

# Effect of rat serum containing Biejiajian oral liquid on proliferation of rat hepatic stellate cells

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## Abstract

**AIM:** Liver fibrosis is a common pathological process of chronic liver diseases. Activation of hepatic stellate cells (HSCs) is the key issue in the occurrence of liver fibrosis. In this study, we observed the inhibitory action of rat serum containing Biejiajian oral liquid (BOL), a decoction of turtle shell, on proliferation of rat HSCs, and to explore the anti-hepatofibrotic mechanisms of BOL.

**METHODS:** A rat model of hepatic fibrosis was induced by subcutaneous injection of  $\text{CCl}_4$ . Serum containing low, medium and high dosages of BOL was prepared respectively. Normal and fibrotic HSCs were isolated and cultured. The effect of sera containing BOL on proliferation of HSCs was determined by  $^3\text{H}$ -TdR incorporation.

**RESULTS:** The inhibitory rate of normal rat HSC proliferation caused by 100 mL/mL sera containing medium and high dosages of BOL showed a remarkable difference as compared with that caused by colchicine (medium dosage group:  $34.56 \pm 4.21\%$  vs  $29.12 \pm 2.85\%$ ,  $P < 0.01$ ; high dosage group:  $37.82 \pm 1.32\%$  vs  $29.12 \pm 2.85\%$ ,  $P < 0.01$ ). The inhibitory rate of fibrotic rat HSC proliferation caused by 100 mL/L serum containing medium and high dosages of BOL showed a remarkable difference as compared with that caused by colchicine (medium dosage group:  $51.31 \pm 3.14\%$  vs  $38.32 \pm 2.65\%$ ,  $P < 0.01$ ; high dosage group:  $60.15 \pm 5.36\%$  vs  $38.32 \pm 2.65\%$ ,  $P < 0.01$ ). The inhibitory rate of normal rat HSC proliferation caused by 100 mL/L and 200 mL/L sera containing a medium dosage of BOL showed a significant difference as compared with that caused by 50 mL/L (100 mL/L group:  $69.02 \pm 9.96\%$  vs  $50.82 \pm 9.28\%$ ,  $P < 0.05$ ; 200 mL/L group:  $81.78 \pm 8.92\%$  vs  $50.82 \pm 9.28\%$ ,  $P < 0.01$ ). The inhibitory rate of fibrotic rat HSC proliferation caused by 100 mL/L and 200 mL/L sera containing a medium dosage of BOL showed a significant difference as compared with that caused by 50 mL/L (100 mL/L group:  $72.19 \pm 10.96\%$  vs  $61.38 \pm 7.16\%$ ,  $P < 0.05$ ; 200 mL/L group:  $87.16 \pm 8.54\%$  vs  $61.38 \pm 7.16\%$ ,  $P < 0.01$ ).

**CONCLUSION:** Rat serum containing BOL can inhibit proliferation of rat HSCs, and the inhibition depends on the dosage and concentration of BOL. The inhibitory effect on

HSC proliferation is one of the main anti-hepatofibrotic mechanisms of BOL.

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## INTRODUCTION

Liver fibrosis is a common pathological process of chronic liver diseases. Injury factors would cause an unbalance between synthesis and degradation of extracellular matrix (ECM), which results in excessive collagen deposition in the liver<sup>[1]</sup>. Liver fibrosis could be reversed before developing into liver cirrhosis. Therefore, it is fundamental to prevent and treat cirrhosis to arrest its progression to liver cancer.

There are several steps in treating liver fibrosis in Western medicine, namely to inhibit activation of HSCs and proliferation of fibroblast-like HSCs and/or synthesis of matrix protein, to promote degradation of matrix protein, to impair activation of cytokines which lead to liver fibrosis, and to give gene therapy<sup>[2,3]</sup>. At the same time, the wide application of Western drugs should be restricted due to their toxicity and side effects.

The anti-hepatofibrotic effect of Biejiajian oral liquid (BOL) has been confirmed in our previous studies<sup>[4-6]</sup>. At present, we investigated the effect of rat serum containing BOL on the proliferation of rat hepatic stellate cells (HSCs) to further explore its anti-hepatofibrotic mechanisms.

## MATERIALS AND METHODS

### Materials

One hundred and forty male Wistar rats, weighing ( $360 \pm 20$ ) g, were supplied by Animal Center of Academy of Medical Sciences of Zhejiang Province. Fodder was maize powder (Hangzhou Sijiqing Feed Factory). Lard (commercially available) and cholesterol were produced by Chemical Branch of Guangzhou Medicinal Company (batch number: 980503). Ethanol (A.R) was purchased from Changyuan Chemical Plant of Changshu City (batch number: 980630). BOL was prepared by the Pharmaceutical Laboratory, Zhejiang College of Traditional Chinese Medicine.

### Methods

**Animal model** Except 30 rats for normal control, the rest of 60 Wistar rats received sc 5 mL/kg  $\text{CCl}_4$  in the first day of experiment, followed by sc 400 mL/L  $\text{CCl}_4$ -liquid paraffin mixture 3 mL/kg daily for 3 d. The normal group received an equal amount of 9 g/L NaCl (NS) daily for 6 wk. Except the normal group, each rat was fed with mixed fodder (maize powder with 5 g/L cholesterol and 200 g/L lard) and drank 200 g/L ethanol only. The normal group was fed with general fodder and water. The time required to complete the induction of model was 6 wk<sup>[7]</sup>.

**Serum containing BOL preparation** Eighty Wistar rats were divided into NS group, colchicine (0.1 mg/kg) group, groups

receiving high (9.2 g/kg), medium (4.6 g/kg) and low dosages (2.3 g/kg) of BOL. Each group received the drug once a day for 7 d. Venous blood was collected from inferior vena cava under asepsis 1 h after the last ig, then serum was separated (3 000 r/min, 20 min, 4 °C), inactivated at 56 °C for 30 min, and stored at -70 °C for use.

**HSC isolation and culture** HSCs were isolated from the livers of normal rats and those with liver fibrosis. Isolation and culture were performed according to the modified Freidman method.

**HSC proliferation determination** Monolayer HSCs appeared in the 24-well culture plate after they were cultured in 1640 culture medium (Gibco BRC) with sera containing different dosages and concentration of BOL for 48 h. <sup>3</sup>H-TdR (Shanghai Institute of Atomic Energy) was added on 92.5 µgBq/well. HSCs were collected after cultured again for 24 h. The HSCs were digested with 2.5 g/L trypsinase-1.1 g/L EDTA (Sigma), fixed by 100 mL/L trichloroacetic acid, dehydrated by alcohol, and then oven dried. Dimethylbenzene solution containing 5 mL/L ppo (Amersham) and 0.2 mL/L PoPop was added to measure CPM value of every sample.

### Statistical analysis

Statistical analysis was performed with ANOVA. Data were presented as mean±SD. Significant differences were determined by using ANOVA. Results were considered statistically significant when  $P<0.05$ .

## RESULTS

### Effect of BOL on rat HSC proliferation

We used 100 mL/L sera containing different dosages of BOL to culture HSCs from normal and hepatofibrotic rats. Results showed that <sup>3</sup>H-TdR incorporated into rat HSCs was inhibited obviously by each dosage group, and the higher the dose, the better the effect (Tables 1, 2).

**Table 1** Effect of different BOL dosage sera on <sup>3</sup>H-TdR incorporation of normal rat HSCs (mean±SD)

Groups	n	Cpm/well	Inhibitory rate (%)
Control	6	2 846.31±218.92	
Colchicine	6	2 018.37±39.31 <sup>b</sup>	29.12±2.85
Low-dose	6	1 968.42±78.32 <sup>b</sup>	30.84±2.71
Intermediate-dose	6	1 870.21±109.45 <sup>b</sup>	34.56±4.21 <sup>a,d</sup>
High-dose	6	1 779.86±61.12 <sup>a,d,f</sup>	37.82±1.32 <sup>d,f</sup>

<sup>a</sup> $P<0.05$  vs low-dose group, <sup>b</sup> $P<0.01$  vs control group, <sup>d</sup> $P<0.01$  vs colchicine group, <sup>f</sup> $P<0.01$  vs low-dose group.

**Table 2** Effect of different BOL dosage sera on <sup>3</sup>H-TdR incorporation of hepatofibrotic rat HSCs (mean±SD)

Groups	n	Cpm/well	Inhibitory rate (%)
Control	6	1 657.82±67.21	
Colchicine	6	1 022.54±12.18 <sup>b</sup>	38.32±2.65
Low-dose	6	994.69±37.86 <sup>b</sup>	40.15±5.36
Intermediate -dose	6	828.91±48.19 <sup>b,d,f</sup>	51.31±3.14 <sup>d,f</sup>
High-dose	6	662.59±17.87 <sup>b,d,f</sup>	60.15±5.36 <sup>d,f</sup>

<sup>b</sup> $P<0.01$  vs control group, <sup>d</sup> $P<0.01$  vs colchicine group, <sup>f</sup> $P<0.01$  vs low-dose group.

### Effect on rat HSC proliferation caused by different concentration sera containing medium dosage of BOL

We used 50 mL/L, 100 mL/L and 200 mL/L sera containing

an intermediate dosage of BOL to culture normal and hepatofibrotic rat HSCs, which were controlled with normal rat sera at the same concentration. We observed that <sup>3</sup>H-TdR incorporation increased gradually as normal serum concentration increased. <sup>3</sup>H-TdR incorporation decreased gradually as drug serum concentration increased. <sup>3</sup>H-TdR incorporation in drug serum group at the same concentration was significantly lower than that in control group ( $P<0.05$ , Tables 3, 4).

**Table 3** Effect of different concentration sera containing intermediate dosage of BOL on <sup>3</sup>H-TdR incorporation of normal rat HSCs (mean±SD)

Concentration	n	Control group (cpm/well)	BOL group (cpm/well)	Inhibitory rate (%)
50 mL/L	6	1 479.79±171.01	701.19±134.25 <sup>a</sup>	50.82±9.28
100 mL/L	6	1 990.80±601.42	640.11±209.69 <sup>a</sup>	69.02±9.96 <sup>c</sup>
200 mL/L	6	2 699.89±789.10	479.59±257.30 <sup>a</sup>	81.78±8.92 <sup>b</sup>

<sup>a</sup> $P<0.05$  vs control group, <sup>b</sup> $P<0.01$  vs 50 mL/L concentration, <sup>c</sup> $P<0.05$  vs 50 mL/L concentration.

**Table 4** Effect of different concentration sera containing intermediate dosage of BOL on <sup>3</sup>H-TdR incorporation of hepatofibrotic rat HSCs (mean±SD)

Concentration	n	Control group (cpm/well)	BOL group (cpm/well)	Inhibitory rate (%)
50 mL/L	6	987.73±154.02	563.07±124.38 <sup>a</sup>	61.38±7.16
100 mL/L	6	1 342.58±701.36	375.92±109.47 <sup>a</sup>	72.19±10.96 <sup>c</sup>
200 mL/L	6	2 306.39±652.14	299.83±123.51 <sup>a</sup>	87.16±8.54 <sup>b</sup>

<sup>a</sup> $P<0.05$  vs control group, <sup>b</sup> $P<0.01$  vs 50 mL/L concentration, <sup>c</sup> $P<0.05$  vs 50 mL/L concentration.

## DISCUSSION

### Mechanism of liver fibrosis

The cells synthesizing extracellular matrix (ECM) in the liver were mainly active HSCs<sup>[8]</sup>. HSCs are situated in the Disse's spaces. As liver cells were injured, HSCs would be activated and might increase, and then converted into myofibroblast-like cells (MFBLC), which could express cytokines, receptors, smooth muscle alpha-actin (alpha-SMA) and synthesize a great deal of ECM. Therefore, activation of HSCs is a key step in the pathogenesis of liver fibrosis<sup>[9-13]</sup>. Activation of local renin-angiotensin system might relate to hepatic fibrosis. Active HSCs could synthesize angiotensin II, which could participate in tissue remodeling in human liver<sup>[14]</sup>. It is the main source of collagen formation. HSC apoptosis could relieve experimental liver fibrosis in rats. Thus, it is an important pathway for preventing liver cirrhosis that inhibits HSC proliferation and collagen synthesis. Moreover, HSC proliferation is a key phase in liver fibrosis. Inhibiting proliferation of HSCs had an important sense for anti-hepatofibrosis<sup>[15,16]</sup>. Basement membrane-like matrix could inhibit proliferation of HSCs, estrogen could relieve liver fibrosis, and lipid could induce HSC proliferation<sup>[17,18]</sup>.

### Recognition of liver fibrosis in TCM

Researches on preventing and treating liver fibrosis in TCM have been increasing<sup>[4-7]</sup>. According to the theory of TCM, liver fibrosis is manifested as weakened body resistance while pathogenic factors prevail, damp-heat and blood stasis coexist, and the liver is depressed due to deficiency of Qi and blood in the spleen and kidney. Liver depression and Qi stagnation can result in blood stasis, blood fails to nourish the liver, and the

key pathogenetic mechanism is blood stasis. At beginning, the pathogenetic mechanism is Qi stagnation and blood stasis. If treated improperly, the disease would evolve into blood stasis, so that body resistance is weakened and excessive superficiality is present. The therapy is to promote the circulation and relieve the stasis, and to strengthen the body resistance to eliminate pathogenic factors, to resolve and soften the hard masses.

### Anti-hepatofibrotic mechanism of BOL

BOL is an improved preparation from Biejiajian Pill that was recorded in an ancient medical book (*Jinkui Yaolue*). Its ingredients include more than twenty herbs, they are Carapax trionycis, Blackberrylily rhizome, Baical skullcap root, Zingiberis, rhizoma, Radix et rhizoma rhei, Ramulus cinnamomi, Folium pyrosiae, Magnoliae cortex, Lilac pink herb, Lagerstroemia indica L, Colla corii asini, Radix bupleuri, Catharsius, Paeonia, Ppaonia suffruticosa, Pruni persicae, semen, Radix ginseng, Pinelliae tuber, Tansymustard seed, Nitrum, Beehive. The combination of ingredients has the effect on promoting circulation and relieving stasis, strengthening body resistance and eliminating pathogenic factors, as well as resolving and softening the hard masses, which is conformable with the principle of treating liver fibrosis. Our previous experiments indicated the mechanisms of anti-hepatofibrosis of BOL as follows. It can prevent hepatocytes from degeneration and necrosis, eliminate liver fibrosis, inhibit the synthesis and secretion of ECM, relieve the capillarization of hepatic sinusoids, and improve the microcirculation of liver. HSCs are sensitive to oxygen and hypoxia could enhance liver fibrosis<sup>[19-22]</sup>. BOL could also regulate the immune function, and reduce the damage of liver cells<sup>[4-6]</sup>.

In conclusion, serum-containing BOL can inhibit HSC proliferation and the inhibition may be dependent with the dosage and concentration of BOL.

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