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Scutellaria baicalensis inhibits liver fibrosis induced by bile duct ligation or carbon tetrachloride in rats

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Abstract

This study was carried out to investigate the antifibrotic effects of methanol extracts from the traditional Chinese medicinal herb, the root of *Scutellaria baicalensis* Georgi, on liver fibrosis induced by bile duct ligation and scission (BDL) or carbon tetrachloride (CCl_4) in rats. Liver fibrosis was assessed by histological observations and by measuring levels of liver hydroxyproline, lipid peroxidation based on malondialdehyde (MDA) production, and serum enzyme activities. The morphological characteristics of liver tissue were examined by Masson's trichrome staining and immunostaining against smooth muscle cell α-actin. In both models, the levels of hydroxyproline and MDA in liver were significantly increased. Treatment with a methanol extract of *S. baicalensis* significantly reduced the levels of liver hydroxyproline and MDA, with improved histological findings. In both models, the liver areas positive for smooth muscle cell α-actin were considerably decreased by treatment with oral methanol extract of *S. baicalensis* (150 mg kg^{-1} daily for 28 days). A methanol extract of *S. baicalensis* root inhibits fibrosis and lipid peroxidation in rat liver induced by BDL or CCl_4.

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Introduction

Liver fibrosis resulting from the repeated repair of chronic liver damage is a common cause of death in humans (Chojkier & Brenner 1988; Park et al 1997). The development of approaches to prevent fibrotic changes in the liver or to repair the fibroses is therefore important (Chojkier & Brenner 1988; Friedman 1993). However, therapeutic antifibrotic drugs are still at an experimental stage (Park et al 1997; Nan et al 2000a). The major problems in developing antifibrotics are toxicity owing to the need for chronic administration and the lowered therapeutic effects when agents are used clinically as compared with in-vitro. Traditional herbal medicine has been established over thousands of years and is based on experience and practice. For these reasons, developing antifibrotics from the natural products used in traditional medicine, known to lack acute toxicity, may lead to improved therapies.

The dried root of *Scutellaria baicalensis* Georgi ("Golden root"; Huang Qin in Chinese; Hwang-geum in Korean) is a traditional herb that has been used for treating inflammation, jaundice, and other liver disorders in China, Japan and Korea. It has been reported to have antiviral, antimycotic, antioxidant, nitric-oxide-inducing, and quinone-reductase-inducing activities (Shao et al 1999; Li et al 2000; Ng et al 2000). The dried root of *S. baicalensis* has a particularly high

flavonoid content (over 35%), which gives it a yellow colour and gives rise to its traditional name of Golden root or Golden skullcap. The most commonly studied flavonoids in *S. baicalensis* root include baicalin, baicalein, wogonin and wogonoside. Multiple biological properties have been described for this herb, including anti-inflammatory, antiviral, anticarcinogenic, free-radical-scavenging, antioxidant and immunostimulatory effects. It has also been reported to have antiproliferative effects on vascular smooth muscle cells and hepatic stellate cells (Gao et al 1999; Inoue & Jackson 1999). It is also one of the main components in Xiao-Chai-Hu-Tang (in Chinese) or Sho-saiko-to (in Japanese), a blended Chinese herbal medicine prescribed for human liver diseases, which has been reported to protect against liver fibrosis induced by dimethylnitrosamine or pig serum (Shimizu et al 1999; Shimizu 2000).

To our knowledge, there have been no in-vivo studies on the effect of this single herb on experimental liver fibrosis. In this study, the antifibrotic and protective effects of *S. baicalensis* root were studied in two experimental models of liver fibrosis in rats, induced either by bile duct ligation and scission (BDL) or by carbon tetrachloride (CCl_4) administration.

Materials and Methods

Animals

Male Sprague–Dawley rats (200–220 g for BDL; 160–180 g for CCl_4) were purchased from Dae Han Laboratory Animal Research and Co. (Choongbuk, Korea), and had free access to normal standard chow diet (Jae Il Chow, Korea) and tap water. Throughout the experiment, the animals were housed, four or five per cage, in laminar flow cages maintained at $22 \pm 2^{\circ}C$, 50–60% relative humidity, under a 12-h light–dark cycle. The animals were kept in these facilities for at least one week before the experiment. Animal care and treatment was carried out in accordance with the Guiding Principles in the Use of Animals in Toxicology adopted by the Society of Toxicology (USA) in July 1989 and revised in March 1999. The Animal Care Committee in Wonkwang University approved the study.

Preparation of methanol extract from *S. baicalensis* root

S. baicalensis root was purchased from Sungboe Farm Co. (Ahnsung, Kyunggi-do, Korea) and was authenticated by Professor Y. C. Kim, College of Pharmacy, Wonkwang University. *S. baicalensis* Georgi root (1 kg) was extracted three times with 3 L methanol under reflux for 3 h. The combined methanol solution was concentrated to give a dark brownish extract (150 g, 15% w/w of starting crude material) under reduced pressure. We used methanol extraction because this extract contains more flavonoids, for example baicalein, than an aqueous extract. The baicalein and baicalin content in this methanol extract was approximately 28% when assayed by HPLC (Lin et al 1999).

Liver fibrosis induced by BDL

Biliary liver fibrosis was induced by BDL operation as described previously (Park et al 1997; Nan et al 2000a). Briefly, rats were anaesthetized with ketamine/xylazine and the common bile duct was exposed and double-ligated using silk sutures. The first ligature was placed below the junction of the hepatic ducts and the second was placed above the entrance of the pancreatic ducts. The common bile duct was then cut between the double ligatures. In sham-operated rats, an incision was made in the abdomen and then closed without any treatment. The numbers of rats used in each group are shown in Table 1.

Liver fibrosis induced by CCl_4

CCl_4 was given to rats orally (1 mL kg^{-1} as carbon tetrachloride, mixed with an equal volume of corn oil) twice a week for 28 days (Bickel et al 1991). Three days after the last dose, rats were killed under ether anaesthesia and blood and liver samples were collected. The numbers of rats used in each group are shown in Table 1.

Treatment with methanol extract of *S. baicalensis* root

Methanol extract was diluted with distilled water and given orally by gavage for 28 days, at a daily dose of 150 mg kg^{-1}, which corresponds to 1.0 g of *S. baicalensis* root. The control groups received equal amounts of distilled water, given orally for 28 days.

Determination of serum biochemical parameters

After 28 days of treatment, rats were anaesthetized with ether and blood was obtained by cardiac puncture for serum biochemical testing. Blood samples were kept

at room temperature for 1 h and centrifuged at 3000 rev min^{-1} for 30 min to obtain serum, which was kept at −20°C until further assay. The levels of serum aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activity, and level of total bilirubin were measured using an Autodry chemistry analyser (Spotchem SP4410; Arkray, Kyoto, Japan).

Determination of hydroxyproline content in liver

The hydroxyproline content in liver was determined by the method of Jamall et al (1981). Briefly, specimens of the liver were weighed and completely hydrolysed in 6 M HCl. A fraction of the samples was derivatized using chloramine T solution and Erhlich's reagent, and optical density was measured at 558 nm. A standard calibration curve was prepared using trans-4-hydroxy-L-proline (Sigma Chemical Co., St Louis, MO).

Determination of lipid peroxidation levels in liver

Lipid peroxidation was quantified by the thiobarbituric acid (TBA) method (Plaa & Charbonneau 1994) using the S9 fraction of liver homogenates as an enzyme source. All manipulations were done on ice to avoid auto-peroxidation. The S9 fraction of homogenate (5 mg protein) was reacted with TBA solution in a boiling water bath for 10 min and then extracted with *n*-butanol for 10 s. The organic phase, containing a coloured complex, was collected for fluorescence measurement. Excitation and emission wavelengths were 532 and 553 nm, respectively. The calibration curve was prepared using 1,1,3,3-tetraethoxypropane (Sigma), a chemical that releases malondialdehyde (MDA) in acidic conditions.

Histological and immunohistochemical examination of liver

Samples of livers were fixed rapidly with 10% neutralized formalin (pH 7.4) and stained with Masson's trichome method to determine collagen deposition in fibrotic liver. Smooth muscle cell α-actin, for the detection of activated hepatic stellate cells, was assessed immunohistochemically by the strepavidin–biotin–peroxidase complex method, using the LSAB 2 Kit (DAKO Co., Carpinteria, USA) and anti-α-smooth muscle cell actin monoclonal antibody (Boehringer Mannheim, Germany).

Image analysis of liver fibrosis

Histomorphometric analysis was performed on a Visus image processor equipped with a digital camera (Sound Vision SVMicro), which provides digital images with a resolution of 960 × 800 pixels. The analyser stores 24-bit colour images. The liver section was placed on the X–Y stage of a Leitz Diaplan microscope after equalization of light intensity. A numeric image of the complete section was stored in the colour image processor at a final magnification of 10×. After interactive thresholding with colour segmentation function, the colour image was divided into two different sections, one for picrosirius autom red and the other for collagen. The area percentage of each section appeared automatically.

The relative area of liver fibrosis was expressed as the mean percentage of fibrosis in the liver sections: (*S. baicalensis*-treated fibrotic liver − control sham-treated liver) × 100/(control fibrotic liver − control sham-treated liver). The scores of nine separate livers from each group were then averaged.

In addition, we assessed the mean value of smooth muscle α-actin-positive area in six ocular fields per specimen as a percentage area at a final magnification of 63× using an image analysis system. As the vessels were stained by anti-smooth muscle α-actin monoclonal antibody, the mean value of seven specimens (six ocular fields per specimen) of normal rat livers was subtracted from each experimental specimen. The relative smooth muscle α-actin-positive area was expressed as the percentage of total area of the specimen: (*S. baicalensis*-treated fibrotic liver − control sham-treated liver) × 100/(control fibrotic liver − control sham-treated liver).

Statistical analysis

All values are expressed as means ± s.d. When groups of more than three were compared, one-way analysis of variance and Turkey's multiple comparison test were performed. Comparison between any two groups was performed using Student's *t*-test. Statistically significant differences between groups were defined as $P < 0.05$. Calculations were performed with the GraphPad Prism program (GraphPad Software, Inc., San Diego, USA).

Results

BDL rats treated with methanol extract of *S. baicalensis* root

BDL rats showed symptoms of jaundice (e.g. urine colour) within five days and developed fibrosis within 28

Table 1 Changes in bodyweight and liver weight in rats with liver fibrosis induced by bile duct ligation and scission (BDL) and carbon tetrachloride (CCl_4), treated orally with a methanol extract of *Scutellaria baicalensis* root (150 mg kg^{-1} daily) for 28 days.

Group	n	Bodyweight (g)		Liver weight (g)	Liver weight/bodyweight
		Day 0	Day 28		
BDL					
Control sham-operated	4	225±12	305±27	8.1±1.1	3.8±0.2
Sham-operated+extract	4	227±17	315±36	8.2±1.4	3.6±0.1
Control BDL	10	230±18	295±26	24.1±3.1*	7.9±0.4*
BDL+extract	9	235±11	321±34	17.1±2.1†	6.3±0.2*†
CCl_4					
Control sham-operated	4	205±13	285±32	7.8±3.3	3.6±0.1
Sham-operated+extract	4	212±14	295±22	8.2±2.5	3.5±0.2
Control CCl_4	10	219±16	275±42	10.1±2.8	4.3±0.2
CCl_4+extract	9	225±12	287±29	9.8±3.1	4.1±0.2

Data are mean±s.d. *$P < 0.001$, significantly different compared with the control sham-operated group. †$P < 0.01$, significantly different compared with the control BDL group.

days of the operation. All of the operated rats showed a slight decrease in bodyweight during the first week, but then returned to a normal weight. There was no significant difference in bodyweight between control rats and rats treated with extract (Table 1). Liver weights were significantly increased in BDL rats compared with sham-operated rats ($P < 0.001$). The liver-to-body weight ratio of BDL rats treated with extract was significantly lower than that of the control BDL rats ($P < 0.01$; Table 1).

Serum aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase levels were significantly elevated in BDL rats ($P < 0.001$; Table 2). Serum bilirubin was extremely elevated ($P < 0.001$). In BDL rats treated with extract, serum aspartate aminotransferase and alanine aminotransferase activities were significantly lower than in control BDL rats ($P < 0.01$; Table 2). There were no significant differences in serum parameters between control sham-operated rats and sham-operated rats treated with extract (Table 2).

As shown in Table 3, levels of hydroxyproline and MDA in the livers of BDL rats increased approximately 3.3-fold ($P < 0.01$) and 2.5-fold ($P < 0.001$), respectively, showing that 28 days of bile duct obstruction markedly increased collagen deposition and lipid peroxidation in the liver. Treatment with *S. baicalensis* extract reduced levels of hydroxyproline and MDA to 64% ($P < 0.01$) and 54% ($P < 0.01$) of control BDL rats, respectively. In sham-operated rats treated with the extract, there were no significant changes in hydroxyproline or MDA levels when compared with control sham-operated rats.

Histological analysis of liver sections showed that after 28 days of BDL, there was an increase in collagen deposition around the portal triad, excessive bile duct proliferation and inflammatory cell deposition, resulting in destruction of the lobular architecture (Figure 1A). In BDL rats treated with extract (Figure 1B), there was a tendency towards less pronounced destruction of the liver architecture, bile duct proliferation and fibrosis when compared with control BDL rat livers. Quantitative morphometric analysis demonstrated that *S. baicalensis* significantly decreased connective tissue deposition from 12.4±2.8% in control BDL livers to 7.1±3.3% in extract-treated BDL livers ($P < 0.01$). The extract-treated sham-operated rat livers showed no histological alterations when compared with control sham-operated rat livers (data not shown).

Using an antibody against smooth muscle α-actin, a marker of stellate cell activation, we assayed expression of this protein in liver immunohistochemically. In sham-operated livers, only the regions of vascular smooth muscle cells around the vessel were positive for smooth muscle α-actin and stellate cells positive for smooth muscle α-actin were only rarely observed (data not shown). Activated hepatic stellate cells, which express smooth muscle α-actin, were remarkably increased in the livers of control BDL rats (Figure 1C). In contrast, treatment with *S. baicalensis* extract markedly reduced the numbers of smooth muscle α-actin-positive areas in BDL-induced fibrotic livers

Table 2 Serum biochemical values in rats with liver fibrosis induced by bile duct ligation and scission (BDL) and carbon tetrachloride (CCl_4), treated orally with a methanol extract of *Scutellaria baicalensis* root (150 mg kg^{-1} daily) for 28 days.

Group	n	Aspartate transaminase (IU L^{-1})	Alanine transaminase (IU L^{-1})	Alanine phosphatase (IU L^{-1})	Total bilirubin (mg dL^{-1})
BDL					
Control sham-operated	4	86±18	34±29	603±75	0.3±0.1
Sham-operated+extract	4	74±16	25±14	548±126	0.3±0.1
Control BDL	10	670±95*	153±25*	1326±95*	8.2±0.6*
BDL+extract	9	468±99*†	109±19*†	893±193*†	7.3±1.2
CCl_4					
Control sham	4	96±12	40±11	376±11	0.3±0.1
Sham+extract	4	76±14	33±13	431±54	0.3±0.2
Control CCl_4	10	985±259#	1033±243#	2238±971#	0.8±0.4
CCl_4+extract	9	427±199#‡	398±189#‡	1395±548#	0.7±0.3

Data are means±s.d. *$P < 0.001$, significantly different from control sham-operated group. #$P < 0.001$, significantly different from control sham group. †$P < 0.01$, significantly different from control BDL group. ‡$P < 0.01$, significantly different from control CCl_4 group.

Table 3 Hydroxyproline and thiobarbituric-reactive substance values in rats with liver fibrosis induced by bile duct ligation and scission (BDL) and carbon tetrachloride (CCl_4), treated orally with a methanol extract of *Scutellaria baicalensis* root (150 mg kg^{-1} daily) for 28 days.

Group	Hydroxyproline (μg (g liver)$^{-1}$)	Thiobarbituric reactive substance (pmol (mg protein)$^{-1}$)
BDL		
Control sham-operated	375±54	156±19
Sham-operated+extract	345±49	107±15
Control BDL	1207±156*	399±46*
BDL+extract	767±108*†	214±32*†
CCl_4		
Control sham-operated	310±38	149±12
Sham-operated+extract	298±45	129±15
Control CCl_4	678±178#	478±58#
CCl_4+extract	479±129#‡	275±49#‡‡

Data are mean±s.d. *$P < 0.001$, significantly different compared with the control sham-operated group. †$P < 0.01$, significantly different compared with the control BDL group. #$P < 0.001$, significantly different from the control sham group. ‡$P < 0.05$, significantly different from the control CCl_4 group. ‡‡$P < 0.01$, significantly different from the control CCl_4 group.

(Figure 1D). Quantitative morphometric analysis of these livers stained against smooth muscle α-actin demonstrated that *S. baicalensis* treatment significantly decreased hepatic stellate cell activation from 5.9±1.7% in control BDL rat livers to 2.8±1.5% in extract-treated BDL rat livers ($P < 0.01$).

CCl_4 rats treated with methanol extract of *S. baicalensis* root

Levels of serum aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase activity were significantly elevated in control CCl_4-treated rats

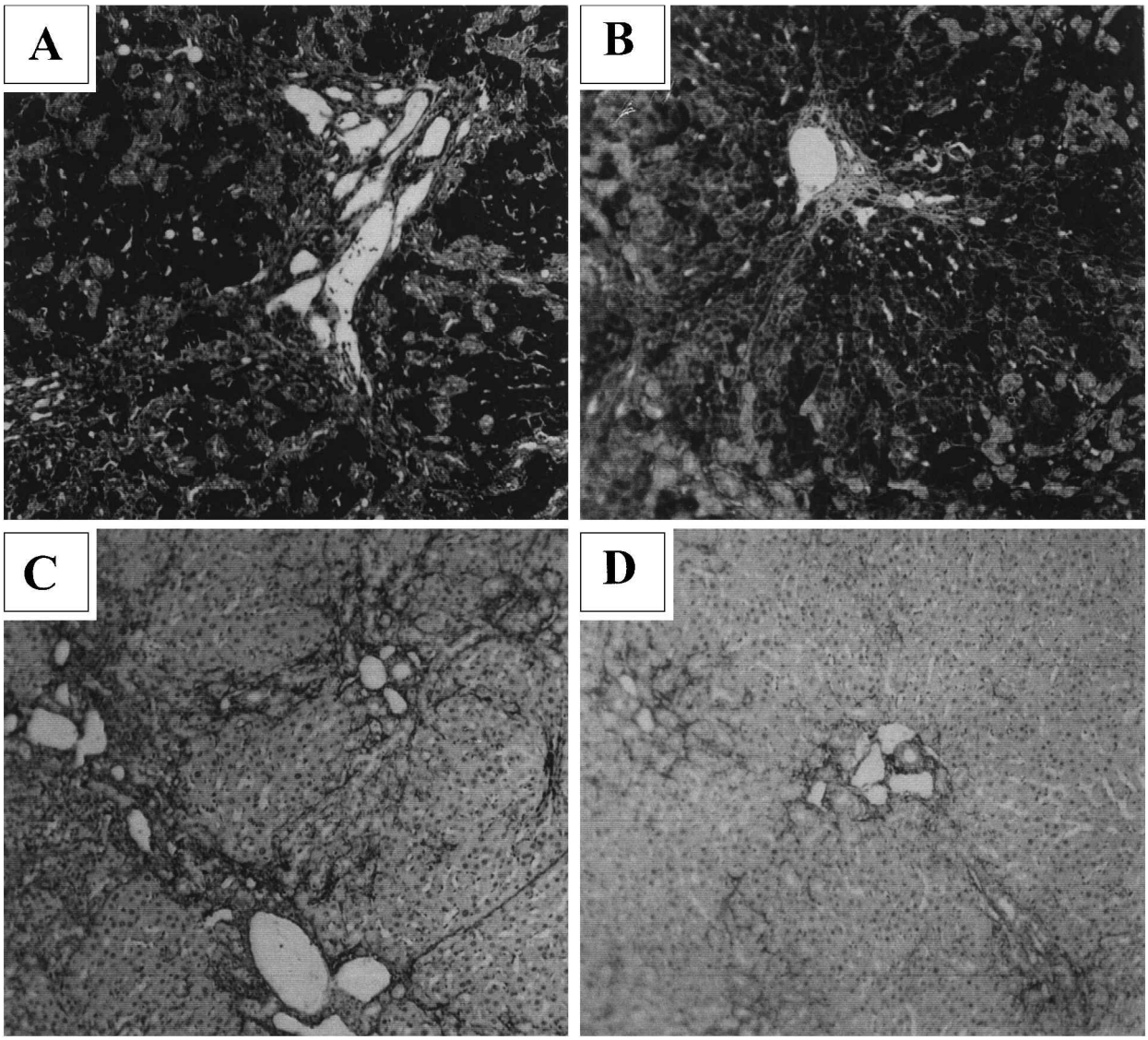

Figure 1 Masson's trichrome staining for collagen and immunohistochemical appearance for smooth muscle α-actin of representative fibrotic liver sections from rats treated with a methanol extract of *Scutellaria baicalensis*. Liver fibrosis was induced by bile duct ligation and scission (BDL) for a duration for four weeks. The methanol extract of *S. baicalensis* (150 mg kg^{-1} daily) was administered orally to rats throughout the duration of BDL (magnification 100×). A. Control BDL liver (Masson's trichrome staining); B. BDL liver treated with *S. baicalensis* (Masson's trichrome staining); C. control BDL liver (smooth muscle α-actin immunostaining); D. BDL liver treated with *S. baicalensis* (smooth muscle α-actin immunostaining).

($P < 0.001$; Table 2). In CCl_4-treated rats that received extract, serum aspartate aminotransferase and alanine aminotransferase activities were significantly lower than in the control CCl_4-treated rats ($P < 0.01$; Table 2), whereas there were no significant differences in serum parameters between extract-treated sham-treated rats and control sham-treated rats (Table 2).

As shown in Table 3, the levels of hydroxyproline increased about 2-fold in control CCl_4-treated rat livers ($P < 0.01$). In *S. baicalensis* extract-treated CCl_4 rat liver, the hydroxyproline level decreased to 71% of levels in control CCl_4 rat liver ($P < 0.01$; Table 3). Liver MDA levels increased to 321% ($P < 0.01$) in control CCl_4-treated rats ($P < 0.01$). Compared with control CCl_4 rats, treatment with extract reduced the MDA level to 58% that of control CCl_4 rats ($P < 0.01$; Table 3).

The livers of rats treated with CCl_4 for 28 days showed inflammation, less pronounced destruction of the liver architecture, and extensive accumulation of connective

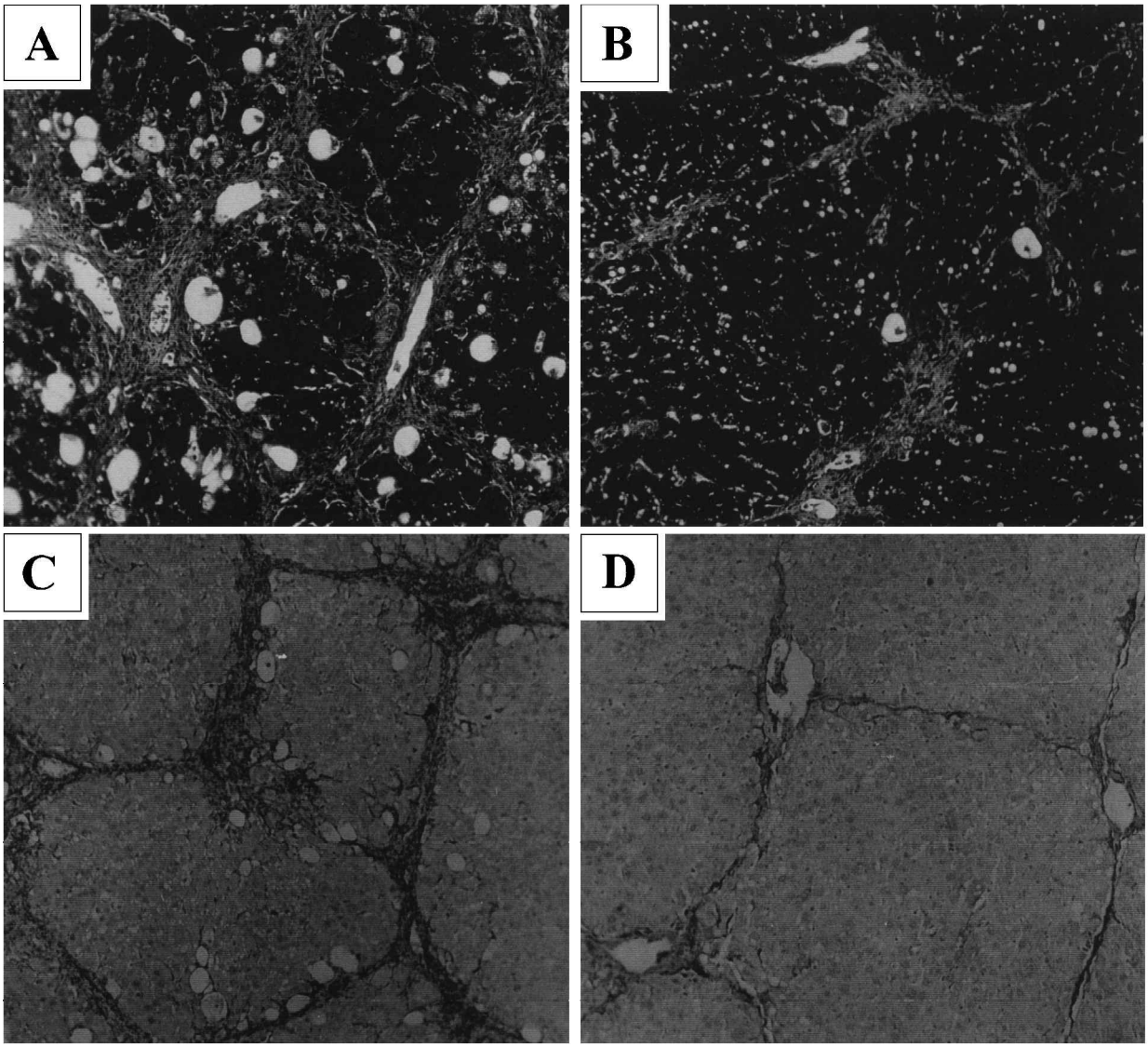

Figure 2 Masson's trichrome staining for collagen and immunohistochemical appearance for smooth muscle α-actin of representative fibrotic liver sections from rats treated with a methanol extract of *Scutellaria baicalensis*. Liver fibrosis was induced by carbon tetrachloride (CCl_4) administered orally (1 mL kg^{-1} as carbon tetrachloride) twice a week for four weeks. The methanol extract of *S. baicalensis* (150 mg kg^{-1} daily) was administered orally to rats during CCl_4 treatment (magnification 100×). A. Control CCl_4 liver (Masson's trichrome staining); B. CCl_4 liver treated with *S. baicalensis* (Masson's trichrome staining); C. control CCl_4 liver (smooth muscle α-actin immunostaining); D. CCl_4 liver treated with *S. baicalensis* (smooth muscle α-actin immunostaining).

tissue resulting in formation of continuous fibrotic septa between the central and portal veins and nodules (Figure 2A). However, co-treatment with *S. baicalensis* extract reduced the accumulation of collagen (Figure 2B) and resulted in less pronounced destruction of the liver architecture. Quantitative morphometric analysis demonstrated that *S. baicalensis* significantly decreased liver connective tissue deposition from $9.8 \pm 3.2\%$ in control CCl_4-treated rats to $5.2 \pm 2.1\%$ in extract-treated CCl_4-treated rats ($P < 0.01$).

Hepatic stellate cell activation and proliferation were examined immunohistochemically using an antibody against smooth muscle α-actin. In vehicle-treated rat livers, only vessels were positive, and positive stellate cells were only rarely observed (data not shown). Smooth muscle α-actin-positive areas increased markedly in control CCl_4-treated rat livers (Figure 2C). In contrast, smooth muscle α-actin-positive stellate cells were markedly reduced in the livers of CCl_4-treated rats co-treated with *S. baicalensis* extract (Figure 2D). Quan-

titative morphometric analysis of livers stained against smooth muscle α-actin demonstrated that *S. baicalensis* treatment significantly decreased liver hepatic stellate cell activation from $4.5 \pm 1.8\%$ in CCl_4-treated rats to $2.2 \pm 1.3\%$ in extract-treated CCl_4-treated rats ($P < 0.01$).

Discussion

Although there has been a lot of effort devoted to finding and developing effective drugs for treating liver fibrosis, there have been no specific therapeutic agents developed that prevent or cure liver fibrosis. For several years, we have screened a number of liver protective and antifibrotic agents from natural products, which are traditionally used for treating liver diseases in oriental folk medicine (Park et al 1997; Song et al 1998; Nan et al 2000a, b, 2001; Park et al 2000a, b). Developing antifibrotics from natural products used in folk medicine may reduce the risk of toxicity and maintain therapeutic effectiveness when the drug is used clinically.

S. baicalensis root is one of the main ingredients of Sho-saiko-to, a commonly used traditional formula for treating chronic hepatitis, chronic gastroenteritis and bronchial asthma in China, Japan, and Korea. It is known that Sho-saiko-to has antifibrotic effects in liver fibrosis induced by dimethylnitrosamine or pig serum (Borchers et al 2000) and the active compounds of this plant, baicalein and baicalin, have antiproliferative effects on fibroblasts in-vitro (Chung et al 1995; Inoue & Jackson 1999). Recently, however, Sho-saiko-to has been suspected of inducing thrombocytopenic purpura and pneumonia when used clinically. As it has been reported that the active ingredients of Sho-saiko-to are baicalein and baicalin, we decided to examine the hepatoprotective and antifibrotic effect of a methanol extract of *S. baicalensis*, which also contains these components.

To evaluate the hepatoprotective and antifibrotic potential of a given agent, selection of an appropriate animal model is important. The most widely used method for inducing liver fibrosis is CCl_4 administration. CCl_4 requires bioactivation by cytochrome P450s, which yields the reactive metabolite, the trichloromethyl radical. This free radical initiates lipid peroxidation, resulting in liver damage. In contrast to models induced by toxicants such as CCl_4, BDL for four weeks leads to fibrosis that resembles the biliary fibrosis seen in humans (Kountouras et al 1984; Milani et al 1990). 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 (Nan et al 2000a, b; Park et al 2000a, b). In the present study, two models of induced liver fibrosis (BDL and CCl_4 treatment), which are aetiologically and pathogenically different, were used to examine the antifibrotic effect of *S. baicalensis*.

In the BDL model, there were marked increases in liver weight, serum bilirubin and the activities of serum aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase, as well as in hydroxyproline and MDA content in liver after 28 days of BDL. Treatment with a methanol extract of *S. baicalensis* root for 28 days significantly reduced stellate cell activation, connective tissue deposition and lipid peroxidation in BDL rat livers and improved serum biochemical parameters. These results indicate that the methanolic extract of *S. baicalensis* Georgi root significantly inhibited collagen accumulation and lipid peroxidation in liver fibrosis induced by biliary obstruction.

In the CCl_4 model, co-treatment with the methanol extract of *S. baicalensis* root also reduced the degree of hepatocellular injury as evidenced by improved serum biochemical parameters and levels of hydroxyproline and MDA in CCl_4-treated rat livers. Morphometric analysis on Masson's trichome-stained sections demonstrated that *S. baicalensis* root extract significantly decreased the fibrotic area and from the immunohistological appearance *S. baicalensis* reduced the smooth muscle cell α-actin-positive areas in liver, showing inhibition of hepatic stellate cell activation during liver injury.

The results of this study indicate that a methanol extract of *S. baicalensis* root alone can effectively inhibit the liver fibrosis induced by BDL or CCl_4, as evidenced by reduced levels of connective tissue, lipid peroxidation and hepatic stellate cell activation in liver.

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