Regulating effect of Chinese herbal medicine on the peritoneal lymphatic stomata in enhancing ascites absorption of experimental hepatofibrotic mice

Ji-Cheng Li, Shi-Ping Ding, Jian Xu

METHODS: Two Chinese herbal composite prescriptions were used separately to treat the carbon tetrachloride-induced mouse model of liver fibrosis. The histo-pathologic changes of the liver sections (HE and VG stainings) were observed. The peritoneal lymphatic stomata was detected by scanning electron microscopy and computer image processing. The changes of urinary volume and sodium ion concentration were measured.

RESULTS: In the model group, lots of fibrous tissue formed in liver and extended into the hepatic lobules to separate them incompletely. In the treated and prevention groups, the histo-pathologic changes of liver was rather milder, only showed much less fibrous tissue proliferation in the hepatic lobules. The peritoneal lymphatic stomata enlarged with increased density in the experimental groups (diameter: PA, 3.07±0.69㎛; PB, 2.82±0.37㎛; TA, 3.25±0.82㎛ and TB, 2.82±0.56㎛; density: PA, 7.11±1.90 stomata-1000㎛²; PB, 8.76±1.45 stomata-1000㎛²; TA, 6.55±1.44 stomata-1000㎛² and TB, 8.76±1.79 stomata-1000㎛²), as compared with the model group (diameter: 2.00±0.52㎛; density: 4.45±1.05 stomata-1000㎛²). After treatment, the urinary volume and sodium ion excretion increased in the experimental groups (PA, 231.28±41.09mmol·L⁻¹; PB, 171.69±27.48mmol·L⁻¹ and TA, 231.44±34.12mmol·L⁻¹), which were significantly different with those in the model group (129.33±36.75mmol·L⁻¹).

CONCLUSION: Chinese herbal medicine has marked effects in alleviating liver fibrosis, regulating peritoneal lymphatic stomata, improving the drainage of ascites from peritoneal cavity and causing increase of urinary volume and sodium ion excretion to reduce the water and sodium retention, and thus have favorable therapeutic effect in treating ascites.

INTRODUCTION

Since von Recklinghausen first reported the peritoneal lymphatic stomata, numerous investigators demonstrated that these are small openings of the subperitoneal lymphatic vessels in animals and in humans[10]. It has also been observed that particles, cells and solutions containing vital dyes are absorbed rapidly by the peritoneal lymphatic stomata. Subsequent researchers suggested that the peritoneal cavity is an integral part of the lymphatic system with enormous absorption powers, functioning primarily by means of the subperitoneal lymphatics via the peritoneal stomata[16-21]. Thus it has played an extremely important role in pathological conditions such as ascites absorption[22-24], peritoneal dialysis[25], intrauterine fetal hemolytic hemorrhage, and neoplastic metastasis of the peritoneal cavity[26].

Liver cirrhosis is a common progressive pathological lesion of chronic liver diseases in response to various liver-damaging factors[20-23]. Among liver cirrhosis of various causes, one common feature is the increased hepatic deposition of extracellular matrix, which consists mainly of collagen, leading to portal hypertension, esophageal varices and ascites. In the treatment of the ascites, the methods such as catharsis, diuresis, diaphoresis were used, but these methods would have side-effects which limit their clinical use[24-26]. In recent years, attention has been paid to the therapeutic effect of Chinese medicine on the ascites[28-40]. With the discovery of the human lymphatic stomata and the study of the lymphatic drainage system in the peritoneal cavity, Li et al confirmed that Chinese medicine can regulate the lymphatic stomata and promote the excretion of substance from the peritoneal cavity, and provided a new approach to the management of liver cirrhosis with ascites[41].

In the present case, based on the previous study of peritoneal lymphatic stomata (PLS) regulation, mouse liver fibrosis model induced by carbon tetrachloride gastrogavage was used for studying the effect of two kinds of Chinese herbal medicine in anti-fibrosis, regulating PLS and promoting urine output and urinary ion excretion, so as to provide an experimental basis for clinical trial in cirrhosis with ascites.

MATERIALS AND METHODS

Animal and grouping
One hundred and two male ICR mice, weighing 18.2-25.4g, provided by Experimental Animal Center of Zhejiang Academy of Medical Sciences, were randomly divided into six groups: 17 in the prevention A group (PA group), 17 in the prevention B group (PB group), 20 in the treatment A group (TA group), 20 in the treatment B group (TB group), 20 in the model group and 8 in the control group.

Drugs
Chinese composite prescription I and II (CP I and II) were supplied by Zhejiang Academy of Traditional Chinese Medicine. The former consisted of radix Salviae Miltiorrhizae, Radix Codonopsis...
Pilosulae, Rhizoma Atractyloidis Alba and Rhizoma Alismatis, and the latter consisted of rhizoma Ligustici Wallichii, Semen Persicae and radix Salviae Miltiorrhizae. The herbal drugs were steeped in 75% alcohol for 24h, then purified with rotatory evaporator (ZFQ85A type, produced by Shanghai 11th Factory of Electron Tube). The crude drug content in CP I was 9Kg·L⁻¹ and in CP II 6Kg·L⁻¹.

Establishment of mouse liver fibrosis model
All the experimental animals were fed freely with 380mg·L⁻¹ pentobarbital solution instead of water for 10d. Excepting the control group, the mice were given 10% CCl₄ in rape-seed oil solution (100ml/900ml) 0.2ml/mouse by gastro-gavage every 4 days for 10 weeks to induce liver fibrosis. CP I or CP II (0.2ml/mouse) was given to the PA and PB groups respectively at the same time of CCl₄ administration. For the TA and TB groups, equal volumes of CP I and CP II were given respectively beginning from wk 7 of the experiment, after liver fibrosis formation was confirmed by pathological examination. The model group was untreated and the control group was given 0.2ml/mouse of normal saline per day. The experiment was lasted to wk10.

Preparation of pathological samples
The livers were taken out after the mice were killed, fixed with Bouin liquid, embedded in paraffin, sectioned, and stained with HE and VG methods for observation under light microscopy.

Preparation of sample for transmission electron microscopic examination
The tissue blocks of about 1-2mm in diameter were fixed with 25mL·L⁻¹ glutaraldehyde solution in 0.1 M phosphate buffer. Then they were postfixed in 10g·L⁻¹ O₃O₄ in 0.1mol·L⁻¹ phosphate buffer for 1h at 4°C. After dehydration in a graded series of ethanol and steeping in propylene oxide, they were embedded in Epon 812. Semi-thin serial sections were made for orientation and identification of the mesothelium, and then fine sections of the mesothelium were cut with Leica Ultracut UCT ultramicrotome and stained with uranyl acetate and lead citrate. The sections were examined with a Philips EM 410 TEM operated at 60 kV.

Preparation of sample for scanning electron microscopic examination
The diaphragmatic peritoneum of mice was cut into pieces 3×3mm in size, double fixed with 25mL·L⁻¹ glutaraldehyde solution and 10g·L⁻¹ O₃O₄, gradiently dehydrated with ethanol, managed by 20g·L⁻¹ tannic acid and 10g·L⁻¹ O₃O₄, then postfixed in 10g·L⁻¹ O₃O₄ in 0.1mol·L⁻¹ phosphate buffer for 1h at 4°C. After dehydration in a graded series of ethanol and steeping in propylene oxide, they were embedded in Epon 812. Semi-thin serial sections were made for orientation and identification of the mesothelium, and then fine sections of the mesothelium were cut with Leica Ultracut UCT ultramicrotome and stained with uranyl acetate and lead citrate. The sections were examined with a Hitachi S-570 scanning electron microscope after metallizing with 20kV of accelerating voltage.

Computer image processing
Computerized digital processor of electronic microscopic image was used. The whole set of instruments consisted of camera, A/D, IBMP II computer, high resolution color display monitor of 64 grey level / 256 false color (including VGA adapter) and application software.

Urinary volume and ionic concentration determination
Urine in 2 hrs was collected with a filter and ionic concentration of Na⁺, K⁺ and Cl⁻ were determined using auto-biochemical analyzer (Beckman CX△7 type).

Statistic analysis
Student’s t-test was adopted.

RESULTS
Body weight and death of CCl₄-treated mice
After intake of CCl₄, the mice generally manifested sluggish motion, loose skin with lusterless hairs, obvious lowering of body weight 24h afterward but recovered gradually in the later 3d until the next CCl₄ medication, and these formed a cyclic change. The lowest number of deaths due to CCl₄ intake occurred in the PA group (3 mice), and then the TA group (7 mice), PB group (11 mice), TB group (13 mice) and the model group (13 mice) in the order. The mortalities in PA and TA group were significantly lower than that in the model group (P<0.05).

Pathological changes of liver
In the model group, the margin of liver was uneven; lots of fibrous tissue formed in portal areas and extended into the hepatic lobules to separate them incompletely; a large amount of inflammatory cells infiltrated in the intralobular and the interlobular regions; the liver structure was disordered with some displacement of central veins, and there were more necrotic and degenerated liver cells compared with the control (Figure 1, 2). Under transmission electron microscopy, there were numerous collagenous fibers around the liver cells (Figure 3). In the two preventive and two treated groups, the pathological changes of liver was rather milder, showing less fibrous tissue proliferation and inflammatory cell infiltration in the interlobular space; the hepatic cell cords arranged radially with less displacement of central veins and less degenerated or necrosis hepatic cells (Figure 4). The pathologic changes in the PA and TA groups were milder than those in the PB and TB groups.
Figure 3 TEM observation of liver in the control (A) and model group (B).
Lot of collagenous fiber (arrows) formed in model group. N: nucleus. A ×12000, B ×13500

Figure 4 Light microscope observation of liver fibrosis tissue in PA group (A) and TA group (B).
The pathological changes of liver was rather lighter compared with the model. ×200

Figure 5 SEM observation of the peritoneal stomata (arrow) in the control group.
The mesothelial cells (C) and locked stomata (triangle) of the diaphragmatic peritoneum in the mice. ×2000

Figure 6 SEM observation of mice diaphragmatic peritoneum in the prevention A group.
Both diameter and distributive density of the peritoneal stomata (arrows) are significantly increased. ×2000

Figure 7 SEM observation of mice diaphragmatic peritoneum in the treatment A group.
Some erythrocytes (arrow) could be seen to pass through the lymphatic stomata. ×3000
Changes of peritoneal lymphatic stomata

Many microvilli on the mesothelial surface of diaphragmatic peritoneum of mice could be seen with electron microscopy. The microvilli seemed to be less at the place where the mesothelial cell with its nucleus protruding to peritoneal cavity and to be more at the junctions neighboring mesothelial cells. Such uneven distribution of the villi drew out the rhomboid or polygonal outlines of mesothelial cells, which were ordinarily of the flat form and the cubical form. Peritoneal lymphatic stomata (PLS) could be found only between the neighboring cubical mesothelial cells, distributed in cluster distribution and were round or elliptical shaped. In physiological condition, only a part of PLS are opened, most are in the locked manner (Figure 5).

Observations in this study showed that the diameters of PLS in the PA and PB groups increased significantly with more stomata opened, and thus resulted in an increase in their density (Table 1, Figure 6). Similar changes also occurred in the TA and TB groups (Table 1, Figure 7). Through PLS, one could see the lymphatic drainage units (LDU) formed by endothelial cytoplasmic protrusions and connective tissue fibers at the bottom of PLS (Figure 8). Intraperitoneal substances could enter through PLS into the subperitoneal lymphatic vessels so as to accelerate the absorption of ascites from the peritoneal cavity.

Table 1. Comparison of Diameter and Density of PLS between Groups (µm, Mean ± SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Diameter/µm</th>
<th>Density/(stomata·1000µm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>2.11±0.78</td>
<td>5.70±1.25</td>
</tr>
<tr>
<td>Model</td>
<td>20</td>
<td>2.00±0.52</td>
<td>4.45±1.05</td>
</tr>
<tr>
<td>PA</td>
<td>17</td>
<td>3.07±0.69</td>
<td>7.11±3.90</td>
</tr>
<tr>
<td>TA</td>
<td>20</td>
<td>3.25±0.82</td>
<td>6.55±1.44</td>
</tr>
<tr>
<td>PB</td>
<td>17</td>
<td>2.82±0.37</td>
<td>8.76±4.15</td>
</tr>
<tr>
<td>TB</td>
<td>20</td>
<td>2.82±0.56</td>
<td>8.76±1.79</td>
</tr>
</tbody>
</table>

aP<0.05, bP<0.01, vs control group; cP<0.01, vs model group.

Comparison of urinary ionic concentration

The excretions of Na⁺, K⁺ and Cl⁻ in the PA and PB group were significantly increased than those in the control group and the model group respectively (P<0.05 or P<0.01) (Table 2). The excretion of these ions in the TA group was also increased, but in the TB group, increase in ionic excretion was only limited to K⁺ and Cl⁻, whereas the Na⁺ excretion was not significantly different from that in the model or the control group (Table 2).

Table 2. Comparison of urinary ionic concentration between groups (mmol·L⁻¹, Mean ± SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Cl⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>117.98±42.29</td>
<td>100.16±31.32</td>
<td>93.43±36.20</td>
</tr>
<tr>
<td>Model</td>
<td>20</td>
<td>129.33±36.75</td>
<td>162.21±39.42</td>
<td>120.49±42.34</td>
</tr>
<tr>
<td>P/A</td>
<td>17</td>
<td>231.28±41.09</td>
<td>241.63±59.43</td>
<td>212.17±44.01</td>
</tr>
<tr>
<td>TA</td>
<td>20</td>
<td>231.44±34.12</td>
<td>306.45±79.53</td>
<td>240.66±31.37</td>
</tr>
<tr>
<td>PB</td>
<td>17</td>
<td>2.82±0.87</td>
<td>188.17±73.91</td>
<td>149.50±43.79</td>
</tr>
</tbody>
</table>

aP<0.05, bP<0.01, vs control group; cP<0.05, vs model group.

Comparison of urine volume between groups

The urine volumes 2h after CP I and CP II gastroagavage in the prevention or treatment groups (average 1250µL) were more than that in the control group (average 550µL), and that in the model group was the least one (average 150µL).

DISCUSSION

It is considered currently that liver fibrosis is the basic pathologic change of chronic liver diseases. No matter what the pathogenic mechanism of different liver diseases is, the ultimate consequence of progressive pathologic processes is the development of cirrhosis, portal hypertension and ascites[42-46]. CCl₃ is a super-hepatotoxin, with which the CCl₃ free radical is produced during metabolic processes and acts on liver cells to covalently conjugate with the cyto-membranous unsaturated lipid to cause lipid peroxidation and necrosis of hepatocytes[47-49].

CP I and CP II are made up according to the traditional Chinese medicinal therapeutic principle in treating “hypochondriac pain”, “lump” and “tympanties”. The former has the effects of activating blood circulation to relieve stasis, strengthening “Spleen”, supplementing Qi, and smoothening Qi to eliminate fullness. The latter is mainly used to activate blood circulation and remove stasis. Salviae miltiorrhiza in the prescriptions could preserve the integrity of hepatocytes, eliminate toxic free radicals, inhibit lipid peroxidation of cytomembrane and relieve necrosis of hepatocytes[46-49]. The two composite Chinese herbal prescriptions contain equal dosage of Salviae miltiorrhiza, aiming at antagonizing the CCl₃, free radical, preserving the integrity of hepatocytes and decreasing the toxic action of CCl₃ on hepatocytes. However, view from the mortality of hepato-fibrosis mice, the mortality in the PA group was the lowest, and that in the PB and TB groups was not significantly different from that in the model group, the results suggested that the effect of CP I in protecting against acute damage of hepatocytes was superior to that of CP II. Wang held that the main mechanisms of Chinese herbal medicine in antagonizing liver fibrosis were: (1) To protect liver cells from degeneration, necrosis and immunological damage so as to eliminate the inducing factors of liver fibrosis; (2) To inhibit the activation and proliferation of hepatic stellate cells; (3) To decrease the expression of the genes responsible for ECM secretion; (4) To promote the activity and production of collagenase[38,49].

According to traditional Chinese medical theory, cirrhotic ascites, one of the serious complications of liver cirrhosis, is caused by insufficiency of Yuan-Qi (vigor) and ferociousness of evil Water in the late decompensatory stage. Radix Codonopsis Pilosulae used in the prescriptions may strengthen “Spleen” and promote its function, activate Yuan-Qi of Middle-Jiao; Rhizoma Atractylodis Alba may strengthen “Spleen” and promote urination, and supplement “Spleen” to remove ascites; and Rhizoma Alismatis has special function in inducing duresis. The collocation of the four drugs has effects not only in strengthening Spleen, supplementing Qi, activating blood circulation to relieve stasis, and inducing diuresis to relieve abdominal distension, but also in antagonizing liver fibrosis and protecting liver.

In the previous study, it was shown that PLS are opening of subperitoneal lymphatic capillary vessels on the surface of peritoneum, possessing an active absorptive function for liquid and granular substances in the peritoneal cavity, and the drugs for treating ascites can accelerate the absorption of peritoneal lymph by PLS. This experiment touched preliminarily upon the function of PLS regulation and changes of urinary volume and ions excretion during prevention and treatment of liver fibrosis with traditional Chinese drugs. It was shown in the experiment that the diameter and density of PLS in the CPII prevention or treatment groups were all higher than those in the control or the model groups respectively. Our previous study also revealed that when the diameter and density of PLS were increased by drugs, the quantity of absorption through PLS was increased significantly[46]. It was further proved that the urinary volume and ionic excretion in the CP I and CP II prevention groups were increased significantly and also increased in the two treatment groups except Na⁺ in CP II group. These results coincide with the regulatory effects of PLS, and indicate that both the two composite prescriptions have the effects in promoting the absorption through PLS on intraperitoneal substances of PLS, so as to accelerate liquid and ion excretion in cirrhotic patients with ascites.

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