

# Influence of BOL on hyaluronic acid, laminin and hyperplasia in hepatofibrotic rats

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## Abstract

**AIM: To study the anti-hepatofibrosis mechanism of Bie Jia Jian oral liquid (BOL).**

**METHODS: The model was induced by subcutaneous injection of CCl<sub>4</sub>. BOL was administered and the change of serum hyaluronic acid (HA) and laminin (LN) was observed and the degeneration of liver cells and the degree of fibre hyperplasia analyzed. Changes of ultra micro-structure in liver cells were observed in some samples.**

**RESULTS: HA was reduced in both the groups with low and high dosage of BOL, which showed a remarkable difference as compared with that of the model group ( low dosage group: 376.15 µg/L±35.48 µg/L vs 806.07 µg/L±98.49 µg/L, P<0.05; high dosage group: 340.14 µg/L±30.18 µg/L vs 806.07 µg/L±98.49 µg/L, P<0.05). The LN content of low and high dosage group of BOL was lower than that of model group (low dosage group: 71.99 µg/L±8.15 µg/L vs 133.94 µg/L±14.45 µg/L, P<0.01; high dosage group: 71.68 µg/L±11.62 µg/L vs 133.94 µg/L±14.45 µg/L, P<0.01) and colchicine group (low dosage group: 71.99 µg/L±8.15 µg/L vs 118.28 µg/L±16.13 µg/L, P<0.05; high dosage group: 71.68 µg/L±11.62 µg/L vs 118.28 µg/L±16.13 µg/L, P<0.05). Examined by Ridit, BOL could reduce the degeneration and necrosis of liver cells ( $\chi^2 = 11.99$  P<0.05), the degree of fibre hyperplasia ( $\chi^2 = 13.24$  P<0.05) and the pathological change of ultra micro-structure as well.**

**CONCLUSION: The BOL has certain therapeutic effect on the experiment hepatofibrosis. Its mechanisms might include: protecting the function of liver cells, inhibiting excessive synthesis and secretion of extracellular matrix from hepatic stellate cells, relieving the capillarization of hepatic sinusoid, improving liver micro-circulation, and regulating immune function.**

**Subject headings** BIEJIA JIAN PILL/administration dosage; BIEJIA JIAN PILL/pharmacology; peroral liquids; liver cirrhosis, experimental/TCD therapy; laminin/analysis; hyaluronic acid/analysis

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## INTRODUCTION

Since 1994, our research group has studied on anti-hepatofibrotic effect of the Biejiajian pill and Biejiajian oral liquid (BOL) in rats. It verified that both of them had fair effect on anti-hepatofibrosis<sup>[1-3]</sup>. Supported by the Natural Science Foundation of Zhejiang Province in 1999, we studied the anti-hepatofibrotic mechanism of BOL in rats again. Now we report the results below.

## MATERIALS AND METHODS

### Materials

Eighty SD rats, weighing (180±20) g, offered by Animal Centre of the Academy of Medical Sciences of Zhejiang Province. Fodder: maize powder (produced by Hangzhou Sijiqing Feed Factory); Lard (commercially supplied); Cholesterol (produced by Chemical Branch of Guangzhou Medicinal Company, batch number: 980503). Ethanol (A.R) (produced by Changyuan Chemical Plant of Changshu City, batch number: 980630). Medicines: All of the 23 ingredients of BOL are purchased from the Out patient Department of Zhejiang College of TCM, such as Biejia, Danpi, etc. appraised by the Department of Pharmacy. Colchicine (produced by Kunming Pharmacy Company, Limited, batch number: 980536). Reagents: CCl<sub>4</sub>(A.R) (produced by Fenshui Synthesis Chemical Plant of Yixing City, batch number: 1995122). Liquid paraffin (C.P) (produced by Hangzhou Chemical Reagents Factory, batch number: 971020). HA and LN radioimmunoassay kits (afforded from Shanghai Navy Institute of Medical Science); main instruments: SN-688γ-counter (Shanghai Atomic Energy Institute); UV-754 ultraviolet-visible spectrophotometer (Shanghai 3rd Analytic Instrument Factory); MR4100 enzyme marker (American Dynatach Company); H-500 transmission electric microscope (Japanese Hitachi, Co).

## METHODS

### Animal model

All groups but the normal group received sc CCl<sub>4</sub> (A.R) 5 mL·kg<sup>-1</sup> in the first day of experiment, afterwards sc 400 mL·L<sup>-1</sup> CCl<sub>4</sub>-liquid paraffin mixture 3 mL·kg<sup>-1</sup> every 3 days; the normal group sc equal amount of 9 g·L<sup>-1</sup> NaCl (NS), lasted 6 weeks. Except the normal group, every group was fed with mixed fodder (maize powder with 5 g·L<sup>-1</sup> cholesterol and 200 g·L<sup>-1</sup> lard) and drank 200 g·L<sup>-1</sup> ethanol only. The normal group was fed with general fodder and water. The time required to complete the induction of model is 6 weeks.

### Grouping

Thirty-seven established rat models were grouped into II-V groups randomly i.e. group II model control (n = 10), group III colchicine (n = 9), group IV low dosage of BOL (n = 9) and group V high dosage of BOL (n = 9). Another 11 normal rats served as normal control in group I.

### Therapy

The BOL afforded by Zhejiang College of TCM, according to the ingredient of Jingui Biejiajian pill, which is recorded in an ancient medical book of TCM (Jinkui Yaolue). It contains crude Chinese

medicine 0.92 g per millilitre (that is 20 times the dosage used in adults). I, II group ig NS 10 mL/kg, III group ig 0.001% colchicine in distilled water of the dosage of 0.1 mg·kg<sup>-1</sup>, IV group ig BOL 4.6 g·kg<sup>-1</sup> and V group ig BOL 9.2 g·kg<sup>-1</sup>. All groups had been treated once a day for 5 weeks and took water and fodder ad lib. For maintaining pathological attack, 400 mL·L<sup>-1</sup> CCl<sub>4</sub>-liquid paraffin mixture 3 mL·kg<sup>-1</sup> once a week until the end of treatment was injected subcutaneously to the models of II-V groups. After the treatment, venous blood was collected from retrobulbar vein, serum was separated and preserved under -70°C for test. HA and LN were examined by radioimmunoassay. The left liver lobe was dissected and fixated by 100 mL·L<sup>-1</sup> formalin and embedded in paraffin, serially sectionalized and then stained with HE observed under optical microscope. In case of observing the collagenous fiber hyperplasia Masson staining was employed. The standards for interpreting the degree of hyperplasia under microscope are in accordance to the criteria of the 5th National Academic Conference of Infection and parasitosis<sup>[4]</sup>, and the degree of degeneration refers to the standard of reference<sup>[5]</sup>.

### Statistical analysis

Data described as  $\bar{x}\pm s$  and analysed by F test, and *q* test was used to compare the data between 2 groups of multiple groups. The semiquantitative data was analysed by Ridit test. *P*<0.05 regarded as significant.

## RESULTS

During the period of treatment, in group II 2 rats died, 1 rat each died in group I, IV and V, and in group III no rat died.

### Influence of BOL on serum HA and LN

Compared with model group, the serum HA of each treated group was lowered significantly (*P*<0.05). There was no significant difference between each treated group (*P*>0.05). This is in accordance with the results of other article<sup>[6]</sup>. Results of the serum LN: the difference between the colchicine group and the model group was not significant (*P*>0.05). Compared with the normal group, the LN of each treated group was raised significantly (*P*<0.01). The LN in both low- and high-dose BOL groups were higher significantly than that of normal group (*P*<0.01) and colchicine group (*P*<0.05, Table 1). The difference between high- and low-dose BOL groups was not significant.

**Table 1** Serum HA and LN in BOL treated SD rats ( $\bar{x}\pm s$ ,  $\mu\text{g}\cdot\text{L}^{-1}$ )

Groups	<i>n</i>	HA	LN
Normal	10	197.77±26.02	42.78±10.05
Model	8	806.07±98.49 <sup>b</sup>	133.94±14.45 <sup>b</sup>
Colchicine	9	402.53±50.78 <sup>a</sup>	118.28±16.13 <sup>b</sup>
Low-dose BOL	8	376.15±35.48 <sup>a</sup>	71.99±8.15 <sup>bdc</sup>
High-dose BOL	8	340.14±30.18 <sup>a</sup>	71.68±11.62 <sup>bdc</sup>

<sup>b</sup>*P*<0.01 vs normal group; <sup>a</sup>*P*<0.05 vs model group, <sup>d</sup>*P*<0.01 vs model group; <sup>c</sup>*P*<0.05 vs colchicine.

### Influence of BOL on liver hyperplasia and degeneration

Being analysed by Ridit ( $\chi^2 = 13.24$ , *P*<0.05, Table 2), we found that the treatment was relevant with the extent of liver hyperplasia. According to the average Ridit, the high-dose BOL group did the best in improving liver hyperplasia.

According to the results of statistical analysis ( $\chi^2 = 11.99$ , *P*<0.05, Table 3), we considered that the extent of liver cell degeneration was related to the treatment. Referring to the average Ridit, the effectiveness of improving the degeneration in high dosed BOL group was best, and model group had the worst result.

**Table 2** Extent of liver hyperplasia in each treated group

Groups	<i>n</i>	Degree of liver fibrosis					Average of Ridit
		-	+	++	+++	++++	
Model	8	0	0	1	4	3	0.7247
Colchicine	9	0	1	2	4	2	0.5894
Low-dose BOL	8	0	2	1	4	1	0.5176
High-dose BOL	8	0	5	2	1	0	0.2364

$\chi^2 = 13.24$ ,  $\chi^2_{(4)} = 9.49$ .

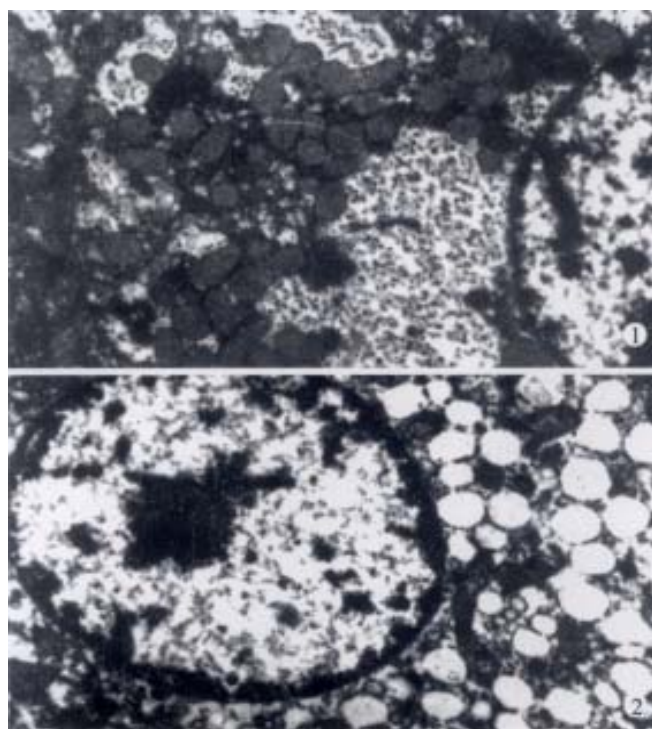
**Table 3** Liver cell degeneration in each treated group

Groups	<i>n</i>	Extent of hyperplasia					Average of Ridit
		-	+	++	+++	++++	
Model	8	0	0	2	3	3	0.7408
Colchicine	9	0	1	4	3	1	0.5584
Low-dose BOL	8	0	1	2	4	1	0.5599
High-dose BOL	8	0	4	3	1	0	0.2576

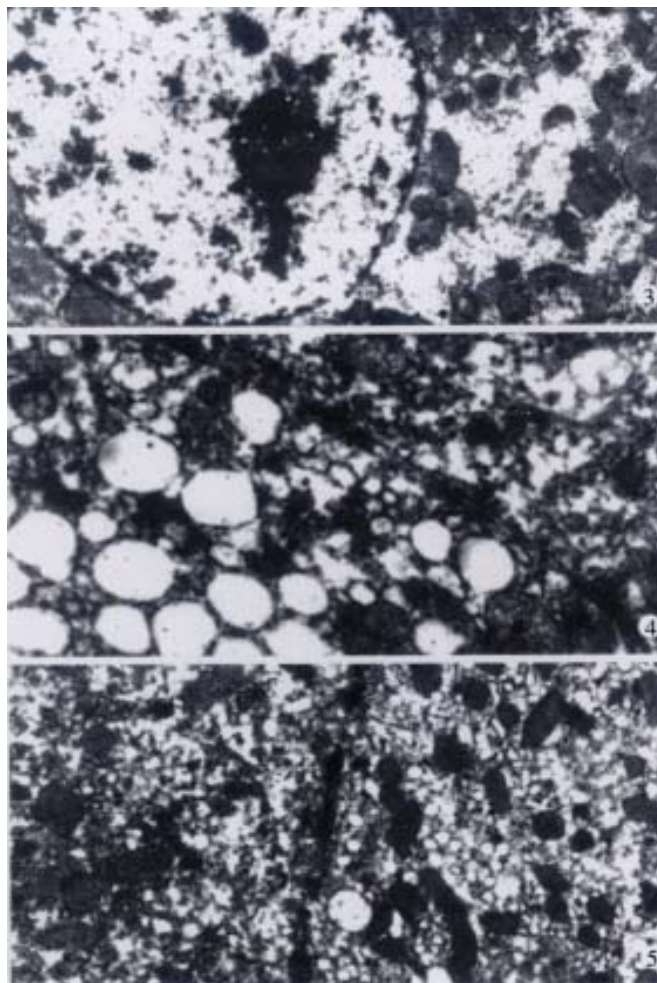
$\chi^2 = 11.99$ ,  $\chi^2_{(4)} = 9.49$ .

### Electron microscopy

The nucleuses in liver cell of normal SD rats were circular or elliptical and located in the center of cell, the mitochondria were circular or elliptical, the ridges were clear, the Ito cell could be found in Disse's spaces, its cytoplasm contained plenty of fat droplets. However, the nucleolus disappeared, rough endoplasmic reticulum expanded obviously, the mitochondria were tumid, the ridges were broken or lacked, there were a great deal of fat droplets in the cytoplasm. Many collagenous fibers accumulated in Disse's spaces. The number of stellate cells and rough endoplasmic reticulum increased and surrounded by a large number of collagenous fibers. Compared with the model group, the fat droplets in liver cell of the high-dose BOL group decreased, the structure of nucleus was clear and the mitochondria were tumid lightly. Being observed under the electronic microscope the results of low dosed BOL group were similar to those of colchicine group. Their damage as lighter and more serious than that in model group and high-dose BOL group respectively (Figures 1-5).



**Figure 1** Normal group Showing clear liver cell structure with plenty of mitochondria and clear ridges, no fat droplets in cytoplasm. EM×6 000  
**Figure 2** Model group Showing few mitochondria without nucleolus, cell structure is not clear, a lot of fat droplets in cytoplasm. EM×4 000



**Figure 3** High dosed BOL group Showing clear nucleus structure, lightly tumid mitochondria and few fat droplets in cytoplasm. EM×4 000

**Figure 4** Model group Showing many deposited fibers. EM×8 000

**Figure 5** High dosed BOL group Showing few deposited fibers. EM×5000

## DISCUSSION

Recently, studies on the prevention and treatment of liver fibrosis are very popular in China. Besides western medicine<sup>[7-11]</sup>, single Chinese drug and its effective ingredients<sup>[12-19]</sup>, anti-hepatofibrotic effect of Chinese complex formula<sup>[20-27]</sup> are also the main project in researches. These studies illustrate the mechanism and clinical effectiveness<sup>[28-32]</sup> in anti-hepatofibrosis from different point of view. Now, we will especially discuss the anti-hepatofibrotic mechanism of BOL.

### *The knowledge of liver fibrosis in TCM*

Though the term, liver fibrosis, is not mentioned in the ancient medical documents of TCM, the corresponding clinical symptoms of this disease in TCM terms such as abdominal mass, costal pain, flatulence, jaundice, blood stasis, etc. are commonly noted. The clinical symptom of liver fibrosis is intricate. According to the theory of TCM, its basic pathogenesis is that: the condition is manifested as body resistance weakened while pathogenic factors prevailed, damp-heat and blood stasis coexisted, liver depressed and deficiency of *qi* and blood in spleen and kidney. Liver depression and *qi* stagnation can result in blood stasis, blood fails to nourish liver, and the key pathogenetic mechanism is blood stasis<sup>[33]</sup>. At the beginning, the pathogenetic mechanism is *qi* stagnation and blood stasis. If being treated improperly, the disease will evolve into blood stasis mainly, then into a condition which manifests as blood stasis, body resistance weakened and excess in superficiality. The therapy is promoting the circulation and relieving the stasis, strengthening the body resistance

to eliminate pathogenic factors, resolving and softening hard mass.

BOL is an improved preparation from Biejiajian Pill which is recorded in an ancient medical book (Jinkui Yaolue). Its ingredients include twenty-three herbs, such as biejia, taoren, dampi, shaoyao, dahuang, caihu, guizhi, banxia, houpu, renshen, e-jiao, huangqin etc. The combination of ingredients has the effect on promoting circulation and relieving stasis, strengthening body resistance and eliminating pathogenic factors simultaneously, resolving and softening the hard mass, which is conformable with the principle of treating liver fibrosis. The therapy of promoting blood circulation and clemishing stasis has been adopted in clinic<sup>[34]</sup>.

### *Inquiring into the anti-hepatofibrotic mechanism of BOL*

At present, the mechanism of liver fibrosis is inferred that the pathogenic factors injure the liver cell and activate Kupffer's cells to secrete certain kinds of cytokines, which act jointly with other cytokines secreted by the platelets, hepatic sinusoid endotheliocytes, hepatocytes and some chemical intermediates to activate stellate cells, converting them into myofibroblasts, secreting a great deal of extracellular matrix (ECM). The increase of secretion and decrease of degradation result in accumulation of ECM in liver, forming liver fibrosis gradually<sup>[35-39]</sup>. According to this experimental result, we suggest the main effect of BOL on treating liver fibrosis in four aspects as follows. ① Preventing the hepatocyte from degeneration and necrosis. The degeneration and necrosis of liver cell are the factors inducing excessive deposition of ECM, which takes an important role in the progression of liver fibrosis<sup>[40,41]</sup>. So, preventing the degeneration and necrosis of liver cell after being hurt can eliminate the promoting factor of liver fibrosis, preventing Kupffer's cells and stellate cells from being activated, inhibiting the progression of liver fibrosis. This experimental result shows that BOL can protect the liver cells from being harmed, reduce their degeneration and necrosis, clean or partly clean the inflammatory reaction (also a promoting factor of liver fibrosis), all these demonstrate the good effect on treating liver fibrosis. ② Inhibiting the liver stellate cells to synthesize and secrete ECM. It is well known that liver stellate cells are the interstitial cells served as the main source of ECM. As the liver cells are injured, they will be activated, and increased, and then convert into the transitive type and myofibroblast which synthesize and secrete the ECM excessively<sup>[40,42-44]</sup>. The collagens are the main elements of ECM. They are formed in rough endoplasmic reticulum of fibroblasts and are secreted as collagenous fibrils<sup>[45]</sup>. Both the number of stellate cells and rough endoplasmic reticulum of high dosed BOL group are less than those of model group, which indicates that inhibiting the activation and hyperplasia of the liver stellate cells maybe one of the effects of BOL to prevent and treat liver fibrosis. ③ Relieving the capillarization of hepatic sinusoid, improving the microcirculation of liver. The fenestrate on the hepatic sinusoid endotheliocyte is the site for exchanging materials between the liver cells and plasma. When liver fibrosis developed, ECM synthesized profoundly, deposited in Disse's spaces, forming the membranous spiral lamina under the hepatic sinusoid endothelium, fenestrate of hepatic sinusoid decreased or vanished and then forming capillarization, resulting in impairment of microcirculation, subsequently, affecting the exchange of the oxygen and substance of the liver cell seriously, thus bringing out or aggravating the damage of liver cell<sup>[45-48]</sup>. LN is the main glycoprotein of the membranous spiral lamina and distributes together with the IV type collagen in the liver. So its serum content is considered as a renewal index of membranous spiral lamina<sup>[49-51]</sup>. This experimental result indicated that: BOL can decrease the LN content in liver fibrosis, thus relieving the capillarization of hepatic sinusoid, improve the microcirculation of liver, reducing the damage of liver cell and accelerating the repairment of liver. This may be an



important effect of BOL on promoting the circulation and transforming stasis. ④ Regulating the immune function, reducing the immunodamage of liver cell. Some researchers<sup>[52]</sup> complain that the liver cirrhosis is the result of the immune function disturbance. According to our previous report<sup>[2]</sup>, we suggest that BOL can adjust humoral immunity, reduce the immunodamage of liver cell, improve the substance metabolism and protect the liver tissue from being injured.

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