Original Research

Anti-fibrotic effect of *Cordyceps sinensis* polysaccharide: Inhibiting HSC activation, TGF-β1/Smad signalling, MMPs and TIMPs

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Abstract

Cordyceps sinensis has been used to treat liver disease in traditional Chinese medicine for thousands of years. Polysaccharide extracted from cultured *Cordyceps sinensis* mycelia (CS-PS) is the major active components of *cordyceps sinensis* with anti-liver injury effects. In the present study, the effects of CS-PS on hepatic stellate cell (HSC) activation, transforming growth factor-β1 (TGF-β1)/Smad pathway, as well as matrix metalloproteinase (MMP) 2, MMP9 and tissue inhibitor of metalloproteinase (TIMP) 1, TIMP2, were investigated in liver fibrosis in rats induced by carbon tetrachloride (CCl_4). Colchicine was used as a positive control. The effect of CS-PS inhibition liver injury and fibrosis was confirmed by decreasing serum alanine aminotransferase, aspartate aminotransferase, total bilirubin, hepatic hydroxyproline and increasing serum albumin, as well as alleviation of histological changes, which was comparable to that of colchicine. With CS-PS treatment, hepatic α-smooth muscle actin, TGF-β1, TGF-β1 receptor (TβR)-I, TβR-II, p-Smad2, p-Smad3 and TIMP2 proteins expression were down-regulated comparing to that in CCl_4 group. The activities of MMP2 and MMP9 in liver tissue were also inhibited in CS-PS-treated group. It is indicated that the effects of CS-PS anti-liver fibrosis are probably associated with the inhibition on HSC activation, TGF-β1/Smads signalling pathway, as well as MMP2, MMP9 activity and TIMP2 expression.

Keywords: *Cordyceps sinensis* polysaccharide, liver fibrosis, hepatic stellate cell (HSC), transforming growth factor-β1 (TGF-β1), matrix metalloproteinase (MMP), tissue inhibitor of metalloproteinase (TIMP)

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Introduction

Cordyceps (*Cordyceps sinensis [Berk.] Sacc.*) is a medicinal fungus used in traditional Chinese medicine to maintain health and treat a wide range of disorders,[1,2] including liver fibrosis.[3,4] The cultured cordyceps mycelium has been demonstrated to have similar pharmacological efficacy to that of natural fungus in many aspects.[5,6] The polysaccharide extracted from cultured *Cordyceps sinensis* mycelia is the major active components and has been found to have anti-fibrotic effect in liver in the previous experimental studies.[7,8] The effective dosage of *Cordyceps sinensis* polysaccharide (CS-PS) on liver fibrosis was screened in a recent study.[9] But, the pharmacological mechanisms of CS-PS inhibition liver fibrosis were rarely disclosed.

Liver fibrosis and cirrhosis represent the common end-stage of various chronic liver diseases, including chronic viral hepatitis, non-alcoholic steatohepatitis, alcoholic liver disease and so on.[10] As the fibrosis progresses, it results in liver failure and portal hypertension and is associated with the increased risk of hepatic carcinoma.[11] Fibrosis and cirrhosis are the major causes of morbidity and mortality worldwide.[12] But, the only curative treatment, available now, for cirrhosis is transplantation.[13]

Fibrosis is an excessive wound healing response to chronic liver injury and characterized by the accumulation of extracellular matrix (ECM) rich in fibrillar collagens (mainly collagen I and collagen III). Investigations on the mechanisms disclosed that the activated hepatic

stellate cell (HSC) is the main fibrogenic cell of injured liver and fibrosis is a dynamic and reversible process.[12] Therefore, the promising targets for anti-fibrotic therapies are focused on HSC activation, signalling pathways activating HSC and molecules modulating fibrolysis and fibrogenesis.[14]

In the present study, the effects of CS-PS on HSC activation, transforming growth factor-β1 (TGF-β1) signalling pathway, matrix metalloproteinases (MMPs) activity and tissue inhibitor of metalloproteinases (TIMPs) expression in liver fibrosis were investigated in rats induced by carbon tetrachloride (CCl_4).

Colchicine is an alkaloid agent and was found to improve laboratory values, fibrosis markers and prolong survival in patients with mild to moderate cirrhosis.[15–17] In the present study, colchicine was used as a positive control.

Material and methods

Preliminary chemical analysis of CS-PS

CS-PS was purchased from Shanghai Kangzhou Fungi Extract Co., Ltd (Shanghai, China). Total carbohydrate content of CS-PS was determined by using the phenol–sulphuric acid method as described previously[18] with some modification. CS-PS (10 mg) was dissolved in 20 mL of deionized water and centrifuged at 1000 *g* for 10 min. Supernatant (3 mL) was removed into a clean tube and diluted to 25 mL with deionized water. Two millilitres of CS-PS prepared sample solution was added into the testing tube for detection. To get the standard curve, 2 mL of glucose aqueous solution (containing glucose 0–80 μg) was used as standard substance. One millilitre of 6% phenol solution was added into the standard and testing tubes and mixed. Then, 5 mL of concentrated H_2SO_4 was added, and the mixture was vortexed and incubated in room temperature for 30 min (Supplementary Table 1). The absorbance was read at 490 nm by ultraviolet visible spectrophotometer (Shanghai Spectrum Instruments Co., Ltd.), repeating three times. Total carbohydrate content of CS-PS was determined to be $38.3 \pm 1.3\%$ according to the calibration curve (Supplementary Figure 1).

Carbohydrate composition of CS-PS was analysed by gas chromatograph-mass spectrometer (GC-MS) (TRACE-DSQ; Thermo Fisher Scientific Inc., FL, USA). For GC-MS analysis, 2 mg of polysaccharide was hydrolysed at 120°C by 3 mL of 2.0 mol/L trifluoroacetic acid for 2 h. The hydrolysate was reacted with 100 mg sodium borohydride and 2 mL double distilled water at room temperature for 4 h. Then, 2 mL acetic anhydride was added ant the mixture was kept at 100°C for another 60 min. Finally, the sample was analysed by GC-MS (TRACE-DSQ). Analysis showed that 100 mg CS-PS contained 4.05 ± 0.34 mg arabinose, 4.87 ± 0.32 mg mannose, 88.19 ± 0.77 mg glucose and 2.89 ± 0.16 mg galactose (Supplementary Figure 2).

Molecular masse of CS-PS was analysed by high-performance gel permeation chromatographic (HPGPC) system (Agilent 1100; Agilent Technologies, Inc., CA, USA)[19] and determined to be $8.80 \pm 0.13 \times 105$ Da (Supplementary Figure 3).

Animals and treatment

Forty-two male Wistar rats weighing 150 ± 10 g were supplied by the experimental animal centre to Chinese academy of science (Shanghai, China). The animals were housed in an air-conditioned room at 25°C with a 12-h dark/light cycle. The experiment was conducted in accordance with the principles for laboratory animal use and care approved by local ethics committee.

Rats firstly were divided into control ($n = 6$) and CCl_4-stimulated group ($n = 36$). Liver fibrosis in rats was induced with a modified method previous reported.[20] Rats were injected subcutaneously with CCl_4 (product of Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) at dosage of 3 mL/kg·d for the first time and then 50% CCl_4 solution (CCl_4 dilution in olive oil, product of Sinopharm Chemical Reagent Co., Ltd), 2 mL/kg·d, twice per week for nine weeks. The rats in control group were injected with equal volume of saline. At the end of the sixth week, CCl_4-stimulated rats were randomly divided into CCl_4 ($n = 12$), CCl_4 plus CS-PS ($n = 11$) and CCl_4 plus colchicine group ($n = 11$). Rats in CCl_4 plus CS-PS and CCl_4 plus colchicine group were orally administrated with CS-PS (commercial product of Kangzhou Fungi Extract Co., Ltd., Shanghai, China) 60 mg/kg·d and colchicine (Kunming Pharmaceutical Corp., Kunming, China) 0.1 mg/kg·d, respectively, the others with equal volume of sterile water, all for three weeks.

At the end of ninth week, rats were anaesthetized with Nembutal (45 mg/kg, i.p.); liver tissue and serum were collected and stored in −80°C for biochemical and Western-blot analysis. Liver tissue specimens taken from the hepatic right median lobe[21–23] (illustrated in Supplementary Figure 4) were fixed in 10% phosphate-buffered formaldehyde routinely processed and embedded in paraffin for histological observation.

Histological examinations

Liver tissue was formalin-fixed and embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin-eosin (H&E; Nanjing Jiancheng Bioengineering institute, Nanjing, China) and examined under light microscope (Olympus Medical Systems Corp., Tokyo, Japan).

Collagen deposition was observed in Sirius red stain section (5 μm thick) under light microscope (Olympus Medical Systems Corp., Tokyo, Japan).

Serum ALT, AST, Alb, TBil and hepatic Hyp assay

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (Alb) and total bilirubin (TBil) were determined with the corresponding biochemical assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Table 1 Antibodies in immunohistochemical assay and immunoblot

Antibody	Species	Brand	Dilution
α-SMA	Mouse IgG2a	SIGMA, MO, USA	1:400
TIMP-1	Mouse IgG1	Lab Vision, USA	2 μg/mL
TIMP-2	Mouse IgG1	Lab Vision, USA	2 μg/mL
TGF-β1	Mouse IgG1	Abcam, UK	1:2000
TβR-I	Rabbit IgG	Cell Signaling, MA, USA	1:1000
TβR-II	Rabbit IgG	Cell Signaling, MA, USA	1:1000
Smad2	Mouse IgG1	Cell Signaling, MA, USA	1:1000
Smad3	Rabbit IgG	Cell Signaling, MA, USA	1:1000
p-Smad2	Rabbit IgG	Abcam, UK	1:300
p-Smad3	Rabbit IgG	Epitomics, CA, USA	1:1000
GAPDH	Mouse LgG1	Kangchen, Shanghai, China	1:5000
HRP-linked antibody	Goat anti rabbit IgG	Santa Cruz, CA, USA	1:5000
HRP-linked antibody	Goat anti mouse IgG	Santa Cruz, CA, USA	1:5000

Hepatic hydroxyproline (Hyp) content was evaluated according to the little modified method previous reported.[24,25] Briefly, 100 mg of liver tissue was homogenized and hydrolysed in 5 mL of 6 N HCL at 110°C for 18 h. After filtration of the hydrolysate through a 0.45-mm Millipore filter (Millipore, Bedford, MA), 100 μL of hydrolysate was removed to a clean tube and dried at 40°C. The samples were then incubated with Ehrlich's reagent (25% [w/v] para-dimethylaminobenzaldehyde and 27.3% [v/v] perchloric acid in isopropanol) at 50°C for 90 min. After cooling to room temperature, the supernatant was read at 558 nm (SpectraMax M5; Molecular Devices, CA, USA) against a reagent blank, and the results were determined from the Hyp standard curve (Nakateyitesuku Company, Japan). The standard value of Hyp and the procedure of Hyp test were presented in the Supplemental Table 2. The results of Hyp were presented as μg/g liver tissue.

Immunohistochemical assessment of hepatic α-SMA

As described in previous study,[26] paraffin sections of liver tissue (4 μm thick) were used for immunohistochemical assessment. Briefly, after endogenous peroxidase blockage and bovine serum albumin blockage, the samples were incubated at 4°C overnight, with α-smooth muscle actin (α-SMA) antibody (Mouse IgG2a α-SMA; SIGMA, MO, USA). Following the processing of the samples incubated with a 1:250 dilution of horseradish peroxidase (HRP)-linked goat anti-mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 1 h at 37°C, diamino benzidine (DAB) was applied as a chromogen and hematoxylin was used for floor staining.

Immunoblot analysis

Protein expression of α-SMA, TGF-β1, TGF-β1 receptor I (TβR-I), TGF-β1 receptor II (TβR-II), Smad2/3, phosphorylated Smad2/3 (p-Smad2/3), TIMP1 and TIMP2 in liver tissue was detected by Western-blot analysis.

As described previously,[26,27] total proteins in liver tissue were extracted, analysed with bicinchoninic acid (BCA) protein concentration assay kit (Beyotime Inst. Biotechnology, Jiangsu, China).

Sample protein was separated by electrophoresis in 10% SDS-PAGE separating gel with Bio-Rad electrophoresis system (BioRad Laboratories, Hercules, CA). The primary antibodies (Table 1) were incubated at 4°C overnight. The corresponding HRP-conjugated secondary antibodies were incubated at room temperature for 1 h.

The ECL kit (Pierce Biotechnology Inc., Rockford, USA) and the Furi FR-980 image analysis system (Shanghai Furi Co., Shanghai, China) were employed for revealing and quantitative analysis of the blots. GAPDH protein was used as the internal control.

Gelatin zymography

MMP-2, 9 activity were assessed by gelatin zymography as previously described.[25,28] Total protein was extracted with RIPA lysates (containing 1M Tris–HCl, 0.5M NaCl, pH 7.0) and separated by electrophoresis in 0.1% gelatin and 8% stacking gel at 4°C. Then, the gel was eluted in eluent (containing 2.5% Triton X-100, 50 mM Tris–HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, pH 7.6) for 45 min, twice, and rinsed (rinse solution containing 50 mM Tris–HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, pH 7.6) for 30 min, twice. After being incubated in the buffer (containing 50 mM Tris–HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% Brij-35, pH 7.6) for 18 h at 37°C on incubator shakers, the gel was stained with mixture of 0.05% Coomassie blue G-250 R-250, 30% methanol and 10% acetic acid for 3 h, then orderly decoloured with solution A (containing 30% methanol and 10% acetic acid), solution B (containing 20% methanol and acetic 10% acid) and solution C (containing 10% methanol and acetic 5% acid). Finally, the intensity of the bands was assayed by the Furi FR-980 image analysis system (Shanghai Furi Co., Shanghai, China).

Table 2 Body weight and liver/spleen weight ratio

Group	*n*	Body weight (g)	Liver/spleen weight ratio
Control	6	394 ± 14	13.04 ± 1.12
CCl_4	10	276 ± 30*	4.03 ± 1.51*
CCl_4 + CS-PS	10	287 ± 43	6.29 ± 2.75†
CCl_4 + Colchicine	11	276 ± 29	5.30 ± 3.20

Values are means ± SD.
*$P < 0.01$, vs. control.
†$P < 0.05$, vs. CCl_4.

Statistical analysis

All results were expressed as mean + SD. The data were analysed using a one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) post hoc test. T-test is employed for the comparison of two parameters. Differences were considered statistically significant if the P value < 0.05.

Results

Mortality, body weight and liver/spleen weight ratio

In the first six weeks, two rats died from CCl_4 stimulation. In the following three weeks, another two rats died in CCl_4 group and one in CCl_4 plus CS-PS group. No death in control group and CCl_4 plus colchicine group.

After CCl_4 administration, body weight decreased obviously as well as the liver/spleen weight ratio comparing to that of control; however, in CS-PS-treated group, the liver/spleen weight ratio increased significantly (Table 2).

Effects of CS-PS on liver injury induced by CCl_4

Liver injury was examined by biomarkers of liver damage and histological changes in liver tissue. H&E staining sections revealed that with the administration of CCl_4 for nine weeks, the normal structures of lobules were destroyed. Comparing to the hepatic histological structure of rats in control group (Figure 1Aa), large amount of fibrous connective tissue was observed and the large or small typical pseudo lobules formed and filled with steatosis hepatocytes (Figure 1Ab). Numerous fibroblasts and inflammatory cells were observed in the septa (Figure 1Ab). The sinusoidal expanded and bile ducts damaged obviously (Figure 1Ab). After CS-PS (Figure 1Ac) and colchicines (Figure 1Ad) treatment, steatosis and inflammatory cells decreased, thinner collagen fibre and fewer complete pseudo lobules were observed.

With CCl_4 administration, serum ALT, AST and TBil increased (ALT, 258.8 + 27.1 vs. 35.7 + 3.7 U/L, $P < 0.01$; AST, 285.6 + 29.5 vs. 64.2 + 3.8 U/L, $P < 0.01$; TBil, 1.02 + 0.25 vs. 0.51 + 0.12 μg/dl, $P < 0.01$) and Alb decreased significantly (24.90 + 5.58 vs. 33.76 + 1.72 g/L, $P < 0.01$) (Figure 1C). After treated with CS-PS, serum ALT, AST and TBil decreased (ALT, 177.5 + 47.0 vs. 258.8 + 27.1 U/L, $P < 0.01$; AST, 209.3 + 40.7 vs. 285.6 + 29.5 U/L, $P < 0.01$; TBil, 0.79 + 0.23 vs. 1.02 + 0.25μg/dl, $P < 0.05$) and Alb increased remarkably (30.15 + 2.68 vs. 24.90 + 5.58 g/L, $P < 0.05$) (Figure 1C). In colchicine-treated group, serum ALT, AST, TBil decreased obviously, either (ALT, 192.6 + 60.9 vs. 258.8 + 27.1 U/L, $P < 0.01$; AST, 220.9 + 60.9 vs. 285.6 + 29.5 U/L, $P < 0.01$; TBil, 0.74 + 0.25 vs. 1.02 + 0.25μg/dl, $P < 0.05$), Alb increased, but not significantly in statistics (29.09 + 4.82 vs. 24.90 + 5.58 g/L, $P > 0.05$) (Figure 1C).

Histological observation in H&E staining sections and serum biomarkers evaluation indicated the effects of CS-PS alleviating liver injury induced by CCl_4 which was comparable to that of colchicine.

Effects of CS-PS on collagen deposition in liver tissue induced by CCl_4

Hepatic collagen deposition was visualized in Sirius red staining sections and determined by Hyp content in liver tissue. Collagen deposition in liver tissue, especially in perisinusoidal areas, was observed in Sirius red staining sections after CCl_4 administration. Fibrous septas between the portal area and central venous as well as numerous complete pseudo lobules were also observed. With the treatment of CS-PS and colchicine, fewer pseudo lobules and thinner collagen deposition were observed (Figure 1B).

Hepatic Hyp content increased obviously in CCl_4 administrated rats compared with that of control (524 + 143 vs. 178 + 21 μg/g liver tissue, $P < 0.01$) and decreased in CS-PS and colchicine-treated rats (CCl_4 + CS-PS group, 371 + 50 vs. 524 + 143 μg/g liver tissue, $P < 0.05$; CCl_4 + colchicine group, 373 + 112 vs. 524 + 143 μg/g liver tissue, $P < 0.05$) (Figure 1C).

The histological observation in Sirius red staining sections and hepatic Hyp content supported that CS-PS inhibited the collagen deposition in liver induced by CCl_4.

Effect of CS-PS on α-SMA expression

It is well demonstrated that HSC activation plays an essential role in liver fibrogenesis.[29] α-SMA protein expression was detected as the marker of activated stellate cells.[30] Immunohistological assay showed that in control group, positive staining only appeared in vessel wall while with CCl_4 administration, positive staining was stronger and distributed in fibrous septa which was faded out after being treated with CS-PS and colchicine (Figure 2A). Similarly, in Western-blot evaluation, protein expression of α-SMA was up-regulated in CCl_4 group comparing to that in control and down-regulated significantly by CS-PS and colchicine treatment (Figure 2B).

Effects of CS-PS on TGF-β1, TβR-I and TβR-II protein expression

TGF-β1 is one of the most important cytokines which activates HSC to promote liver fibrosis.[31] In the present research, with CCl_4 administration for nine weeks, TGF-β1, TβR-I and TβR-II protein expression in liver tissue were all up-regulated obviously and down-regulated remarkably after treatment with CS-PS. Colchincine also down-regulated the protein expression of TGF-β, while no

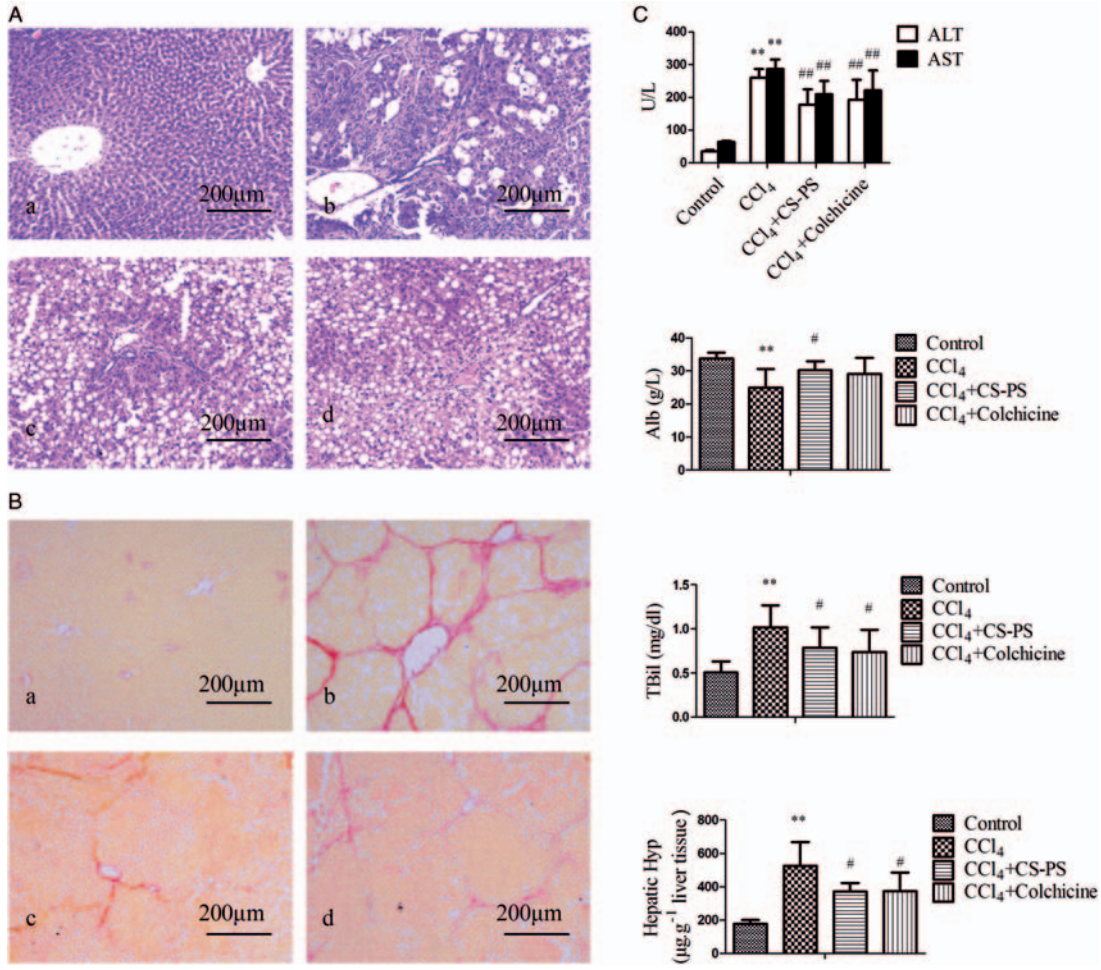

Figure 1 Effects of *Cordyceps sinensis* polysaccharide (CS-PS) on liver injury and collagen deposition. Liver fibrosis in rats was induced by carbon tetrachloride (CCl_4) injection subcutaneously for nine weeks: (a) Control, (b) CCl_4, (c) CCl_4 + CS-PS and (d) CCl_4 + Colchicine. Rats were administrated orally with CS-PS 60 mg/kg·d and colchicine 0.1 mg/kg·d, respectively, from seventh week to the end of the experiment. (A) Liver sections were subjected to hematoxylin-eosin (H&E) staining to observe the hepatic histological changes. (B) Collagen deposition in liver tissue was observed in Sirius red staining sections. (C) Alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (Alb) and total bilirubin (TBil) were determined with the corresponding biochemical assay kits. Hepatic hydroxyproline (Hyp) content was evaluated according to the little modified method previous reported.[24,25] Results were presented as the means + SD. $^*P < 0.05$, as compared with control group; $P < 0.01$, as compared with control group; $\#P < 0.05$, as compared with CCl_4 group; $\#\#P < 0.01$, as compared with CCl_4 group

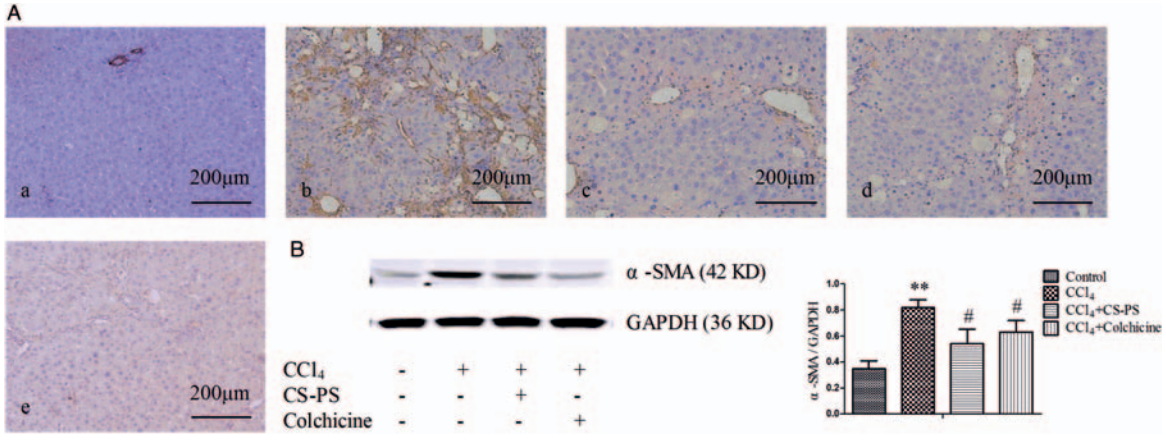

Figure 2 Effect of *Cordyceps sinensis* polysaccharide (CS-PS) on α-smooth muscle actin (α-SMA) expression in liver tissue. Liver fibrosis in rats was induced by carbon tetrachloride (CCl_4) injection subcutaneously for nine weeks: (a) Control, (b) CCl_4, (c) CCl_4 + CS-PS and (d) CCl_4 + Colchicine. α-SMA protein expression was detected by immunohistology (A) and Western blot (B). Rats were administrated orally with CS-PS 60 mg/kg·d and colchicine 0.1 mg/kg·d, respectively, from seventh week to the end of the experiment. Results were presented as the means + SD. $P < 0.01$, as compared with control group; $\#P < 0.05$, as compared with CCl_4 group. The data were representative of three independent experiments

significant inhibition on TβR-I and TβR-II protein expression was observed (Figure 3A).

Effects of CS-PS on Smad2/p-Smad2, Smad3/p-Smad3 protein expression

TGF-β1 signalling depends on TβR-I and TβR-II and the down-stream Smad proteins phosphorylation. TGF-β1 binding to TβR-II enables the formation and stabilization of TβR-I/TβR-II complex. The TβR-II kinase then phosphorylates TβR-I and activates Smad2 and Smad3. The phosphorylated Smad2 and Smad3 form a complex and translocate into the nucleus, binding to DNA, then activate the target gene expression.[31] In the present research, p-Smad2 and p-Smad3 protein expression were all up-regulated with CCl_4 administration for nine weeks, while

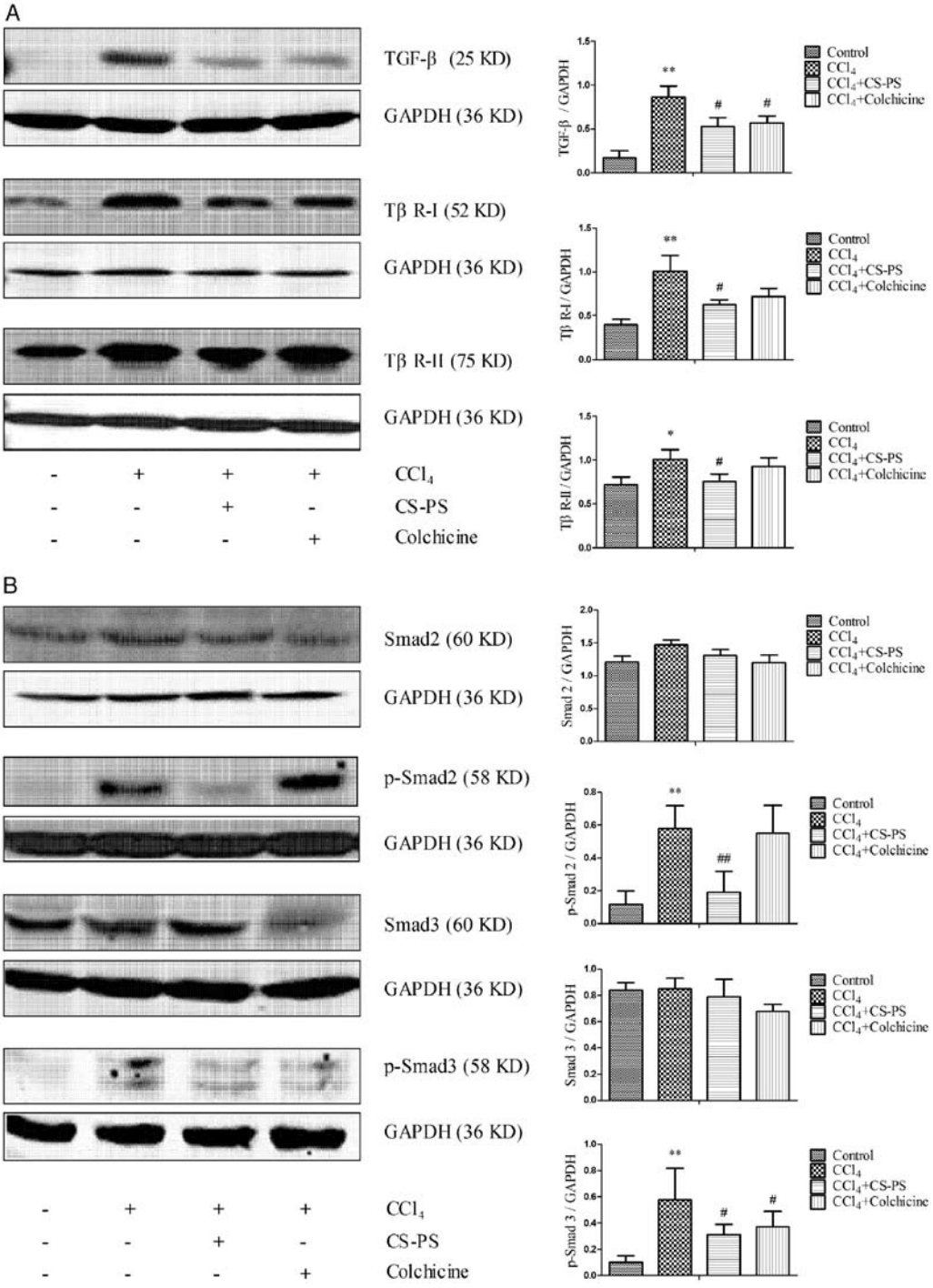

Figure 3 Protein expression of transforming growth factor-β1 (TGF-β1)/Smad signal pathway in liver tissue by Western-blot analysis. Liver fibrosis in rats was induced by carbon tetrachloride (CCl_4) injection subcutaneously for nine weeks. Rats were administrated orally with CS-PS 60 mg/kg·d and colchicine 0.1 mg/kg·d, respectively, from seventh week to the end of the experiment. Results were presented as the means + SD. $P < 0.01$, as compared with control group; $\#P < 0.05$, as compared with CCl_4 group; $\#\#P < 0.01$, as compared with CCl_4 group. The data were representative of three independent experiments

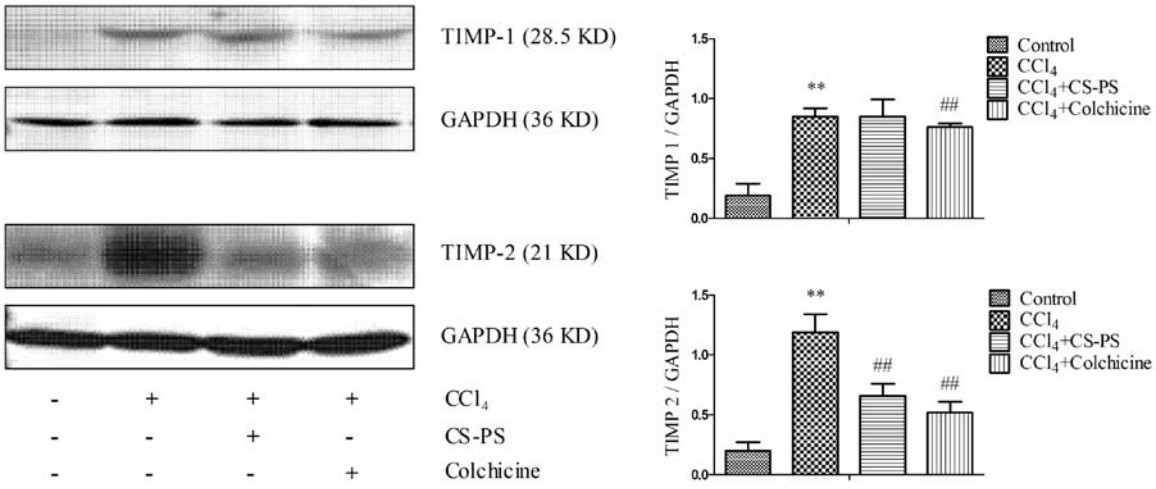

Figure 4 Protein expression of TIMP-1/TIMP-2 in liver tissue by Western-blot analysis. Liver fibrosis in rats was induced by carbon tetrachloride (CCl_4) injection subcutaneously for nine weeks. Rats were administrated orally with CS-PS 60 mg/kg·d and colchicine 0.1 mg/kg·d, respectively, from seventh week to the end of the experiment. Results were presented as the means + SD. $P < 0.01$, as compared with control group; $\#\#P < 0.01$, as compared with CCl_4 group. The data were representative of three independent experiments

no obvious changes were observed in Smad2 and Smad3 protein expression. CS-PS inhibited both p-Smad2 and p-Smad3 protein expression remarkably (Figure 3B). Colchicine inhibited p-Smad3 protein expression but no significant decrease of p-Smad2 protein expression was detected in the colchicine-treated animals (Figure 3B).

Effects of CS-PS on MMP2 and MMP9 activity

Liver fibrosis has been demonstrated as a dynamic and bidirectional pathological process which involves increased matrix synthesis and changes in the regulation of matrix degradation.[32] The balance of MMPs action and TIMPs, a family of tissue inhibitors of metalloproteinases, plays an important role in the progression of liver fibrosis. MMP-2 and MMP-9 are capable of degrading normal liver matrix.[32] In the present study, the activities of MMP-2 and MMP-9 in liver tissue were found increased remarkably with CCl_4 administration for nine weeks and decreased in CS-PS-treated animals while no inhibitory effects were observed with colchicine treatment (Figure 5).

Effects of CS-PS on Protein expression of TIMP-1/TIMP-2

Protein expression of TIMP-1 and TIMP-2 in liver tissue was up-regulated with CCl_4 administration for nine weeks (Figure 4). CS-PS treatment down-regulated TIMP-2 protein expression while both TIMP-1 and TIMP-2 protein expression were all down-regulated in colchicines-treated animals (Figure 4).

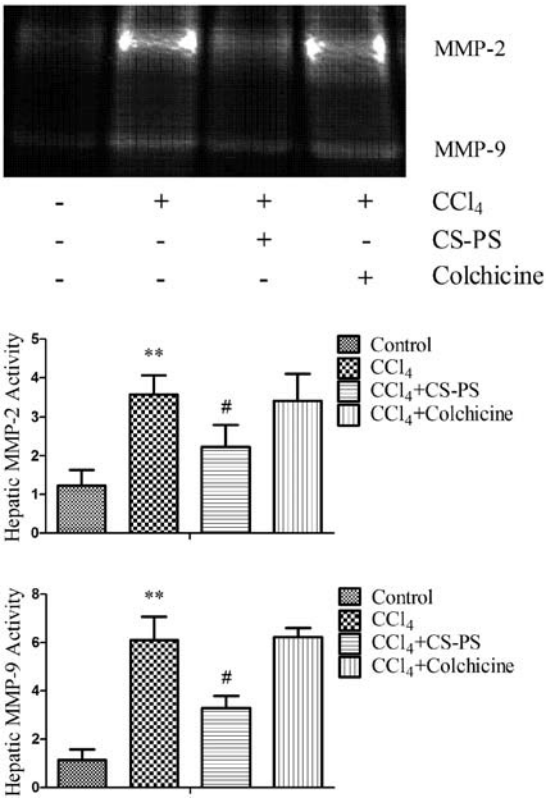

Figure 5 Activity of MMP-2 and MMP-9 in liver tissue by Gelatin zymography. Liver fibrosis in rats was induced by carbon tetrachloride (CCl_4) injection subcutaneously for nine weeks. Rats were administrated orally with CS-PS 60 mg/kg·d and colchicine 0.1 mg/kg·d, respectively, from seventh week to the end of the experiment. Results were presented as the means + SD. $P < 0.01$, as compared with control group; $\#P < 0.05$, as compared with CCl_4 group. The data were representative of three independent experiments

Discussion

Cordyceps sinensis as a precious traditional Chinese medicine was used to maintain health and treat disease for thousands of years in China. Recently, *Cordyceps sinensis* was demonstrated to be protective on liver disease, including prolonging survival of patients with hepatocellular carcinoma[33] and liver fibrosis in rodents.[4] But the application of *Cordyceps sinensis* was limited because the source of natural fungus is rare and expensive. *Cordyceps* mycelia extract is a promising source of therapeutic medicines because it can be mass-produced and it has a chemical composition similar to

that of the fruiting body of *Cordyceps sinensis*.[5,6] A number of bioactive constituents from *cordyceps* species have been reported,[34] including cordycepin, antibacterial and antitumor adenosine derivatives, sterols, polyphenolics and polysaccharides. Polysaccharides isolated from *cordyceps* species are major antioxidant phytochemicals[35] and recently, the effect of polysaccharides from *cordyceps* species on liver fibrosis was sporadically reported,[7,8,36] while the potential pharmacological mechanism deserved further investigation.

In the present research, CS-PS was demonstrated to inhibit liver fibrosis induced by CCl_4 in rats. After treatment with CS-PS or colchicine, the hepatic Hyp content, histological changes as well as biomarkers of liver function were all improved, which confirmed the effects of CS-PS on fibrosis reported in the our previous research[7,9] and the pharmacological effects are comparable to colchicine.

HSC activation is crucial in liver fibrogenesis.[29,37,38] Activation of HSC can be divided into two phase: initiation and perpetuation.[38] Initiation refers to early changes in gene expression that result primarily from paracrine stimuli derived from damaged resident liver cells (hepatocytes, endothelial and Kupffer cells) and platelets.[39,40] Persistent of the initiation stimuli accompanying sustained injury leads to a perpetuation phase. When HSCs is activated and converted to myofibroblasts, α-SMA is expressed.[41] In the present study, hepatic α-SMA protein expression was up-regulated significantly with CCl_4 administration and down-regulated by CS-PS or colchicine treatment, which indicated HSC activation in liver fibrosis induced by CCl_4 was inhibited by CS-PS or colchicine.

Perpetuation involves at least seven distinct changes in HSC behaviour, including proliferation, chemotaxis, fibrogenesis, contractility, altered matrix degradation, retinoid loss and inflammatory signalling.[42] Increased fibrogenesis is the most direct way in which HSC contributes to hepatic fibrosis and TGF-β1 is the most potent fibrogenic factor.[42] Sources of TGF-β1 are multiple, such as Kupffer cells,[40] endothelial cells and platelet,[39] but the HSC autocrine expression is among the most important.[43,44]

Once being activated, TGF-β1 signals through a complex of two related receptors, TβR-I and TβR-II. Binding of the homodimer TGF-β1 to TβRII enables the formation and stabilization of TβRI/TβII complex. The TβRII kinase then phosphorylates TβRI and activates Smad2 and Smad3. These phosphorylated Smads then form heterodimer complexes with the Smad4 and move into the nucleus to promote the target genes expression.[45] The TGF-β1/TβR/Smads signal pathway, especially the TβR function and phosphorylation of Smad2 and Smad3, is the key molecular mechanism of liver fibrosis[31] and therefore, the potential operative targets of anti-fibrotic treatment. In the present research, the protein expressions of TGF-β1, TβRI, TβRII as well as p-Smad2 and p-Smad3 were all up-regulated significantly in the fibrotic animals induced by CCl_4 accompanying with HSC activation, which were all down-regulated by CS-PS treatment remarkably accompanying with the amelioration of liver fibrosis and HSC activation. Colchicine inhibited TGF-β1 and p-Smad3 protein expression obviously while no significant inhibitory effects on TβRI, TβRII and p-Smad2 were observed.

An imbalance between ECM degradation and accumulation always exists in the progress of fibrosis. ECM consists of three main families: collagen, glycoprotein and proteoglycan.[46] In normal liver, the Disse space contains a basement membrane-like matrix which is composed of non-fibril-forming collagens including types IV, VI and XIV, glycoprotein and proteoglycan.[47,48] The interstitial ECM, composed of fibril-forming collagens and fibronectin, is largely confined around large vessels and in the portal areas.[29] As the liver becomes fibrosis, the total content of collagens and non-collagenous components increases three to eight-fold,[49] which was confirmed by the hepatic Hyp content in the present study. The outcome of fibrogenesis is the conversion of normal, low-density basement membrane-like matrix to high-density interstitial type matrix, which represents a change in both the quality and quantity of the ECM.[12,29] The factors responsible for ECM remodeling include the MMPs and their inhibitors TIMPs.

The degradation of normal liver matrix was demonstrated to contribute to the pathogenesis of liver fibrosis, particularly in the early stages of response to liver injury.[32] MMP-2 and MMP-9 are included in the most relevant MMPs capable of degrading normal liver matrix.[32] MMP2 is secreted by activated HSC[50] and the principal cellular source of MMP9 is Kupffer cell.[51] MMP2 activation depends on the interaction of membrane type1-MMP and TIMP2. MMP9 is activated by plasmin and stromelysin. It has been observed that the activity of MMP2 and MMP9 in liver fibrosis progression increases as HSCs become more activated both in human and animals.[10,12,32] In the present study, activities of MMP2 and MMP9 in hepatic tissue were found to be increased obviously after CCl_4 administration for nine weeks. With CS-PS treatment, activities of MMP2 and MMP9 in the liver tissue were down-regulated obviously accompanying with alleviation of liver fibrosis and HSC activation, while no obvious inhibitory effects were observed in colchicine-treated group.

Besides involvement into the activation of MMP2, TIMP2 and TIMP1 also inhibit the activity of MMPs capable of degradation of fibrotic liver matrix, such as MMP1 in human, MMP13 in rats and MMP8. TIMP1 and TIMP2 have been found increased in patients with sclerosing cholangitis, biliary atresia, primary biliary cirrhosis and autoimmune chronic active hepatitis.[32] In the present study, the protein expression of TIMP1 and TIMP2 in the hepatic tissue was found to be up-regulated significantly after administration with CCl_4 for nine weeks. Since liver fibrosis progression has been demonstrated to be associated with the inhibition of matrix degradation,[32] the alteration of TIMPs protein expression in the CCl_4-stimulated rats in the present study is consistent with the previous researches.[25,52,53] With CS-PS treatment, the protein expression of TIMP2 was down-regulated remarkably accompanying with amelioration of liver fibrosis, while no significant variation on TIMP1 protein expression was observed comparing to that in CCl_4 group. On the other hand, both TIMP1 and TIMP2 protein expressions were down-regulated by colchicine treatment.

Another arresting point in this model is related to the mechanism of CCl_4 inducing liver injury which ultimately promotes liver fibrosis. CCl_4 is activated by cytochrome (CYP) 2E1, CYP2B1 or CYP2B2, and possibly CYP3A,[54] and then initiates the chain reaction of lipid peroxidation, which attacks and destroys polyunsaturated fatty acids, in particular those associated with phospholipids. This affects the permeabilities of mitochondrial, endoplasmic reticulum and plasma membranes, resulting in the loss of cellular calcium sequestration and homeostasis, which can contribute heavily to subsequent cell damage.[54] Colchicine was demonstrated to reduce hepatic CYP450 in CCl_4-induced liver injury.[55] *Cordyceps sinensis* mycelium was found to be a inducer of CYP1A2 and CYP3A4 and has few effect on CYP2E1.[56] Interestingly, in our previous preliminary study, CS-PS was found to decrease malonaldehyde and increase glutathione in the liver tissue in CCl_4-stimulated rats. The antioxidative activity of CS-PS probably contributes to its inhibition of lipid peroxidation induced by CCl_4. Since CYP450 is important in mediating CCl_4 toxicity, the effects of CS-PS on CYP450 deserves further investigation.

Colchicine is a plant alkaloid that was tested in clinical trials for anti-fibrotic treatments and was found to improve laboratory values, fibrosis markers and prolong survival in patients with mild to moderate cirrhosis,[15–17] But, colchicine is criticized because of the significantly increasing risk of adverse events and is recommended for cirrhosis treatment.[57]

In the present study, CS-PS, the major active components of the mass-produced cultured *cordyceps sinensis*, has been demonstrated to ameliorate liver fibrosis induced by CCl_4 in rats significantly and the anti-fibrotic effects of CS-PS was comparable to that of colchicine. Furthermore, the inhibition on liver fibrosis by CS-PS is also demonstrated to be associated with its down-regulation on HSCs activation, protein expression of hepatic TGF-β1, TβRI, TβRII, p-Smad2, p-Smad3 and TIMP2, as well as the activities of MMP2 and MMP9 in the present study. In the consideration of the urgent needs of development of effective and safe reagents on liver fibrosis, the mechanisms of CS-PS inhibition liver fibrosis deserve further investigation.

Author contributions: YH, JP and QF designed the study; XL, LC and LX conducted the experiments; JP and XL analysed data; and JP wrote the manuscript.

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