Protective effects of sesamin on liver fibrosis through antioxidative and anti-inflammatory activities in rats

Xiaowei Chen¹, Xiaozhou Ying², Lu Chen³, Weiwei Zhang⁴, and Youcai Zhang³

¹Department of Ultrasound Imaging, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China, ²Department of Orthopaedic Surgery, the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, China, ³Department of Infectious Diseases, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China, and ⁴Department of Infectious Diseases, the Third Affiliated Hospital of Wenzhou Medical University, Wenzhou, China

Abstract

Context: Sesamin (Ses) from Sesamum indicum seeds has potent antioxidants and anti-inflammatory effects.

Objective: This study focused on the antioxidant and anti-inflammatory effects of Ses on Carbon tetrachloride (CCL₄)-induced hepatic fibrosis in experimental rats and the potential mechanism underlying the activation of NF-kB pathway.

Materials and methods: Hepatic fibrosis was induced by interperitoneally (i.p.) administered with 20% CCL₄ in corn oil (2 mL/kg for 8 weeks) in rats. After 8 weeks, activities of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL) were checked. The levels of protein carbonyls and antioxidant enzymes such as superoxide dismutase (SOD) and GSH-Px were determined after Ses administration. H&E and Masson’s trichrome staining for histopathological changes of liver tissues were observed. Western blotting was used to detect expression of IL-6, cyclooxygenase-2 (COX-2), and NF-kB activation. Finally, the levels of hydroxyproline in liver tissues were also determined.

Results: Ses decreased the release of liver enzymes – ALT, AST, and TBIL, reduced protein carbonyls, attenuated the reduction of SOD and GSH-Px activities induced by CCL₄ in the liver tissue. It also significantly reduced the levels IL-6 and COX-2 in the liver caused by CCL₄ by inhibition of NF-kB activation. Histological results indicated that Ses significantly improved the pathological lesions of liver fibrosis.

Conclusions: Ses exerted hepatoprotective effects possibly due to the antioxidant effect and suppressing the NF-kB activation.

Introduction

Liver fibrosis is a wound-healing response to chronic liver injury caused by a variety of etiological factors including viruses, alcohol, and autoimmune disease. Iredale et al.¹ found that liver fibrosis is reversible and an effective treatment would probably reverse the process. Recently, some studies have shown that oxidative stress caused by an imbalance between the oxidant and antioxidant systems of the body should be a major apoptotic stimulus in the different types of acute and chronic liver injury². Carbon tetrachloride (CCL₄) is widely used for animal models of liver injury and liver fibrosis. Some studies have demonstrated that antioxidant agents prevent CCL₄ hepatotoxicity, by inhibiting oxidative and increasing the activities of antioxidant enzymes³.

Hepatic inflammation is regarded as a hallmark of fibrosis, which can progress to cirrhosis. Damage associated with hepatic inflammation is mediated by the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and cyclooxygenase-2 (COX-2)⁴. The chronic liver damage induced by CCL₄ in rats produces liver inflammatory and biochemical patterns that resemble rat liver cirrhosis⁵. Despite efforts to develop anti-fibrotic agents, till date no drug has been approved as an anti-fibrotic agent in human. Therefore, it is urgent to develop an approach for the prevention or treatment of hepatic fibrosis.

Sesame lignans from Sesamum indicum seeds are potent antioxidants and anti-inflammatory effects. Sesamin (Ses) is the most abundant lignan in sesame seed oil⁶. Past studies found that Ses exerts antioxidative⁷, anti-inflammatory⁸, anti-hypertensive¹⁰, cholesterol-lowering¹¹, hepatoprotective⁷,¹²,¹³, neuroprotective effects¹⁴, and chondroprotective effects¹⁵. Nevertheless, the possible protective effect of Ses against CCL₄-induced hepatic fibrosis and the underlying mechanisms remain unclear. Therefore, we currently evaluated the ability of Ses to prevent the development and progression of CCL₄-induced liver fibrosis in rats, which is more similar with human liver fibrosis¹⁶,¹⁷.
**Materials and methods**

**Chemicals and reagents**

Sesamin (≥95% purity), CCl₄, silymarin was obtained from Sigma Chemicals Company (St. Louis, MO). Sesamin was suspended in 0.1% carboxymethyl cellulose (CMC) in this study. Diagnostic kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBIL), superoxide dismutase (SOD) and protein carbonyls were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Protein concentration were measured using the bicinchoninic acid (BCA) assay kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); IL-6 antibody, COX-2 antibody, NF-kB, and IκB-α antibody are from Santa Cruz Biotechnology (Santa Cruz, CA). Corn oil was purchased from the local market, sealed and stored at room temperature after high temperature sterilization.

**Animals and treatment**

Male Sprague-Dawley rats (150–200 g) were obtained from Wenzhou Medical University and were quarantined and allowed to acclimate for a week prior to experimentation. The rats were housed under normal laboratory conditions (21 ± 2°C, 12/12 h light–dark cycle) with free access to standard pellet diet and water ad libitum. All animal procedures were conducted in accordance with the standards set forth in the guidelines for the care and use of experimental animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals and the National Institutes of Health. The study protocol was approved by the Animal Ethics Committee of Wenzhou Medical University.

The models were established using previously described methods with slight modification. Briefly, the animals were randomly divided into six groups of eight. Ses was suspended in 0.1% CMC and was used in this study. Group I served as the normal control and was orally with CMC daily with i.p. administered corn oil (1 ml/kg body weight) twice per week for 8 weeks. Group II served as the Ses (100 mg/kg) alone group and was oral administration of Ses (100 mg/kg/d) in CMC daily with i.p. administered corn oil (1 ml/kg body weight) twice per week for 8 weeks. To induce oxidative stress and hepatic fibrosis, animals of Groups III, IV, V, and VI were i.p. administered 2 ml/kg body weight of CCl₄ (20% in corn oil) twice per week for 8 weeks. Group III served as the CCl₄ control and was orally administered CMC daily. Group IV served as the positive control and was orally administered silymarin (200 mg/kg) daily for 8 weeks. Many studies showed that silymarin was an effective agent for CCl₄-induced liver injury and hepatic fibrosis. Groups V and VI were orally administered Ses in CMC daily at doses of 50 and 100 mg/kg, respectively.

**Determination enzyme activity in serum and hepatic homogenate**

The serum was collected and the liver was made into tissue homogenate at the end of the experiment, and ALT, AST, and TBIL in serum were analyzed according to manufacturer’s procedure.

**Determination of SOD GSH-Px and protein carbonyls activity**

Liver was excised immediately after the animals were sacrificed. The liver, except a portion of the left lobe to be used for histopathological sections, was frozen quickly and stored at −80°C. Prior to determinations, thawed tissue samples were homogenized in nine volumes of ice cold 50 mM phosphate buffer (pH 7.4), centrifuged at 2500 rpm for 20 min at 4°C. The supernatant was used for the determination of GSH-Px and protein carbonyls using commercially available diagnostic kits (Jian Cheng Bioengineering Institute, Nanjing, China). Superoxide dismutase (SOD) activity was determined by the method of Kakkar et al. GSH-Px activity was based on the reaction of GSH transforms into GSSG. Reduced glutathione in liver homogenate was determined by reaction with 1,2-dithio-bis nitro benzoic acid (DTNB). Briefly, 1 mol DTNB with 2 mol GSH reacted together with 1 mol 5,5′-dithiobis (2-nitrobenzoic acid) with an intense yellow color, measured spectrophotometrically at 412 nm using a Synergy2 Automatic microplate reader (BioTek Instruments, Inc., Winooski, VT).

**Western blot analysis**

Nuclear and cytoplasmic extracts for western blotting were obtained by using a nuclear/cytoplasmic isolation kit (Beyotime Institute of Biotechnology, Beijing, China). Protein levels were determined using the BCA assay kit. Samples (80 µg each) were separated by denaturing SDS-PAGE and transferred to a PVDF membrane by electrophoretic transfer. The membrane was pre-blocked with 5% BSA and 0.1% Tween-20 in Tris-buffered saline (TBST) and incubated overnight with the primary antibody (in TBST with 5% BSA). Each membrane was washed three times for 15 min and incubated with the secondary horseradish peroxidase-linked antibodies. Quantitation of detected bands was performed with the Scion Image analysis software (Scion Corp., Frederick, MD). To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody. Each density was normalized using each corresponding β-actin density as an internal control and averaged from three samples.

**Determination of TNF-α level**

TNF-α was assayed using ELISA kit (Genzyme Diagnostics, Cambridge, MA). Briefly, liver tissues were homogenized in PBS (pH 7.4) for 30 s. They were then centrifuged at 12,000 rpm for 20 min. About 100 µl of the supernatant was added to the 96-well microtiter plate pre-coated with monoclonal TNF-α antibody (coupled to horse radish peroxidase) and incubated for 2 h at 37°C. After thorough washing, the substrate solution was added. Color development was allowed for 10 min and the reaction was stopped by application of stop solution. Color absorbance was read in a microplate reader (Dynex Technologies, Chantilly, VA) at 450 nm. Protein was determined using the Lowry method.
Rats were eight in each group. Each value represents the mean ± SD.

### Effects of Ses on CCl₄

The hydroxyproline levels in the livers were determined by a determination of hydroxyproline level.

### Determination of hydroxyproline level

The hydroxyproline levels in the livers were determined by a modified version of the previous method. The liver samples were weighed and completely hydrolysed in 6 M HCl. After hydrolysis, the samples were derivatised using chloramine T solution and Erhlich’s reagent, and the optical density was measured at 558 nm. A standard calibration curve was prepared using trans-4-hydroxy-L-proline.

### Liver histopathology

Liver samples were fixed in 10% buffered formaldehyde solution, processed by the paraffin slice technique. Sections about 4 μm thick were stained with haematoxylin and eosin (HE) and Masson’s trichrome staining to investigate liver histological and fibrotic changes. The degree of liver damage was examined blindly by a special pathologist under a light Olympus microscope (Olympus, Hamburg, Germany).

### Statistical analysis

Data bars represent the means ± SD (standard deviations) for at least three independent experiments in all cases. One-way analysis of variance (one-way ANOVA) was used to compare the differences in means of more than two groups, followed by Dunnett multiple comparison tests to determine significant differences among the pairs. p Value of 0.05 or less was considered statistically significant.

### Results

#### Effects of Ses on CCl₄-induced hepatotoxicity

Serum ALT, AST, and TBIL were elevated by CCl₄ treatment and had a significant difference when compared with the control group; Treatment with Ses at 50–100 mg/kg or silymarin for 8 weeks significantly reversed the increase of ALT, AST, and TBIL. However, Ses (100 mg/kg) alone did not modify the liver index when compared to the control group (Table 1).

Superoxide dismutase (SOD), GSH-Px, and protein carbonyls in hepatic homogenate were measured and the results showed that CCl₄ induced the decline of the antioxidant activity of SOD and GSH-Px (p<0.05), and induced the increase of the pro-oxidant activity of protein carbonyls (p<0.05); however, Ses at 50–100 mg/kg reversed the changes of SOD, GSH-Px, and protein carbonyls induced by CCl₄ (Figure 1).

#### Effects of Ses on inflammatory cytokines and NF-κB/IκB-α expressions

As shown in Figure 2, CCl₄ treatment caused significant increase in the expression levels of IL-6 and COX-2 in mouse livers as compared with those of the control group. However, treatment with Ses in CCl₄-treated mice significantly decreased the expression levels of IL-6 and COX-2 (p<0.05).

To investigate whether Ses represses the production of TNF-α, which play central role in inflammatory disease, TNF-α level was detected in mice stimulated with CCl₄ in the presence or absence of Ses. After treatment with CCl₄, serum TNF-α level significantly increased compared with that in the normal group (p<0.05). Pre-treatment with Ses resulted in a significant decrease in TNF-α production (p<0.05) (Figure 3). Single Ses administration also did not affect TNF-α levels compared with the normal group.

To further investigate the molecular mechanism of inflammation in mouse liver, we explored the NF-κB/IκB-α signaling pathway in CCl₄-induced rats. We found that the IκB-α protein level significantly decreased in CCl₄-treated rats compared with the control group. Following IκB-α degradation, NF-κB was released from the physical restriction imposed by IκB-α and translocated to the nucleus. Treatment with Ses strongly inhibited the translocation of NF-κB from the cytosol to the nuclear fraction (Figure 4).

#### Effects of Ses on histopathologic characteristics

The protective effects exerted by Ses against CCl₄-induced hepatotoxicity were further confirmed by conventional histological assessment. HE staining for the normal control groups and Ses (100 mg/kg) alone groups showed normal architecture, whereas the CCl₄-treated groups exhibited fatty degeneration, necrosis, and inflammation of hepatocytes. However, treatment with Ses at 50–100 or 200 mg/kg of silymarin markedly improved hepatic morphology and architecture, with less pseudo-lobules and inflammatory cell infiltration compared with the CCl₄-treated group (Figure 5).

#### Effect of Ses on collagen accumulation in Masson’s trichromic stain and hydroxyproline content

The levels of hydroxyproline in CCl₄-treated group were significantly higher than those in the control group (Figure 6). However, treatment with Ses at 50–100 mg/kg significantly inhibited the elevation in hydroxyproline levels following CCl₄ administration. The collagen of these fibrotic tissues had a blue color when stained by Masson’s trichrome (Figure 7). In the normal control groups and Ses (100 mg/kg) alone groups, the liver sections showed normal hepatic cells without fibrosis (Figure 7A and B). The livers of rats which were treated with CCl₄ showed extensive accumulation of thick fibrotic tissue, resulting in the formation of continuous fibrotic septa, nodules of regeneration, and noticeable alterations in the central vein compared with the normal control (Figure 7C). The lesions of silymarin-treated rats were present to a lesser degree (Figure 7D) than those found in the CCl₄-treated group. A lesser degree of hepatocyte fibrosis was

### Table 1. Effects of Ses on ALT, AST, and TBIL in serum in CCl₄-induced liver fibrosis model of rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>TBIL (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>44.3 ± 17.1</td>
<td>62.2 ± 13.2</td>
<td>5.3 ± 3.2</td>
</tr>
<tr>
<td>Ses 100 mg/kg</td>
<td>45.2 ± 12.6</td>
<td>59.98 ± 15.7</td>
<td>6.4 ± 2.8</td>
</tr>
<tr>
<td>CCl₄ control</td>
<td>397.6 ± 38.2#</td>
<td>422.8 ± 61.1#</td>
<td>24.4 ± 4.1#</td>
</tr>
<tr>
<td>Silymarin</td>
<td>147.8 ± 27.7*</td>
<td>202.4 ± 45.5*</td>
<td>12.84 ± 5.3*</td>
</tr>
<tr>
<td>200 mg/kg + CCl₄</td>
<td>190.7 ± 47.3*</td>
<td>212.3 ± 55.6*</td>
<td>16.5 ± 7.9</td>
</tr>
<tr>
<td>Ses 50 mg/kg + CCl₄</td>
<td>160.3 ± 40.7*</td>
<td>183.8 ± 31.9*</td>
<td>11.42 ± 5.3*</td>
</tr>
<tr>
<td>100 mg/kg + CCl₄</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Rats were eight in each group. Each value represents the mean ± SD. Significance was determined by one-way ANOVA. #p<0.05 as compared with the normal group. *p<0.05 as compared with the CCl₄ control group.

DOI: 10.3109/08923973.2015.1085064
Figure 1. Effects of Ses on SOD (A), protein carbonyl (B), and GSH-Px (C) in hepatic homogenate in CCl₄-induced liver fibrosis model of rats. #p < 0.05 as compared with the normal group; *p < 0.05 as compared with the CCl₄-treated control group.

Figure 2. Effects of Ses on IL-6 and COX-2 production in CCl₄ intoxicated rats. β-actin was probed as an internal control in relative density analysis. The vehicle control is set as 1.0. (I) Normal control; (II) Ses 100 mg/kg; (III) CCl₄ control; (IV) silymarin 200 mg/kg + CCl₄; (V) Ses 50 mg/kg + CCl₄; (VI) Ses 100 mg/kg + CCl₄. The panels (bars) denote the mean ± SD of three experiments for each condition determined from densitometry relative to β-actin. #p < 0.05 as compared with the normal group, *p < 0.05 as compared with the CCl₄-treated control group.
observed in the livers of Ses-treated rats at doses of 50 (Figure 7E) and 100 mg/kg (Figure 7F).

Discussion

Hepatic fibrosis is thought to be reversible; however, there is no satisfied agent in the clinical practice to reverse the pathological process yet. In this study, we found that Ses possessed the protective effects on CCl₄-induced liver fibrosis by anti-oxidative and anti-inflammatory effects in rats. Also, we found that Ses inhibited liver CCl₄-induced inflammation probably through regulating NF-kB/İkB-α signaling pathway.

The CCl₄-induced liver injury model has been extensively used to investigate the efficacy and mechanisms of hepatoprotective drugs. It is known that CCl₄ is metabolized primarily by the cytochrome P4502E1 component of the hepatic mixed function oxidase system, the resulting metabolites of trichloromethyl and trichloromethylperoxyl radicals trigger the peroxidation of polyunsaturated fatty acids in the endoplasmic reticulum and mitochondria, which subsequently leads to the generation of reactive oxygen species (ROS) and activation of immune cells. Therefore, administration of CCl₄ with a repeating and persistent pattern for different time courses has been reported to duplicate a series of well-characterized models from acute liver injury to chronic hepatic fibrosis in numerous studies. In parallel with the alteration of liver function markers, these phenomena were also confirmed by histological observation. HE staining showed that Ses can relieve the inflammatory cell infiltration, hydropic degeneration, and fibrosis around the central vein as well as focal necrosis to some extent, which is induced by the administration of CCl₄.

Many studies suggest that one possible molecular mechanism involved in CCl₄ hepatotoxicity is the disruption of delicate oxidant/antioxidant balance, which can lead to liver injury via oxidative damage. Superoxide dismutase (SOD) was an effective defense enzyme that catalyzes the dismutation of superoxide anions into hydrogen peroxide (H₂O₂). GSH-Px was an important enzyme that catalyzed the reduction of H₂O₂ and hydroperoxides to non-toxic products and terminated the chain reaction of lipid peroxidation by removing lipid hydroperoxides from the cell membrane. Lipid peroxides or reactive oxygen species could easily inactivate these antioxidant enzymes in toxicity. The results of the present study showed that SOD and GSH-Px activities were significantly decreased in the liver in response to CCl₄ treatment compared with control group rats, indicating increased oxidative damage to liver. However, treatment with Ses significantly increased SOD and GSH-Px activities.

![Graph](Image)  
**Figure 3.** Effect of Ses on serum TNF-α levels in CCl₄-induced liver fibrosis model of rats. Data are represented as the mean ± S.D. *p < 0.05 as compared with the normal group, *p < 0.05 as compared with the CCl₄-treated control group.

![Graph](Image)  
**Figure 4.** Effect of Ses on NF-KB/İkB-α signaling pathway in CCl₄-induced rats. The panels (bars) denote the mean ± SD of three experiments for each condition determined from densitometry relative to β-actin. *p < 0.05 as compared with the normal group; *p < 0.05 as compared with the CCl₄-treated control group.
activity of liver tissue, decreased protein carbonyls level in CCl₄-induced liver fibrosis rats. Su et al.⁹ found that Ses is able to restore the decreased expression of Mn-SOD in doxorubicin-induced cardiac injury rats. Liu et al.⁷ showed that Ses could attenuate CCl₄-induced acute hepatic injury by inhibiting ROS generation and increasing liver total antioxidant capacity level in male ICR mice. CCl₄ is metabolized to produce highly toxic trichloromethyl free radical (•CCl₃) and/or trichloro methyl peroxy (•OOCCL₃) free radicals by cytochrome P450 enzyme and causes damage to hepatocytes within the body. Both trichloromethyl and its peroxyl radical are capable of binding to proteins or lipids, or of abstracting a hydrogen atom from an unsaturated lipid, initiating lipid peroxidation, and liver damage. Reactive oxygen species (ROS) generated by metabolic intermediates of xenobiotics via induction of CYP450 families as well as activated inflammatory cells through NADPH oxidases promote the accumulation of lipid derived oxidation products that cause liver injury, resulting in cell necrosis. Further research needs to study the effect of Ses on CCl₄-induced expression of P450 enzyme.

Accumulating evidences have revealed that CCl₄ and excessive oxidative stress induced by CCl₄ can stimulate circulating monocytes and tissue macrophages, which lead to the synthesis and release of a variety of pro-inflammatory cytokines. Many studies also demonstrated that IL-6 and COX-2 play a key role in the development and maintenance of inflammatory and those cytokines elevation is associated with many liver diseases. Our study showed that CCl₄ treatment significantly up-regulated the expression of IL-6 and COX-2 in the liver of rats. However, Ses significantly inhibited the up-regulation of IL-6 and COX-2. These results suggested that Ses could alleviate liver fibrosis caused by CCl₄ partly through suppressing inflammatory response. NF-kB is a nuclear transcription factor that regulates expression of a large number of genes that are critical for the regulation of apoptosis and inflammation. Excessive ROS induced by CCl₄ intermediates can function as signaling messengers to NF-kB, and ultimately lead to increase the expression of...
COX-2 and IL-6. Recently, Lee et al.\textsuperscript{9} showed that Ses could inhibit the production of VEGF and MMP-9 via inhibiting NF-κB-mediated pathways in breast cancer cells. In this study, the translocation of activated NF-κB to the nucleus was increased after CCl\textsubscript{4} treatment in mouse liver. However, the NF-κB activation was inhibited by Ses, implying that inhibition of NF-κB activation was tightly involved in the anti-inflammatory action of Ses. Further studies are required to define the exact mechanism underlying the anti-oxidative and anti-inflammatory effects of Ses.

Some studies reported that an increase in hydroxyproline levels in the liver indicates enhanced hepatic fibrosis, which is associated with the exacerbation of lipid peroxidation and the depletion of antioxidant status after treatment with CCl\textsubscript{4}\textsuperscript{25}. In this study, treatment with Ses or silymarin significantly inhibited the elevation in hydroxyproline levels following CCl\textsubscript{4} administration. In clinical diagnoses and experimental examinations, the detection of liver fibrosis often depends on the detection of collagen fibers. Masson’s trichrome stain is a routine staining technique for detecting collagen fibres in liver tissue\textsuperscript{34}. In our study, histopathological examination of collagen deposition using Masson’s trichromic stain supported these findings.

Conclusions

The present study showed that Ses exerted beneficially hepatoprotective and antifibrotic effects on oxidative damage and inflammatory cytokines induced by CCl\textsubscript{4}. The protective effects against hepatic fibrosis may be, at least in part, due to the antioxidant effect and suppressing the NF-κB activation. These findings may benefit the development of new strategies in prevention of hepatic fibrosis using safe natural substances.

Declaration of interest

The authors declare no conflict of interests.

References


Figure 7. Histopathological changes of fibrosis occurred in CCl\textsubscript{4}-intoxication and prevention by the treatment with Ses (Masson trichrome stain). (A) Normal control; (B) Ses 100 mg/kg; (C) CCl\textsubscript{4} control; (D) silymarin 200 mg/kg + CCl\textsubscript{4}; (E) Ses 50 mg/kg + CCl\textsubscript{4}; (F) Ses 100 mg/kg + CCl\textsubscript{4}. DOI: 10.3109/08923973.2015.1085064


