Liver fibrosis in mice treated with Yue-Ju-Bao-He pills

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Abstract

Background: The aim of this study was to investigate the protective effect and antioxidant mechanism of Yue-Ju-Bao-He pills (YJBH) on liver fibrosis induced by carbon tetrachloride in mice. Methods: Thirty C57BL/6 mice were randomly divided into control group, model group, and YJBH group. The control group received 0.2 mL olive oil intraperitoneally injected twice per week for six weeks. Four weeks after injection, the control group received oral treatment of 0.2 mL normal saline once per day for three weeks. The model and YJBH groups received 20% carbon tetrachloride olive oil solution (2 mL/kg) intraperitoneal injections twice per week for 6 weeks to induce liver fibrosis. After 4 weeks of carbon tetrachloride injections, the model and YJBH groups received oral treatment of either 0.2 mL normal saline or YJBH (0.9 mg/kg) once per day for 3 weeks, respectively. After three weeks of treatment, liver sections from mice were used for hematoxylin-eosin staining and Masson’s Trichrome staining to observe the status of the liver tissue. The therapeutic effect of YJBH on liver fibrosis was studied by determining alanine transaminase and aspartate aminotransferase and pathological changes in livers. Determining the malondialdehydelevel, superoxide dismutase, and glutathione peroxidase activities in liver homogenates were done to observe antioxidative mechanisms. Results: Compared with the control group, the body weight of mice in the model group decreased significantly and the liver index increased. After YJBH treatment, the body weight of mice increased and the liver index decreased compared with the model group. In addition, liver function indexes aspartate aminotransferase and alanine transaminase were significantly improved. The level of malondialdehyde in liver tissue was significantly decreased, and the expression of glutathione peroxidase and superoxide dismutase were increased. The pathological examination showed that liver cell injury of YJBH group was significantly reduced by the infiltration of inflammatory cells into collagen fibers. Conclusion: YJBH can effectively delay the progress of liver fibrosis induced by carbon tetrachloride, and the mechanism may be related to oxidative stress.

Keywords: Yue-Ju-Bao-He pills, liver fibrosis, carbon tetrachloride, oxidative stress

Competing interests:
The authors declare no conflicts of interest.

Abbreviations:
YJBH, Yue-Ju-Bao-He pills; CCl4, carbon tetrachloride; H&E, hematoxylin-eosin; ALT, alanine transaminase; AST, aspartate aminotransferase; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-PX, glutathione peroxidase; SD, standard deviation.

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Background

Liver fibrosis is caused by viral hepatitis, chemical or drug-induced liver disease, alcoholic or non-alcoholic fatty liver disease, and autoimmune liver disease [1]. The main pathological features of this disease are excessive deposition of extracellular matrix and dysplasia of connective tissue [2]. If appropriate attention and treatment are not given in time, liver fibrosis will further develop toward cirrhosis, portal hypertension, and liver failure [3]. Fortunately, due to the powerful regenerative ability of liver cells, liver fibrosis is partially reversible. Therefore, active and effective anti-fibrosis therapy should be taken in time to inhibit further deterioration of various chronic liver diseases. This is necessary to prolong the survival time and improve the quality of life of patients [4].

Traditional Chinese medicine has shown good effects in anti-liver fibrosis treatment. Modern pharmacological studies have confirmed that ligustazine can reduce the inflammatory response and the severity of liver cell damage, and then restore the normal function of hepatic sinusoid endothelial cells [5]. An extract of Qing-Gan recipe, a traditional Chinese medicine, can treat liver fibrosis. Its mechanism is to reduce the activity of hepatic stellate cells and the expression of inflammatory factors [6]. In addition, Xiao-Cheng-Qi decoction can also alleviate liver injury by reducing the oxidative stress induced by carbon tetrachloride (CCl4) in mice [7]. Traditional Chinese medicine believes that the main cause of liver fibrosis is liver loss and drainage. Depressed liver and Qi obstruction leads to the accumulation of dampness and heat, blood stasis, phlegm, and other pathological products. Therefore, “the liver loses its catharsis function” is the key to its pathogenesis [8]. Yue-Ju-Bao-He pills (YJBH) come from the Danxi’s Mastery of Medicine. It has the effect of invigorating spleen, promoting the circulation of Qi, and resolving depression. The purpose of this study was to investigate the preventive and therapeutic effects of YJBH on CCl4 induced liver fibrosis in mice and to explore the possible mechanism of its anti-liver fibrosis.

Methods

Reagents and instruments

YJBH was purchased from Beijing Tongrentang Pharmaceutical Co., Ltd. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH-PX) test kits were purchased from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China). Fixation solution of 4% paraformaldehyde and Masson’s Trocolor staining kit were obtained from Solarbio Biotechnology Co., Ltd. (Beijing, China).

Animals and treatment

Male C57BL/6 mice, weighing (20.0 ± 2.0) g, were purchased from Beijing HFK Bioscience Co., Ltd. After adaptive feeding for three days, 30 C57BL/6 mice were divided into control group (n = 10), model group (n = 10), and YJBH group (n = 10). Mice in the control group were intraperitoneally injected with olive oil (2 mL/kg body weight) twice a week, for 6 weeks. The model group and YJBH group were injected with 20% CCl4 olive oil solution (2 mL/kg) in the same way as the control group. After 4 weeks, the control group and model group were gavaged with normal saline (0.5 mL/kg body weight) every day for 3 weeks. The YJBH group was gavaged with YJBH (0.9 ml/kg body weight) every day for 3 weeks. Body weights of mice in each group were recorded during the treatment (Figure 1).

Serum biochemical markers assay

After standing for 30 minutes at room temperature, blood was centrifuged, and the serum was stored at −80°C for analysis. The indexes of ALT and AST in the serum of mice in each group was determined by biochemical kit. Frozen liver tissue (0.1 g) was weighed and placed in 900 μL normal saline and vortexed 30 seconds, then centrifuged to prepare 10% liver tissue homogenate. The activities of SOD and GSH-PX and the level of MDA in the tissues of mice in each group were determined.

Liver biochemical analysis

At the end of the experiment liver tissues of each group were collected, fixed with formalin solution, embedded in paraffin, and sectioned into 3 μm tissues. Hematoxylin and eosin (H&E) staining and Masson’s trichrome staining were performed. The damage of liver cells and the distribution of collagen fibers in liver sections of mice in each group were examined by light microscope. Image J was used to quantify the positive areas of fiber staining, detect the integral optical density, and calculate the positive expression areas.

Liver biochemical analysis

All numerical results were expressed as mean ± standard deviation. Statistical differences were determined using one-way ANOVA. P-value < 0.05 was considered statistically significant.

Results

Effects of YJBH on general condition, body weight, and liver index in liver fibrosis model mice

During the whole experiment, the control group showed good mental state, smooth coat color, good performance in daily activities such as eating and drinking water, and average body weight increased
steadily over the course of the experiment. On the contrary, compared with the control group, the model group was depressed, the amount of food and water was decreased, the coat color was darkened. and the body weight was not increased. YJBH group was improved in terms of coat color and body weight. At the end of the experiment, the body weight of mice in the model group was significantly lower than that in the control group \((P < 0.01)\), while the body weight of mice in YJBH group was significantly higher than the model group \((P < 0.01)\). The liver index of the model group was dramatically increased and the liver index was significantly decreased after YJBH administration \((P < 0.01)\) (Figure 2 and Figure 3).

**Figure 1 Experimental design.** YJBH, Yue-Ju-Bao-He pills; CCI4, carbon tetrachloride; H&E, hematoxylin-eosin; ALT, alanine transaminase; AST, aspartate aminotransferase; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-PX, glutathione peroxidase.

**Figure 2 Changes of body weight after YJBH treatment.** Mice were divided into control group \((n = 10)\), model group \((n = 10)\), and YJBH group \((n = 10)\). Data are presented as mean ± standard deviation. \(*P < 0.01\) compared with the control group; \(**P < 0.01\) compared with the model group. YJBH, Yue-Ju-Bao-He pills,
Effects of YJBH on liver function in liver fibrosis model mice
At the end of the experiment, the serum levels of AST (P < 0.01) and ALT (P < 0.05) were significantly increased in the model group compared with the control group. Compared with model group, the serum levels of AST and ALT were significantly decreased in the YJBH group (P < 0.01) (Table 1).

Effects of YJBH on oxidative stress levels in liver

The SOD, MDA, and GSH-PX in liver tissue is an important index to evaluate its antioxidant capacity. Our results showed that the levels of MDA (P < 0.05) was higher in the model group compared to the control group, and the index of GSH-PX (P < 0.05) and SOD (P < 0.01) were significantly lower. Compared with the model group, the level of MDA (P < 0.05) was decreased in YJBH group, and the concentration of GSH-PX (P < 0.05) and SOD (P < 0.01) were increased (Table 2).

Table 1 Concentration serum liver function biomarkers after YJBH treatment. (m ± SD, n = 10)

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>155.64 ± 7.22</td>
<td>63.84 ± 8.43</td>
</tr>
<tr>
<td>Model</td>
<td>222 ± 15.94##</td>
<td>85.92 ± 4.06#</td>
</tr>
<tr>
<td>YJBH</td>
<td>175.32 ± 12.22**</td>
<td>78.12 ± 6.23**</td>
</tr>
</tbody>
</table>

##P < 0.01 compared with the control group; #P < 0.01 compared with model group; *P < 0.05 compared with control group. YJBH, Yue-Ju-Bao-He pills. AST, aspartate aminotransferase; ALT, alanine transaminase; m, mean; SD, standard deviation.

Table 2 The activities of SOD and GSH-Px, and the levels of MDA in mice liver homogenate after YJBH treatment. (x ± s, n = 10)

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol/mg protein)</th>
<th>GSH-P (U/mg protein)</th>
<th>SOD (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.59 ± 0.93</td>
<td>49.82 ± 13.87</td>
<td>297.78 ± 37.9</td>
</tr>
<tr>
<td>Model</td>
<td>4.65 ± 1.53*</td>
<td>28.01 ± 10.33*</td>
<td>211.84 ± 16.37###</td>
</tr>
<tr>
<td>YJBH</td>
<td>2.77 ± 0.81*</td>
<td>43.13 ± 4.35*</td>
<td>263.94 ± 14.72**</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with control group; **P < 0.01 compared with the control group; *P < 0.01 compared with model group. YJBH, Yue-Ju-Bao-He pills; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-PX, glutathione peroxidase; m, mean; SD, standard deviation.

Figure 3 Changes of liver indexes after YJBH treatment. Mice were divided into control group (n = 10), model group (n = 10), and YJBH group (n = 10). Data are presented as mean ± standard deviation. ##P < 0.01 compared with the control group; **P < 0.01 compared with the model group. YJBH, Yue-Ju-Bao-He pills.
Figure 4 Pathological changes in mouse liver tissue by H&E staining (100×). Black arrows indicate the inflammatory cell infiltration. Red arrows indicate the cellular swelling of hepatocytes. YJBH, Yue-Ju-Bao-He pills; H&E, hematoxylin-eosin.

Effects of YJBH on H&E staining of liver tissue in liver fibrosis model mice

H&E staining showed that the structure of liver lobules was clear and complete in the control group was complete, and the liver cell cords were arranged radially and neatly with the central vein as the center. No degeneration or necrosis were observed in the liver cells, and the nuclear structure was clear. In the model group, liver tissue was obviously damaged, liver lobule structure destroyed, and liver cells cord-like arrangement was destroyed. A large number of inflammatory cells were identified, liver cells were swollen and disordered, and fatty changes were observed. In the YJBH administration group, the degree of inflammatory infiltration and hepatocyte necrosis were reduced and the pseudolobules were significantly less than those in the model group (Figure 4).

Masson’s trichrome staining of liver tissue

Pathological examination results of Masson’s staining showed that the structure of liver lobules was clear and complete in the control group. Hepatic cell cords were arranged radially around the central vein; no obvious pathological changes were observed; and only a small number of blue fibers around blood vessels were seen. In the model group, a large number of inflammatory cells and necrotic cells were observed in the liver tissue; the hepatic cord arrangement was disordered; the structure of hepatic lobules was fuzzy; a large number of fibroblasts were proliferated, the fibrous septum was thickened, a large number of fibrous Bridges and pseudolobules were formed, and a large number of fibers were stained blue by Masson’s staining ($P < 0.01$). In the YJBH treatment group, liver tissue lesions were significantly reduced. Liver lobular structure was clear, a small amount of liver cells were deformed and necrotic, and a small amount of fibers were generated ($P < 0.05$). Masson staining only showed that a small amount of liver cells around the fibrous hepatic cord (stained blue) (Figure 5).

Discussion

Liver fibrosis is a repair response to chronic liver injury. It is the common pathologic basis and leads to the development of chronic liver disease from liver cirrhosis to liver cancer [9]. Studies have shown that the occurrence and development of liver fibrosis is closely related to the activation of hepatic stellate cells in liver tissue. Hepatic stellate cells mainly exist in the parasinus space between endothelial cells and liver cells. Its main functions are fat storage, vitamin A metabolism, synthesis and secretion of collagen glycoprotein proteoglycan matrix metalloproteinases and tissue inhibitors, and other matrix components. In addition, it can also participate in the regulation of hepatic sinus blood flow with the expression of cytokines and their receptors [10, 11]. In chronic liver injury, liver parenchymal cells are damaged and a large number of inflammatory cells infiltrate, promoting the activation of hepatic stellate cells [12]. Activated hepatic stellate cells have low lipid and vitamin A content and enhanced proliferation and migration ability, which can be converted into myofibroblasts [13]. Myofibroblasts secretes extracellular matrix mainly composed of collagen fibers, leading to massive deposition of extracellular matrix and leading to liver fibrosis [14].

In traditional Chinese medicine, liver fibrosis is included in the category of hypocholic pain, jaundice, and swollen hepatocclusion. The common clinical manifestations are chest pain, ascites, jaundice, silence, and loss of appetite. The chief causes are liver and spleen disease. The pathogenesis is liver stagnation, spleen deficiency, blood stasis, and dampness [15]. Therefore, in the comprehensive analysis of the pathogenesis of liver fibrosis, the core problem is that the Qi of the liver is not smooth. The smooth flow of Qi ensures the normal function of blood storage in the liver. Therefore, to treat the liver, first treat Qi. YJBH, a traditional Chinese medicine compound, is made of Gardenoside, Medicated Leaven, Rhizoma Cyperi, Szechuan Lovage Rhizome, Atractylodes Lancea,
Radix Aucklandiae, and Betelnut. Gardenias clear heat and light fire. Rhizoma Cyperi promotes blood circulation and promotes liver circulation. Szechuan Lovage Rhizome enhances blood flow and reduces blood stasis. Atractylodes has the effect of reducing dampness and strengthening spleen. All traditional Chinese medicine regulates Qi to help the body. In recent years, animal experiments have proved that YJBH has an effect on chronic inflammation by effect by lipid peroxidation [16, 17].

CCL4 mixed solution is the most widely used method for liver fibrosis model induction at present. It is straightforward and has high similarity with human liver fibrosis [18]. As an exogenous toxin, CCL4 can be metabolized into trichloromethyl free radicals in the body, causing liver damage. Failure to remove these free radicals can lead to cell damage, erosion of the cell membrane, and loss of cellular integrity [19]. Experimental results show that the pathological manifestations of the liver in the model group were obvious lipid peroxidation, massive secretion, and accumulation of collagen fibers, indicating liver cell damage.

ALT and AST are important indicators to evaluate liver function. Under normal physiological conditions, there are a large number of enzymes in liver cells. ALT is mainly present in the cytoplasm of liver cells, and AST is present in the mitochondria of liver cells [20]. After CCL4 entered the body, the membrane permeability of hepatocytes targeted at the liver was increased. Liver cells infiltrate a large number of enzymes into the blood, and the concentration of ALT and AST in the blood increases sharply [21]. Therefore, ALT and AST in blood can reflect the degree of liver injury [22]. In this experiment, YJBH administration reduced ALT and AST concentration in the blood of mice. This suggests that YJBH can improve liver function.

It is well-known that lipid peroxidation is also one of the main mechanisms of CCL4-mediated liver injury [23]. After CCL4 enters the body, it is transformed into free radicals, which attack unsaturated fatty acids on the cell membrane and trigger lipid peroxidation reactions [24]. MDA is one of the final products of lipid peroxidation and an indicator of lipid peroxidation. It can harden cell membranes, reduce fluidity, increase permeability, and lead to swelling and necrosis of liver cells [25]. As an important oxidase and free radical scavenger, SOD can catalyze the disproportionation of superoxide anions into
hydrogen peroxide and oxygen, thereby reducing the concentration of hydrogen peroxide [26]. GSH-PX is the main antioxidant in cells, which can combine with the metabolite of cytochrome P450 to achieve the scavenging effect of free radicals, thus protecting the liver [27]. The results of this study showed that YJBH administration group could effectively reduce MDA level and increase SOD and GSH-PX activities. These results indicate that YJBH can reduce oxidative stress in vivo.

Conclusion

In summary, YJBH can improve liver function, restore liver histopathological morphology, and alleviate the development of liver fibrosis. The mechanism of action may be related to the reduction of oxidative stress by YJBH.

References


