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Sho-saiko-to Prevents Liver Fibrosis Induced by Bile Duct Ligation in Rats

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Abstract: Hepatic fibrosis is an over-accumulation of extracellular matrix (ECM). It is a result of an imbalance between collagen synthesis and degradation. Matrix metalloproteinase (MMP) has degradative activity against collagen, but tissue inhibitors of metalloproteinase (TIMP) control the active forms of MMP by blocking the active site of MMP. In our study, we established the bile duct ligated model (BDL) in rats to evaluate anti-fibrotic potential of Chinese medicine sho-saiko-to (TJ-9). We assessed the drug's potential in inhibiting collagen accumulation, suppressing procollagen α1 types (I) and (III), and TIMP-1 mRNA expression. After administration of TJ-9, hyperbilirubinemia reduced approximately four-fold when compared with BDL-untreated group. TJ-9 also significantly reduced the collagen content and fibrogenic score, as well as downregulated elevated procollagen α1 types (I) and (III) and TIMP-1 mRNA level. Finally, we concluded that (1) TJ-9 significantly reduced cholestasis in rats with BDL, (2) TJ-9 markedly reduced the collagen content by 50%, and (3) TJ-9 exerted its antifibrogenic effect by downregulation of the mRNA expression of procollagen α1 types (I) and (III), and TIMP-1 in liver tissue.

Keywords: Liver Fibrosis; Bile Duct Ligation; Sho-saiko-to (TJ-9); Bupleurum; Pinellia; Scutellaria; Jujube; Ginseng; Glycyrrhiza; Ginger.

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Introduction

Hepatic fibrosis is a wound healing or scarring process in response to liver injury. There are five progressive findings in understanding the mechanism of hepatic fibrosis. First, fibrosis is an over-accumulation of extracellular matrix (ECM), including collagen types I and III, glycoproteins and proteoglycans. Second, hepatic stellate cell (Ito cell) is the major source of ECM. Third, the activation of Ito cell is regulated by a growth factor (TGF-β1) and cytokine (IL-1,4,6). Fourth, it is an imbalance between matrix degradation (MMP) and accumulation (TIMP). Finally, apoptosis in Ito cell clearance of ECM (Li and Friedman, 1999). Thus, the antifibrotic therapy included (1) removal of injury stimulation, (2) suppression of hepatic inflammation, (3) downregulation of Ito cell activation, and (4) promotion of matrix degradation (MMP) or inhibition of ECM accumulation (TIMP).

Herbal medicine, sho-saiko-to (TJ-9; Xiao-Chai-Hu-Tang in Chinese), is the most popular herbal medicine in China and Japan. TJ-9 has been used in the treatment of chronic liver disease, such as viral hepatitis and liver cirrhosis. It improved subjective symptoms and abnormal liver functions with low side effects (Gibo *et al*., 1994). TJ-9 consists of the extracts of seven herbal components (*Bupleurum* root, *Pinellia* tuber, *Scutellaria* root, *Jujube* fruit, *Ginseng* root, *Glycyrrhiza* root and *Ginger* rhizome). Many studies have indicated its cytoprotective effects on various liver injury experiments, including CCl4 and D-galactosamine (Sakae *et al*., 1989; Araki *et al*., 1988). Furthermore, several reports assayed the preventive and therapeutic effects of TJ-9 on the experimental hepatic fibrosis induced in rats by dimethylnitrosamine (DMN) and pig serum (Amagaya *et al*., 1988; Shimizu *et al*., 1999).

Sakaida *et al*. (1998) reported that TJ-9 prevented hepatic fibrosis, reduced the expression of type III procollagen alpha-1 mRNA and inhibited the activation of Ito cell in the choline-deficient diet. Miyamura *et al*. (1998) and Ono *et al*. (1999) reported that TJ-9 extract was useful in suppressing the activation of Ito cell and inhibition of collagen accumulation.

In addition, Shimizu *et al*. (1999) also confirmed the antifibrotic effect of TJ-9 in reducing the collagen type I synthesis, α-smooth muscle actin (SMA) expression, cell proliferation and production of reactive oxygen species in hepatic stellate cell culture. However, among these previous reports, no one had ever assayed the antifibrotic effect of TJ-9 on the rat's BDL model.

The BDL experimental model in rats had been examined extensively, because it could induce progressive portal fibrosis and finally develop secondary biliary cirrhosis (Koutouras *et al*., 1984). Compared with the toxin-induced chronic liver damage, such as CCl4, there was little liver necrosis and inflammation. Therefore, it resembled human biliary liver fibrosis and allowed the detection of antifibrotic effects that were not obscured by radical scavenging or anti-inflammatory properties of the toxic agent (Boigk *et al*., 1997).

The aim of the present study was to assay the preventive and therapeutic effects of TJ-9 on rat hepatic fibrosis induced by BDL. In order to understand the mechanism of TJ-9 to prevent hepatic fibrosis, we also tested the drug's effect on the collagen content, the mRNA expression of procollagen α1 types (I) and (III), and TIMP-1. At the end, we used the α-SMA immunohistochemical stain to detect the drug's suppressing effect to the activated Ito cell.

Materials and Methods

Preparation of TJ-9 Extract

TJ-9 extract powder was kindly provided by Ko-Da Pharmaceutical Co., Taiwan. Briefly, 1000 g of Chinese herbal drug (containing 333 g *Bupleurum* root, 133 g *Scutellaria* root, 133 g *Ginseng* root, 67 g *Glycyrrhiza* root, 67 g *Ginger* rhizome, 133 g *Pinellia* tuber and 133 g *Jujube* fruit) were decocted with 7 liters of boiling water in a stainless oven for 1 hour (Table 1). The decoction was filtered and decocted again for another 50 minutes. Then, the filtrate was concentrated under reduced pressure (60–80 mmHg) at 55°C by a rotary vacuum evaporator, and freeze-dried at –45°C. Subsequently, the productive rate of TJ-9 extract was 25.47%, and the stock solution was 50 mg/ml.

Table 1. Composition of Sho-saiko-to Extract (TJ-9)

Plant Name	Family	Part Used	Composition
Bupleurum chinese DC	Umbelliferae	Radix	5 (33.4%)
Pinellia ternata thunb. Breit.	Araceae	Rhizoma	2 (13.3%)
Panax ginseng C.A. Meyer	Araliaceae	Radix	2 (13.3%)
Scutellaria baicalensis Georgi	Labiatae	Radix	2 (13.3%)
Glycyrrhiza uralensis Fischer	Leguminosae	Radix	1 (6.67%)
Zingiber officinale Roscoe	Zingiberaceae	Rhizoma	1 (6.67%)
Zizyphus jujuba Mill.	Rhamnaceae	Fructus	2 (13.3%)
Total: 1000 g Productive rate: 25.47%			15 (100%)

Provided by Ko-Da Pharmaceutical Co., Taiwan, R.O.C.

Animals

Sixty male Wistar albino rats weighing approximately 200–250 g were purchased from the National Animal Center and kept on a standard rat diet with free access to tap water, with a 12-hour light-dark cycle. This experiment has been reviewed by the committee of ethics on animal experimentation in Chiayi Christian Hospital. After accommodation for two weeks, the rats were randomly assigned into three groups: (1) sham-operation, (2) bile duct ligation (BDL), or (3) BDL and treated with 0.5 g/kg sho-saiko-to (TJ-9) by parenteral gavage. Rats were anaesthetized with 0.1 ml/100 g Zoletil (tietamine and zolezetam, Virbac, France) by intraperitoneal injection. A midline abdominal incision was made and the common bile duct was identified, doubly ligated with 3-0 black silk, and transected between two ligatures. Two milliliters of sterile saline were instilled into the peritoneum at the end of surgery, and the abdomen was closed in two layers with 3-0 black silk (Pleobani *et al*., 1999). A sham-operation was performed identical to the BDL procedure except the bile duct was not ligated or transected. The TJ-9 treatment group also underwent bile duct ligation, and was treated with 0.5 g/kg dosage of TJ-9 by parenteral gavage everyday except Sundays for 6 weeks. After 42 days, all of the animals were sacrificed under Zoletil anesthesia, and blood was

collected from carotid artery. Their liver, spleen and kidney were also removed, fixed in 10% formalin for histological examination, and were immediately snap-frozen in liquid nitrogen for extraction of total RNA and collagen determination.

Serum Assays

Serum alanine aminotransferase, alkaline phosphatase, r-glutamyl transferase, total bilirubin, direct bilirubin, total protein, albumin and albumin/globumin ratio were determined with standardized test kit (Wako & Shino Co., Japan) by an automated analyzer (Hitachi 7150, Japan).

Hydroxyproline Determination

Hydroxyproline (HYP) is an amino acid unique to all collagen and represents approximately 12% of amino acids in the major fibrillar collagen types I and III (Cho *et al*., 2000). Thus, we measured the content of hydroxyproline in the liver to estimate the collagen content and the anti-fibrotic property of these drugs by using the modified method (Woessner, 1961).

At first, liver tissues of the right lobe were dehydrated by 95% alcohol for 5–6 hours, and defatted with acetone for 2 days. This defatted tissue was dried in the oven at 110°C, then ground into powder. Thirty micrograms of liver tissue powder were placed in a test tube. Then 3 ml of 6 M HCℓ was added and hydrolyzed at 110°C for 12 hours. Hydrolysates were filtered and diluted to 50 ml, and the solution was neutralized to pH = 6 by 6 M NaOH. Two microliters of the dilution (pH = 6) and 1 ml of 0.05 M chloramine-T were placed in a new tube, shaken vigorously and left at room temperature for 20 minutes, followed by the addition of 1 ml of 3.15 M perchloric acid and 1 ml of 10% p-dimethylamino-benzaldehyde, and then incubated at 60ºC for 20 minutes. Finally, the tube was cooled in an ice bath for 5 minutes to stop the reaction, and then the absorption was read at 550 nm against a reagent blank by means of a spectrophotometer (Beckman DU650, USA).

RNA Extraction and cDNA Preparation

The total RNA was extracted by the Miniprep system (Viogene, Viogene-Biotek Co., Taiwan, ROC) according to manufacturer's instructions. Then, 0.5–1.0 μg of total RNA was reverse-transcribed using Im Prom-II™- reverse transcriptase (Promega Co. Madison, WI, USA) at 42ºC (Iredale, 1996). By adding DEPC water, total RNA equals to 10 μL. Oligo (dT) 15 primer (1 μl) was added and heated at 70ºC for 5 minutes. Reaction buffer (5x, 5 μl), $MgCl_2$ (25 Mm, 5 μl), dNTP mix (10 mM each) 1.0 μl, recombinant RNasin® Ribonuclease inhibitor (0.5 μl), Im Prom-II™ reverse transcriptase (1.0 μl) were added. It was incubated at 25ºC for 5 minutes, 42ºC for 1 hour, 70ºC for 15 minutes.

Quantitative Analysis of Procollagen Types (I) and (III) and TIMP-1

Polymerase chain reaction (PCR) was performed with specific primers as follows: procollagen (I) product size 100 base pairs (bp), sense primer: 5′CCCGAGGAAACAATGGTGC-3′; antisense primer: 5′-ATTCCCTGAAGACCGGGGGC-3′. procollagen (III) product size 303 bp, sense primer: 5′-TTTTACTGCTGAGGGGATGG-3′; antisense primer: 5′-TGGACTGCTGTGCCAAAATA-3′. TIMP-1 product size 511 bp, sense: 5′-TCCCCAGAAATCATCGAGAC-3′, antisense: 5′-GTTCAGGGTTCAGCTTTTGC-3′. GAPDH (167 bp as internal control): sense primer: 5′-CTACCCACGGCAAGTTCAAT-3′, antisense primer: 5′-TACTCAGCACCAGCATCACC-3′. PCR was performed in a total volume of 50 μl, each reaction contained 4 μl of cDNA template, 20 pmole of each primer, 0.5 mM of each dNTP, 10x Pro-Taq DNA polymerase buffer and 2 units of Pro-Taq DNA polymerase (Promega, WI, USA). After an initial denaturation at 95ºC for 5 minutes, 35 cycles of 95ºC for 40 seconds, 54ºC for 1 minute, 72ºC for 1.5 minutes, and finally, 72ºC for 7 minutes to extend the reaction by using a DNA thermocycler (Applied Biosystems Gene Amp PCR System 2400). The PCR products were electrophoresized to separate DNA on a 5% acrylamide gel (Lee *et al*., 2001). The gel stained with ethidium bromide and visualization on a UV transilluminator. By using Kodak Digital Science ID 3.02 and DC40/DC120 Camera, PCR products were quantified. The target mRNA signals were normalized to the GAPDH mRNA and expressed as relative abundance.

Histological and Immunohistochemical Examination

Formalin-fixed hepatic tissue was stained with hematoxylin-eosin to define hepatic architecture, using Masson's staining to highlight the areas of hepatic fibrosis. Finally, we used the α-SMA to detect the activated stellate cells (Shimizu *et al*., 1999). After incubation with 1% H_2O_2 in methanol, the samples were digested with 0.1% trypsin for 30 minutes at 37ºC. Samples were washed twice with PBS at room temperature, and incubated for 1 hour at 37ºC with a 1:100 dilution of α-SMA antibody (Dako, Denmark). After washing, the samples were incubated with a 1:200 dilution of biotin-conjugated goat anti-rabbit IgG. They were finally incubated with the avidin-biotin complex (Dako, Denmark) and the antigen–antibody complex was visualized with a light microscope.

Histological Semi-quantitative Scoring System

We used a histological semi-quantitative scoring system (SSS) to evaluate hepatic fibrosis, whereby four main sites of fibrotic deposit were analyzed: centrilobular vein (CLV), portal tract (PT), peri-sinusoidal space (PS), width (WS) and number (NS) of septa (Chevallier *et al*., 1994). This histological score is detailed as follows: (1) centrilobular vein (grade 0: normal vein, 1: thickened wall with stellate aspect of vein wall, and 2: markedly thickened wall with fibrous extension between hepatocytes); (2) portal tract (grade 0: absent of fibrosis,

1: enlarged without septa, 2: enlarged with septa, and 3: cirrhosis); (3) peri-sinusoidal space (grade 0: absent of fibrosis, 1: localized fibrosis, and 2: diffused fibrosis); (4) number of septa (grade 0: absent, 1: 6 ≤ septa per 10 nm, 2: > 6 septa per 10 nm, and 3: nodular organization); and (5) width of septa (grade 1: thin or incomplete septa, 2: thick with loose connective matrix, 3: very thick and dense connective matrix, and 4: > 2/3 area. Subsequently, this score is expressed as SSS = CLV + PS + PT + 2(WS * NS).

Statistical Methods

Results were expressed as mean ± SD. The data obtained was evaluated by ANOVA as appropriate. The pairwise comparison was performed by Bonferroni test, and the level of significance was set at 5% for each analysis.

Results

Assessment of Liver Function after BDL for 6 Weeks

In the BDL rat model, evidence of cholestasis caused hepatic injury and stimulated fibrogenesis or fibrosis in the liver. As presented in Table 2, we observed that the serum total bilirubin of BDL group elevated 16-fold (9.4 ± 1.2 mg/dl) compared with the sham-operation group (0.6 ± 0.4 mg/dl), and the direct bilirubin of BDL group elevated 20-fold (4.0 ± 0.5 mg/dl) compared with the sham-operation group (0.2 ± 0.2 mg/dl). On the contrary, TJ-9 significantly reduced the level of cholestasis about four-fold. For another aspect of cholestasis, TJ-9 dramatically abated the increasing tendency of alkaline phosphatase and r-glutamyl transferase. All rats that received BDL did not display a significant increase in alanine aminotransminase level. For the preservation of liver function, TJ-9 also demonstrated its hepatoprotective effect against cholestasis injury by exhibiting a significant improvement of serum albumin.

Table 2. Effect of Sho-saiko-to (TJ-9) on Serum Biochemical Indices in Rat with Bile Duct Ligation for 6 Weeks

	T-Bil	D-Bil	GPT	ALK-P	γ GT	ALB	A/G
Sham-op	0.6 ± 0.4‡	0.2 ± 0.2‡	48 ± 7	172 ± 19	0 ± 0†	4.4 ± 0.2†	2.9 ± 0.6
BDL	9.4 ± 1.2	4.0 ± 0.5	89 ± 41	223 ± 39	34 ± 13	2.8 ± 0.2	1.0 ± 0.3
BDL + TJ-9	2.6 ± 3.2†	1.1 ± 1.4†	87 ± 36	160 ± 66	7 ± 14†	3.7 ± 0.8*	1.4 ± 0.5

Values are means ± SD. Significance: $^{*}p < 0.05$, $^{†}p < 0.01$, $^{‡}p < 0.001$, compared with the BDL-untreated group. Sham-op: Sham-operated group, BDL: bile duct ligated group (untreated), BDL + TJ-9: bile duct ligated group treated with Sho-saiko-to (TJ-9) at a dose of 0.5 g/kg body weight/day, T-Bil:total bilirubin, D-Bil: direct bilirubin, GPT: alanine aminotransferase, ALK-P: alkaline phosphatase, GT: γ-glutamyl transferase, ALB: albumin, A/G: albumin/globumin ratio.

Quantitative Analysis of Collagen Content

Extent of liver fibrosis was measured biochemically as HYP, which is unique to collagen content. Thus, HYP could serve as a gold standard of fibrosis. The HYP content of the BDL untreated group led to an almost six-fold increase when compared with the sham-operated group (45.1 ± 10.5 versus 7.1 ± 2.4 mg HYP/g dry liver, $p < 0.05$), whereas TJ-9 at a dose of 0.5 g/kg/day significantly reduced HYP by 50% to 60% (Table 3).

Table 3. Parameters of Fibrogenesis in Bile Duct Ligated and Sham-operated Rats

	HYP	Pathol. SSS	Procollagen(I)	Procollagen (III)	TIMP-1
Sham-op	7.1 ± 2.4‡	0 ± 0†	0.235 ± 0.018†	0.183 ± 0.022*	0.284 ± 0.131‡
BDL	45.1 ± 10.5	10.8 ± 0.7	0.589 ± 0.147	0.631 ± 0.270	1.176 ± 0.163
BDL + TJ-9	16.1 ± 7.7‡	5.1 ± 2.6†	0.361 ± 0.178*	0.339 ± 0.113*	0.520 ± 0.396*

Values are means ± SD. Significance: $^{*}p < 0.05$, $^{†}p < 0.01$, $^{‡}p < 0.001$, compared with the BDL-untreated group. Sham-op: Sham-operated group, BDL: bile duct ligated group (untreated), BDL + TJ-9: bile duct ligated group treated with Sho-saiko-to (TJ-9) at a dose of 0.5 g/kg body weight/day, HYP: hydroxyproline (a quantitative analysis of collagen contend), Pathol. SSS: histological semi-quantitative scoring system as described in methods, Procollagen α1 (I), (III) and TIMP-1 (tissue inhibitor of metalloproteinase-1): all of these mRNA signals were normalized to GAPDH and expressed as relative abundance.

Histological Assessment

At the end of 6 weeks, all of the BDL-untreated rats became icteric, experienced abdominal extension and excreted dark urine. After sacrificing, the livers of the BDL-untreated rats were unusually enlarged, and their splenomegaly was obvious, as well as massive ascites, but these phenomena were less significant in the TJ-9-treated group. All of the BDL rats showed obvious dilation of choledochal duct, no matter whether they received TJ-9 treatment or not.

There was significant difference in the hepatic histological picture between the TJ-9 treated and untreated BDL rats (Fig. 1). In the BDL-untreated rats, the most prominent change of liver was massive portal bile ductular proliferation and bridging fibrosis. Using the Masson stain, fibrosis was demonstrated in the portal areas around proliferate bile ductules and in the perivenular areas around the central vein with stellate radiating fibrosis between liver plates. The bridging fibrosis was complete or nearly complete and formed nodular cirrhotic pattern in BDL-untreated rats (Fig. 2). Meanwhile, for the BDL-untreated group, spindle-shaped α-SMA-positive cell increased markedly in number and surrounded the newly formed bile duct in portal tract. It was not found in the sinusoid space of the sham-operated group (Fig. 3). However, there was only little inflammation and necrosis or apoptosis in the hepatocytes.

In the BDL rats treated with TJ-9 at a dose of 0.5 g/kg BW, there was a significant reduction in the bile ductular proliferation and fibrosis as well as the activation of stellate cells (Figs. 1 to 3). We may conclude that the treatment of TJ-9 reduced fibrosis in the BDL model, because its possible route to treatment is related to inhibiting the activation of stellate cells.

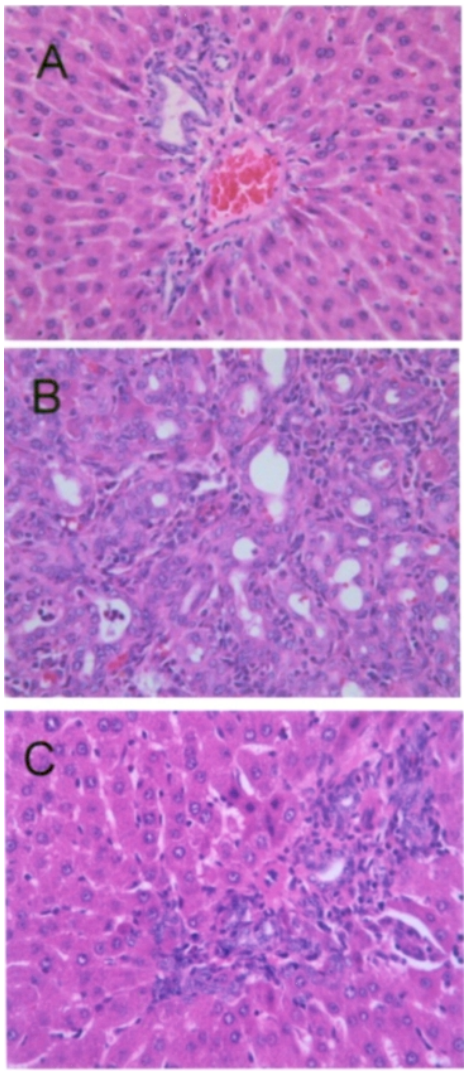

Figure 1. Representative histological finding of liver tissue in rats after BDL for 6 weeks and stained with hematoxylin-eosin (H.E.), original magnification ×200. (A) Sham-operated rat, (B) BDL without treatment, and (C) BDL and treated with TJ-9 at a dose of 0.5 g/kg BW/day.

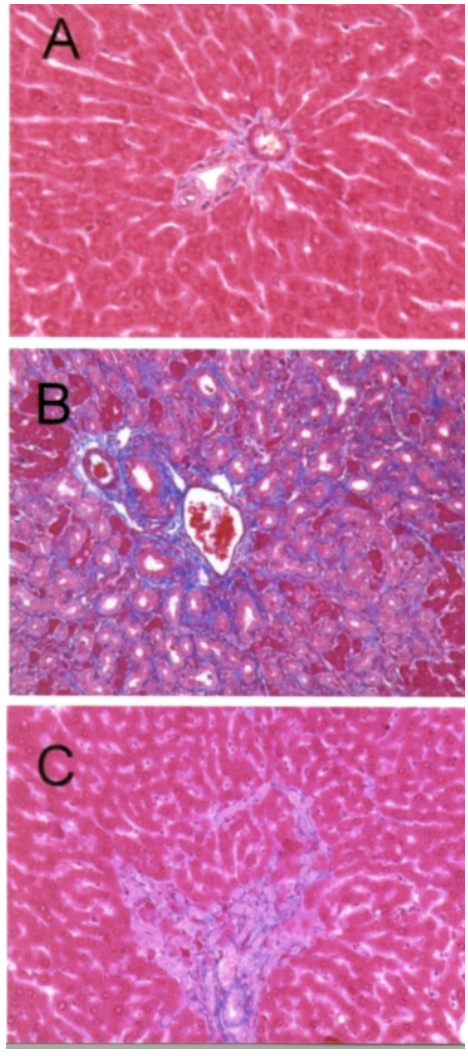

Figure 2. Representative histological finding of liver tissue in rats after BDL for 6 weeks and stained with Masson, original magnification ×100. (A) Sham-operated rat, (B) BDL without treatment: a large amount of dark collagen bonds around proliferate bile duct and radiating blue fibers between liver plates, and (C) BDL and treated with TJ-9 at a dose of 0.5 g/kg BW/day, only localized fibrosis was seen at portal tract.

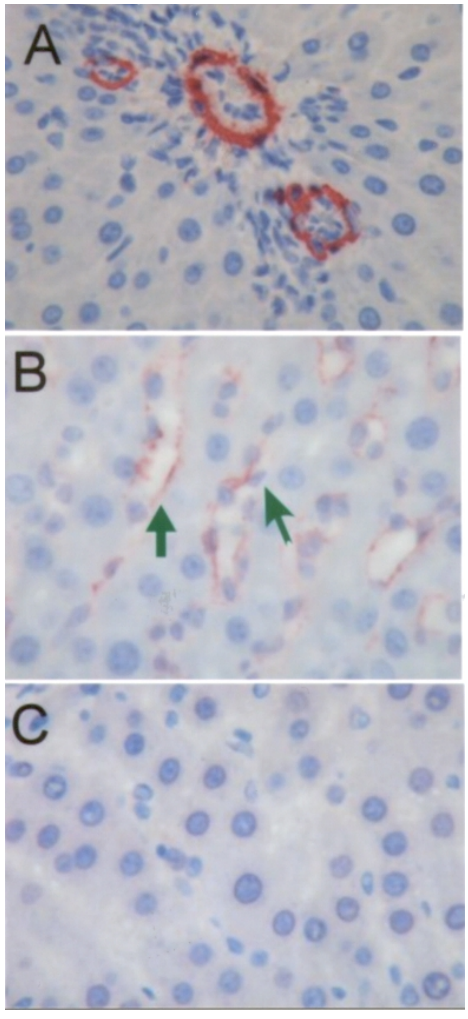

Figure 3. Representative histological finding of liver tissue in rats after BDL for 6 weeks and stained with α-SMA antibody, original magnification ×400. (A) Sham-operated rat: dark rings indicate the muscular wall of portal vein, hepatic artery and bile duct; (B) BDL without treatment: dark lines indicate the activated Ito cell, and (C) BDL and treated with TJ-9 at a dose of 0.5 g/kg BW/day: no obvious dark line.

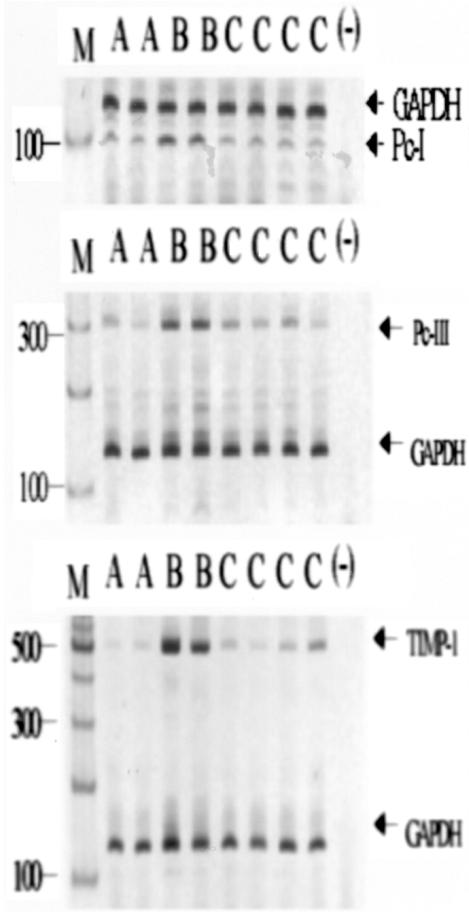

Figure 4. The mRNA expression of procollagen α1 types (I) and (III) and TIMP-1 was performed by RT-PCR analysis. The total RNA was extracted from liver tissue in rat after BDL for 6 weeks. Lane (M): the marker, Lane (A): sham-operated rat, Lane (B): BDL without treatment, Lane (C): BDL and treated with TJ-9 at a dose of 0.5 g/kg BW/day. Pc-I: The pocollagen α1 type (I) and Pc-III: the procollagen α1 type (III). GAPDH is the internal control.

Histological Semi-quantitative Scoring System (SSS)

This SSS design represents a reliable and convenient method for fibrosis evaluation, because it analyzes the four main sites of fibrotic deposit: centrilobular vein, portal tract, peri-sinusoidal space and number of septa. Using the SSS design, the score of the sham-operation group is zero, while the BDL-untreated group received the significantly increasing score of 10.8. In contrast, TJ-9-treated group received the significantly decreased score of 5.1 when compared with the BDL-untreated group. Therefore, we are convinced that TJ-9 dramatically reduced liver fibrosis in rat secondary biliary fibrosis, a good model refractory to the most potential antifibrotic agent.

Expression of Procollagen α1 Types (I) and (II) and TIMP-1

The mRNA expression of procollagen α1 types (I) and (II) and TIMP-1 was determined by RT-PCR (Fig. 4). Procollagen α1 type (I) (100 bp), procollagen α1 type (III) (303 bp) and TIMP-1 (511 bp) were clearly shown at the expected size. There was no equivalent band in the negative control. After bile duct ligation, the relative abundance of hepatic procollagen α1 types (I) and (III) and TIMP-1 transcripts were markedly increased when compared with the sham-operated control group. TJ-9 significantly reduced procollagen α1 types (I) and (III) and TIMP-1 levels by 40%, 50% and 55%, respectively.

Discussion

The BDL experimental model in rats induced bile duct proliferation and dilation. It also led to periportal and perineoductular fibrosis, and eight- to 12-fold increased hepatic collagen content with a little inflammation and necrosis (Gerling *et al*., 1996). In our pilot study, we found that TJ-9 at a dose of 0.5 g/kg was more useful than 0.25 g/kg and 1.0 g/kg dose on anti-cholestasis and antifibrotic effect (data not shown). Ono *et al*. (1999) has proven that 1.5% TJ-9 showed better effect than 0.75% and 3.0% on inhibition of collagen accumulation in dimethylnitrosamine model. In addition, Miyamura *et al*. (1998) also reported that 1.5% TJ-9 in the pellet food was the best dosage than 0.75% and 3% for suppressing the activation of the Ito cell. Thus, we estimated the average daily weight of pellet food, and found that 1.5% TJ-9 in pellet food was approximately near our dosage of 0.5 g/kg/day.

In the BDL rat model of cholestasis, evidence of oxidant injury (lipid peroxidation) has been correlated with extent of hepatic injury and fibrosis (Parola *et al*., 1996). Shimizu *et al*. (1999) has confirmed the preventive effect of TJ-9 on DMN and the pig serum animal model, because TJ-9 inhibited lipid peroxidation, collagen (I) accumulation and α-SMA expression. Our data also showed that TJ-9 reduced cholestasis and collagen content, and we presumed that its possible mechanism is by means of inhibition of lipid peroxidation.

The Ito cell (hepatic stellate cell) has the central role in hepatic fibrogenesis. It also is the major source of fibrilla and nonfibrilla matrix proteins. When the Ito cell was activated, it would transfer into a myofibroblast-like cell, accompanied by loss of cellular retinol and

synthesis of a large amount of α-SMA and extracellular matrix. Therefore, the expression of α-SMA has been reported to be a marker of stellate cell activation (Rocky *et al*., 1992). Sakaida *et al*. (1998) has indicated that TJ-9 prevented liver fibrosis by means of inhibiting the activation of stellate cell. Using the α-SMA immunohistologic stain, we also confirmed that phenomenon. For the mechanism of inhibiting the activation of stellate cell, Kayano *et al*. (1998) have clarified that TJ-9 significantly accumulated the stellate cell in the G0/G1 phase and decreased cell numbers in G2/M phase. Shimizu *et al*. (1999) also used the Western blotting analysis to prove that TJ-9 inhibited α-SMA expression during the culture of stellate cell.

Liver fibrosis is an imbalance between diminished matrix degradation (MMPs) and enhanced matrix accumulation (TIMPs) (Alcolado *et al*., 1997). MMPs divide into three groups according to their substrate specificity: (1) interstitial collagenases (MMP-1, -8 and -13) cleave collagen I, III, II and X; (2) gelatinases (MMP-2 and -9) degrade gelatin and collagen IV; and (3) stromelysins (MMP-3 and -11) degrade proteoglycan and collagen III and IV (Benyon *et al*., 2001). In contrast, TIMP-1 and -2 are thought to result in a net inhibition of matrix breakdown and an accumulation of fibrilla collagen (Benyon *et al*., 1996). Iredale *et al*. (1996) has shown that the expression of TIMP-1 mRNA increased in the BDL model, so as to inhibit interstitial collagenase activity. TIMP-1 inhibits activation of the progelatinase and blocks the active site of MMPs, especially MMP-1 and MMP-13, which are the key enzymes in the degradation of interstitial collagen I, III, II, VII and X (Iredale *et al*., 1992). Therefore, we asserted that TJ-9 reduced the collagen content and presented its antifibrogenic effect by means of down-regulating the mRNA expression of procollagen α1 types (I) and (III) and TIMP-1 in rat's liver with BDL model. In addition, TJ-9 blocked TIMP-1 mRNA expression so as to relieve the suppression of ECM degradation (MMPs).

Furthermore, there are many cytokines involved in liver fibrogenesis. They regulate the activation of stellate cell and are divided into two groups: (1) mitogenic (transforming growth factor-α, platelet-derived growth factor, interleukin-1, tumor necrosis factor-α and insulin-like growth factor) and (2) fibrogenic (transforming growth factor-β and interleukin-6) (Tsukamato, 1999). Yamashiki *et al*. (1996) has reported that TJ-9 strongly induces the production of IL-1β, IL-10, tumor necrosis factor (TNF-α) and granulocyte colony-stimulating factor (G-CSF) by peripheral mononuclear cell. The mechanisms of TJ-9 in reducing liver fibrosis by means of regulating the cytokines are now in progress.

Shimizu *et al*. (1999) provided evidence that TJ-9 functions as a potent anti-fibrosuppressant via the inhibition of oxidative stress in hepatocytes and Ito cells, and its active components are baicalin and baicalein. These two flavonoids are similar to silybinin and quercetin; both have been reported to have antifibrogenic properties (Geerts and Rogiers, 1999). Silybinin prevents the lipid peroxidation of hepatocyte membranes by acting as an oxygen radical scavenger (Pietrangelo *et al*., 1995). Boigk *et al*. (1997) reported that silymarin retarded collagen accumulation in BDL rats. Recently, Jia *et al*. (2001) also confirmed that the antifibrotic effect of silymarin in rat secondary biliary fibrosis is mediated by down-regulating of procollagen α1 type (I) and TIMP-1. These findings can support the results of our research.

Finally, we concluded that (1) TJ-9 at a dose of 0.5 g/kg significantly reduced cholestasis in rats with BDL; (2) TJ-9 markedly reduced the collagen content by 50% in rat model of secondary biliary fibrosis; and (3) TJ-9 exerted its antifibrogenic effect by down-regulating the mRNA expression of procollagen α1 types (I) and (III) and TIMP-1 in liver tissue.

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