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RAPID COMMUNICATION

Preventive effect of *Qianggan-Rongxian* Decoction on rat liver fibrosis

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Abstract

AIM: To study the preventive effects of *Qianggan-Rongxian* Decoction on liver fibrosis induced by dimethylnitrosamine (DMN) in rats.

METHODS: Male Wistar rats were randomly divided into hepatic fibrosis model group, control group and 3 treatment groups (12 rats in each group). Except for the normal control group, all the rats received 1% DMN (10 μL/kg body weight, i.p), 3 times a week for 4 wk. The rats in the 3 treatment groups including a high-dose DMN group (10 mL/kg), a medium-dose DMN group (7 mL/kg), and a low-dose DMN group (4 mL/kg) were daily gavaged with *Qianggan-Rongxian* Decoction, and the rats in the model and normal control groups were given saline vehicle. Enzyme-linked immunosorbent assay (ELISA) was used to determine the changes in serum hyaluronic acid (HA), laminin (LN), and type Ⅳ collagen levels. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using routine laboratory methods. Pathologic changes, particularly fibrosis, were examined by hematoxylin and eosin (HE) and Sirius red staining. Hepatic stellate cells (HSC) were examined by transmission electron microscopy.

RESULTS: Compared with the model control group, the serum levels of HA, LN, type Ⅳ collagen, ALT and AST were decreased markedly in the other groups after treatment with *Qianggan-Rongxian* Decoction, especially in the medium-dose DMN group ($P < 0.05$). Moreover, the area-density percentage of collagen fibrosis was lower in the *Qianggan-Rongxian* Decoction treatment groups than in the model group, and a more significant drop was observed in the medium-dose DMN group ($P < 0.05$).

CONCLUSION: *Qianggan-Rongxian* Decoction can inhibit hepatic fibrosis due to chronic liver injury, delay the development of cirrhosis, and notably ameliorate liver function. It may be used as a safe and effective thera-peutic drug for patients with fibrosis.

Key words: Liver fibrosis; *Qianggan-Rongxian* Decoction; Prevention; Rat model; Dimethylnitrosamine

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INTRODUCTION

In China, the incidence of liver cirrhosis is still high[1]. Hepatic cirrhosis results from fibrosis[2-4]. Many factors can lead to chronic liver disease and hepatic fibrosis[5-9]. Hepatic fibrosis is associated with a number of morphological and biochemical changes leading to structural and metabolic abnormalities in the liver. Hepatic stellate cells (HSC) play a major role in various types of liver fibrosis through initial myofibroblast transformation. Although new therapeutic approaches have recently been proposed, there is no established therapy for liver fibrosis[10,11]. *Qianggan-Rongxian* Decoction is a traditional Chinese medicine. The aim of the present study was to investigate its protective effects on rat liver fibrosis induced by dimethylnitrosamine (DMN).

MATERIALS AND METHODS

Composition of Qianggan-Rongxian Decoction

The compositions of *Qianggan-Rongxian* Decoction

mainly include 13 Chinese herbs, including 15 g *Pig Bile powder*, 10 g *Bupleuri*, 10 g *Baical Skullcap Root*, 10 g *Pinellia Tuber*, 10 g *Chinese Angelica*, 10 g *Barbary Wolfberry Fruit*, 10 g *Nutgrass Galingale*, 10 g *Oriental Waterplantain Rhizome*, 3 g Pangolin Scale, 153 g *Danshen Root*, 10 g *White Peony Alba*, 10 g *Radix Glycyrrhizae* ,15 g *Tangshen*.

Animals and experiment protocol

Male Wistar rats weighing 175-200 g were obtained from the Experimental Animal Center of Chengde Medical College. The rats were randomly divided into control group, model group, and 3 treatment groups (12 rats in each group). Except for the normal control group, all the rats were abdominally injected with 1% DMN (10 μL/kg body weight, i.p.), 3 times a week for 4 wk, while the rats in the control group were abdominally injected with an equivalent amount of saline. The rats in the 3 treatment groups, including a high-dose DMN group (10 mL/kg), a medium-dose DMN group (7 mL/kg), and a low-dose DMN group (4 mL/kg), were given *Qianggan-Rongxian* Decoction daily *via* a gastric tube, once a day for 4 wk. After 4 wk, except for the dead, all the rats were anesthetized with 200 g/L urethane (5 mL/kg, abdominal injection). Blood was taken from the abdominal aorta, centrifuged at 4℃, and plasma was kept at -20℃ for assay.

Measurement of serum levels of hyaluronic acid, type Ⅳ collagen and laminin

Quantitative enzyme-linked immunoabsorbent assay (ELISA) was used to determine serum levels of hyaluronic acid (HA), type Ⅳ collagen, and laminin (LN).

Measurement of plasma levels of alanine aminotransferase and aspartate aminotrans-ferase

Plasma levels of alanine aminotransferase (ALT) and aspartate aminotrans-ferase (AST) were measured using routine laboratory methods.

Sirius-red and HE staining

Formalin-fixed and paraffin-embedded liver tissues were cut into 4-μm thick sections which were stained with hematoxylin and eosin (HE) and Siriusred. HE staining was used to observe liver pathologic structures, Siriusred staining and CMIAS image analysis system (Beihang, China) were used to determine the area-density percentage of collagen fibrosis in hepatic tissue. At least five high-power (× 400) fields were chosen and positive collagen fibrosis (red staining) was determined. The area-density percentage of collagen fibrosis was calculated by dividing the number of positive collagen fibroses (positive optical density) over the total number of collagen fibroses (integrated optical density).

Electron microscopy

Fresh liver tissue sections (1 mm × 1 mm × 1 mm) were fixed in 10% paraform fixative, dehydrated and embedded in Epon-812 resin, and then stained with uranyl acetate and lead citrate for 15 min, respectively. Liver "transitional" HSC were observed under JEM-1200EX, 80 kV electron microscope (JEOL, Japan).

Table 1 Serum levels of HA, LN, and type Ⅳ collagen (mean ± SD)

Groups	*n*	HA (ng/mL)	LN (ng/mL)	Type *Ⅳ* collagen (ng/mL)
Control	12	19.81 ± 2.86	11.02 ± 1.70	13.49 ± 2.49
Model	10	44.64 ± 3.09[c]	33.27 ± 5.81[c]	62.71 ± 19.16[c]
High-dose DMN group	10	23.14 ± 4.58[a,c]	14.02 ± 2.63[a,c]	22.10 ± 2.44[a,c]
Medium-dose DMN group	12	22.58 ± 3.60[a]	13.87 ± 1.45[a]	25.64 ± 4.68[a,c]
Low-dose DMN group	10	26.08 ± 5.62[a,c]	19.12 ± 5.02[a,c]	27.64 ± 4.68[a,c]

[a]$P < 0.05$ *vs* model group; [c]$P < 0.05$ *vs* control group.

RESULTS

Changes in serum HA, LN levels and type *Ⅳ* collagen levels

The serum levels of HA, LN, and type Ⅳ collagen were markedly increased in the model group compared with the control group ($P < 0.05$). Compared with the model group, the serum levels of HA, LN, and type Ⅳ collagen were significantly decreased in the 3 treatment groups ($P < 0.05$) (Table 1).

Siriusred and HE staining

At the end of the study, the liver of control rats had no appreciable alterations in the model group (Figure 1A), more fibrous tissues formed and extended into the hepatic lobules to separate them incompletely and thick intralobular septa were evident (Figure 1B). While in the 3 treatment groups, especially in the medium-dose DMN group, the pathological changes in liver were rather milder, showing less fibrous tissue proliferation (Figure 1C). The rat liver was stained with Siriusred. The area occupied by the fibrotic septa was markedly increased in the model group compared with the control group ($P < 0.05$). Compared with the model group, the area occupied by the fibrotic septa was significantly decreased in the 3 treatment groups ($P < 0.05$) (Table 2).

Plasma levels of ALT and AST

Plasma levels of ALT and AST were higher in the model group than in the control group ($P < 0.05$), while ALT and AST levels were significantly lower in the *Qianggan-Rongxian* Decoction treatment groups than in the model group. No difference was found in the serum levels of ALT and AST between the medium-dose DMN group and normal group (Table 3).

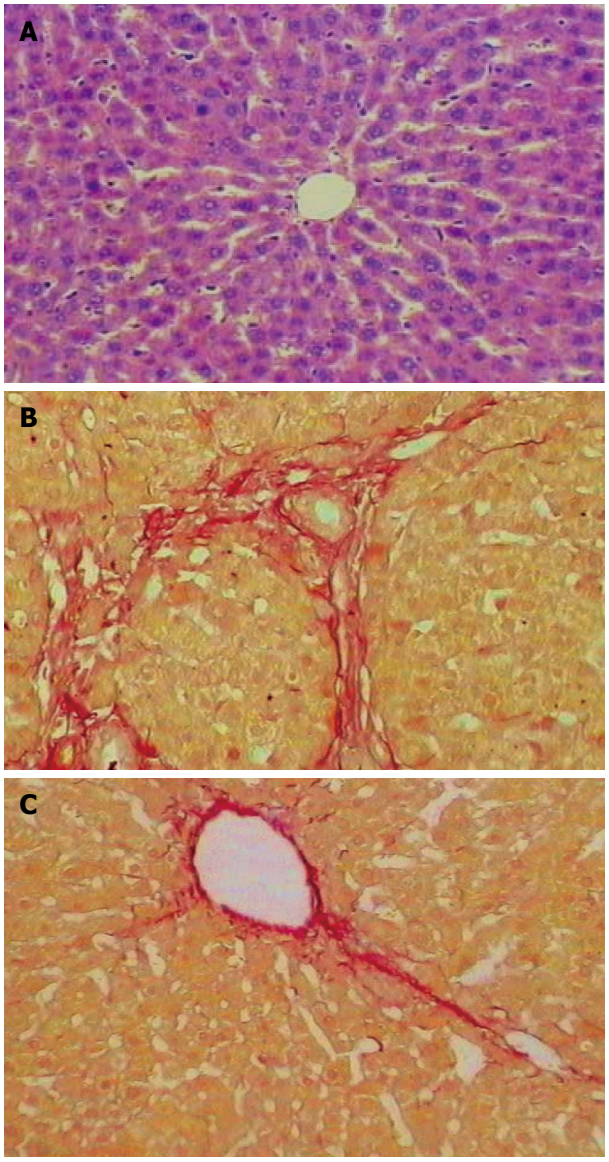

Figure 1 Light microscopy showing normal liver tissue in the control group (HE staining, × 100) (**A**), liver fibrosis tissue and formation of more fibrous tissue as well as a large amount of inflammatory cells soaked in intralobules and interlobules in model group (van Gieson staining, × 100) (**B**), and liver fibrosis tissue in *Qianggan-Rongxian* Decoction treatment group (**C**). The pathological change in liver was rather milder compared with the model group (van Gieson staining, × 100).

Ultrastructure observation under electron microscope

HSC were normal in the control group and typical myofibroblasts were observed in the fibrous septum of the model group (Figure 2A). The elongated cell body contained indented nuclei and numerous microfilaments outlined by a lamina-like structure. Collagen fibers of variable size were seen all around the myofibroblasts (Figure 2B). "Transitional" HSC could be observed under the electron microscope (Figure 2C).

DISCUSSION

Hepatic fibrosis at the intermediate and crucial stage is characterized by reversibility. If treated properly at this stage, cirrhosis could be successfully prevented. However, it remains a problem to prevent cirrhosis or

Table 2 Area occupied by the fibrotic septa and its ratio to the total area examined (mean ± SD)

Groups	*n*	Area covered by fibrotic septa (μm^2)	Ratio (%)
Control	12	10.33 ± 6.89	0.12 ± 0.08
Model	10	221.23 ± 51.21[c]	2.00 ± 0.20[c]
High-dose DMN group	10	59.32 ± 10.41[a,c]	1.60 ± 0.24[a,c]
Medium-dose DMN group	12	21.73 ± 15.42[a,c]	1.06 ± 0.13[a,c]
Low-dose DMN group	10	61.73 ± 15.42[a,c]	1.68 ± 0.14[a,c]

[a]$P < 0.05$ *vs* model group; [c]$P < 0.05$ *vs* control group.

Table 3 Serum levels of ALT and AST (mean ± SD)

Groups	*n*	Area covered by fibrotic septa (μm^2)	Ratio (%)
Control	12	63.0 ± 11.9	307 ± 23
Model	10	1931 ± 552[c]	2696 ± 764[c]
High-dose DMN group	10	960 ± 557[a,c]	1560 ± 965[a,c]
Medium-dose DMN group	12	739 ± 345[a,c]	1239 ± 725[a,c]
Low-dose DMN group	10	983 ± 460[a,c]	1631 ± 859[a,c]

[a]$P < 0.05$ *vs* model group; [c]$P < 0.05$ *vs* control group.

to control its progression. Great efforts have been made to find safe and effective drugs. Recent clinical and experimental observations demonstrated that Chinese medicines have some preventive and therapeutic values against fibrosis[12-14]. *Astragalus*, one of the compositions of *Qianggan-Rongxian* Decoction, can relieve stasis by activating blood circulation, and eliminate fullness by strengthening the "spleen", supplementing and smoothening "Qi", reinforcing the body's immunological function. It also could preserve the integrity of hepatocytes, eliminate toxic free radicals, inhibit lipid peroxidation of cytomembrane, relieve necrosis of hepatocytes, and reduce fibrosis[15-19]. Thoroughfare is mainly used to activate blood circulation, remove stasis, and dredge the liver[20]. *Qianggan-Rongxian* Decoction has been used for 20 years to prevent liver fibrosis in clinical practice. However, its effect and associated mechanisms need further study. DMN-induced experimental model may be helpful in understanding the relationship between liver injury and development of hepatic fibrosis[21]. As estimated by histological analysis of liver tissue stained with Sirius red[22], it can be used to detect different degrees of hepatic fibrosis, and examination of the liver can reveal a progressive increase in fibrosis scores and expansion of fibrous septa[23]. Serum HA, LN and collagen type Ⅳ levels were significantly increased in the rats as detected by ELISA, showing that simultaneous determination of HA, LN and collagen type Ⅳ levels is an optimal choice[24]. We treated the rat liver fibrosis induced by injection of DMN with *Qianggan-Rongxian* Decoction. After 4 wk, no appreciable alterations were found in the control

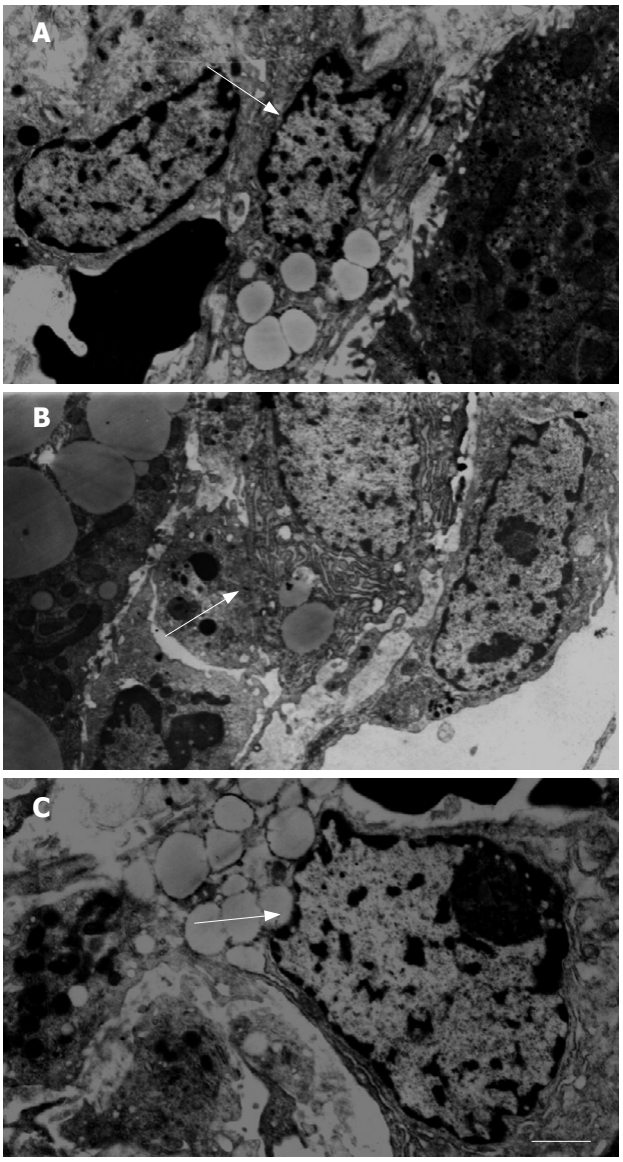

Figure 2 Electron microscopy showing normal HSC in the model group (**A**), typical myofibroblasts in the control group (**B**) (× 5000, bar = 1 μm), and "transitional" HSC (× 6000, bar = 1 μm) in *Qianggan-Rongxian* Decoction treatment group (**C**).

group. However, the rats in the model group had an almost integrity fibrosis septum, and pseudolobules could be seen in nearly all sections. While the rats that received *Qianggan-Rongxian* Decoction had less fibrosis, reticular fibrosis in the interlobular septum was limited and no pseudo-lobules could be seen. HSC play a central role in the pathogenesis of liver fibrosis and are able to regulate matrix degradation in the liver. Following liver injury, HSC become activated and express extracellular matrix. *Qianggan-Rongxian* Decoction can inhibit transition from HSC to myofibroblasts and fibroblasts. In addition, *Qianggan-Rongxian* Decoction could decrease the area-density percentage of collagen fibrosis. HA, LN, and type Ⅳ collagen are good serum markers of hepatic fibrosis. In this study, the serum levels of these 3 markers in the model group were much higher than those in the control group ($P < 0.05$). The serum levels of HA, LN and type Ⅳ collagen were significantly lower in the *Qianggan-Rongxian* Decoction treatment groups than in the control group. ALT and AST are the indexes of liver functions. Since ALT in cytoplasm of liver cells is discharged into blood when degeneration, hyper permeability and necrosis of liver cells occur, increased serum ALT levels reflect the degree of liver cell injury. Our study showed that *Qianggan-Rongxian* Decoction could decrease the serum levels of ALT and AST in rats with hepatic injury induced by DMN, indicating that *Qianggan-Rongxian* Decoction may work by protecting liver against fibrosis. The mechanism underlying rat liver fibrosis induced by DMN is associated with immune function, which is similar to the mechanism underlying human liver fibrosis[25]. Thus, DMN-induced rat liver fibrosis may be a useful model for determination of liver fibrosis during drug screening. The mechanism of *Qianggan-Rongxian* Decoction may need further study.

In summary, *Qianggan-Rongxian* Decoction may play a role in anti-fibrotic therapy by protecting liver cells and inhibiting the deposition of collagen fibers in liver, thus providing a safe and effective strategy for inhibition of cirrhosis in clinic practice.

COMMENTS

Background

In China, the incidence of liver cirrhosis is still high, liver fibrosis and cirrhosis are due to chronic liver injury. Although new therapeutic approaches have recently been proposed, there is no established therapy for liver fibrosis. The authors investigated the preventive effect of *Qianggan-Rongxian* Decoction on rat liver fibrosis induced by dimethylnitrosamine (DMN).

Research frontiers

Qianggan-Rongxian Decoction can protect the liver against fibrosis induced by DMN in rats.

Innovations and breakthroughs

Enzyme-linked immunosorbent assay (ELISA) and hematoxylin and eosin as well as Sirius red staining and transmission electron microscopy demonstrated that *Qianggan-Rongxian* Decoction could prevent liver against fibrosis.

Applications

ELISA can determine the changes in serum levels of hyaluronic acid (HA), laminin (LA), and type Ⅳ collagen. Pathologic changes, particularly fibrosis can be examined by light microscopy. Hepatic stellate cells (HSC) can be examined by transmission electron microscopy

Terminology

Qianggan-Rongxian Decoction is a kind of Chinese medicine, which may protect the liver against fibrosis.

Peer review

This is an interesting article. *Qianggan-Rongxian* Decoction can protect the liver against fibrosis and inhibit the deposition of collagen fibers in liver, thus providing a safe and effective strategy for inhibition of cirrhosis in clinical practice. The paper is well organized and the results are clearly described and commented.

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