

• BASIC RESEARCH •

Effect of Danshao Huaxian capsule on expression of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 in fibrotic liver of rats

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Supported by the Foundation of Traditional Chinese Medicine Modernization Project of Guizhou Province, No. 200409

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Received: 2004-10-30 Accepted: 2004-11-19

CONCLUSION: DSHX enhances the expression of MMP-1 but decreases that of TIMP-1 in liver tissues of CCl₄-induced hepatic fibrotic rats, which may result in its elevated activity that contributes to fighting against hepatic fibrosis.

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Key words: Danshao Huaxian capsule; Hepatic fibrosis; MMP-1; TIMP-1; Collagen

Yang Q, Xie RJ, Geng XX, Luo XH, Han B, Cheng ML. Effect of Danshao Huaxian capsule on expression of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 in fibrotic liver of rats. *World J Gastroenterol* 2005; 11 (32): 4953-4956

<http://www.wjgnet.com/1007-9327/11/4953.asp>

Abstract

AIM: To investigate the effects of Danshao Huaxian (DSHX) capsules, a preparation of traditional Chinese medicine, on the expression of matrix metalloproteinase-1 (MMP-1), and tissue inhibitor of metalloproteinase-1 (TIMP-1) in the fibrous livers of rats.

METHODS: Eighty male Wistar rats were randomly divided into normal control group (group A), CCl₄-induced hepatic fibrosis group (group B), non-DSHX-treated group (group C), low dose-treated group (group D), and high dose-treated group (group E). Fibrous liver models in rats were induced by subcutaneous injection of CCl₄, oral administration of alcohol and high-lipid/low-protein diet for 8 wk. After the models were established, the rats in groups D and E were orally given a low dose (0.5 g/kg) and a high dose (1.0 g/kg) of DSHX daily for 8 wk, respectively. Then, the liver indexes, serum hyaluronic acid (HA) and alanine aminotransferase (ALT) were examined. The degree of hepatic fibrosis was evaluated by optical microscopy. Hydroxyproline (Hyp) in the urine was determined, and the expression of MMP-1 and TIMP-1 was detected by immunohistochemical techniques.

RESULTS: In groups D and E, the liver indexes, levels of serum HA and ALT reduced and development of hepatic fibrosis weakened significantly. The urinary Hyp and expression of MMP-1 in the liver tissues elevated, but the expression of TIMP-1 decreased obviously, as compared to groups B and C.

INTRODUCTION

Hepatic fibrosis is a common result of any chronic injury to the liver. It is characterized by excessive deposition of extracellular matrix (ECM), especially collagens I and III^[1-4]. Matrix metalloproteinase (MMP), is a member of zinc-dependent endopeptidase family, that degrades various components of ECM. In liver tissues, 8 members of MMP have been discovered^[5]. One of them is matrix metalloproteinase-1 (MMP-1) with its specific inhibitor, tissue inhibitor of metalloproteinase-1 (TIMP-1) closely correlated with liver fibrosis due to the accumulation and degradation balance of collagens I and III^[6-8]. In this study, we examined the therapeutic effect of Danshao Huaxian (DSHX) capsule on CCl₄-induced hepatic fibrosis in rats and explored its mechanism.

MATERIALS AND METHODS

Experimental animals

Eighty male Wistar rats weighing 180-220 g were provided by the Experimental Animal Center of Guiyang Medical College. The rats were randomly divided into five groups: normal control group (group A), CCl₄-induced hepatic fibrosis group (group B), non-DSHX-treated group (group C), low dose DSHX-treated group (group D), and high dose DSHX-treated group (group E). Each group consisted of 16 rats.

Chemicals and reagents

DSHX capsule containing five Chinese herbal medicines (Tetrandrine, *Radix Salviae Miltiorrhizae*, *Radix Paeoniae Rubra*,

Astragalus Membranaceus and Ginkgo leaf) was supplied by Guiyang Pharmaceutical Company. Carbon tetrachloride (CCl₄) was obtained from Chongqing Inorganic Chemistry Factory and cholesterol was from Beijing Chemical Reagent Company. Immunohistochemical kits for MMP-1 and TIMP-1, hyaluronic acid (HA) and hydroxyproline (Hyp) kits were purchased from Wuhan Boster Biological Engineering Ltd Co., Beijing Northern Biological Technical Research Institute and Nanjing Jiancheng Biological Engineering Research Institute, respectively.

Instruments

Automatic biochemical analytic instrument (Hitachi 7170A) and Olympus BX41 Microimage Collecting System were employed in the study.

CCl₄-induced liver fibrosis and sample collection

Liver fibrosis was induced in four groups by a complex method^[9]. The rats in groups B, C, D, and E received subcutaneous injections of 40% CCl₄ solution (a mixture of pure CCl₄ and peanut oil) and 0.3 mL/100 g, twice a week for 8 wk (the first dose was pure CCl₄ 0.5 mL/100 g). Meanwhile, they were fed with high-lipid/low-protein diet (79.5% corn farina, 20% fat, and 0.5% cholesterol) daily, and orally supplemented with 30% alcohol every other day. Rats in group A were fed only with normal diet. After liver fibrosis was produced, the rats in group B (by this time, only 12 rats survived) were killed and their blood and livers were collected. The wet livers were weighed and liver samples were fixed in 40 g/L formaldehyde. The serum was centrifuged at 1 500 r/min for 5 min and stored at -80 °C. DSHX capsules were then given to the rats in group D (0.5 g/kg, p.o., daily) and group E (1.0 g/kg, p.o., daily) for 8 wk. The rats in group C were given normal saline instead of DSHX. At the end of the experiment (by this time, the rat survivors in group A and groups C-E were 16, 10, 10, and 10, respectively), all the rats were killed, the liver and serum samples were handled in the same way as mentioned above.

Liver index calculation

Liver index was calculated according to the formula (liver weight/body weight)×100%.

Measurements of serum HA and ALT

Concentrations of serum HA and alanine aminotransferase (ALT) were measured by radioimmunoassay or automatic biochemical analytic instrument (Hitachi 7170A).

Histopathological examination

An equal portion of liver from each rat (1 cm×1 cm×0.5 cm) was fixed in 40 g/L formaldehyde for 48 h, embedded in paraffin, and sectioned with a microtome. The 5-μm-thick sections were stained with hematoxylin and eosin for general histopathology examination. Van-Gieson staining was performed for evaluating the severity of liver fibrosis. Liver fibrosis was classified as previously described^[10].

Urinary Hyp determination

The 24 h urine of the rats in each experimental group was collected for determination of urinary Hyp in the 8th wk

and at the end of the experiment, respectively.

Expression of MMP-1 and TIMP-1

The expressions of MMP-1 and TIMP-1 were detected by the streptavidin-biotin complex (SABC) immunohistochemical technique strictly following the directions offered. PBS was used as the negative control to produce MMP-1 and TIMP-1 polyclonal rabbit IgG. Finally, the number of positive cells per field of vision at 400× magnification was counted.

Statistical analysis

Quantitative data were expressed as mean±SD and subjected to one-way analysis of variance, followed by *t* test for multiple comparisons. Ordinal data were analyzed by Radit analysis. *P*<0.05 was considered statistically significant.

RESULTS

Effect of DSHX on liver index, concentration of serum HA and ALT in rats

As shown in Table 1, compared to group A, the liver index and concentrations of serum HA and ALT of rats in group B and group C increased greatly (*P*<0.05). After 8 wk of treatment with DSHX, the liver index and concentration of serum HA and ALT in groups D and E, reduced obviously compared to those in groups B and C (*P*<0.05).

Table 1 Liver index, serum HA, and ALT in rats of each group (mean±SD)

Group	<i>n</i>	Liver index (relative liver weight)	HA (ng/mL)	ALT (U/L)
A	16	0.0249±0.0027	192.52±41.97	32.40±2.30
B	12	0.0423±0.0044 ^b	316.17±78.48 ^a	174.50±6.02 ^b
C	10	0.0295±0.0019 ^{b,d}	300.86±72.73 ^a	104.75±6.54 ^{b,d}
D	10	0.0268±0.0028 ^{d,e}	224.92±36.62 ^{c,e}	96.13±4.94 ^{d,e}
E	10	0.0267±0.0017 ^{d,f}	200.78±31.71 ^{c,e}	93.13±5.79 ^{d,f}

^a*P*<0.05, ^b*P*<0.01 vs normal control group; ^c*P*<0.05, ^d*P*<0.01 vs hepatic fibrosis group; ^e*P*<0.05, ^f*P*<0.01 vs non-DSHX-treated group.

Effect of DSHX on degree of hepatic fibrosis

After HE and V-G staining, hepatocytes of the normal control rats were arrayed radially along the central vein and there were no collagen fibers. The lobular structure of the liver was destroyed and the hepatic cords were disordered in the rats of group B. Also, the fibrous connective tissues containing numerous inflammatory cells regenerated in the portal area. Meanwhile, collagen fibers expanded into the hepatic parenchyma and there appeared fibrous septa surrounding and separating the normal lobules. The degree of hepatic fibrosis in this group was significantly serious compared to that in group A (*P*<0.01). The hepatic fibrosis in group C was alleviated and the fibrous septa were thinner than those in group B. Except for the obvious expansion of collagen fibers, pseudo lobules were also found in some severe samples from group C. The lobular structure in groups D and E was ameliorated significantly, and regeneration of fibrous connective tissues and septa reduced as compared to that in groups B and C. The degree of the hepatic fibrosis in each group is shown in Table 2.

Table 2 Degree of hepatic fibrosis in rats of each group

Group	n	Degree of hepatic fibrosis						Average
		0	I	II	III	IV	V	
A	16	16	0	0	0	0	0	0
B	12	0	0	0	1	7	4	4.25 ^d
C	10	0	1	4	3	2	0	2.60 ^{df}
D	10	1	3	5	1	0	0	1.60 ^{af}
E	10	1	6	2	1	0	0	1.30 ^{bf}

^a*P*<0.05 and ^b*P*<0.01 *vs* non-DSHX-treated group; ^d*P*<0.01 *vs* normal control group; ^f*P*<0.01 *vs* hepatic fibrosis group.

Effect of DSHX on urinary excretion of Hyp

Urinary Hyp is an useful index to evaluate the degradation of hepatic collagen. Compared to group A, the Hyp excretion increased in groups B and C. After being treated with DSHX, Hyp in the urine of groups D and E was more than that in groups B and C, suggesting that DSHX capsule could facilitate degradation of collagen (Table 3).

Table 3 Urinary excretion of Hyp (μg/24 h) in rats of each group (mean±SD)

Group	n	Urinary excretion of Hyp
A	16	47.80±5.76
B	12	62.00±6.40 ^b
C	10	182.44±30.83 ^b
D	10	242.76±49.76 ^d
E	10	541.09±73.39 ^{df}

^b*P*<0.01 *vs* normal control group; ^d*P*<0.01 *vs* hepatic fibrosis group; ^f*P*<0.01 *vs* non-DSHX-treated group.

Effect of DSHX on expression of MMP-1 and TIMP-1

Immunohistochemical (SABC) study showed that there were only a small number of cells with positive light yellow staining of MMP-1 and TIMP-1 in the normal livers. MMP-1 was observed only in some hepatocytes and stromal cells, whereas TIMP-1 was mainly distributed in vascular endothelial cells and some fibroblasts in the portal area. In the livers of groups B, C, D, and E, the cells with MMP-1 and TIMP-1 positive staining were detected in the fibrous septa and stained dark yellow. The expression of MMP-1 and TIMP-1 in group B was higher than that in group A (*P*<0.01). Compared to groups B and C, the expression of MMP-1 in groups D and E increased significantly, whereas that of TIMP-1 decreased sharply. The results are shown in Table 4.

Table 4 Percentage of expression of MMP-1 and TIMP-1 in each group (mean±SD)

Group	n	MMP-1 (%)	TIMP-1 (%)
A	16	0.595±0.87	1.08±0.68
B	12	5.956±1.85 ^f	9.04±1.60 ^f
C	10	5.104±2.20 ^f	6.46±1.77 ^{bf}
D	10	8.271±3.29 ^{ad}	5.07±0.69 ^{bc}
E	10	8.518±1.88 ^{ad}	4.32±0.63 ^{bd}

^a*P*<0.05 and ^b*P*<0.01 *vs* the hepatic fibrosis group; ^c*P*<0.05 and ^d*P*<0.01 *vs* non-DSHX-treated group; ^f*P*<0.01 *vs* normal control group.

DISCUSSION

DSHX capsule is a mixed preparation, composed of five traditional Chinese herbal medicines. We previously reported that DSHX is effective in prevention of hepatic fibrosis^[11]. In this study, we found that after DSHX treatment of rats with liver fibrosis for 8 wk, the relative liver weight and concentration of serum HA and ALT significantly reduced and liver fibrosis was alleviated, suggesting that DSHX possesses a therapeutic effect on CCl₄-induced hepatic fibrosis in rats by attenuating liver inflammation, preventing necrosis of hepatocytes and promoting their generation.

Hepatic fibrosis is a common consequence of enhanced ECM synthesis and weakened breakdown of proteins in the connective tissue, which lead to increased deposition of ECM in the extracellular matrix. The main component of ECM in normal liver is collagen, which is divided into types I, III-VI. Except for these collagen fibers, there are many other non-collagen components, including fibronectin, laminin, tenascin, and entactin^[12]. In fibrous liver, overaccumulated ECM is mainly interstitial collagens (types I and III). Activated hepatic stellate cells (HSCs) are the main source of ECM^[13-17] during liver fibrosis. HSCs in the space of Disse are in a quiescent form without fibrogenic activity, in part because they are in contact with a complex ECM composed of collagen type IV, laminin, and proteoglycans^[18]. When separated from these factors, they are activated into a pro-fibrogenic myofibroblastic phenotype. The expression of MMP-2 increases obviously after liver injury, which may result in excess degradation of type IV collagen and disorder of the microenvironment of the space of Disse, and then HSCs become activated. Meanwhile, Kupffer cells generate cytokines in response to liver injury, in which HSCs are activated and ECM is overexpressed. Normally, deposition of matrix components in liver is well controlled through constant remodeling by matrix-degrading enzymes. MMP is the most important among ECM-degrading enzymes. MMP, a zinc-dependent endopeptidase, can degrade specific components of ECM and its biological activity can be suppressed by TIMPs^[19]. MMPs are released by a variety of cells (macrophages, neutrophils, endothelial cells, etc.) and participate in such physiological and pathological processes as ECM degradation, tissue remodeling, angiogenesis, and tumor invasion^[20-22].

Till now, 26 members of the family of MMPs have been identified^[22] and with the following characteristics^[23-25]. (1) Structurally, there is a zinc atom at the active site; (2) they are often in an inactivated form, when produced; (3) their primary structures contain two highly conserved sequences, a N-terminal peptide domain and a catalytic domain; and (4) they can be inhibited by specific inhibitors known as TIMPs. The activities of MMPs are regulated at the transcriptional level, through zymogen activation and suppression by a family of inhibitory proteins, TIMPs. Four members of the TIMP family have been identified so far, including TIMPs 1-4, which are named in the order of their discovery. Their sequences possess a highly conserved secondary structure interacting with proteins via six conserved disulfide bonds. It seems that there are two domains in the TIMP molecule, a N-terminal domain that is endowed with inhibition of metalloproteinase and a C-terminal domain

that may be important in protein location or combination with progelatinases^[26]. TIMPs act widely and their most important action is to inhibit the activity of MMPs. TIMPs can interact with MMPs to inhibit their activities at 1:1 stoichiometry^[27-29]. MMP-1, a member of the MMP family that specifically degrades native collagen types III and I, plays an important role in the accumulation and degradation balance of ECM. The level of MMP-1 increases transiently in the early stage of liver fibrosis, but becomes undetectable in the stage of cirrhosis^[30]. In contrast, the level of TIMP-1, a specific tissue inhibitor of MMP-1, elevates consistently in the fibrosis process and reaches its peak in the stage of cirrhosis. Upregulation of TIMP-1 and downregulation of MMP-1 result in the inhibition of degradation of collagen types I and III, which leads to overexpression and deposition of ECM in the extracellular matrix.

In addition to inhibiting the degradation of matrix, TIMP-1 plays a significant role in regulation of the apoptosis of such cells such as B lymphocytes, breast epithelial cells and HSCs. TIMP-1 inhibits the apoptosis of HSCs, indicating another possible mechanism whereby it is implicated in the pathogenesis of liver fibrosis. In this study, we found that MMP-1 in the two groups treated with DSHX was overexpressed, whereas the expression of TIMP-1 reduced significantly. We conclude that DSHX can reverse the process of liver fibrosis by upregulating the gene expression and generation of MMP-1 and downregulating the TIMP-1 expression. These results may provide a therapeutic strategy to combat hepatic fibrosis by targeting MMP-1 and TIMP-1.

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