

Antifibrotic Effect of Extracellular Biopolymer from Submerged Mycelial Cultures of *Cordyceps militaris* on Liver Fibrosis Induced by Bile Duct Ligation and Scission in Rats

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The antifibrotic effects of hot water extract (WEC), intracellular biopolymer (IPC) and extracellular biopolymers (EPC) from mycelial liquid culture of *Cordyceps militaris* on liver fibrosis were studied. Liver fibrosis was induced by a bile duct ligation and scission (BDL/S) operation, duration of 4 weeks in rats. In BDL/S rats, the levels of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin in serum and hydroxyproline content in liver were dramatically increased. The WEC or IPC treatment (30 mg/kg/day for 4 weeks, p.o.) in BDL/S rats reduced the serum AST, ALT and ALP levels significantly ($p < 0.01$). The EPC treatment (30 mg/kg/day for 4 weeks, p.o.) reduced the serum ALT, AST and ALP levels significantly ($p < 0.01$). Malondialdehyde contents in liver treated with WEC, IPC or EPC were significantly reduced ($p < 0.05$). But Liver hydroxyproline content was decreased only in EPC treated BDL/S rats to 55% that of BDL/S control rats ($p < 0.01$). The morphological characteristics and expression of alpha smooth muscle like actin in fibrotic liver, which appeared in BDL/S control group were improved in EPC treated fibrotic liver. These results indicate that EPC (30 mg/kg/day for 4 weeks, p.o.) has an antifibrotic effect on fibrotic rats induced by BDL/S.

Key words: Bile duct ligation and scission, *Cordyceps militaris*, Liver fibrosis, Lipid peroxidation, Alpha-smooth muscle like actin

INTRODUCTION

Although there has been a lot of effort devoted to finding effective drugs for liver fibrosis, there has been no specific therapeutics that can prevent or cure liver fibrosis. It has been reported that corticosteroids (Guzelian *et al.*, 1984), penicillamine (Brenner *et al.*, 1990), colchicines (Brenner *et al.*, 1990; Rodriguez, L., 1998), proline hydroxylase inhibitors (Boker *et al.*, 1991; Bicker *et al.*, 1998), prostaglandins (Brenner *et al.*, 1990), interferon-gamma (Rockey *et al.*, 1992), proline analogues (Ala-Kokka *et al.*, 1989), retinoids (Brenner *et al.*, 1990) and malotilate (Ala-Kokka *et al.*, 1989) have antifibrotic effects, but there

are few promising therapeutics that are safe and effective in patients with liver fibrosis.

As part of the ongoing multidisciplinary research project in our research center, we have screened candidates for antifibrotics from natural products, which have been used for liver diseases or as a general health tonic in oriental folk medicine, for the past several years (Park *et al.*, 1997; Song *et al.*, 1998; Nan *et al.*, 2000a; Nan *et al.*, 2000b; Park *et al.*, 2000a; Park *et al.*, 2000b). The *Cordyceps* fungi species (Clavicipitaceae) are one of the natural products that have been classified as a general health tonic in oriental folk medicine as well as a very effective herb for treating circulatory, respiratory, immune, sexual dysfunction (Sung *et al.*, 1993). A number of bioactive constituents from *Cordyceps* species have been reported, recently. These are : cordycepin and other antibacterial and antitumor adenosine derivatives, ophicordin, an antifungal agent, a biopolymer shown to have anti-

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tumor activity, an immunopotentiating galactomannan, and L-tryptophan (Bok *et al.*, 1999). Until now studies have been focused on *C. sinensis*, which has been used as genuine *Cordyceps* fungi in China. But because of the harsh environment and very small production amounts, wild type of *C. sinensis* is very rare and expensive. In Korea, it was reported that 5 species of wild *Cordyceps* were collected: *C. militaris*, *C. nutans*, *C. sphecocephala*, *Isaria japonica* and *Torrubiella* species. From the studies reported until now, components and pharmacological effects of *C. militaris*, which is easily found in Korea and cheaper than *C. sinensis*, are similar to those of *C. sinensis* (Ikumoto *et al.*, 1991; Zhao *et al.*, 2000).

In order to evaluate the antifibrotic potential of a given agent, selection of appropriate animal models that most closely reproduce human chronic liver diseases is important (Park *et al.*, 1997; Nan *et al.*, 2000a; Nan *et al.*, 2000b; Nan *et al.*, 2000c). Extrahepatic obstruction of the bile duct leads to dilation of bile canaliculi, reduction of canalicular microvilli, widening of the pericanalicular space and increased lysosomal activity. Biliary obstruction for 4 weeks or more leads to fibrosis and resembles the biliary fibrosis/cirrhosis of human in contrast to other models induced by toxicants. Based on these principles, liver fibrosis was induced by bile duct ligation and scission in rats in this study (Park *et al.*, 1997; Nan *et al.*, 2000a; Nan *et al.*, 2000b; Nan *et al.*, 2001).

We report herein the antifibrotic effect of the extracellular biopolymer from submerged mycelial cultures of *Cordyceps militaris* on liver fibrosis induced by bile duct ligation and scission in rats and as far as we know, this is the first report that biopolymer from *Cordyceps militaris* has antifibrotic effect *in vivo*.

MATERIALS AND METHODS

Submerged mycelial culture of *Cordyceps militaris*

Cordyceps militaris was obtained from the Rural Development Administration (Suwon, Korea). The composition of the synthetic medium for mycelial growth was as follows (g/L): galactose 1, sucrose 9, xylose 1, glucose 9, yeast extract 0.5, peptone 2, potato dextrose broth 2, $\text{NH}_4\text{H}_2\text{PO}_4$ 0.5, DL-serine 0.5, KH_2PO_4 1, CaCl_2 0.6, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 2, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.02. The pH was adjusted to 5.0 before sterilization. The submerged mycelial culture was carried out in 500 ml flask containing 200 ml of medium on a rotary shaker (120 rpm, 10 days) or in a 5 l air-lift fermenter at 20°C with an air flow rate of 1.0 vvm.

Preparation of extracellular biopolymer of *C. militaris* (EPC)

The submerged mycelial culture was carried out in a 5 l jar fermenter (working volume: 3 l, pH: 5, temperature:

25°C, agitation speed:100 rpm and aeration rate:1 vvm) for 10 days. Culture broth was harvested by centrifugation (10,447g for 20 min) and supernatant was treated with four times (v/v) of ethanol to collect the exopolymers by centrifugation (10,447 × g for 20 min). The precipitate (EPC) was redissolved in distilled water (10 mg of EPC/ml), dialysed for 3 days and then lyophilized.

Preparation of intracellular biopolymer of *C. militaris* (IPC)

The submerged mycelial culture was carried out in 500 ml flasks containing 200 ml of media on a rotary shaker (120 rpm, 10 days). Mycelia were harvested by centrifugation (10,447 × g/20 min), washed with distilled water and lyophilized for extraction of the intracellular biopolymer. Dried mycelia were extracted with hot water, then hot water extract was centrifuged (10,447 × g for 20 min). IPC was precipitated by adding 4-fold volume of ethanol to the supernatant. Ethanol precipitated only high molecular weight carbohydrates or carbohydrate protein complexes (i.e. polysaccharides or protein bound polysaccharides) from all of the water soluble components in hot water extract. For purification, the precipitate (IPC) was redissolved in distilled water (10 mg of IPC/ml). After centrifugation (10,447 × g for 20 min), supernatant was dialysed for 3 days and lyophilized.

Preparation of hot water extract of cultured *C. militaris* mycelia (WEC)

The submerged mycelial culture was carried out in 500 ml flasks containing 200 ml of media on a rotary shaker (120 rpm, 10 days). Mycelia were harvested by centrifugation (10,447 × g/20min), washed with distilled water and lyophilized. Dried mycelia were extracted with hot water and hot water extract was centrifuged (10,447 × g for 20 min). The supernatant was lyophilized and the dried product was redissolved in distilled water (10 mg of dried product/ml). After centrifugation (10,447 × g for 5 min) the supernatant was dialysed for 3 days and lyophilized. WEC consisted low molecular weight sugars, amino acids or peptides as well as high molecular weight carbohydrates or carbohydrate protein complexes.

Animals

Male Sprague-Dawley rats (initial body weight: 200-250 g) were used. They received normal food and water *ad libitum* and were maintained under 12 h light-dark cycles at 20~23°C and 50~60% relative humidity throughout the experiment.

Liver fibrosis induction and animal treatment

Rats were anesthetized with Ketamine/Rompun and double ligatures were performed on the common bile

duct with a section between the ligatures. In sham rats, only an incision was made in the abdomen, which was then closed without any treatment. The number of rats used in each group is shown in Table I. Lyophilized samples were dissolved with distilled water, and given orally using intubation needle to rats in each group from the beginning of the experiment up to 28 day, at a dose of 30 mg/kg/day of corresponding sample. The control groups received the equal amounts of distilled water for 28 days, orally by using intubation needle. After 28 day of treatment, rats were anesthetized with ether and blood was obtained by cardiac puncture for serum biochemical testing. Immediately, the liver was removed and weighed. A portion of the liver was fixed in 10% neutralized formalin (pH 7.4) for morphological examination and the rest was kept at -20°C for determination of hydroxyproline.

Serum biochemical parameters

Rats were weighed weekly for the duration of the experiment. After 28 days of treatment, the rats were anesthetized with ether and blood was obtained by cardiac puncture for serum biochemical testing. Sera were obtained by centrifuging blood samples at 3000 rpm after incubation at room temperature for one hour. Sera were subsequently kept at -20°C until further analysis. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) activities and levels of total-cholesterol and total-bilirubin were measured using an Autodry chemistry analyzer (SPOTCHEM™ SP4410, Arkray, Japan).

Liver hydroxyproline content measurement

Liver collagen concentrations were estimated by measuring hydroxyproline (Jamall *et al.*, 1981). In brief, specimens

of the liver were weighed and hydrolyzed completely in 6 M hydrochloric acid. Aliquots of the samples were derivatized using Chloramine T solution and Ehrlich reagent and measured at 558 nm. A standard calibration curve was prepared using trans-4-hydroxy-L-proline (Sigma Chem. Co., USA).

Measurement of lipid peroxidation in liver

Lipid peroxidation was quantified by the thiobarbituric acid (TBA) method (Plaa *et al.*, 1994) using S9 fraction of liver homogenates as enzyme source; all manipulations were made on ice and rapidly to avoid peroxidation. S9 fraction of homogenate (approximately 5 mg protein) was reacted with TBA solution in a boiling water bath for 10 min and then extracted with n-butanol for 10 sec. The organic phase containing colored complex was collected for fluorescence measurement. 532 nm and 553 nm were used for excitation and emission wavelengths, respectively. The calibration curve was prepared using 1,1,3,3-tetraethoxypropane (Sigma Chemical Co., USA), a chemical releasing malondialdehyde (MDA) in acidic conditions.

Histochemical and immunohistochemical examination

The portion of removed liver was rapidly fixed with 10% neutralized formalin (pH 7.4). Alpha-smooth muscle like actin (alpha-SMA) for detection of activated hepatic stellate cells was immunohistologically assessed by the streptavidin-biotin-peroxidase complex method using LSAB®2 Kit (DAKO Co., USA) and anti-alpha-smooth muscle like actin monoclonal antibody (Boehringer Mannheim, Germany).

Statistical analysis

Table I. Serum biochemical values in fibrotic rats induced by bile duct ligation and scission (BDL/S) treated with polysaccharides or hot water extract from submerged mycelial cultures of *Cordyceps militaris* for 4 weeks

Group	n	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	T-chol (mg/dl)	T-bil (mg/dl)
Control-Sham	4	86 ± 18	49 ± 6	603 ± 75	84 ± 23	0.3 ± 0.2
Control-BDL/S	4	670 ± 95*	153 ± 11*	1326 ± 27*	133 ± 11*	8.2 ± 1.2*
WEC-BDL/S	4	413 ± 85*.#	90 ± 21*.#	818 ± 102*.#,†,‡	101 ± 21	7.8 ± 0.5*
IPC-BDL/S	8	369 ± 69*.#	82 ± 11*.#	934 ± 116*.#	114 ± 14*	6.8 ± 3.9*
EPC-BDL/S	8	475 ± 69*.#	105 ± 22*.#	1014 ± 61*.#	108 ± 11	7.7 ± 1.9*

Results represent the mean ± S.D.

*: Significantly different from each sham group ($p < 0.01$). #: Significantly different from control-BDL/S group ($p < 0.01$). †: Significantly different from IPC-BDL/S group ($p < 0.05$). ‡: Significantly different from EPC-BDL/S group ($p < 0.01$).

n: number of rats.

WEC: Treated with hot water extract of submerged mycelial cultures of *C. militaris* (30 mg/kg/d, p.o.).

IPC: Treated with intracellular biopolymer of submerged mycelial cultures of *C. militaris* (30 mg/kg/d, p.o.).

EPC: Treated with extracellular biopolymer of submerged mycelial cultures of *C. militaris* (30 mg/kg/d, p.o.).

AST: Aspartate transaminase.

ALT: Alanine transaminase.

ALP: Alkaline phosphatase.

T-bil: Total-bilirubin.

T-chol: Total-cholesterol.

Results were expressed as means \pm S.D. Statistical differences were determined between groups using a one-way ANOVA and Tukey's multiple comparison test. Values of $p < 0.05$ were considered a significant.

RESULTS AND DISCUSSION

Serum biochemical testing

Serum biochemical parameters are shown in Table I. Activities of serum AST, ALT, ALP and levels of total-bilirubin were elevated significantly in control BDL/S rats ($p < 0.01$). In BDL/S rats treated with WEC (30 mg/kg/day), serum AST, ALT and ALP activities were reduced to 62%, 59% and 62% that of control BDL/S rats, respectively ($p < 0.01$). In IPC treated BDL/S rats, serum AST, ALT and ALP activities were reduced to 55%, 54% and 70% that of control rats ($p < 0.01$). In BDL/S rats treated with EPC, serum ALT, AST and ALP activities were decreased to 69%, 71% and 77%, respectively, that of control BDL/S rats ($p < 0.01$). WEC significantly lowered ALP activities when compared to IPC ($p < 0.05$) or EPC ($p < 0.01$), while there were no significant differences between the treated groups in other serum biochemical parameters.

Hydroxyproline contents in liver

As shown in Table II, liver hydroxyproline increased to about 3.3-fold 28 days after BDL/S ($p < 0.01$). Compared with the control BDL/S rats, treatment with EPC (30 mg/kg/day) reduced the hydroxyproline content in the liver up to 45% ($p < 0.01$). In contrast, WEC (30 mg/kg/day) and IPC (30 mg/kg/day) had little effect on the hydroxyproline content of fibrotic rat liver although WEC and IPC improved the serum biochemical parameters. In EPC-, IPC- or WEC- treated sham rats, there were no significant changes in hydroxyproline content compared with that of control sham rats (data not shown).

Malondialdehyde contents in liver

Malondialdehyde values in liver of BDL/S control rats were increased to 174% ($p < 0.001$) that of sham, showing that 4 weeks of biliary obstruction increases lipid peroxidation in liver, significantly. Malondialdehyde values of sham rats treated with EPC, IPC or WEC were similar to that of normal control rats. WEC, IPC and EPC treatment decreased lipid peroxidation in liver to 74%, 78% and 66%, respectively, that of BDL/S liver ($p < 0.05$) (Table II).

Morphological changes in liver

Histology of BDL/S rat liver stained with H&E showed excessive bile duct proliferation and inflammation throughout the liver, connective tissue deposition near portal triad resulting in destruction of the lobular architecture (Fig. 1, upper panel). In EPC-treated rats, there was a tendency towards less pronounced destruction of the liver architecture and degree of bile duct proliferation, inflammation and fibrosis through out the liver when compared with control BDL/S rat liver. In both WEC- and IPC-treated BDL/S rats the liver histology was similar to that of BDL/S rats (data not shown). Expression of alpha-SMA was reduced in EPC treated fibrotic liver (Fig. 1, lower panel), while WEC or IPC had no inhibiting effect on alpha-SMA expression (data not shown), indicating EPC inhibited hepatic stellate cell activation during liver fibrogenesis. Hepatic stellate cells are non-parenchymal cells in liver that are activated during liver injury and synthesize extracellular matrix leading to fibrosis in chronic liver injury. From the result above it is evident that EPC inhibited liver fibrosis by attenuating hepatic stellate cell activation during liver fibrosis caused by biliary obstruction. In contrast either WEC or IPC had no inhibition effect on hepatic stellate cell activation and this may be the reason why WEC or IPC did not reduce the hydroxyproline content in liver though they improved the serum biochemical parameters.

Table II. Hydroxyproline and thiobarbituric acid reactive substance in liver of fibrotic rats induced by bile duct ligation and scission treated with biopolymers or hot water extract from submerged mycelial cultures of *Cordyceps militaris* for 4 weeks

Group	n	Hydroxyproline ($\mu\text{g/g}$ liver)	Thiobarbituric acid reactive substance (pmole/mg protein)
Control-sham	4	298 \pm 48	286 \pm 78
Control-BDL/S	8	965 \pm 147*	497 \pm 76*
WEC-BDL/S	8	762 \pm 187*	370 \pm 54#
IPC-BDL/S	8	810 \pm 136*	387 \pm 87#
EPC-BDL/S	8	531 \pm 105*.#	329 \pm 65#

Results represent the mean \pm S.D.

*: Significantly different from each sham group ($p < 0.01$).

#: Significantly different from control-BDL/S group ($p < 0.01$).

n: number of rats.

WEC: Treated with hot water extract of submerged mycelial cultures of *C. militaris* (30 mg/kg/d, p.o.).

IPC: Treated with intracellular biopolymer of submerged mycelial cultures of *C. militaris* (30 mg/kg/d, p.o.).

EPC: Treated with extracellular biopolymer of submerged mycelial cultures of *C. militaris* (30 mg/kg/d, p.o.).

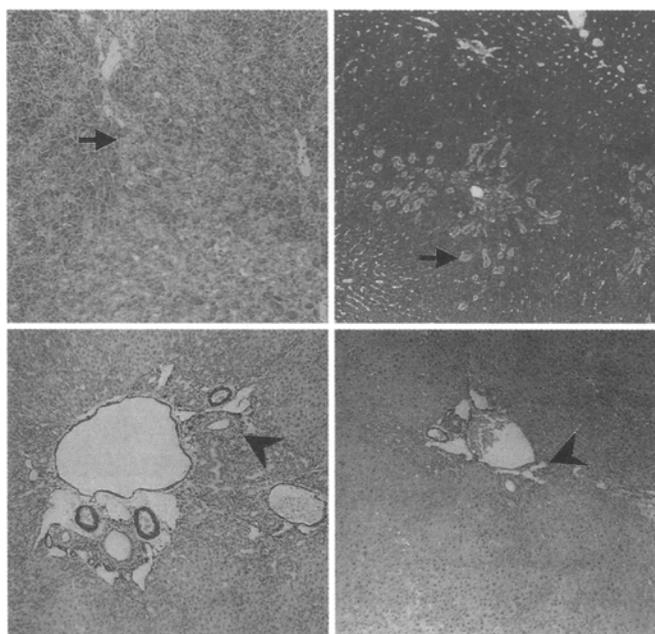


Fig. 1. Light microscopical appearance of fibrotic rat liver induced by bile duct ligation and scission treated with extracellular biopolymers from submerged mycelial cultures of *Cordyceps militaris* for 4 weeks (30 mg/kg/d, p.o.). Upper left: Fibrotic rat liver (Hematoxylin and eosin staining, 100 \times). Upper right: Fibrotic rat liver treated with extracellular biopolymer (H&E, 100 \times). Lower left: Fibrotic rat liver (Immunohisto-chemical staining against alpha smooth muscle cell like actin, 100 \times). Lower right: Fibrotic rat liver treated with extracellular biopolymer (Immunohistochemical staining against alpha smooth muscle cell like actin, 100 \times). Arrows indicate proliferated bile ducts. Arrow heads indicate the activated hepatic stellate cells expressing alpha-smooth muscle actin.

Cordyceps species are generally known as the ‘caterpillar fungus’ due to this characteristic parasitism of the living larvae of insects (Sung *et al.*, 1993). In Chinese medicine, it is classified as a general health tonic because of ability of *Cordyceps* to improve energy, stamina, appetite, endurance and sleep patterns. *C. militaris*, one of *Cordyceps* species which is cheap and widely used in Korea, is reported to show anticarcinogenic and hormonal effects (Liu *et al.*, 1997).

For the past several years, we have screened a number of antifibrotic agents from natural products, which are used traditionally for liver diseases or used as tonic in Asia (Park *et al.*, 1997; Song *et al.*, 1998). Developing antifibrotics from natural products used in traditional medicine may reduce the risk of toxicity and maintain therapeutic effectiveness when the drug is used clinically. But the uniformity of the natural product quality was problem because the contents of their active compounds were different due to the harvesting season and area. To maintain the uniform quality of *C. militaris* we used submerged mycelial cultures in this study.

From the results of present work, WEC and IPC

significantly lowered the serum ALT, AST, ALP activities and lipid peroxidation in liver, but they had no effect on collagen deposition or alpha-SMA expression in liver. Either WEC or IPC treatment in dose of 30 mg/kg/day had hepatoprotective effect as shown in improved serum biochemical parameters but they had no antifibrotic effect on biliary fibrosis in rats. EPC lowered the activities of serum ALT, AST and ALP and inhibited the collagen deposition and lipid peroxidation in liver. EPC attenuated hepatic stellate cell activation as shown in immunostaining against alpha-SMA. These results gave us the evidence that EPC treatment in dose of 30 mg/kg/day had antifibrotic effect via inhibiting hepatic stellate cell activation in rats with biliary obstruction. Total carbohydrate content in EPC was about 40.9% which, consisted of five different kinds of sugar. They were ramnose, ribose, mannose, galactose and glucose in the molar percent of 0.1, 53.8, 5.4, 5.0 and 36.1, respectively (Song *et al.*, 1998). The uronic acid content was 3.1%. Total protein content was only about 4.4%, and the major amino acids were glutamic acid (10.2%), aspartic acid (9.5%), serine (9.4%), and glycine (8.4%) (Song *et al.*, 1998). But further study is needed to clarify the active constituents responsible for these effects and to understand the antifibrotic mechanism of of extracellular biopolymer of submerged mycelial cultures of *C. militaris*.

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