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• *BASIC RESEARCH* •

Effects of Chinese traditional compound, JinSanE, on expression of TGF-β1 and TGF-β1 type II receptor mRNA, Smad3 and Smad7 on experimental hepatic fibrosis *in vivo*

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Abstract

AIM: The transforming growth factor-beta (TGF-β)/Smad signaling pathway system plays a prominent role in the control of cell growth and extracellular matrix formation in the progression of liver fibrogenesis. Smad proteins can either positively or negatively regulate TGF-β responses. In this study, the therapeutic effects of Chinese traditional compound decoction, JinSanE, and the changes of TGF-β/Smad signaling pathway system in carbon tetrachloride (CCl_4)-induced rat experimental liver fibrosis were examined.

METHODS: Seventy-two healthy Wistar rats were assigned to groups including normal control group, CCl_4 model group, JinSanE treatment group I and JinSanE treatment group II. Each group contained 18 rats. All groups, except the normal control group, received CCl_4 subcutaneous injection for 8 wk. Rats in JinSanE groups I and II were orally treated with JinSanE daily at the 1st and 5th wk, respectively, after exposure to CCl_4. The expression of TGF-β1 and TGF-β1 type II receptor (TRII) mRNA in the liver was determined by reverse transcription polymerase chain reaction, and the expression of TGF-β1, Smad3 and Smad7 by immunohistochemistry. The liver histopathology was also examined by HE staining and observed under electron microscope. The activities of several serum fibrosis-associated enzymes, alanine aminotransferase (ALT), aspartate aminotransferase (AST), the levels of serum hyaluronic acid (HA) were assayed.

RESULTS: Hepatic fibrosis caused by CCl_4 was significantly inhibited in the JinSanE-treated groups. The degrees of necrosis/degeneration and fibrosis scores were significantly lower in the JinSanE-treated groups than in the model control group. The expression of TGF-β1, TRII and Smad3 was significantly higher in the model group than that in the JinSanE-treated groups, and the active/total TGF-β1 ratio in the JinSanE groups was suppressed. Expression of TRII mRNA and Smad3 proteins showed a distribution pattern similar to that of TGF-β1 with a direct correlation in terms of the degree of hepatic fibrosis. The amount of positive staining Smad7 cells was significantly less in the model group than in the JinSanE-treated groups and the normal group. The contents of ALT, AST and HA were significantly lower in the JinSanE-treated groups than those in the model group.

CONCLUSION: Traditional Chinese medicine, JinSanE, prevents the progression of hepatic damage and fibrosis through the inhibition of TGF-β1, TRII and Smad3 signal proteins, and increases expression of Smad7 signal protein *in vivo*.

Key words: TGF-β; Liver fibrogenesis

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INTRODUCTION

Hepatic fibrosis is a common characteristic of the chronic liver disease, which relates to the abnormal accumulation of extracellular matrix (ECM). It is also the major cause of morbidity and mortality due to the development of cirrhosis and its complications including hepatocellular carcinoma[1,2]. Hepatic stellate cells (HSCs) are the primary cell type responsible for matrix deposition in hepatic fibrosis, undergoing a process of transdifferentiation into fibrogenic myofibroblasts (MFBs)[3]. HSCs are a major target of the profibrogenic agent, transforming growth factor-beta (TGF-β). TGF-β has not only multiple profibrogenic, but also anti-inflammatory and immunosuppressive effects[4,5]. In addition to its fibrogenic action leading to transdifferentiation of HSCs into MFBs, TGF-β is also an important negative

regulator of proliferation and an inducer of apoptosis[6]. TGF-β superfamily members signal through transmembrane Ser-Thr kinase receptors that directly regulate the intracellular Smad pathway[7]. Smads are a unique family of signal transduction molecules that can transmit signals directly from the cell surface receptors to the nucleus. In addition, there is recent evidence that Smads can either positively or negatively regulate the transcription of specific genes in response to TGF-β signaling[8,9]. The TGF-β/Smad signaling pathway plays a prominent role in the activation of HSCs and the regulation of the production, degradation, and accumulation of ECM proteins. On the other hand, genetic and biochemical studies in mammals have firmly established the TGF-β/Smad signaling pathway as a pivotal means for the intracellular signaling of TGF-β[10]. Thus, the TGF-β signal transduction pathway has become a new effective target for the prevention and treatment of hepatic fibrosis[11,12].

Chinese herbal medicine has been used for treatment of acute and chronic hepatitis in China and other countries for thousands of years[13-16]. The traditional Chinese compound, JinSanE, is designed to reduce hepatic fibrosis. It is mainly composed of *Radix curcumae*, *Rhizoma sparganii*, and *Rhizoma zedoariae*, which are widely administered to patients with acute or chronic liver diseases in China, and demonstrate therapeutic effects. However, little is known about the effects and mechanism by which JinSanE protects against hepatic fibrosis. We designed this study to investigate whether JinSanE has an inhibitory effect on the development of hepatic fibrosis in a carbon tetrachloride (CCl_4)-induced hepatic fibrosis model in Wistar rats.

MATERIALS AND METHODS

Animals and tissue specimens

Seventy-two Wistar rats, weighting 180-220 g, were obtained from Renmin Hospital of Wuhan University (Wuhan, China). The rats were randomly divided into four groups: normal group, model group, JinSanE group I and JinSanE group II, with 18 rats in each group. Except those in the normal group, all rats were administered with subcutaneous injection of 400 mL/L CCl_4 dissolved in castor oil (Shanghai Changjiang Chemical Plant, Shanghai, China) 3 mL/kg body weight twice per week for 8 wk. The rats in JinSanE groups I and II were treated with traditional Chinese compound decoction, JinSanE, 6 g/kg body weight, daily, via gastrogavage at the 1st and 4th wk, respectively. Once a week, the rats were weighed and the dosage of CCl_4 and JinSanE was adjusted. The rats were maintained under controlled conditions (24 ℃, 58% humidity, and 12-h day/night rhythm) with alternating 12-h dark/light cycles, fed laboratory chow diet, and had free access to food and water. All rats received humane care, and the study protocols comply with the guidelines of Wuhan University. At the end of the 8-wk experimental period, all rats were killed by bleeding from ophthalmic artery and vein. The blood and liver of all rats were collected for further examinations.

Detection of mRNA by reverse-transcription polymerase chain reaction

Isolation of RNA and reverse-transcription polymerase chain reaction (RT-PCR) were performed as described previously[17]. Isolation of total RNA from liver tissue was performed using the Catrimox-14™ RNA Purification kit (Takara BIO Inc., Tokyo, Japan). RNA concentrations were determined by ultraviolet spectrophotometric measurements at wavelengths of 260/280 nm. mRNA was transcribed into complementary DNA using AMV reverse transcriptase (Takara BIO Inc.). Random primers and Taq polymerase for subsequent PCR were also obtained from Takara BIO Inc. Total RNA (1 μg) was reverse transcribed into cDNA. The cDNA was amplified by PCR with the following forward and reverse primers: TGF-β1, 5'-CAC CA T CCATGACATG AACC-3' and 5'-TCATGTTGGACAAC-TGCTCC-3', respectively, with a product size of 404 bp; TGFβ1-type II receptor (TRII), 5'-CTACAAGGCCA-AGCTGAAGC-3' and 5'-A GCCATGGAGTAGACATC CG -3', respectively, with a product size of 580 bp; and GADPH, 5'-T CCCTCAACATTGTCAGCAA-3' and 5'-AGCTCCACAACGGATACATT-3', respectively, with a product size of 309 bp. The 50 μL PCR reaction mix contained 10 mmol/L dNTP, 2.5 mmol/L $MgCl_2$, 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 30 pmol/L levels of sense and antisense primers, and 5 U of Taq DNA Polymerase (Takara BIO Inc.). The thermal profile that was used consisted of denaturation at 94 ℃ for 1 min, annealing at 55 ℃ for 1 min, and extension at 72 ℃ for 1 min for 35 times for TGF-β1, TRII and GAPDH. In all experiments, possible contamination with genomic DNA was excluded by PCR amplification in the absence of reverse transcriptase. The PCR products were electrophoresed on 2% agarose gel electrophoresis. Semiquantitative evaluation was performed using the Gel Doc 2000 System (BioRad Laboratories GmbH, München, Germany). GAPDH was used as a positive internal control, and was equally positive for all specimens. GAPDH expression was used as a correction factor for TGF-β1 and TRII mRNA, thus, the results were expressed as the number of TGF-β1 and TRII cDNA molecules per GAPDH cDNA molecules.

Immunohistochemistry

Rat monoclonal anti-TGF-β1 antibody, rat polyclonal anti-Smad3 antibody and anti-Smad7 antibody were obtained from Santa Cruz (Los Angeles, USA), and the peroxides method was used to stain TGF-β1-positive, Smad3-positive and Smad7-positive cells. The ultrasensitive TM S-P (Rabbit) kit was obtained from Maxin-Bio (Maxin-Bio, Fuzhou, China). Liver tissues were fixed in 40 g/L formaldehyde, embedded in paraffin, and cut into 4-μm thick sections. The sections were deparaffinized, treated with 0.3% endogenous peroxidase blocking solution for 20 min, and treated sequentially with normal nonimmun goat serum for 20 min at room temperature, then incubated with rabbit anti-TGF-β1 antibody at a previously defined optimal dilution of 1:200, or rabbit anti-Smad3 1:100, or rabbit anti-Smad7 1:100 at room temperature for 1.5 h. All antibodies were diluted in PBS with 1% PBS with 1% bovine serum albumin (BSA), and PBS-1% BSA was used as a negative control. The slides were washed thrice with PBS. Primary antibodies were detected with biotin-labeled anti-rabbit immunoglobulin G. Counterstaining was performed

with hematoxylin. Samples were analyzed by confocal microscopy using 40× objective (Olympus, Japan).

Analytical methods for serum samples

Serum content of hyaluronic acid (HA) was assessed by radioimmunoassay (RIA) using a commercial kit (Shanghai NAVY Medical Institute, Shanghai, China). Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), albumin (ALB) and globulin (GLB) were determined by routine laboratory methods using a Hitachi Automatic Analyzer.

Histopathological examination

Liver tissues were fixed in 40 g/L formaldehyde, embedded in paraffin, cut into 4-μm thick sections and stained with hematoxylin-eosin (H&E). The consensus by the Hepatic Fibrosis Study Group of Chinese Liver Diseases Association[18] was used to evaluate hepatic fibrosis degree and necroinflammatory activity by two independent pathologists blindly. Liver tissues fixed in 2% buffer glutaraldehyde were observed with Hitachi H-300 electron microscope.

Statistical analysis

All data were presented as mean±SE. Differences among groups were assessed using unpaired Student's *t* test and one-way ANOVA. A *P* value of less than 0.05 was considered to be statistically significant. Calculations were performed with SPSS11.0 software package of statistical programs.

RESULTS

Detection of TGF-β1 and TRII mRNA by RT-PCR

Detectable levels of TGF-β1 and TRII mRNA were noted in the normal group, but the expression levels in the model group were significantly increased, compared with those in the normal group (both $P<0.01$) (Table 1, Figures 1 and 2). The expression levels of TGF-β1 mRNA (0.590±0.053, 0.693±0.057, respectively) and TRII mRNA (0.462±0.009, 0.539±0.010, respectively) in JinSanE groups I and II were significantly lower compared with the model group livers (1.410±0.094, 0.906±0.03, respectively) (all $P<0.01$) (Table 1). There was no significant difference in the expression level of TGF-β1 mRNA between the normal group (0.401±0.059) and JinSanE group II ($P>0.05$). Similarly, there was no significant difference in the expression level of TRII mRNA between the normal group (0.275±0.027) and JinSanE group I ($P>0.05$). However, the expression level of TRII mRNA in JinSanE group II was significantly greater than that in JinSanE group I ($P<0.01$).

Table 1 **Comparison of semiquantitative RT-PCR results in the four experimental groups (mean±SD)**

Groups	*n*	Expression levels	
		$TGF\beta_1$mRNA	TRII mRNA
Normal	18	0.401±0.059	0.275±0.027
Model	15	1.410±0.094[b]	1.215±0.079[b]
JinSanE I	17	0.590±0.053[d]	0.461±0.029[d]
JinSanE II	16	0.693±0.057[b,d]	0.651±0.035[b,d,f]

[b]$P<0.01$ *vs* compared with the normal group; [d]$P<0.01$ *vs* compared with the model group; and [f]$P<0.01$ *vs* compared with JinSanE group I.

Detection of TGF-β1, Smad3 and Smad7 by immunohistochemistry

The distribution of TGF-β1, Smad3 and Smad7 was observed in cytoplasm of rat livers in all groups, but nuclei were devoid of staining (Figures 3-5). In normal control livers, the cytoplasm of interstitial tissue and liver sinusoidal were positive for TGF-β1, whereas TGF-β1 was scattered along the sinusoidal walls. Smad3 was rarely observed, but intense Smad7 expression was extensively observed in hepatocytes of normal rats. In contrast, many TGF-β1 and Smad3-positive cells were detected in sinus portal, areas fibrous, septum, hepatocytes in pseudolobes and necrotic areas of severe hepatitis in the model rats, and the amount of TGF-β1-positive cells was obviously larger than that of Smad3-positive cells. In fibrotic liver tissues, weak immunostaining for Smad7 was detected in hepatocytes, fibroblasts and occasional mononuclear infiltrates. In contrast, livers of rats fed with JinSanE showed markedly decreased numbers of TGF-β1 and Smad3-positive HSCs and fibroblasts, and these positive cells in livers of the JinSanE group I were less than those in the JinSanE group II. JinSanE also obviously enhanced numbers of Smad7-positive hepatocytes and fibroblasts, and exhibited stronger Smad7 positivity in the livers of JinSanE group I than in JinSanE group II (Figures 3-5). Generally, the staining patterns for TGF-β1 and Smad3 were similarly distributed, but staining for TGF-β1 was much stronger than that for Smad3 (Figures 3 and 4).

Effect of JinSanE on HA and liver function

Serum content of HA was significantly higher in the model group than that in the normal group, but this marker was markedly lowered in two treated groups compared with the model group. Compared with the normal group, the serum levels of ALT and AST in the model group were significantly raised, and serum levels of ALB and ALB/GLB were significantly diminished. Compared with the model group, the levels of ALT and AST were markedly reduced, and ALB and ALB/GLB levels were significantly increased in JinSanE groups I and II (Table 2).

Table 2 **Biochemical data in the four experimental groups (mean±SD)**

Groups	*n*	ALT (IU/L)	AST (IU/L)	ALB (g/L)	A/G	HA (ng/L)
Normal rats	18	50.17±2.37	77.8±13.36	35.4±1.5	1.30±0.09	116.04±17.81
Model rats	15	276.0±45.4[b]	554.8±68.3[b]	24.7±3.15[b]	0.57±0.48[b]	318.65±20.83[b]
JinSanE I rats	17	105.3±15.1[b,d]	331.5±47.9[b,d]	30.1±2.3[b,d]	0.89±0.04[b,d]	138.20±11.39[d]
JinSanE II rats	16	109.5±28.5[b,d]	248.5±9.42[b,d]	32.7±1.8[b,d]	0.94±0.07[b,d]	129.50±17.04[d]

ALT, alanine transaminase; AST, aspartate transaminase; ALB, albumin; A/G, albumin/globulin ratio; and HA, hyaluronic acid. [b]$P<0.01$ *vs* compared with the normal group; [d]$P<0.01$ *vs* compared with the model group.

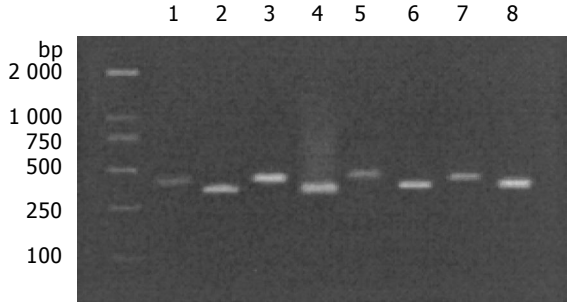

Figure 1 Gel electrophoresis of PCR products for TGF-b1 and GAPDH in rat liver. Total RNA isolated from liver specimens was reverse transcribed and amplified by PCR with specific primers. As a positive control, the housekeeping GAPDH mRNA was also amplified. M (Authors: add M in the photo), Takara markers. Lines 1, 3, 5, 7 are PCR products for TGF-b1, representing the normal, model, JinSanE I and JinSanE II groups, respectively. Lines 2, 4, 6 and 8 are PCR products of GAPDH, representing the normal, model, JinSanE I and JinSanE II groups, respectively.

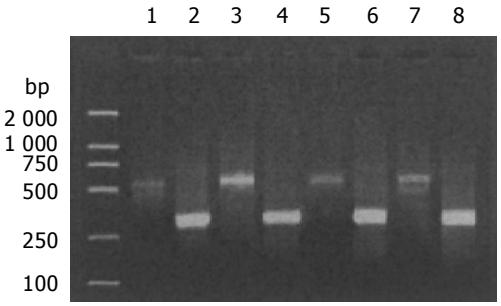

Figure 2 Gel electrophoresis of PCR products for TRII and GAPDH in rat liver. Total RNA isolated from liver specimens was reverse transcribed and amplified by PCR with specific primers. As a positive control, the housekeeping GAPDH mRNA was also amplified. M (Authors: add M in the photo), Takara markers. Lines 1, 3, 5, 7 are PCR products for TRII, representing the normal, model, JinSanE I and JinSanE II groups, respectively. Lines 2, 4, 6 and 8 are PCR products of GAPDH, representing the normal, model, JinSanE I and JinSanE II groups, respectively.

Liver histopathology

The livers in the normal group showed normal lobular architecture with central veins and radiating hepatic cords with irregular sinusoids, and a normal distribution of collagen with a variable amount in portal tracts and a thin rim around central veins. Livers in the model group showed disorderly hepatocyte cords, fatty degeneration, necrosis and infiltration of inflammatory cells and collagen deposition extending from central veins or portal tracts, with thick or thin fibrotic septa and even formation of pseudolobuli. Treatment with JinSanE resulted in apparent amelioration of hepatocyte degeneration, necrosis and infiltration of inflammatory cells, and marked reduction of collagen deposition with no obvious formation of pseudolobuli. Histologic examination revealed that JinSanE-treated rats showed less infiltration of inflammatory cells and less severe progression of liver fibrosis compared with the model rats. Scores of liver necroinflammation and fibrosis are shown in Table 3.

In the livers of the normal rats, the result of electron microscopy showed that HSCs, fat droplet-rich and with pseudopodium, were distributed amongst the hepatocytes. A mass of accumulation of ECM proteins in the Disse's spaces was a prominent finding in fibrotic liver, and the hypertrophied endoplasmic reticulum in HSCs and swollen mitochondrion in hepatocyte were also observed in this group. In the livers of JinSanE-treated rats, the result of electron microscopy showed obviously less accumulation of ECM proteins in the Disse's spaces, fat droplet-rich HSCs, and regenerated and normal hepatocytes.

Table 3 **Effects of JinSanE on liver necroinflammatory scores and fibrosis scores in the four experimental groups (mean±SD)**

Groups	*n*	Necroinflammatory scores	Fibrosis scores
Normal	18	0	0
Model	15	5.28±1.31	7.44±1.59
JinSanE I	17	3.18±0.95[a]	4.40±1.27[a]
JinSanE II	16	4.05±0.92[bc]	5.57±1.20[b]

[b]$P<0.01$ *vs* compared with the model group; [a]$P<0.05$ *vs* compared with the model group; and [c]$P<0.05$ *vs* compared with JinSanE group I.

DISCUSSION

Our study showed that TGF-β1 mRNA and protein increased as fibrosis developed in CCl_4-induced fibrotic liver in a rat model. Our study also demonstrated that ingestion with JinSanE not only suppressed the development of CCl_4-induced hepatic fibrosis, but also decreased the TGF-β1 mRNA and protein levels. The CCl_4-induced hepatic fibrogenesis may be associated with the proliferation of HSCs and ECM accumulation. During the development of chronic liver injury, including inflammation, fibrosis and regeneration, TGF-β1 superfamily plays a prominent role in stimulating liver fibrogenesis by MFBs derived from HSCs[19]. TGF-β1 family regulates many aspects of the cellular function, and consequently exhibits different effects on a variety of cell types and tissues. Hepatocyte apoptosis and HSC activation are both features of chronic liver diseases. TGF-β1 inhibits hepatocyte proliferation and induces hepatocyte apoptosis, and is also the most important cytokine involved in HSC activation, acting via both paracrine and autocrine pathways[20]. TGF-β1 is probably the most decisive cytokine, and HSCs are the most significant cells involved in stimulating ECM synthesis, inhibiting ECM degradation and enhancing accumulation of ECM in the liver[21,22]. Proteolytic release and activation of latent TGF-β by HSCs are key events for pathogenesis of hepatic fibrosis[23]. Cells, such as HSCs, Kupffer cells, MFBs, endothelial cells and invading mononuclear cells, could synthesize and release TGF-β. Many studies have created a strong rationale for an antifibrotic strategy in which the principal objective is blocking TGF-β1.

Active TGF-β binds to specific, high-affinity receptors present on most cells, initiating a signaling cascade that results in biologic effects[24,25]. TGF-β signals through the heteromeric complexes of type I and type II transmembrane Ser/Thr kinase receptors[19,26], which activate the downstream Smad signal transduction pathway. The extracellular domain of the type II receptor binds the ligand, causing formation of heteromeric complexes incorporating type I and type II receptors. The type II receptor then transphosphorylates the type I receptor, activating its kinase and initiating downstream signaling. The type II receptor transmits the

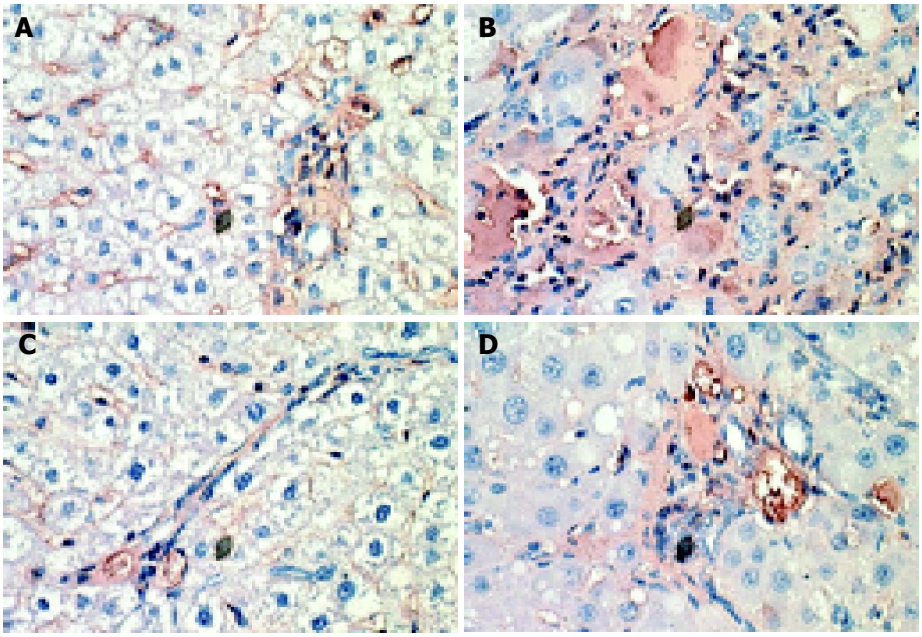

Figure 3 Immunohistochemical staining for TGF-β1 in liver sections. ×400 for **A**, **B**, **C** and **D**. (**A**) TGF-β1 immunoreactivity was evenly distributed within the interstitial tissue and liver sinusoidal in the normal group; (**B**) Stout connective tissue septa were strongly immunoreactive for TGF-β1 in the model group; (**C**) The staining intensity was much weaker in JinSanE group I than that in the model group; and (**D**) The staining positivity in JinSanE group II was stronger than that in the JinSanE group II, but still less than that in the model group.

signal from all isoforms of TGF-β, and it appears to be essential for the biological activity of TGF-β *in vivo*. Thus, its blockade could result in multiple effects[27-32]. Our results indicated that rats treated with JinSanE have reduced expression of TGF-β, TRII mRNA and TGF-β1 protein. This effect may provide an advantage to liver to escape the promoting fibrosis growth signals of TGF-β1, and may be linked to critical steps in the progression of wound repair in liver.

The Smad signaling pathway is critical for TGF-β superfamily signals from the cell surface to the nucleus[33]. Smad proteins are essential components of the intracellular signaling pathways utilized by members of the superfamily. Smad proteins are classified according to their structure and function in signaling by TGF-β family members: the receptor-regulated Smad2 and Smad3 (R-Smads); the common-mediator Smad4 (co-Smads), and the antagonistic or inhibitory Smad6 and Smad7 (I-Smads, part of a negative

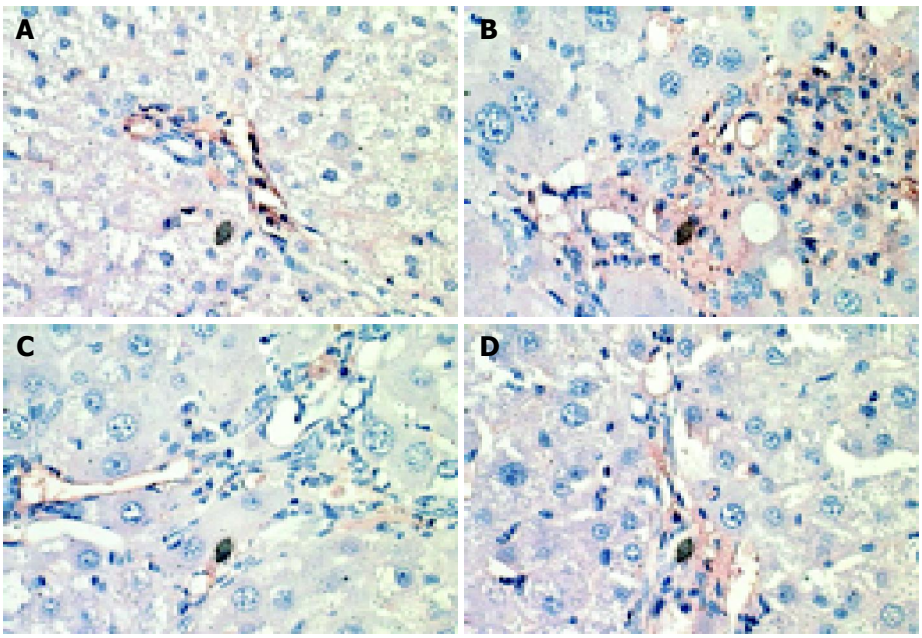

Figure 4 Immunohistochemical staining for Smad3 in liver sections. ×400 for **A**, **B**, **C** and **D**. (**A**) Several cells were positive for Smad3 in the normal group; (**B**) As TGF-β1, similarly increased Smad3 immunoreactivity was detected in the model group, but the staining degree was weak; (**C**) The expression was decreased in JinSanE group I, compared with the model group; and (**D**) Although the staining pattern for Smad3 in JinSanE group II was comparable with that observed for TGF-β1, the staining intensity was much weaker.

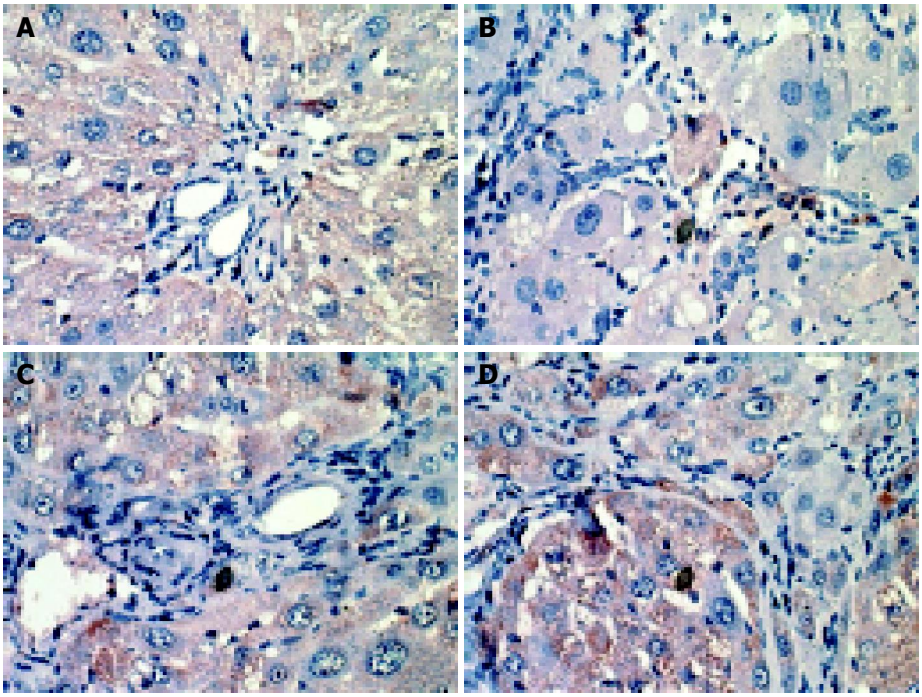

Figure 5 Immunohistochemical staining for Smad7 in liver sections. ×400 for **A**, **B**, **C** and **D**. (**A**) Normal liver displayed ubiquitous and strong staining for Smad7 in hepatocytes; (**B**) Weak staining of Smad7 was observed in the model group, which was in contrast to the abundant expression of TGF-β1 and Smad3 in liver of the group; (**C**) The staining intensity in JinSanE group I was much stronger than that in the model group, and the slender connective tissue septa were strongly immunoreactive; and (**D**) Strong staining for the Smad7 in JinSanE group II was observed.

feedback loop)[34,35]. In quiescent HSCs, TGF-β signaling involves TGF-β type I receptor (TRI)-mediated phosphorylation of serine residues within the conserved SSXS motif at the C-terminus of Smad2 and Smad3, specific R-Smads[9]. Phosphorylation of the R-Smads causes dissociation from the receptor, and induces assembly into complexes with Smad4, a co-Smad. This heteromeric complex then translocates into the nucleus, where the Smads function as transcriptional co-modulators by recruiting co-activators or co-repressors to Smad DNA binding partners[9,19,36]. Thus, Smads transmit signals directly from the receptor kinase into the nucleus, and such a complex accumulates in the nucleus, Smads regulate transcriptional responses by recruiting co-activators and co-repressors to a wide array of DNA-binding partners[37]. In addition, Smad3 phosphorylation mediated by the activated TRI is impaired severely in MFBs during chronic liver injury. Smad3 phosphorylation promotes ECM production in MFBs both *in vitro* and *in vivo*[38]. On the other hand, Smad6 and Smad7 antagonize TGF-β-induced activation of signal-transducing Smads (2 and 3) by preventing the activation of signal-transducing Smad complexes, and participating in a negative feedback loop to control TGF-β responses[19,39-41]. Smad7 interacts stably with activated TRI, thereby preventing R-Smads from binding to and being phosphorylated by these receptors, inhibits the nuclear accumulation of Smad2, and blocks the association, phosphorylation, and activation of Smad2[42,43]. Abnormal expression of anti-sense Smad7 RNA has been found to advance the effect of TGF-β1, supporting the role of Smad7 as a negative regulator in TGF-β signaling[44], thus, ectopic expression of Smad7 in HSCs leads to inhibition of R-Smads and Smad4 phosphorylation and abrogates TGF-β response[45,46]. Smad7 may function as a general inhibitor of TGF-β family signaling, and Smad6 preferentially antagonizes the bone morphogenic protein signaling pathway[47]. In our study, both level and extent of expressions of TGF-β1-inducible Smad7 were remarkably attenuated, whereas Smad3 expression was up-regulated in model fibrotic rats, indicating that Smad7 is a potent *in vivo* inhibitor for signal transduction of the TGF-β superfamily during the development and maintenance of homeostasis of liver tissues. We also observed down-regulation of Smad3 expression and up-regulation of Smad7 expression in JinSanE-treated fibrotic rats, suggesting that JinSanE regulates the balance between Smads3 and Smad7, and thus may decrease activation of an autocrine TGF-β loop that contributes to the HSC change into fibroblasts in hepatic fibrosis.

The advantage of Chinese herbal medicine in treating chronic hepatic diseases has been demonstrated by the results of experimental and clinical studies[2,15,48-50]. Chinese herbal medicine has become of interest to more and more practitioners of western medicine. Fibrosis in the Disse's space may block the exchange of molecules between the sinusoidal space and the hepatocytes[51]. The diminished fibrosis may have led to an attenuation of the hepatic "injury" at a later stage in the disease process, and thus, to a lessening of the liver dysfunction[52]. Our study indicated that traditional Chinese compound decoction, JinSanE, inhibited hepatic fibrosis, which was accompanied by a reduction of liver dysfunction, reduced the serum ALT and AST elevation, and raised the serum levels of ALB and ALB/GLB in rats exposed to CCl_4. Anti-fibrotic therapy with JinSanE is preferentially targeted to the activated mesenchymal cells in the liver that synthesize an excess of

matrix proteins, and resemble the MFBs of healing wounds. Compared with the model rats, there were only slender connective tissue septa and smaller fibrous areas and septum in the livers of rats treated with JinSanE, and the discrimination of liver necroinflammatory scores and fibrosis scores between the rats treated and untreated with JinSanE also demonstrated that JinSanE was effective in protecting against injury. This antifibrotic effect was related to early and long-term use of JinSanE, as confirmed with hepatic histology in our study.

In conclusion, our results demonstrate that TGF-β plays a critical role in the progression of hepatic fibrosis, since TGF-β1, TRII and Smad3 were up-regulated in a rat model of hepatic fibrosis, and the increase in mRNA expression of TGF- β1 and TRII correlated with histological progression to fibrosis. Our study suggests that traditional Chinese compound decoction, JinSanE, should be therapeutic in already-established fibrotic livers *in vivo*. Moreover, our findings demonstrate that the mechanism of antifibrogenic activity of JinSanE is associated with the regulation of ECM proteins, including TGF-β, TRII and Smad expression. The traditional Chinese compound decoction, JinSanE, could inhibit collagen proliferation and facilitate hepatocyte regeneration. Long-term studies in animal models and cells are the next step to establish its safety and clinical potentials.

REFERENCES

1 **Bedossa P,** Paradis V. Liver extracellular matrix in health and disease. *J Pathol* 2003; **200**: 504-515
2 **Shimizu I,** Ma YR, Mizobuchi Y, Liu F, Miura T, Nakai Y, Yasuda M, Shiba M, Horie T, Amagaya S, Kawada N, Hori H, Ito S. Effects of Sho-saiko-to, a Japanese herbal medicine, on hepatic fibrosis in rats. *Hepatology* 1999; **29**: 149-160
3 **Liu C,** Gaca MD, Swenson ES, Vellucci VF, Reiss M, Wells RG. Smads 2 and 3 are differentially activated by transforming growth factor-beta (TGF-beta) in quiescent and activated hepatic stellate cells. Constitutive nuclear localization of Smads in activated cells is TGF-beta-independent. *J Biol Chem* 2003; **278**: 11721-11728
4 **Bissell DM,** Roulot D, George J. Transforming growth factor beta and the liver. *Hepatology* 2001; **34**: 859-867
5 **Galli A,** Crabb DW, Ceni E, Salzano R, Mello T, Svegliati-Baroni G, Ridolfi F, Trozzi L, Surrenti C, Casini A. Antidiabetic thiazolidinediones inhibit collagen synthesis and hepatic stellate cell activation *in vivo* and *in vitro*. *Gastroenterology* 2002; **122**: 1924-1940
6 **Gressner AM,** Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. *Front Biosci* 2002; **7**: d793-d807
7 **Tahashi Y,** Matsuzaki K, Date M, Yoshida K, Furukawa F, Sugano Y, Matsushita M, Himeno Y, Inagaki Y, Inoue K. Differential regulation of TGF-beta signal in hepatic stellate cells between acute and chronic rat liver injury. *Hepatology* 2002; **35**: 49-61
8 **Mehra A,** Wrana JL. TGF-beta and the Smad signal transduction pathway. *Biochem Cell Biol* 2002; **80**: 605-622
9 **Wrana JL.** Phosphoserine-dependent regulation of protein-protein interactions in the Smad pathway. *Structure* 2002; **10**: 5-7
10 **Massaguè J.** TGF-beta signal transduction. *Annu Rev Biochem* 1998; **67**: 753-791
11 **Yoshiji H,** Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Nakatani T, Tsujinoue H, Fukui H. Angiotensin-II type 1 receptor interaction is a major regulator for liver fibrosis development in rats. *Hepatology* 2001; **34**: 745-750
12 **Dooley S,** Hamzavi J, Breitkopf K, Wiercinska E, Said HM, Lorenzen J, Ten Dijke P, Gressner AM. Smad7 prevents activation of hepatic stellate cells and liver fibrosis in rats. *Gastroenterology* 2003; **125**: 178-191
13 **Zhang M,** Song G, Minuk GY. Effects of hepatic stimulator substance, herbal medicine, selenium/vitamin E, and ciprofloxacin on cirrhosis in the rat. *Gastroenterology* 1996; **110**: 1150-1155
14 **Li J,** Yuan S, Zhao J. Study on the regulatory effect of Chinese herbal medicine on peritoneal lymphatic stomata and in enhancing drainage of ascites in mice with liver fibrosis. *Zhongguo Zhongxiyi Jiehe Zazhi* 2000; **20**: 753-756
15 **Kusunose M,** Qiu B, Cui T, Hamada A, Yoshioka S, Ono M, Miyamura M, Kyotani S, Nishioka Y. Effect of Sho-saiko-to extract on hepatic inflammation and fibrosis in dimethylnitrosamine induced liver injury rats. *Biol Pharm Bull* 2002; **25**: 1417-1421
16 **Li JC,** Ding SP, Xu J. Regulating effect of Chinese herbal medicine on the peritoneal lymphatic stomata in enhancing ascites absorption of experimental hepatofibrotic mice. *World J Gastroenterol* 2002; **8**: 333-337
17 **Okuno M,** Akita K, Moriwaki H, Kawada N, Ikeda K, Kaneda K, Suzuki Y, Kojima S. Prevention of rat hepatic fibrosis by the protease inhibitor, camostat mesilate, via reduced generation of active TGF-beta. *Gastroenterology* 2001; **120**: 1784-1800
18 Hepatic Fibrosis Study Group of Chinese Liver Diseases Association. Consensus on evaluation of the diagnosis and efficacy of hepatic fibrosis. *Chin J Infect Dis* 2002; **10**: 327-328
19 **Tahashi Y,** Matsuzaki K, Date M, Yoshida K, Furukawa F, Sugano Y, Matsushita M, Himeno Y, Inagaki Y, Inoue K. Differential regulation of TGF-beta signal in hepatic stellate cells between acute and chronic rat liver injury. *Hepatology* 2002; **35**: 49-61
20 **Napoli J,** Prentice D, Niinami C, Bishop GA, Desmond P, McCaughan GW. Sequential increases in the intrahepatic expression of epidermal growth factor, basic fibroblast growth factor, and transforming growth factor beta in a bile duct ligated rat model of cirrhosis. *Hepatology 1997*; **26**: 624-633
21 **Le Pabic H,** Bonnier D, Wewer UM, Coutand A, Musso O, Baffet G, Clement B, Theret N. ADAM12 in human liver cancers: TGF-beta-regulated expression in stellate cells is associated with matrix remodeling. *Hepatology* 2003; **37**: 1056-1066
22 **Cui X,** Shimizu I, Lu G, Itonaga M, Inoue H, Shono M, Tamaki K, Fukuno H, Ueno H, Ito S. Inhibitory effect of a soluble transforming growth factor beta type II receptor on the activation of rat hepatic stellate cells in primary culture. *J Hepatol* 2003; **39**: 731-737
23 **Gaca MD,** Zhou X, Issa R, Kiriella K, Iredale JP, Benyon RC. Basement membrane-like matrix inhibits proliferation and collagen synthesis by activated rat hepatic stellate cells: evidence for matrix-dependent deactivation of stellate cells. *Matrix Biol* 2003; **22**: 229-239
24 **Tsunawaki S,** Sporn M, Ding A, Nathan C. Deactivation of macrophages by transforming growth factor-beta. *Nature* 1988; **334**: 260-262
25 **Balza E,** Borsi L, Allemanni G, Zardi L. Transforming growth factor beta regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts. *FEBS Lett* 1988; **228**: 42-44
26 **Roulot D,** Sevcsik AM, Coste T, Strosberg AD, Marullo S. Role of transforming growth factor beta type II receptor in hepatic fibrosis: studies of human chronic hepatitis C and experimental fibrosis in rats. *Hepatology* 1999; **29**: 1730-1738
27 **Tahashi Y,** Matsuzaki K, Date M, Yoshida K, Furukawa F, Sugano Y, Matsushita M, Himeno Y, Inagaki Y, Inoue K. Differential regulation of TGF-beta signal in hepatic stellate cells between acute and chronic rat liver injury. *Hepatology* 2002; **35**: 49-61
28 **Yata Y,** Gotwals P, Koteliansky V, Rockey DC. Dose-dependent inhibition of hepatic fibrosis in mice by a TGF-beta soluble receptor: implications for antifibrotic therapy. *Hepatology* 2002; **35**: 1022-1030
29 **Wrana JL,** Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massague J. TGF beta signals through a

heteromeric protein kinase receptor complex. *Cell* 1992; **71**: 1003-1014

30 **Inagaki M,** Moustakas A, Lin HY, Lodish HF, Carr BI. Growth inhibition by transforming growth factor beta (TGF-beta) type I is restored in TGF-beta-resistant hepatoma cells after expression of TGF-beta receptor type II cDNA. *Proc Natl Acad Sci USA* 1993; **90**: 5359-5363

31 **Bhushan A,** Lin HY, Lodish HF, Kintner CR. The transforming growth factor beta type II receptor can replace the activin type II receptor in inducing mesoderm. *Mol Cell Biol* 1994; **14**: 4280-4285

32 **Bassing CH,** Yingling JM, Howe DJ, Wang T, He WW, Gustafson ML, Shah P, Donahoe PK, Wang XF. A transforming growth factor beta type I receptor that signals to activate gene expression. *Science* 1994; **263**: 87-89

33 **Attisano L,** Wrana JL. Signal transduction by the TGF-beta superfamily. *Science* 2002; **296**: 1646-1647

34 **Dong C,** Zhu S, Wang T, Yoon W, Li Z, Alvarez RJ, ten Dijke P, White B, Wigley FM, Goldschmidt-Clermont PJ. Deficient Smad7 expression: a putative molecular defect in scleroderma. *Proc Natl Acad Sci USA* 2002; **99**: 3908-3913

35 **Imamura T,** Takase M, Nishihara A, Oeda E, Hanai J, Kawabata M, Miyazono K. Smad6 inhibits signalling by the TGF-beta superfamily. *Nature* 1997; **389**: 622-626

36 **Carcamo J,** Weis FM, Ventura F, Wieser R, Wrana JL, Attisano L, Massague J. Type I receptors specify growth-inhibitory and transcriptional responses to transforming growth factor beta and activin. *Mol Cell Biol* 1994; **14**: 3810-3821

37 **Zhang Y,** Feng XH, Derynck R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. *Nature* 1998; **394**: 909-913

38 **Furukawa F,** Matsuzaki K, Mori S, Tahashi Y, Yoshida K, Sugano Y, Yamagata H, Matsushita M, Seki T, Inagaki Y, Nishizawa M, Fujisawa J, Inoue K. p38 MAPK mediates fibrogenic signal through Smad3 phosphorylation in rat myofibroblasts. *Hepatology* 2003; **38**: 879-889

39 **Stopa M,** Benes V, Ansorge W, Gressner AM, Dooley S. Genomic locus and promoter region of rat Smad7, an important antagonist of TGFbeta signaling. *Mamm Genome* 2000; **11**: 169-176

40 **Li X,** Ionescu AM, Schwarz EM, Zhang X, Drissi H, Puzas JE, Rosier RN, Zuscik MJ, O'Keefe RJ. Smad6 is induced by BMP-2 and modulates chondrocyte differentiation. *J Orthop Res* 2003; **21**: 908-913

41 **Ishisaki A,** Yamato K, Hashimoto S, Nakao A, Tamaki K, Nonaka K, ten Dijke P, Sugino H, Nishihara T. Differential inhibition of Smad6 and Smad7 on bone morphogenetic protein- and activin-mediated growth arrest and apoptosis in B cells. *J Biol Chem* 1999; **274**: 13637-13642

42 **Hayashi H,** Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Wrana JL, Falb D. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* 1997; **89**: 1165-1173

43 **Souchelnytskyi S,** Nakayama T, Nakao A, Morén A, Heldin CH, Christian JL, ten Dijke P. Physical and functional interaction of murine and Xenopus Smad7 with bone morphogenetic protein receptors and transforming growth factor-beta receptors. *J Biol Chem* 1998: **273**: 25364-25370

44 **Afrakhte M,** Moren A, Jossan S, Itoh S, Sampath K, Westermark B, Heldin CH, Heldin NE, ten Dijke P. Induction of inhibitory Smad6 and Smad7 mRNA by TGF-beta family members. *Biochem Biophys Res Commun* 1998; **249**: 505-511

45 **Dooley S,** Delvoux B, Streckert M, Bonzel L, Stopa M, ten Dijke P, Gressner AM. Transforming growth factor beta signal transduction in hepatic stellate cells via Smad2/3 phosphorylation, a pathway that is abrogated during *in vitro* progression to myofibroblasts. TGFbeta signal transduction during transdifferentiation of hepatic stellate cells. *FEBS Lett* 2001; **502**: 4-10

46 **Nakao A,** Afrakhte M, Moren A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin NE, Heldin CH, ten Dijke P. Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* 1997; **389**: 631-635

47 **Datta PK,** Moses HL. STRAP and Smad7 synergize in the inhibition of transforming growth factor beta signaling. *Mol Cell Biol* 2000; **20**: 3157-3167

48 **Yao XX,** Tang YW, Yao DM, Xiu HM. Effects of Yigan Decoction on proliferation and apoptosis of hepatic stellate cells. *World J Gastroenterol* 2002; **8**: 511-514

49 **Zhang M,** Song G, Minuk GY. Effects of hepatic stimulator substance, herbal medicine, selenium/vitamin E, and ciprofloxacin on cirrhosis in the rat. *Gastroenterology* 1996; **110**: 1150-1155

50 **Li MY,** Ryan P, Batey RG. Traditional Chinese medicine prevents inflammation in CCl_4-related liver injury in mice. *Am J Chin Med* 2003; **31**: 119-127

51 **Friedman SL.** Seminars in medicine of the Beth Israel Hospital, Boston. The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. *N Engl J Med* 1993; **328**: 1828-1835

52 **Qi Z,** Atsuchi N, Ooshima A, Takeshita A, Ueno H. Blockade of type beta transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat. *Proc Natl Acad Sci USA* 1999; **96**: 2345-2349

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