Efficacy of Chinese medicine *Yi-gan-kang* granule in prophylaxis and treatment of liver fibrosis in rats

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Abstract

**AIM:** To investigate the efficacy of a Chinese medicine, *Yi-gan-kang* granule (granules for benefiting the liver), in prophylaxis and treatment of liver fibrosis in rats and its possible mechanism.

**METHODS:** One hundred and forty Sprague-Dawley rats were randomly divided into seven groups (20 each): group 1, blank control group without any interference during the study; group 2, CCl₄-induced liver fibrosis group; group 3, pig serum-induced liver fibrosis group; group 4, prophylaxis group of CCl₄-induced liver fibrosis by *Yi-gan-kang*; group 5, prophylaxis group of pig serum-induced liver fibrosis by *Yi-gan-kang*; group 6, treatment group of CCl₄-induced liver fibrosis by *Yi-gan-kang*; group 7, treatment group of CCl₄-induced liver fibrosis by *Yi-gan-kang*. At wk 6, 10, 14 and 20 (baseline for CCl₄ or pig serum induction), five rats in each group were anesthetized and their livers were removed for pathological studies including immunohistochemical studies for α-SMA, type I collagen and in situ hybridization of tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA of hepatic stellate cells (HSCs). Anti-lipid peroxidation in isolated mitochondria, cell proliferation and apoptosis in cultured HSCs were also studied.

**RESULTS:** The mean number of pseudolobuli at wk 10, 14 and 20 in the prophylaxis group was significantly less than that in the control group (P<0.05 or P<0.01). The effect of prophylaxis at wk 14 in CCl₄ rats and at wk 10 in pig serum-induced rats was much better than that of treatment group (P<0.01). The thickness (in μm) of fibers both in pig serum-induced prophylaxis and in treatment groups at wk 14 and 20 was significantly less than that in control group (P<0.05). The number of fibers both in prophylaxis and in treatment groups from wk 10 or 14 to 20 was significantly less than that in control group (P<0.05 or P<0.01). The tissue HSC positive rates of type I collagen, α-SMA and TIMP-1 mRNA, which represented the active phenotype of HSCs in tissues, remained very high from wk 6 to the end of model making in control group. While in prophylaxis group, they were at a relatively low level. In treatment group, there was a gradual decreasing trend. Time- and dose-dependent effects of anti-lipid peroxidation on isolated mitochondria, cell proliferation and apoptosis in cultured HSCs were also observed during the study.

**CONCLUSION:** *Yi-gan-kang* can effectively inhibit or inverse the course of liver fibrogenesis in CCl₄- and pig serum-induced rat models.

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Key words: Liver fibrosis; *Yi-gan-kang* granule; Prophylaxis and treatment; Rat model


INTRODUCTION

Chronic liver diseases mainly caused by hepatitis B and C viruses are very common in China and can lead to liver fibrosis[1-3]. Prophylaxis and reversion of liver fibrosis are one of the key steps in the prevention and treatment of chronic liver diseases. *Yi-gan* infusion is a recipe of traditional Chinese medicine designed by professor Xi-Xian Yao, who is a famous expert of gastroenterology in China. The medicine is commonly used in clinics because of its fewer side-effects and excellent clinical efficacies[4,5]. *Yi-gan-kang* is a new generation of *Yi-gan* infusion for therapy of liver fibrosis[6]. In order to explore its antifibrotic mechanism, rat fibrotic models induced by carbon tetrachloride and pig serum were used to mimic the liver fibrosis caused by human viral hepatitis[7].

**MATERIALS AND METHODS**

**Composition of Yi-gan-kang**

The compositions of *Yi-gan-kang* mainly include nine Chinese herbs, such as *Radix Salviae Miltiorrhizae*, *Radix Angelicae Sinensis*, *Radix Paeoniae Rubra*, etc.
Animal models

One hundred and forty Sprague-Dawley male rats, weighting 180-220 g, were randomly divided into seven groups (20 each): group 1, blank control group without any interference during the study; group 2, CCl_4 model control group; group 3, pig serum-induced liver fibrosis group; group 4, prophylaxis group of CCl_4-induced liver fibrosis by Yi-gan-kang; group 5, prophylaxis group of pig serum-induced liver fibrosis by Yi-gan-kang; group 6, treatment group of CCl_4-induced liver fibrosis by Yi-gan-kang; group 7, treatment group of CCl_4-induced liver fibrosis by Yi-gan-kang. Rats in groups 2, 4 and 6 were given 400 mL/L CCl_4 peanut oil solution at the dose of 2 mL/kg (subcutaneous injection), twice weekly for 10 wk. Rats in groups 3, 5 and 7 were given pig serum (from fresh mix blood of adult pigs without condensation or dilution) at the dose of 2 mL/kg (intraperitoneal injection), twice weekly for 10 wk. Rats in groups 4 and 5 were fed with forage containing 10% of Yi-gan-kang, 4 wk prior to the beginning of model making, while rats in groups 6 and 7 were fed with forage containing 10% of Yi-gan-kang, 4 wk before the end of model making. The duration of feeding forage containing 10% of Yi-gan-kang was 14 wk both in prophylaxis and in treatment groups. All rats had free access to sterile water at room temperature of 25 °C in a 12-h light and dark cycle. At wk 6, 10, 14 and 20, five rats in each group were anesthetized and their livers were harvested.

Extraction of water-soluble compositions in Yi-gan-kang

Yi-gan-kang granules were dissolved in water and filtered, the filtrates were combined and centrifuged at 4 000 r/min for 15 min. The supernatant was mixed with 95% ethanol, stored at 4 °C overnight, then dried on a rotatory evaporator, twice weekly for 10 wk. Rats in groups 2, 4 and 6 were given 400 mL/L CCl_4 peanut oil solution at the dose of 2 mL/kg, (subcutaneous injection), twice weekly for 10 wk. Rats in groups 3, 5 and 7 were given pig serum (from fresh mix blood of adult pigs without condensation or dilution) at the dose of 2 mL/kg (intraperitoneal injection), twice weekly for 10 wk. Rats in groups 4 and 5 were fed with forage containing 10% of Yi-gan-kang, 4 wk prior to the beginning of model making, while rats in groups 6 and 7 were fed with forage containing 10% of Yi-gan-kang, 4 wk before the end of model making. The duration of feeding forage containing 10% of Yi-gan-kang was 14 wk both in prophylaxis and in treatment groups. All rats had free access to sterile water at room temperature of 25 °C in a 12-h light and dark cycle. At wk 6, 10, 14 and 20, five rats in each group were anesthetized and their livers were harvested.

Tissue and specimen

For liver specimens, one piece of liver tissue was fixed in 10% neutral formaldehyde and embedded in paraffin for histological section, while another piece was immediately stored in liquid nitrogen for frozen section. The paraffin-embedded liver tissue was sectioned routinely for HE and stored in liquid nitrogen for frozen section. The paraffin-embedded liver tissue was sectioned for HE and Masson staining.

Semi-quantitative score of cell degeneration and collagen proliferation

Cell degeneration was scored as follows: 0, no hepatocyte degeneration; 1, the number of degenerated hepatocytes being less than 1/4 of the total cells; 2, the number of degenerated hepatocytes being 1/4 or 1/2 of the total cells; 3, the number of degenerated hepatocytes being 1/2 or 3/4 of the total cells; 4, the number of degenerated hepatocytes being not less than 3/4 of the total cells.

Collagen proliferation was scored as follows: 0, no obvious collagen; 1, slight but no septation; 2, moderate with incomplete septation; 3, obvious with thin complete septation; 4, severe with thick complete septation.

Immunohistochemical analysis of α-smooth muscle actin and type I collagen

The specimens embedded in paraffin were sectioned and placed on polylysine-coated glass slides and dehydrated at 60 °C for 8 h in alcohol, then transferred into 3% H_2O_2 for 30 min and digested in digest solution for 10 min. After the unspecified binding sites were blocked with normal goat serum for 2 h, α-SMA monoclonal antibody or rabbit anti-collagen type I polyclonal antibody (Boster Biotech Co. Ltd) was applied to the sections at 4 °C overnight. After being washed with PBS, the sections were incubated with IgG/biotin at 37 °C for 15 min, washed, and further incubated with avidin/HRP at 37 °C for another 15 min. After being rinsed thrice, tissue sections were stained with DAB for 10 min, and the positive cells were visualized under microscope.

Detection of TIMP-1 mRNA by in situ hybridization

In order to observe the mRNA expression of tissue inhibitor of metalloproteinase-1 (TIMP-1), another very important parameter reflecting the degree of liver fibrosis, in situ hybridization of TIMP-1 mRNA was carried out. Briefly, frozen sections on polylysine-coated glass slides were fixed in 3% polyformaldehyde containing 0.1% DEPC at room temperature for 30 min. After being rinsed thrice in water and dried, the sections were stored at -20 °C for several days. Then, the sections were immersed in 0.5% H_2O_2 in methanol for 30 min. After being rinsed thrice, the sections were digested by pepsin at 37 °C for 60 s. After being rinsed thrice in PBS and once in water, the sections were transferred into a moister chamber containing 20 mL of 20% glycerin for prehybridization at 37 °C for 4 h, then 20 μL specific probe solution (Boster Biotech Co. Ltd) was applied to the sections, the sections were covered with special coverslips for in situ hybridization and kept at 42 °C overnight. The probe sequences of the products were as follows: 5′-ACAGC AACAA CAGGA TGCCA GAAGC CAGGG-3′ and 5′-TCTCC ATGCC TGAGG GTGAG ACGAA CCGGA-3′. After removal of the coverslips, the sections were rinsed twice in 2× SSC each for 5 min, once in 0.5× SSC for 15 min, and twice in 0.2× each for 10 min, and blocked by normal goat serum for 60 min. Then after being washed four times with PBS, the sections were incubated with streptavidin-biotin complex at 37 °C for 20 min. After being stained with DAB, restained with hematoxylin, and washed with water, positive cells in the sections could be visualized under optical microscope.

Isolation of liver mitochondria

According to Myers et al [8], liver mitochondria were isolated as follows. Briefly, the rats were killed, the abdominal cavity was opened immediately, and the livers were removed. Part of liver tissues was cut into small pieces and put into a 0.25 mol/L ice-cooled sucrose water. The liver tissues were homogenized at medium speed for 30 s and centrifuged at 1 000 r/min for 10 min. Two-thirds of the upper supernatant was obtained and centrifuged at 7 000 r/min for another 10 min. The deposition was dissolved in ice-cold sucrose water, mixed for 10 s, and centrifuged at 18 000 g for 20 min. The condensed deposition was mitochondria.

Lipoperoxidation of mitochondria

Taking malondialdehyde (MDA) and superoxide dismutase
(SOD) as the test parameters, we determined the dose- and time-dependent effect of the extraction of Radix Salviae Miltiorrhizae root, the main component of Yi-gan-kang, on anti-lipid peroxidation of mitochondria. Mitochondria were isolated according to Myers et al. [9]. The MDA and SOD kits were bought from Nanjing Biological Corporation and the experiments were performed according to the manufacturer’s instructions. During the experiments, the extraction was further diluted into different concentrations: 0.5, 1.0, 2.0, 5.0, and 10.0 mg/mL. Mitochondria at the concentration of 1.5 mg/mL, ADP of 1 mmol/L, FeSO4 and Tris-HCl of 10 mmol/L were added to each of the above concentrations with the total volumes of 4 and 1.65 mL respectively. MDA and SOD were determined at 5, 10, 15, 20 and 30 min during the reaction.

HSC culture

HSC lines (established by Professor Greenwel) were provided by Southwest Hospital, Third Military Medical University. The phenotype was activated hepatic stellate cells (HSCs) [9, 10]. Cells were cultured in RPMI1640 (Gibco) medium supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 mg/L streptomycin, and kept in a controlled atmosphere (50 mL/L CO2) incubator at 37 °C. Exponentially growing cells were seeded in plates for 24 h and treated with Yi-gan-kang extraction at various concentrations (144, 72, 18, 9 and 4.5 mg/mL) for 24, 48 and 72 h respectively. The cells not treated with this drug served as control.

Dose-dependent effects of Yi-gan-kang on cell proliferation

Colorimetric MTT assays [11] were used to observe cell proliferation. HSCs were incubated in 96-well plates. The concentration of cells was adjusted to 1×10^4/L. After being cultured for 24 h, Yi-gan-kang extraction was added in different concentrations such as 144, 72, 18, 9 and 4.5 g/L and incubated for 48 h. Triplicate wells of cells were used. After the supernatant was extracted, 10 μL of 0.05% MTT solution was added to all wells and incubated for 4 h followed by incubation with dimethylsulfoxide for coloration. After a few minutes at room temperature to ensure that all crystals were dissolved, the optic density (A) was read on an ELISA reader at test wavelength of 570 nm and reference wavelength of 630 nm.

Apoptosis analysis

In situ cell death detection kits (TUNEL) were purchased from Boehringer Mannheim Company, Germany. Briefly, the cells were adjusted to a density of 2×10^5/cm^2, and added to 24-well plates with coverslips in 0.5 mL each well. After being incubated with 18 mg/mL Yi-gan-kang extraction (Yi-gan-kang at 18 mg/mL had the best inhibitory effect on proliferation of cultured HSCs by MTT analysis) for 48 h, the glass slides were taken out, rinsed, fixed and stained. The negative control with omission of TUNEL enzyme was designed according to the manufacturer’s instructions. The cells stained with dark brown nuclei were considered as positive cells. About 500-1 000 cells were selected randomly and counted in each coverslip under the high magnification (400×) microscope. Apoptosis index was calculated as (apoptotic cells/total cells)×100%. Flow cytometry analysis of HSC apoptosis at 24, 48 and 72 h was also carried out in this study. Briefly, cells were digested by 2.5 mg/mL trypsin, washed with PBS, fixed in cold ethanol at 4 °C and stained with propidium iodide, and then analyzed by flow cytometry. Also, centrifuged cells fixed in glutaraldehyde were observed under transmission electron microscope.

Immunohistochemical image analysis

Computerized image analysis system (East China University) was used to get the mean positive cell rate in immunohistochemical and in situ hybridization sections. Values were determined by the same observer.

Statistical analysis

Using the statistical analysis software, SPSS 10.0, the values of mean positive cell rates were entered into an analysis database. ANOVA Student-Newman-Keuls and Student’s t tests were used to analyze the differences between mean positive cell rates of each group at different time points.

RESULTS

Pathology of Masson staining between groups and time points

Three parameters of pathologic images were used in the comparison between groups and time points. They were the mean number of pseudonodules, the mean number of tissue separating fibers per 200× field, and the mean thickness of the fibers. The results are shown in Figures 1 and 2.

![Figure 1](image1.png)

**Figure 1** Comparison of liver pathological changes in three groups of pig serum-induced model rats at wk 10 (A-C), 14 (D-F), 20 (G-I) (Masson 100×).

Immunohistochemical analysis of α-SMA and type I collagen, and in situ hybridization of TIMP-1 mRNA

The dynamic changes of type I collagen, α-SMA and...
TIMP-1 mRNA were observed in liver tissues of both CCl₄ and PS fibrotic models of rats. These three parameters could represent the active phenotype of HSCs in tissues. In model control group, the positive cell rate of active phenotype was very high from wk 6 to the end of model making. In prophylaxis group, it was relatively low. In treatment group, there was a gradual decreasing trend from high levels (Figures 3 and 4).

**Electron microscope**

Normal hepatocytes were in fusiform shape and relatively big and regular. The nucleus was in oval-round shape and membrane was clear. Typical cytoplasm organelles were observed around the vicinity of nuclei. Lipid droplets were visible in HSCs. In model animals, hepatocytes became enlarged with nuclei shrunken and membrane obscure. Lipid droplets with different sizes were easily found in cytoplasm and nuclei. The number of organelles decreased. More collagen fibers and fibroblasts were observed in Dissi space. Around the area of hepatocyte necrosis was the infiltration of single-nucleus cells and deposition of bulky collagen fibers. In prophylaxis group, mitochondria were normal and endoplasmic reticulum was slightly enlarged. Degeneration of hepatocytes was significantly improved. Only a minimum quantity of collagen fibers was observed in Dissi space. There was no obvious proliferation of HSCs and apoptosis of HSCs was easily seen with chromatin towards nucleus margin (Figure 5).

**Anti-lipoperoxidation effect of Yi-gan-kang**

Radix *Salviae Miltiorrhizae* showed a dose- and time-dependent effect on MDA production and SOD activity in mitochondria reaction (Figure 6).

**Dose- and time-dependent inhibitory effects of Yi-gan-kang on cell proliferation**

The inhibitory effects of *Yi-gan-kang* at concentrations of 144 and 72 g/L were stronger than those at concentrations of 36, 18 and 9 g/L. No obvious inhibitory effect was found at the concentration of 4.5 g/L. At the concentration of 18 g/L, HSCs were significantly inhibited compared to those in control group. The effect was time-dependent, and reached its peak at 72 h ($P<0.01$, Tables 1 and 2).

**Apoptosis of cultured HSCs incubated with Yi-gan-kang**

A dose of 18 mg/mL of *Yi-gan-kang* and a 48-h acting period were selected as the experimental conditions to compare the effect of the herb on HSC lines. The AI was significantly

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**Figure 2** Comparisons of liver pathologic features in three groups of CCl₄-induced model rats at wk 10 (A-C), 14 (D-F), 20 (G-I) (Masson, 100×).
higher than that in control group (22.5±7.1% vs 4.3±1.3%, *P*<0.01) (Tables 3 and 4). Apoptotic cells were characterized by compaction of nuclear chromatin and condensation of cytoplasm. Most chromatin-condensed cells demonstrated evidence of DNA fragmentation and were strongly stained by TUNEL staining (Figure 7).

Small round cells were observed on the surface of the monolayer, which could be displaced by agitation of the tissue culture plate, demonstrating that they were very loosely adhered to the monolayer and some of them were detached floating in the culture supernatant (Figure 8). These findings suggested that HSCs have undergone programmed cell death or apoptosis[12]. Under transmission electron microscope, the apoptotic HSCs were found, i.e., the dilated endoplasmic reticulum, irregular nuclei, chromatin condensation and heterochromatin ranked along inside of nuclear membrane. All these features were the characteristics of apoptosis and could distinguish programmed cell death from necrosis (Figure 9).

Flow cytometry analysis also showed that, after HSCs were treated with *Yi-gan-kang* extraction at different concentrations of 36, 18 and 9 mg/mL for 48 h, the apoptosis rate was significantly higher than that in control group (*P*<0.01) and *Yi-gan-kang* could increase the apoptosis rate in a dose- and time-dependent manner compared to the control group (Figure 10).
Chronic liver diseases are quite common in China. Elucidation of their pathogenesis of hepatic fibrosis may provide the basis of anti-fibrosis therapy. But so far, there is no good therapy for hepatic fibrosis. The key to treatment of chronic liver diseases is to stop and reverse the occurrence and development of hepatic fibrosis. Anti-fibrosis therapies especially those with huo-xue-hua-yu (refreshing blood and eliminating stasis) herbs including Radix Salviae Miltiorrhizae, Radix Angelicae Sinensis, Radix Paeoniae Rubra, etc. have achieved good effects. Yi-gan-kang granule, the main composition of which is Radix Salviae Miltiorrhizae, is a new generation of Yi-gan infusion, widely used in China for nearly 20 years. Recent years, evidence has shown that traditional Chinese medicines including huo-xue-hua-yu herbs have obvious anti-fibrotic efficacies in clinical and experimental studies. To identify the efficacies of Yi-gan-kang granules on hepatic fibrosis, CCl4- and pig serum-induced animal models and HSCs were used in this experimental study. CCl4 and pig serum have different mechanisms in generating fibrosis of animal models. Concurrent use of the two kinds of fibrotic models indicates the antifibrosis efficacies of Yi-gan-kang. Furthermore, we performed the pathologic image studies using computed image analysis system. HSCs are a kind of the key cells in liver fibrogenesis. The antifibrosis efficacies of herbs can be shown by observing the dynamic changes of HSC phenotypes and extracellular matrix deposition. Chinese herbal medicines including Yi-gan-kang granules have multiphasic mechanisms in treating fibrosis. Proliferation, activation, secretion, decomposition, contraction, and apoptosis of HSCs are the main targets of therapies. Our study showed that Yi-gan-kang granules could effectively inhibit or inverse the course of fibrogenesis. Comparisons of the number of pseudonodule, the thickness of separating...
fibers and the width of fibers in different groups and at different time points in CCl\textsubscript{4} and pig serum-induced model rats demonstrated that using Yi-gan-kang granules 4 wk prior to model making to pre-inhibit fibrogenesis was superior to using the medicine 4 wk before the end of model making to treat fibrosis. The effect in prophylaxis and treatment groups was significantly different from that in model control. These findings indicate that Yi-gan-kang granules used at early stage or for the prophylactic purpose should be advocated in treating hepatic fibrosis. Due to the differences in fibrogenesis between human viral hepatitis and experimental animal models, our study could not clearly tell the definite time when Yi-gan-kang granules are used in patients with chronic liver diseases. However, Yi-gan-kang granule is an effective antifibrotic drug against chronic liver diseases.

HSCs are distributed in portal area and perisinusoid space. In the condition of acute and chronic liver impairment, HSCs undergo phenotype changes from quiescence to activation\cite{3}. The activated HSCs, expressing \( \alpha \)-smooth muscle actin, are the key cells in hepatic fibrogenesis during inflammatory reactions. The change of this phenotype increases synthesis and deposition of extracellular matrix, in which type I collagen is the main component. During the activation, HSCs also express matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs\cite{31,33}. In the early stage of HSC activation, the matrix collagen can be decomposed because of predominant activity of MMPs. In the late stage or in pathologic conditions, the synthesis of TIMPs, especially TIMP-1 is predominant, thus down-regulating the decomposition of matrix collagen. Therefore, \( \alpha \)-SMA and TIMP-1 are the two main factors modulating the synthesis and deposition of matrix collagen. We could see from our immunohistochemical or in situ hybridization studies that Yi-gan-kang granules could significantly inhibit or reduce the expression of type I collagen in rats with liver fibrosis. These results attribute to the restraining of activation of HSCs and the expression of TIMP-1. The dynamic observation of \( \alpha \)-SMA, TIMP-1 and type I collagen expression in rats with liver fibrosis showed that prophylaxis groups had better results than control or treatment groups. This result is in accord with the above pathologic image study. Therefore, \( \alpha \)-SMA and TIMP-1 are two main targets of anti-fibrogenesis by huo-xue-hua-yu herbs.

The number of HSCs is controlled by apoptosis and proliferation during progressive fibrosis, particularly during recovery from fibrosis\cite{34,36}. The event in the process of injury-fibrosis recovery is the key of activated HSCs mediated by apoptosis\cite{37}. Therefore, to induce apoptosis of activated HSCs is one of the most important therapeutic strategies for liver fibrosis. Recent evidence has demonstrated that a mechanism to eliminate activated HSCs in culture and in animal models is apoptosis-induction. Interestingly, activated HSCs are more sensitive to apoptotic cells than quiescent HSCs. Such a mechanism may serve in the future to eliminate the undesirable activated HSCs without affecting their normal quiescent counterparts. The present results suggest that Yi-gan-kang granules can significantly inhibit HSC proliferation and increase the apoptosis rate of HSCs in a dose- and time-dependent manner. Our findings strongly suggest that inhibiting HSC proliferation and inducing HSC apoptosis may play an important role in the antifibrotic actions of Yi-gan-kang granules.

It was reported that HSC proliferation, activation and apoptosis, as well as synthesis of ECM are closely related with lipid peroxidation\cite{38,39}. Anti-lipid peroxidation could possibly be the anti-fibrosis mechanism of Chinese herbs. Radix Salviae Miltiorrhizae is a representative herb with anti-peroxidation effects. It exerts its anti-peroxidation effect by
increasing SOD activity to eliminate free oxygen radicals. The extraction of anti-lipid peroxidation components from \textit{Radix Salviae Miltiorrhizae} and other herbs with appropriate modification of the components could enhance the anti-fibrosis effect.

In conclusion, Yi-gan-kang granules effectively inhibit or reverse the course of liver fibrogenesis in CCl\textsubscript{4} and pig serum-induced rat models and are expected to become one of the effective anti-hepatic fibrosis drugs.

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