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Antifibrotic Effect of *Stephania tetrandra* on Experimental Liver Fibrosis Induced by Bile Duct Ligation and Scission in Rats

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We examined the antifibrotic effect of a methanol extract from *Stephania tetrandra* (ST) on experimental liver fibrosis. Liver fibrosis was induced by bile duct ligation and scission (BDL/S) in rats. In BDL/S rats, activity levels of aspartate transaminase (AST), alanine transaminse (ALT), alkaline phosphatase (ALP), concentration of total bilirubin in serum, and hydroxyproline content of the liver were significantly increased. The ST treatment (either 100 mg/kg/day or 200 mg/kg/day, p.o. for 4 weeks) in BDL/S rats reduced the serum AST, ALT and ALP activity levels significantly ($p<0.01$). Similarly, when compared to the control group, the concentration of hydroxyproline in the livers of the BDL/S rats treated with 100mg or 200mg ST treated rats decreased by 40% and 33% respectively, when compared to the BDL/S control group ($p<0.01$). The morphological characteristics of fibrotic liver that were observed in the BDL/S control group, improved in the ST treated BDL/S group. In the fibrotic liver of BDL/S rats treated with ST, a marked reduction in the numbers of alpha smooth muscle cell actin positive stellate cells was observed. These results indicate that doses of either 100 or 200 mg/kg/day of methanol extract from *S. tetrandra*, had an antifibrotic effect in rats with liver fibrosis induced by bile duct ligation and scission.

Key words: Bile duct ligation and scission, *Stephania tetrandra*, Liver fibrosis.

INTRODUCTION

Chronic liver injury leads to excessive deposition of collagen that often results in fibrosis and cirrhosis; one of the most common causes of all deaths (Anthony *et al.*, 1978; Sherlock 1989). Prevention or suppression of fibrotic changes in the liver or liver cirrhosis, is therefore very important. Unfortunately however, therapeutic attempts with antifibrotic drugs are still at an experimental stage (Park *et al.* 1997). The problems associated with developing antifibrotics are associated with the toxicity associated with chronic administration, as well as the decreased therapeutic effects of antifibrotics when tested in clinical studies. Given that the development of antifibrotics from the natural products may reduce the risk of toxicity and therapeutic effectiveness when the drug is used clinically, we screened antifibrotic candidates that have traditionally been used for treating liver diseases in Oriental medicine, (Nan *et al.*, 2000; Park *et al.*, 1997; Park *et al.*, 2000a; Park *et al.*, 2000b; Song *et al.*, 1998).

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In this study, the antifibrotic effect of methanol extract from the dried root of *Stephania tetrandra* S. Moore (Menispermaceae) was examined for its antifibrotic effect on liver fibrosis induced by bile duct ligation and scission in rats. The dried root of *Stephania tetrandra* has traditionally been used by the Chinese to treat human liver fibrosis and cirrhosis (Cheng *et al.*, 1997). Furthermore, it has also been reported to have an anti-inflammatory effect in experimental inflammatory disease models (Kang *et al.*, 1996).

MATERIALS AND METHODS

Plant material

S. tetrandra was purchased from Yanban Yi Yao Gong Shi (Yanji, China), and was identified by Dr. Jin Wen Yi (Faculty of Pharmacy, Yanbian University). The aerial parts of *S. tetrandra* (1 kg) were extracted three times with 3l of methanol under reflux for 3 h. The combined methanol solution was concentrated, yielding a dark brownish extract (46 g) under reduced pressure.

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-hour light-dark cycles at 20~23°C and 50~60% relative humidity throughout the experiment.

Liver fibrosis induction and animal treatment

The effects of bile duct ligation and scission upon the induction liver fibrosis was monitored over a 4 week period (Park et al., 1999). Briefly, 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, an incision was made in the abdomen, which was then closed without any treatment. The number of the rats used in each group is shown in Table I. The concentrated methanol extracts of *S. tetrandra* (ST) were diluted with distilled water and given orally to rats for 28 days following the onset of the experiment, at a dose of 100 mg/kg/day or 200 mg/kg/day of ST. The control groups received equal amounts of distilled water orally for 28 days.

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 3000rpm after incubation at room temperature for one hour. Sera were subsequently kept at -20°C until further analysis. Serum aspartate aminotransferase (AST), alanine aminotrans-ferase (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 6M hydrochloride. Aliquots of the samples were derivatized using Chloramine T solution and Erhlich reagent and measured at 558 nm. A standard calibration curve was prepared using trans-4-hydroxy-L-proline (Sigma Chem. Co., USA).

Histochemical and immunohistochemical examination

Liver specimens were routinely fixed in 10% formalin, embedded in paraffin, and 4-5 μm thick sections were cut and stained using hematoxylin and eosin. For microscopic observation of inflammation, necrosis bile duct proliferation and fibrosis change were graded as being mild(+), moderate(++) or severe(+++). In order to detect hepatic stellate cell activation, the occurrence of alpha-smooth muscle cell actin in liver sections was assayed immunohistologically using the strepavidin-biotin-peroxidase complex method using LSAB® 2kit (DAKO Co., USA) and anti-alpha-smooth muscle cell actin monoclonal antibody (Boehringer Mannheim, Germany). Hepatic stellate cells are non-parenchymal liver cells that activated during liver fibrosis and responsible for connective tissue production in the fibrogenic process.

Statistical analysis

Results were expressed as means ± 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

Effects of ST on body and liver weights

All the rats subjected to the BDL/S operation exhibited jaundice symptoms within 5 days and developed fibrosis

Table I. Body weight and liver weight changes in rats with bile duct ligation and scission (BDL/S) treated with methanol extract of *Stephania tetrandra* for 4 weeks.

Group	*n*	Body weight (g) 0 day	Body weight (g) 28 day	Liver weight (g)	Liver wt. Per 100 g body wt.
Control-Sham	4	247 ± 11	329 ± 29	11.8 ± 1.2	3.7 ± 0.3
ST 100-Sham	4	242 ± 11	326 ± 29	11.9 ± 1.4	3.7 ± 0.2
ST 200-Sham	4	246 ± 5	342 ± 20	12.3 ± 0.5	3.6 ± 0.2
Control-BDL/S	8	240 ± 20	302 ± 16	23.2 ± 3.5*	7.7 ± 0.9*
ST 100-BDL/S	8	240 ± 15	308 ± 28	17.9 ± 2.8*,##	5.9 ± 0.8*,##
ST 200-BDL/S	8	243 ± 10	285 ± 32	17.5 ± 2.9*,##	6.2 ± 0.9*,#

Results represent the mean ± S.D.
*: Significantly different from each control sham group ($p<0.001$).
#: Significantly different from each control BDL/S group ($p<0.05$).
##: Significantly different from each control BDL/S group ($p<0.01$).
n: number of rats.
ST-100: Treated with methanol extract of *S. tetrandra* (100 mg/kg/day, p.o.).
ST-200: Treated with methanol extract of *S. tetrandra* (200 mg/kg/day, p.o.).

28 days after the operation. Furthermore, rats were observed increase in weight for one week subsequent to the operation after which time they returned to normal. However, no significant difference in body weight was noted between control BDL/S rats, and ST-treated BDL/S rats. The mass of the liver in BDL/S rats increased markedly when compared with sham-operated rats ($p< 0.001$). The liver to body weight ratio in ST treated BDL/S rats (either 100 mg/kg/day or 200 mg/kg/day) was significantly lower than that observed in the control BDL/S rats ($p< 0.05$) (Table I).

Serum biochemical testing

Serum biochemical parameters assayed in this study are depicted in Table II. Activities of serum AST, ALT, ALP and levels of total-bilirubin were significantly elevated in control BDL/S rats ($p<0.01$). In BDL/S rats treated with ST (100 mg/kg/day), serum AST, ALT and ALP activities were reduced by 63%, 60% and 67% that of control BDL/S rats, respectively ($p<0.01$). In ST treated BDL/S rats (200 mg/kg/day), serum AST, ALT and ALP activities were reduced by 54%, 55% and 66% that of the control BDL/S rats, respectively ($p<0.01$). In ST treated sham rats (either 100 mg/kg/day or 200 mg/kg/day) however, no significant changes in serum parameters were observed when compared to control sham rats (Table II).

Hydroxyproline contents in liver

As shown in Fig. 1, the content of hydroxyproline in the liver increased approximately 3.5-fold 28 days after the BDL/S operation ($p<0.01$). Compared with control BDL/S rats, treatment with either ST treatments (100 or 200 mg/kg/day) reduced hydroxyproline concentrations in the liver by as much as 40% and 33%, respectively ($p<0.01$). There were no significant changes in hydroxyproline content in ST treated sham rats (either 100 mg/kg/day or 200 mg/kg/day), compared with that of control sham rats (data not shown).

Morphological changes in liver

Histology of BDL/S rat livers showed excessive bile duct proliferation, inflammation and connective tissue deposition resulting in destruction of the lobular architecture (Fig. 2B). In ST treated BDL/S rats (either 100 mg/kg/day or 200 mg/kg/day), there was a tendency towards less pronounced destruction of the liver architecture and degree of bile duct proliferation and fibrosis when compared

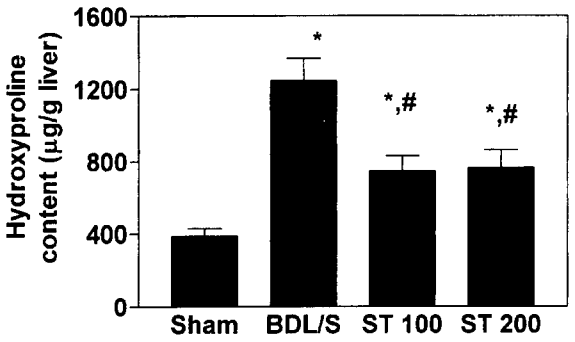

Fig. 1. Liver hydroxyproline content of the bile duct ligation and scission (BDL/S) operated rats treated with methanol extract from *Stephania tetrandra* (ST; 100 mg/kg/day or 200 mg/kg/day, p.o.) for 4 weeks. Result presents the mean ± S.D. *: Significantly different from control-sham group ($p<0.01$). #: Significantly different from control-BDL/S group ($p<0.01$).

Table II. Serum biochemical values in fibrotic rats induced by bile duct ligation and scission (BDL/S) treated with methanol extracts of *Stephania tetrandra* 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	95 ± 7	34 ± 5	756 ± 38	82 ± 8	0.3 ± 0.2
ST 100-Sham	4	70 ± 21	17 ± 4	694 ± 173	77 ± 14	0.3 ± 0.2
ST 200-Sham	4	94 ± 46	17 ± 5	635 ± 202	79 ± 7	0.3 ± 0.1
Control-BDL/S	8	593 ± 108*	145 ± 28*	1265 ± 172*	143 ± 15*	8.7 ± 0.7*
ST 100-BDL/S	8	374 ± 96*,#	88 ± 18*,#	919 ± 158*,#	138 ± 13	7.9 ± 0.5
ST 200-BDL/S	8	323 ± 81*,#	80 ± 20*,#	838 ± 140*,#	116 ± 23	6.9 ± 2.1

Results represent the mean ± S.D.
*: Significantly different from each control sham group ($p<0.001$)
#: Significantly different from control-BDL/S group ($p<0.01$).
n: number of rats.
ST 100: Treated with methanol extract of *S. tetrandra* (100 mg/kg/day, p.o.).
ST 200: Treated with methanol extract of *S. tetrandra* (200 mg/kg/day, p.o.).
AST: Aspartate transaminase.
ALT: Alanine transaminase.
ALP: Alkaline phosphatase.
T-bil: Total-bilirubin.
T-chol: Total-cholesterol.

with control BDL/S rat liver (Fig. 2C, 2D and Table III). Using an antibody against alpha smooth muscle cell actin, a phenotypic marker of hepatic stellate cell activation, we assayed expression of this protein in fibrotic liver immunohistochemically. In control sham livers, only vascular smooth muscle cells and pericytes were strongly positive for alpha smooth muscle cell actin, whereas stellate cells positive for alpha smooth muscle cell actin were rarely observed (Fig. 3A). In the control BDL/S rats, many alpha smooth muscle cell actin-positive stellate cells were detected in areas of centrilobular and periportal fibrotic bands (Fig. 3B). In contrast, BDL/S rats treated with ST (either 100 mg/kg/day or 200 mg/kg/day), showed markedly reduced numbers of alpha smooth muscle cell actin positive stellate cells (Fig. 3C and D).

Although a lot of effort has been devoted to finding effective drugs for the treatment of liver fibrosis, no specific therapeutics have been found that can prevent, or cure liver fibrosis, despite the fact that it is one of the most common causes of death. It has been reported that pentoxifylline (Peterson *et al.*, 1996), oestradiol (Shimizu *et al.*, 1999), colchicine (Rodriguez *et al.*, 1998), prolyl 4-hydroxylase inhibitors (Bickel *et al.*, 1998), hepatocyte growth factor (Yasuda *et al.*, 1996), interferon-gamma (Rockey *et*

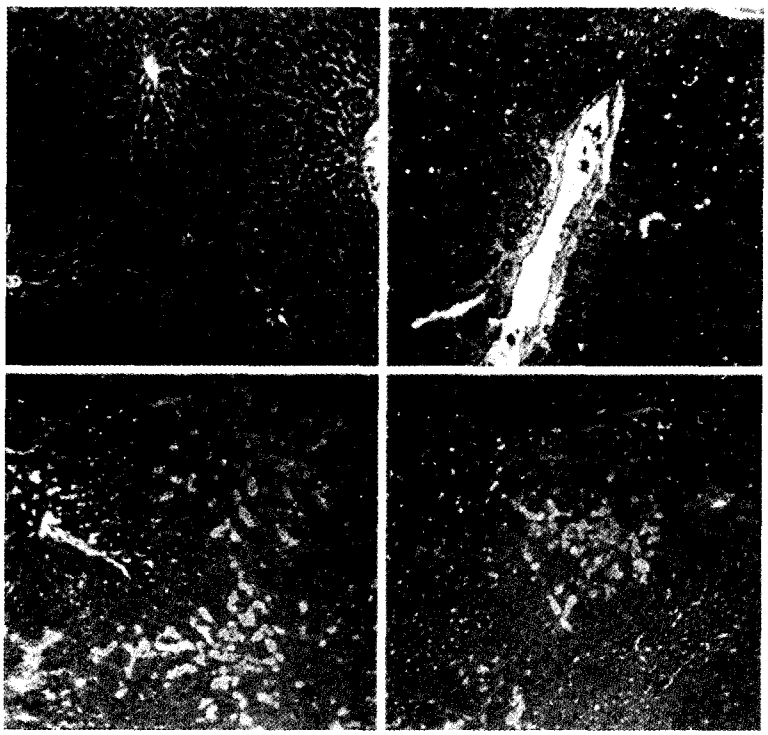

Fig. 2. Light microscopical appearance of fibrotic rat liver induced by bile duct ligation and scission (BDL/S) treated with methanol extract from *Stephania tetrandra* (100 mg/kg/day or 200 mg/kg/day p.o.). for 4 weeks (H&E, magnification × 100). A: Control Sham group. B: Control BDL/S group. C: BDL/S group treated with methanol extract from *S. tetrandra* (100 mg/kg/day). D: BDL/S group treated with methanol extract from *S. tetrandra* (200 mg/kg/day).

Table III. Histological assessment of fibrosis, inflammation, necrosis and bile duct proliferation in methanol extract of *S. tetrandra* treated- fibrotic liver induced by bile duct ligation and scission (BDL/S), duration of 4 weeks.

Group	Fibrosis	Inflammation	Necrosis	Bile duct proliferation
Control-BDL/S	++ n=6	+++ n=6	++ n=1	+++ n=6
	+ n=2	++ n=2	+ n=7	++ n=2
ST 100-BDL/S	++ n=1	++ n=1	+ n=8	++ n=4
	+ n=7	+ n=7		+ n=4
ST 200-BDL/S	++ n=2	++ n=1	+ n=8	++ n=5
	+ n=6	+ n=7		+ n=3

n: number of rats.
ST 100: Treated with methanol extract of *S. tetrandra* (100 mg/kg/day, p.o.).
ST 200: reated with methanol extract of *S. tetrandra* (200 mg/kg/day, p.o.).
mild (+), moderate (++) or severe (+++)

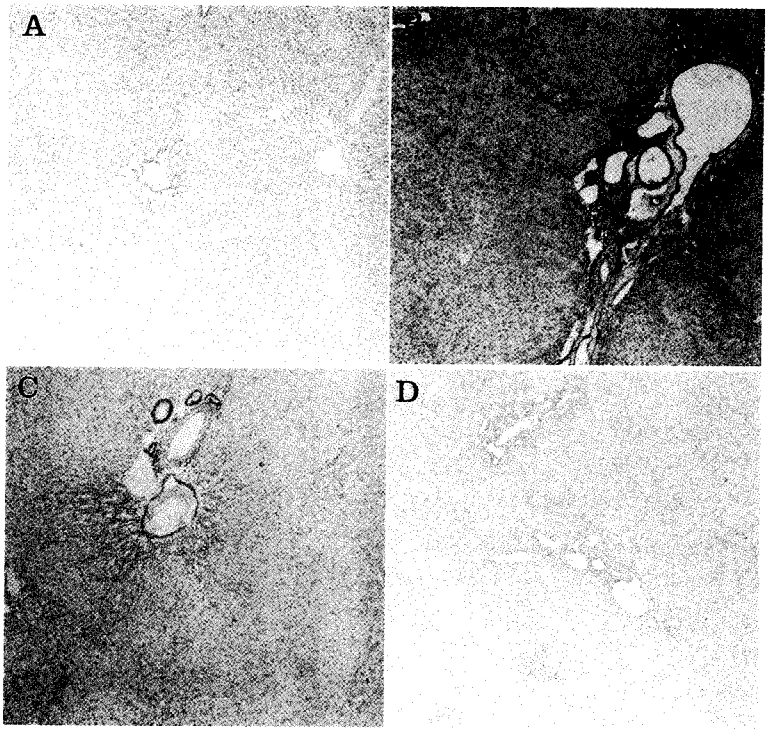

Fig. 3. Reaction of representative liver sections with antibody to alpha-smooth muscle actin. (A) Control Sham group; (B) Control bile duct ligation and scission (BDL/S) group; (C) BDL/S group treated with methanol extract from *Stephania. tetrandra* (100 mg/kg/day); (D) BDL/S group treated with methanol extract from *Stephania tetrandra* (200 mg/kg/day) (magnification × 63).

al., 1992), retinoids (Brenner *et al.*, 1990), penicillamine (Brenner *et al.*, 1990), corticosteroids (Guzelian *et al.*, 1984), prostaglandins (Brenner *et al.*, 1990) and malotilate (Ala-Kokka *et al.*, 1989) all have antifibrotic effects in animal models. However, few promising therapeutics have been discovered that are safe and effective in clinical situations; a consequence of their adverse effects or lack of therapeutic effect when used clinically. For the past several years, we have screened a number of antifibrotic agents from natural products that have traditionally been used to treat liver diseases in Asia. Developing antifibrotics from natural products used in traditional medicine may reduce the risk of toxicity and increase therapeutic effectiveness when the drug is used clinically (Mandali *et al.*, 1998; Nan *et al.*, 2000).

In this study, methanol extract from dried roots of *Stephania tetrandra* (ST) was screened for its antifibrotic effect on liver fibrosis induced by bile duct ligation and scission. The roots of *S. tetrandra* have traditionally been used as antifibrotics, antirheumatics, antihypertensives, analgesics and antipyretics in China (Jiangsu College of New Medicine, 1975; Hu, 1994). In experimental liver fibrosis, induced by bile duct ligation and scission, methanol extract of *S. tetrandra* lowered the activities of serum AST, ALT and levels of ALP and significantly inhibited collagen deposition in liver tissue. Furthermore, immunohistological evidence of a reduction in the alpha smooth muscle cell actin-positive area induced by treatment with methanol derived from *S. tetrandra*, would seem to indicate that the methanol extract of *S. tetrandra* inhibits hepatic stellate cellular activation during fibrosis. Furthermore, these results suggest that treatment methanol extract of *S. tetrandra* exhibits antifibrotic effects by inhibiting hepatic stellate cell activation in liver fibrosis induced by bile duct ligation and scission. However, further investigations are required to fully understand the antifibrotic mechanism of the methanol extract of *S. tetrandra*.

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