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• BASIC RESEARCH •

Inhibitive effect of cordyceps sinensis on experimental hepatic fibrosis and its possible mechanism

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Abstract

AIM: To investigate the inhibitive effect and its possible mechanism of Cordyceps Sinensis (CS) on CCl_4-plus ethanol-induced hepatic fibrogenesis in experimental rats.

METHODS: Rats were randomly allocated into a normal control group, a model control group and a CS group. The latter two groups were administered with CCl_4 and ethanol solution at the beginning of the experiment to induce hepatic fibrosis. The CS group was also treated with CS 10 days after the beginning of CCl_4 and ethanol administration. All control groups were given corresponding placebo at the same time. At the end of the 9th week, rats in each group were humanely sacrificed. Blood and tissue specimens were taken. Biochemical, radioimmunological, immunohistochemical and molecular biological examinations were used to determine the level change of ALT, AST, HA, LN content in serum and $TGF\beta_1$, PDGF, collagen I and III expression in tissue at either protein or mRNA level or both of them.

RESULTS: As compared with the model control group, serum ALT, AST, HA, and LN content levels were markedly dropped in CS group (86.0±34.4 *vs* 224.3±178.9, 146.7±60.2 *vs* 272.6±130.1, 202.0±79.3 *vs* 316.5±94.1 and 50.4±3.0 *vs* 59.7±9.8, respectively, $P<0.05$). Tissue expression of $TGF\beta_1$ and its mRNA, collagen I mRNA were also markedly decreased (0.2±0.14 *vs* 1.73±1.40, 1.68±0.47 *vs* 3.17±1.17, 1.10±0.84 *vs* 2.64±1.40, respectively, $P<0.05$). More dramatical drop could be seen in PDGF expression (0.87±0.43 *vs* 1.91±0.74, $P<0.01$). Although there was no statistical significance, it was still strongly suggested that collagen III mRNA expression was also decreased in CS group as compared with model control group (0.36±0.27 *vs* 0.95±0.65, $P=0.0615$). In this experiment, no significant change could be found in PDGF mRNA expression between two groups (0.35±0.34 *vs* 0.70±0.46, $P>0.05$).

CONCLUSION: Cordyceps sinensis could inhibit hepatic fibrogenesis derived from chronic liver injury, retard the development of cirrhosis, and notably ameliorate the liver function. Its possible mechanism involves inhibiting $TGF\beta_1$ expression, and thereby, down regulating PDGF expression, preventing HSC activation and deposition of procollagen I and III.

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INTRODUCTION

The incidence rate of chronic in China is high, which afflicts the patients by progressively developing into irreversible cirrhosis[1,2]. Hepatic fibrosis is the intermediate and crucial stage of this process, characterized by reversibility. If treated properly in this stage, cirrhosis could be successfully prevented[3].

Clinical observation and experimental data suggested that liver fibrosis could be reabsorbed under certain conditions. Chinese herbs, well known for their definite effectiveness, cheap prices and negligible side effects, have particular advantages in therapeutic research of hepatic fibrogenesis. Several herbs were suggested recently by some reports to have preventive effect on hepatic fibrosis[4-12], and cordycep sinensis (CS) is one of them[4,5]. However, its exact effectiveness and detailed mechanisms have not been elaborated. In this study, we established the animal model of chronic liver injury-hepatic fibrosis-cirrhosis, intervened with CS, and observed its inhibitive effect. An array of indexes in protein and mRNA levels was established in order to thoroughly investigate its possible mechanism.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing between 200 g and 300 g were obtained from Experimental Animal Center of ChongQing University of Medical Science, China. The rats were housed 3 or 4 per cage and subjected to 12-day/12-night cycle with unrestrictive access to basic food. All animals were treated humanely according to the national guideline for the care of animals in the country.

Preparation for CS suspension

CS was purchased from Bao Ding Pharmaceutical Company, China.

The CS and double-distilled water were mixed in proportion of 1:3 and subject to full vibration.

Reagents

$TGF\beta_1$, PDGF, procollagen I and III RNA probe and detection kit for *in-situ* hybridization were purchased from Boster Biologic Technology Company, China. Anti- $TGF\beta_1$ monoclonal antibody, anti-PDGF multiclonal antibody and its detection kit for immunohistochemical assay were purchased from Santa Cluz biologic technology Company, USA. Serum ALT, AST, HA, and LN examinations were performed by the Laboratory Department of Chong Qing University of Medical Sciences, China.

Establishment of animal model: carbon tetrachloride (CCL_4)-plus-ethanol induced hepatic fibrosis

Sixty-six male Wista rats were randomly assigned to a normal control group, a model control group and a CS group. At the beginning of the experiment, rats in model control group and CS group were subjected to hypodermic injection of (40 % in bean oil) at a dose of 0.3 ml/100 g of body weight twice a week. Besides, rats in these two groups also received 5 %

ethanol solution as the only fluid to drink. Rats in normal control group received hypodermic injection of bean oil at the same dose and frequency as the other two groups and received water ad libitum. Ten days after the CCL_4 administration (for 3 times), CS group was given CS suspension orally at a dose of 1 ml/100 g body weight daily. In the meantime, three rats in model control group were randomly sacrificed to evaluate the liver histological change at this moment while other rats along with rats in normal control group were given saline orally at a dose of 1 ml/100 g body weight daily. All the administrations lasted 9 weeks.

Collection of specimens

At the end of the 9th week, rats in each group were humanely sacrificed by amobarbital sodium anesthesia. Midline laparotomy was performed. Livers were excised and blood was collected through cardiopuncture.

Histological grading

Liver tissues were fixed in formalin and embedded in paraffin blocks according to standard procedures (glass slide was cleaned with 95 % ethanol, treated with APES solution and air dried.) four to six micron thick tissue sections were cut using microtome and applied to slides; And deparafinized in xylenes using three changes for 5 minutes each. Hydrate sections gradually passed through graded alcohols: washing in 100 % ethanol twice, then 95 % ethanol twice for 10 minutes each; and washing in deodorized water for 1 minute. Hemotoxylin and eosin (HE) staining was performed according to the standard procedure.

Fibro-proliferation degree of liver sections were graded numerically based on the criterion described below: grade 0: normal liver; grade 1: few collagen fibrils extend from the central vein and portal tract; grade 2: collagen fibrils extension are obvious but do not encompass the whole lobule; grade 3: collagen fibrils extend and encompass the whole lobule; grade 4: collagen fibrils extend and separate lobule into pseudo-lobule, but mainly shaped in square form; and grade 5: pseudo-lobule shaped mainly in circle form.

Two pathologists who had no knowledge of their sources and each other's assessment examined stained slide independently.

Immunohistochemistry

Liver tissues were fixed in formalin and embedded in paraffin blocks according to standard procedures. Glass slide was cleaned with 95 % ethanol, treated with subbing solution and air-dried. Tissue sections of 4-6 micron thick were cut using microtome and applied to slides; and deparafinized in xylenes using three changes for 5 minutes each. Hydrate sections gradually through graded alcohols: washing in 100 % ethanol twice, then 95 % ethanol twice for 10 minutes each; and washing in deironized water for 1 minutes. Incubate sections for 15 minutes in 0.1 % pepsin at room temperature to expose the antigen masked by formalin fixation and paraffin embedding. Incubate sections with 3 % H_2O_2 and normal non-immunal goat serum for 10 minutes respectively to inactivate endogeneous peroxidase and biotin; incubate sections with primary antibody overnight at 4 ℃. Optimal antibody concentration was determined previously. Wash with three changes of PBS for 5 minutes each. Incubate for 20 minutes with biotin-conjugated secondary antibody and avidin biotin enzyme reagent respectively. Wash with three changes of PBS for 5 minutes each. Incubate in peroxidase substrate for 5 minutes, after that dehydrate through alcohols and xylenes. Immediately add 1-2 drops of permanent mounting medium and covered with glass coverlip.

In situ hybridization

The sequence of RNA probe is as follows:

1. TGFβ_1mRNA
(1) 5' -CGTTT CACCA GCTCC ATGTC GATGG TGTTG CAGGT-3'
(2) 5' -CTTGA TTTTA ATCTC TGCAA GCGCA GCTCT GCACG-3'
(3) 5' -TTGGT ATCCA GGGCT CTCCG GTGCC GTGAG CTGTG-3'
2. PDGF mRNA
(1) 5' -CTCGG CTTCC TCGGC CAGAA CATGG GCGAG GTATC-3'
(2) 5' -AACCT CACCT GGACT TCTTT CAATT TTGGC TTCTT-3'
(3) 5' -TTGCA CTCGG CGATC ATGGC CGGCT CAGCA ATGGT-3'
(4) 5' -GGCTC CAAGG GTCTC CTTCA GTGCC GTCTT GTCAT-3'
3. Col-I mRNA
(1) 5' -CACAG ATCAC GTCAT CGCAC AACAC CTTGC CGTTG-3'
(2) 5' -AGCTT CACCG GGACG ACCAG CTTCA CCAGG AGATC-3'
(3) 5' -TCACT CCTTC TACAT TATAT TCAAA CTGGC PGCCA-3'
4. Col-III mRNA
(1) 5' -ATTAA CAGAC TTGAG TGAAG TCATA ATCTC ATCGG-3'
(2) 5' -AGAAT ACAAT CTGTG TTTCT GACCA GGTGA GGTAG-3'
(3) 5' -GAAGG AGGAG AATCC CGTGG CATGC CAATG AATCT-3'

In situ hybridization was performed as described elsewhere. Briefly, formalin-fixed and paraffin-embedded liver section slides were pretreated by incubation with 0.1 % pepsin, 3 % H_2O_2 and normal non-immunal bovine serum for 10-15 minutes respectively to expose signals masked by formalin fixation and paraffin embedding and inactivate endogeneous peroxidase and biotin. Antisense RNA probe was then added to the sections and incubated together in humidified chamber overnight at 37 ℃. After washing with three changes of 2×standard saline citrate (SSC) and 0.2×SSC for 10 minutes each, the sections were subject to incubation with biotin-conjugated secondary antibody and avidin biotin enzyme reagent respectively. Wash with three changes of PBS for 5 minutes each, incubate in peroxidase substrate for 20 minutes, and dehydrate through alcohols and xylenes. One to two drops of permanent mounting medium were immediately added and covered with glass coverlip.

Semiquantitative image analysis

Computer morphometry (CM-2000B Medical Image Analysis Software, Beijing Medical Software Company, China) was used to quantify the optical density of the signal generated by the immunohistochemical and *in situ* hybridization examination. The exact method is described as follows: The video image was generated with a video camera and digitalized for image analysis at 256 gray levels. An optical threshold and filter combination was set to select positive stains. The structure of interest was discriminated automatically by computer and measured for its optical intensity and total area. Staining index was obtained by multiplying the quantified number of optical intensity and total area.

Statistical analysis

Data were analyzed with SAS software. Quantitative data were presented as means ±SD and were compared using *t* test procedure. Frequency data were compared using NPAR1WAY procedure.

RESULTS

CS inhibits fibril deposition and ameliorates liver function of chronic hepatitis

After 10 days (3 times) of CCL4 administration, rats suffered hepatocyte lipoid degeneration, narcosis, and inflammatory cells infiltration, which fulfilled the diagnostic standard for chronic hepatitis.

Specimens from model control group showed apparent formation of fibrotic septa, encompassing regenerated hepatocytes into pseudo-lobules. Regenerated hepatocytes

underwent severe lipraoid degeneration. Specimens from CS group showed only slight fibrogenesis without pseudo-lobule formation.

Although statistical analysis failed to present any significant disparity between quantitative data of histological grading of the two groups, it still indicated that fibrogenesis of CS group was much less severe than that of model control group (Table 1). Compared with model control group, serum contents of HA and LN in CS group were markedly decreased ($P<0.05$), which indicates from another perspective that CS could inhibit hepatic fibrogenesis (Table 2).

Serum contents of ALT and AST in model control group were significantly elevated compared with both normal control and CS groups. However, there was no significant statistical difference between normal control and CS groups ($P>0.05$), which showed that CS could notably ameliorate liver function(Table 2).

CS reduces procollagen I and III mRNA synthesis

Procollagen I and III mRNA synthesis level in liver tissues were determined by *in situ* hybridization and quantified by computer image analyzing system. Positive staining could only be seen at central vein and periportal areas in normal control group. As the model control and CS groups, positive stains were distributed mainly along fibrotic septa other than central vein and periportal areas.

Compared with model control group, staining index of CS group remarkably dropped ($P<0.05$), which suggested that procollagen I mRNA synthesis was strongly inhibited by CS treatment. On the part of procollagen III, despite that there was no significant difference between the two groups, it was also reasonably suggested that CS could inhibit the synthesis of procollagen III mRNA on the consideration of P value (0.0695) (Table 3).

CS reduces TGFb_1 expression

TGFβ_1 expression in protein and mRNA level were determined by immunohistochemistry and *in situ* hybridization, respectively. Positive stains were quantified with computer image analyzing system. For the sections of normal control group, positive staining of TGFβ_1 and its mRNA was found at central vein and periportal areas. In the sections of model control group and CS group, positive staining could be seen at interstitial cells, inflammatory cells, impaired hepatocytes as well as normal hepatocytes. Fibrotic septa were only slightly stained. TGFβ_1 mRNA positive stain distribution was not completely consistent with that of TGFβ_1. More stain was found at fibrotic septa and less at impaired and normal hepatocytes.

Compared with model control group, the staining index of both TGFβ_1 and TGFβ_1mRNA in CS group was markedly decreased ($P<0.05$, respectively), indicating that CS could inhibit TGFβ_1 mRNA transcription and, thereby, reducing the TGFβ_1 expression (Table 3).

CS reduces PDGF expression

PDGF expression in protein and mRNA level was determined, like TGFβ_1, by immunohistochemistry and *in situ* hybridization respectively. Positive staining was quantified with computer image analyzing system. For the sections of normal control group, positive stains of PDGF and its mRNA could be seen at central vein and periportal areas. In the sections of model control group and CS group, positive stains mainly appeared at fibrotic septa.

PDGF expression in CS group dropped dramatically compared with model control group ($P<0.01$). But for PDGF mRNA, there was no significant difference by statistical analysis. In spite of this, the means of two groups still suggested difference. To make sure of this, further studies are needed (Table 3).

Table 1 Histological grading

Group	Grade						
	0	1	2	3	4	5	6
Normal	7						
Model		3	1	2	2	2[a]	
CS		4	5[ab]				

[a]$P<0.05$ *vs* normal control group, [b]$P<0.05$ *vs* model control group.

Table 2 Serum content of HA, LN, ALT and AST ($\bar{x}\pm s$)

Groups	HA (μg/L)	LN (μg/L)	ALT (U/L)	AST(U/L)
Normal	142.4±51.0	41.6 ±2.2	63.6±11.9	108.6±27.7
Model	316.5±94.1[a]	59.7±9.8[a]	224.3±178. 9[a]	272.6±130.1[a]
CS	202.0±79.3[ab]	50.4±3.0[ab]	86.0±34.4[b]	146.7±60.2[b]

[a]$P<0.05$ *vs* normal control group, [b]$P<0.05$ *vs* model control group.

Table 3 Staining index of procollagen I, III mRNA, TGFβ_1, TGFβ_1 mRNA, PDGF and PDGF mRNA in liver tissues ($\bar{x}\pm s$)

Groups	ProcolI mRNA	ProcolIII mRNA	TGFβ_1	TGF$_1$ mRNA	PDGF	PDGF mRNA
Model	2.64±1.40	0.95±0.65	1.73±1.40	3.17±1.17	1.91±0.74	0.70±0.46
CS	1.10±0.84[a]	0.36±0.27[c]	0.2±0.14[a]	1.68±0.47[a]	0.87±0.43[b]	0.35±0.34[c]

[a]$P<0.05$, [b]$P<0.01$, and [c]$P>0.05$, *vs* model control group.

DISCUSSION

In this study, we demonstrated that CS could inhibit hepatic fibrogenesis and retard the development of cirrhosis by evaluating histological grading and serum contents of HA and LN. Its possible mechanism involves inhibiting the synthesis of TGFβ_1mRNA and thereby downregulating the expression of TGFβ_1 and PDGF, reducing the deposition of collagen I and III.

CS is a type of traditional Chinese tonic that has already been demonstrated by modern pharmacological researches to have extensively positive effect on a few systems of human body[13-19]. Recently, some reports suggested that this herb might also have preventive effect on hepatic fibrosis[4,5]. But the real effect and mechanisms have not been elaborated. This study was designed to evaluate its exact effect on hepatic fibrosis and to investigate its possible mechanism.

Various kinds of chronic liver injury widely spread all over the world and afflicted patients greatly. Effective ways to inhibit fibrogenesis and prevent the development of cirrhosis are of great clinical and academic significance. Although many new agents were being tested, no satisfactory agent with ascertained effectiveness and negligible side effects has appeared as yet. Traditional Chinese herbs were well known for their cheap prices and negligible side effects. Exploration in this area is promising.

We started to treat the rats with CS after 10 days of CCL4 administration. Pathological evaluation showed that rats suffered chronic liver injury in this moment. CS treatment based on this disorder presented its inhibitive effect on preventing the development of cirrhosis. HA and LN are good serum markers of hepatic fibrogenesis[20]. In this study, serum contents of HA and LN in CS group markedly dropped compared with model control group, which indicates that CS could successfully prevent hepatic fibrogenesis. Histological grading also supported this conclusion.

To address the ways in which this herb yielded in a

significant reduction in fibrosis, we investigated the effect of CS treatment in the expression of TGFβ_1 as well as its mRNA. Overexpression of this cytokine was associated closely with fibrogenesis in many ways[21-25]. It can promote HSC to synthesize collagen I and III, and simultaneously inhibit their decomposition by upregulating the expression of Tissue Inhibitor of Metalproteinase (TIMP), which neutralize the activity of Matrix Metalproteinase (MMP)[26-28], an important degrading enzyme of collagen I and III. In addition, TGFβ_1 could also indirectly promote the HSC proliferation by enhancing the expression of PDGF and its receptor[21]. One strategy in the development of antifibrotic drug is the exploration of TGFβ_1 inhibitors[21]. Because TGFβ_1 expression was regulated by diverse factors in transcription, post-transcription, secretion and releasing levels, the expression of its protein and mRNA varied considerably[22]. Consequently, analyses in two levels were indispensable. In this study, we determined the expression of this cytokine by immunohistochemistry and *in situ* hybridization to investigate the effect of CS on TGFβ_1 expression in the both levels. The results showed that both TGFβ_1 and its mRNA expression remarkably decreased in CS group, indicating that CS could downregulate the expression of this important cytokine, which possibly contributed to the reduction of fibrosis.

PDGF is another important cytokine that influences the development of fibrosis. According to the previous reports[29-33], it is the most potent HSC-proliferation promoter, which plays an important role in fibrogenesis. In spite of its earlier identification and isolation, few pharmacological studies observed the effect of the potential agents on this cytokine. In this study, we initially observed the change of this cytokine responding to CS treatment. Compared with model control group, PDGF expression level in protein of CS group dramatically dropped, indicating that CS could inhibit the PDGF expression. Statistical analysis showed no significant difference of PDGFmRNA expression between the two groups. Whether CS exerts inhibitive effect on PDGF expression in mRNA or directly in protein still remains unclear. Further studies are needed to elucidate the detailed mechanisms.

Pathological feature of hepatic fibrosis is the excessive deposition of ECM components[34-36]. As the medium of parenchyma cells, constancy of ECM component is essential to the maintenance of liver function. Changes of proportion of ECM components, and the change of their quantities, cause the damage of hepatocytes and the deterioration of liver function. Syntheses of Collagen I and III increase greatly when fibrogenesis occurs, which are mainly responsible for the adverse effects brought about by ECM. As a result, Collagen I and III overshadow other components and become the most important ECM in the development of fibrosis. After nine weeks of CS treatment, expression of procollagen I and III decreased. On one hand, this result further manifests that CS has inhibitive effects on fibrosis; on the other hand, it might be one of the possible explanations for the amelioration of liver function.

CS is a cheap and widely available herb that is well tolerated and has been used for centuries in traditional Chinese medicine without any side effect reported. In this study, we demonstrated that, administered at the stage of chronic hepatitis, CS could successfully inhibit hepatic fibrogenesis and retard the development of cirrhosis. Moreover, it can strikingly ameliorate the liver function. Therefore, we suggest that this herb should serve as a promising antifibrotic agent and deserves further investigations.

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