difficult to cultivate in a green house, thus, it is expensive to obtain fruiting bodies. Therefore, using a submerged culture method to obtain useful cellular materials, or to produce effective substances from cultured mycelia might be a possible way to overcome the disadvantage of the retarded growth of fruiting bodies. In Taiwan, several biotechnology companies have developed the submerged culture method for *A. camphorata*. In the market of Taiwan, the yield of mycelia or culture filtrate of fermented mycelia...
Male Wistar rats were obtained from the National Laboratory Animals. The concentration of phenolic groups in FMAC was 39.71 μg/mg. The metabolism of CCl₄ involves the production of free radicals through its activation by drug metabolizing enzymes located in the endoplasmic reticulum. It has been found that the formation of CCl₄ involves the production of free radicals through its activation by drug metabolizing enzymes located in the endoplasmic reticulum. CCl₄ is capable of causing liver lipid peroxidation, resulting in liver fibrosis. Hsiao et al. reported that *A. camphorata* extract exerted protection against chronic chemical-induced hepatic injury in mice. In addition, Song et al. showed that FMAC possessed a protective activity against acute liver injury induced by CCl₄. However, the effect of FMAC in chronic liver disease is still unknown. In the present study, we attempted to assess the effect of FMAC on chronic CCl₄-induced liver fibrosis in rats.

**MATERIALS AND METHODS**

**Preparation of test substance**

FMAC was provided by Food Industry Research & Development Institute, Hsinchu, Taiwan. Culture of *Antrodia camphorata* BCRC 930032 was inoculated onto potato dextrose agar (PDA) and incubated at 30°C for 15 to 20 d. The whole colony was then cut and put into the bottle with 50 mL sterile water. After homogenization, the fragmented mycelia suspension was used as inoculum. The seed culture was prepared in a 20 L fermentor (BioTop) agitated at 150 r/min with aeration rate of 0.2vvm and temperature of 30°C. A 5-d culture of 15 L mycelia inoculum was inoculated into a 250 L agitated fermentor (BioTop). The fermentation condition was the same as the seed fermentation but operating with an aeration rate of 0.075 vvm. The deep red culture filtrate was separated from the broth harvested at the 331st hour and poured through the non-woven fabric on a 20-mesh sieve. FMAC was concentrated about 20 fold (450 g/L) under reduced pressure at 50°C, and stored at -30°C until use. FMAC was suspended in distilled water and administered orally to each rat at a volume of 10 mL/kg body weight.

Since antioxidant and anti-radical properties of plant extracts have been attributed to most phenolic compounds, it is expected that the effectiveness of the extracts is related to their phenolic content. To guarantee the reproducibility of pharmacological experiments, the phenolic compounds in FMAC were determined by a modification of the method of Barness et al. using catechin as the standard. The concentration of phenolic groups in FMAC was 39.71 μg/mg.

**Animals**

Male Wistar rats were obtained from the National Laboratory of Animal Breeding and Research Center, National Science Council, and fed with a standard laboratory chow and tap water ad libitum. The experimental animals were housed in air-conditioned room of 21-24°C with 12 h of light. The rats were allowed free access to powdered feed, and main water that was supplied through an automatic watering system. When they reached 250-300 g, the rats were used for experiments. Rats were divided randomly into control and model groups according to the body weight in proper range one day before administration of the test substance. All animals received humane care and the study protocols were in compliance with our institution’s guidelines for use of laboratory animals.

**CCl₄-induced liver fibrosis**

Fibrosis was induced in thirty rats by an oral administration of 2 mL/kg body weight of 200 mL/L CCl₄ (diluted in olive oil) twice a week for 8 wk. At the end of 4 th wk after CCl₄ treatment, the CCl₄-treated rats were further divided into three groups based on the plasma alanine aminotransferase (ALT) level, since the plasma ALT is the major parameter for liver injury. The plasma ALT levels for normal control and 3 CCl₄-treated subgroups were 675 ± 62, 8856 ± 1321, 9005 ± 1659 and 8208 ± 1324 (nkat/L), respectively. The animals received CCl₄ with distilled water or FMAC (0.5, 1.0 g/kg; po, daily) which was added at the last four wk of the treatment. The time interval between CCl₄ and FMAC administrations were 5 h to avoid the disturbance of absorption of each other. After blood was drawn from rats at the eighth week, the animals were sacrificed at the same time and the liver and spleen were quickly taken off. They were then weighed after being clearly washed with cold normal saline and sucked up of the moisture. The largest lobe of liver was divided into four parts, and the same parts were 1) submerged in 40 g/L neutral formaldehyde for the preparation of pathological sections; 2) after weighed, the liver was completely dried at 100°C for the determination of collagen content; 3) the samples for RT-PCR analysis were kept in liquid nitrogen; 4) other sample was stored at -80°C until assay.

**Assessment of liver functions**

The blood was centrifuged at 4700 r/min (Jouan BR4i, France) at 4°C for 15 min to separate the plasma. The levels of plasma ALT and albumin were assayed using clinical test kits (Roche Diagnostics) spectrophotometrically (Cobas Mira; Roche, Rotkreuz, Switzerland). Prothrombin time was measured using a coagulation analyzer (Sysmex-CA1000) and reagent (Dade thromboplastin C plus).

**Assays of hepatic lipid peroxidation and hydroxyproline**

Livers were homogenized in nine volumes of ice-cold 0.15 mol/L KCl, 1.9 mmol/L ethylenediaminetetraacetic acid. The homogenate was used for the determination of lipid peroxidation. Lipid peroxidation was measured by the methods of Ohkawa et al. using 2-thiobarbituric acid. The lipid peroxidation was expressed as malondialdehyde (MDA) μmol/g protein. Protein was measured by the method of Lowry et al. using bovine serum albumin as the standard. Hydroxyproline (HP) determination fol-
lowed a method designed by Neuman et al.13. Dried liver tissue after hydrolysis was oxidized by H2O2 and colored by p-dimethylaminobenzoaldehyde and absorbance was determined at 540 nm. The amount of HP is expressed in mg/g wet tissue.

**RNA extraction and RT-PCR analysis**

Total RNA was isolated from livers of the rats using the acid guanidium thiocyanate-phenol-chloroform extraction methods as described by Chomczynski et al.14. Five micrograms of total RNA from each liver sample were subjected to reverse transcription (RT) by MMuLV reverse transcriptase in a 50 μL reaction volume. Aliquots of the reverse transcription mix were used for amplification by polymerase chain reaction (PCR) of fragments specific to collagen I, transforming growth factor (TGF)-β1 and tissue inhibitor of matrix metalloproteinase (TIMP)-1 using the primer pairs listed in Table 1. The levels of expression of all the transcripts were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the same tissue sample. PCR product was run on a 20 g/L agarose gel recorded by polarid densitometer.

**Pathological examinations**

For histopathological examination, the formalin-fixed liver was embedded in paraffin, cut into 4-5 μm thick sections, stained with Masson’s trichrome. Fibrosis was graded according to the method of Ruwart et al.15, 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; 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 without knowledge of sample treatment.

**Statistical analysis**

Data were presented as mean ± SD. All other experimental data, except the pathological findings, were treated 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. The significance level was set at P < 0.05.

**RESULTS**

**Concentrations of plasma albumin and prothrombin time**

The plasma albumin concentrations were lower in rats treated by FMAC (1 g/kg) than that in control group (Table 2). While in the rats treated by FMAC (1 g/kg), the levels of plasma albumin was markedly higher than that in the CCl4 model group. The prothrombin time in the CCl4 model group was much longer than that in control group. FMAC (1 g/kg) significantly shortened the prothrombin time.

**Weights of spleen**

Marked splenomegaly was caused by CCl4 treatment; the weight of spleen in the CCl4-treated group was about 245% of the control group (Table 3). The increase of spleen weight by CCl4 treatment was significantly reduced by FMAC (1 g/kg).

**Liver MDA and HP contents**

CCl4 induced liver fibrosis to the rats resulting in a marked increase of hepatic MDA and HP contents (Table 3). FMAC (1 g/kg) treatment significantly reduced the increase of hepatic MDA and HP contents caused by CCl4.

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**Table 1** Primer sequences for PCR amplification

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequence</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>Sense 5' CGA CTA AGT TGG AGG GAA CGG TC 3'</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>Antisense 5' TGG CAT GGT GCT AGG CAC GAC 3'</td>
<td></td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Sense 5' TCC CTT GCA AAG TGG AGA GT 3'</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>Antisense 5' GTC ATC GAG ACC CCA AGG TA 3'</td>
<td></td>
</tr>
<tr>
<td>TGF-b1</td>
<td>Sense 5' TAT AGC AAC AAT TCC TGG CG 3'</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>Antisense 5' TGG TGT CAC AGC AGC AGTG 3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense 5' CTT CAT TGA CCT CAA CTA CAT GGT CTA 3'</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Antisense 5' GATG ACA AGC TTC CCA TTC TCA G 3'</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2** Effect of FMAC on plasma albumin concentration and prothrombin time in CCl4-treated rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (g/kg per d)</th>
<th>Albumin (g/L)</th>
<th>Prothrombin time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>36.0 ± 1.3</td>
<td>17.7 ± 0.9</td>
</tr>
<tr>
<td>CCl4 + H2O</td>
<td>–</td>
<td>22.7 ± 1.0a</td>
<td>36.7 ± 7.2b</td>
</tr>
<tr>
<td>CCl4 + FMAC</td>
<td>0.5</td>
<td>23.1 ± 5.1</td>
<td>28.5 ± 9.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>30.7 ± 2.5c</td>
<td>25.1 ± 10.2c</td>
</tr>
</tbody>
</table>

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

**Table 3** Effect of FMAC on spleen weight, hepatic malondialdehyde and hydroxyproline contents in CCl4-treated rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (g/kg per d)</th>
<th>Spleen (g)</th>
<th>Malondialdehyde (μmol/g tissue)</th>
<th>Hydroxyproline (μmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>1.1 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>645.0 ± 64.5</td>
</tr>
<tr>
<td>CCl4 + H2O</td>
<td>–</td>
<td>2.7 ± 0.1a</td>
<td>3.9 ± 0.1a</td>
<td>1730.7 ± 258.0a</td>
</tr>
<tr>
<td>CCl4 + FMAC</td>
<td>0.5</td>
<td>2.8 ± 0.2</td>
<td>2.7 ± 0.1</td>
<td>1741.5 ± 257.1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.4 ± 0.2</td>
<td>2.4 ± 0.6</td>
<td>1311.5 ± 238.8</td>
</tr>
</tbody>
</table>

*P<0.05, *P<0.01 vs CCl4 + H2O group; *P<0.001 vs control group.
RT-PCR analysis of liver tissue

Fragments specific to collagen I, TIMP-1 and TGF-β1 were amplified by RT-PCR (Figure 1). The values from densitometric analysis, after normalization against the corresponding GAPDH transcript were expressed as the collagen I/GAPDH, TIMP-1/GAPDH and TGF-β1/GAPDH ratios. The levels of collagen I, TIMP-1 and TGF-β1 mRNA in rat liver were significantly increased by CCl4 treatment (Table 4), while the administration of FMAC (1 g/kg) significantly decreased the levels of collagen I, TIMP-1 and TGF-β1 mRNA.

Pathological examination

CCl4 induced liver damage of the rats. Masson’s stain showed clear nodular fibrosis at the central vein and the portal vein area (Figure 2B). Treatment of FMAC (1 g/kg) showed marked improvement of these pathological changes of the tissues (Figure 2C and Table 5).

DISCUSSION

The results of the present study indicate that even after the initiation of hepatic fibrosis in a rat model of CCl4-induced liver damage, FMAC administration reduced liver fibrosis, as demonstrated by smaller increases in hepatic collagen and lower mRNA expression of collagen I compared with CCl4 model group. These effects were mainly observed when FMAC was administered from wk 5 to wk 8 of CCl4 treatment. Both plasma albumin and blood clotting factors were mainly synthesized in the liver. When the chronic liver damage led to fibrosis, the albumin contents dropped and prothrombin time prolonged[10,17]. In this experiment, CCl4 induced chronic liver lesions in rats and there appeared a decrease of plasma albumin and an increase of prothrombin time. FMAC clearly counteracted both the decrease of albumin content in the plasma and the prolongation of prothrombin time. These results showed that FMAC ameliorated the decline of liver synthetic functions caused by chronic liver injuries.

Liver fibrosis or cirrhosis leads to blockage of blood flow into the liver and causes portal hypertension and it also influences the blood flow of spleen and gives rise to splenomegaliia[18]. CCl4 in this experiment induced chronic...
hepatic fibrosis as well as splenomegaly. FMAC could improve splenomegaly, indicating that it might ameliorate portal hypertension.

It is well known that liver fibrosis is a result of increased collagen synthesis[19], and HP is the unique component in collagen[19]. The amount of collagen can be reflected by the contents of HP and can be used to express the extent of fibrosis[19]. When CCl4 was applied in this experiment to induce liver fibrosis, the content of HP in liver obviously increased. FMAC could reduce the content of HP, which was confirmed by the histopathological examinations. Many studies have shown that level of collagen I increases during liver fibrosis[20]. Therefore, we also investigated the effect of FMAC on the mRNA expression of collagen I. Treatment with FMAC was effective in reducing the amount of collagen I mRNA expression. This result further confirmed that FMAC could remit hepatic fibrosis.

Regardless of the etiologic factors, gross remodeling of extracellular matrix in the fibrotic liver is regulated by a balance of synthesis and enzymatic degradation of extracellular matrix[21]. Matrix degradation is catalyzed by the activity of matrix metalloproteinases. The activities of matrix metalloproteinases are inhibited by tissue inhibitors of metalloproteinases (TIMPs). The expression of TIMPs drastically increased or decreased with time during liver fibrogenesis and fibrosis resolution, respectively[22]. Four members of the TIMP family have been characterized so far and designated as TIMP-1 to TIMP-4[23]. It has been suggested that TIMP-1 plays an important role in the pathogenesis of liver fibrosis[24]. Consistent with previously published work[25], we observed elevated levels of TIMP-1 upon treatment with CCl4. Treatment with FMAC was effective in reducing the level of TIMP-1 expression, indicating liver fibrosis resolution might be enhanced. This result supported that FMAC could suppress liver fibrotic progression caused by CCl4.

TGF-β1 is a profibrogenic cytokine, because it directly stimulates extracellular matrix production by both Kupffer cells and stellate cells[26,27]. Increased levels of TGF-β1 mRNA expression have been found in patients with liver fibrosis as well as in experimental models of liver fibrosis[24,28]. Blockade of TGF-β1 signaling, 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[29]. In this experiment, CCl4 treatment increased, while FMAC significantly reduced TGF-β1 mRNA expression. This result suggested that FMAC ameliorated liver fibrosis perhaps by reducing TGF-β1 secretion.

Increased free radical production and lipid peroxidation have been proposed as a major cellular mechanism involved in CCl4 hepatotoxicity[30]. Furthermore, a close relationship has been reported between lipid peroxidation and fibrogenesis in rats, in which fibrosis was induced by CCl4 administration[31]. Our results confirmed these findings that hepatic lipid peroxidation is increased during hepatic fibrogenesis. We also found that FMAC inhibited CCl4-induced hepatic lipid peroxidation. These results indicated that FMAC might inhibit lipid peroxidation, and consequently attenuate the development of liver fibrosis.

A large number of studies indicated that FMAC is a good free radical scavenger[32,33]. In conclusion, the present study has demonstrated that FMAC retards the progression of liver fibrosis in CCl4-treated rats possibly by scavenging free radicals formed in the liver. It may be expected that FMAC has preventive potentials in liver fibrosis.

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