Original Article

The Protective Effect of Gan Shen Fu Fang on Liver Endothelial Cells in Common Bile Duct-ligated Rats

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Abstract

Objective: To elucidate the protective effect of Gan Shen Fu Fang (GSFF) on liver endothelial cells in common bile duct-ligated (CBDL) rats. Materials and Methods: Cirrhosis was induced by common bile duct ligation. The rats were divided into three groups: sham group, CBDL group, and GSFF group. After 2 weeks of ligation, rats in the GSFF group were administered GSFF. After 4 weeks, the hydroxyproline (HyP) content of liver tissues was spectrophotometrically determined. The histological changes were evaluated by H and E and Masson staining. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used to observe the ultrastructural changes in the liver, especially in the liver sinusoidal endothelial cells (LSECs). Results: HyP synthesis was significantly inhibited by GSFF, which agreed with the results from H and E and Masson staining for liver fibrosis. The TEM observations of CBDL rats revealed reduced hepatocyte microvilli and deposited fibrous tissue underneath LSECs. SEM confirmed the TEM findings and showed that the fenestrae of LSECs decreased and even disappeared in CBDL rats. The morphological results indicated hepatic sinusoid capillarization. GSFF promoted the restoration of fenestrae and reversed hepatic sinusoid capillarization. Conclusion: GSFF can inhibit HyP synthesis, restore the fenestrae of LSECs, and reverse hepatic sinusoid capillarization in CBDL rats. These results provide a basis for future detailed investigations of the mechanism of action of GSFF in LSECs.

Keywords: Cirrhosis, Gan Shen Fu Fang, hepatic sinusoid capillarization, liver sinusoidal endothelial cells

INTRODUCTION

Cirrhosis is the advanced stage of many different forms of chronic liver diseases, including viral hepatitis, alcoholic, and nonalcoholic fatty liver diseases. It is considered to be an irreversible pathological alteration and is often accompanied by many other severe complications, such as portal hypertension (PHT), hepatic failure, and even hepatocellular carcinoma. Therefore, antifibrotic medicines for liver fibrosis therapy are urgently needed.

Pathologically, cirrhosis is characterized by the replacement of normal liver tissue with fibrous scar tissue, which disrupts normal liver architecture and forms numerous abnormal nodules. More specifically, cirrhosis is an aberrant form of wound healing and the complicated underlying mechanism remains unclear. Hepatic stellate cells (HSCs) have been demonstrated to be the major source of hepatic myofibroblasts in liver fibrosis. In normal conditions, HSCs are in a quiescent state. However, various insults, including viral infection or hepatic toxins, will lead to hepatocyte injury, which subsequently activates quiescent HSCs. Activated HSCs proliferate and undergo phenotypical and morphological transitions to myofibroblasts. Therefore, the activation of HSCs is thought to be the central event in liver fibrosis, but the latter leads to cirrhosis formation. Recent research has shown that liver sinusoidal endothelial cells (LSECs) also play an important role in cirrhosis. HSCs and LSECs are mesenchymal cells. Normal LSECs (differentiated LSECs) are unique. They contain fenestrae, nondiaphragmed pores that transverse the cytoplasm and lack a basement membrane. Once the liver is injured, the fenestrae on LSECs are reduced

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and may even disappear. Meanwhile, an organized basement membrane is developed. This process is termed hepatic sinusoid capillarization. LSECs, with lost fenestrae, are de-differentiated or capillarized cells.[9]

Previous studies have shown that capillarization precedes the activation of HSCs and the onset of hepatic fibrosis. In physiological conditions, LSECs can maintain HSC quiescence and inhibit activation through paracrine factors.[5] However, capillarized LSECs no longer prevent HSC activation, but instead permit or promote the activation of HSCs.[6] The co-culture of activated HSCs and differentiated LSECs revealed that the activated HSCs could be returned to quiescence.[7] The different effects of LSECs on HSC activation suggest that LSECs may act as a gatekeeper for the promotion of HSC quiescence. Based on these findings, the restoration of the LSEC phenotype has been proposed as an alternative approach for the inhibition of fibrosis and cirrhosis.[8]

Gan Shen Fu Fang (GSFF, also known as Glytan), based on traditional Chinese medicine theory, is composed of salvianolic acid B (SA-B) and diammonium glycyrrhizinate (DG). The previous studies on GSFF have focused on PHT. We have shown that GSFF can reduce portal pressure and portal territory blood flow and increase mean arterial pressure and splanchic vascular resistance.[9] We also showed that GSFF improved liver function and inhibited pseudolobule formation in rats with cirrhosis.[10] However, the mechanism by which GSFF prevented the progression of cirrhosis was not clear. In this study, we investigated the effect of GSFF on LSECs to elucidate the mechanism underlying the biological effects.

**Materials and Methods**

**Animal models**

Male Sprague-Dawley rats (approximately 250 g; Vital River Laboratory Animal Technology Co. Ltd., Beijing, China) underwent sham surgery or common bile duct ligation. In brief, a median laparotomy exposed the common bile ducts of the rats, which was then ligated twice.[11] In each animal, the segment between the two ligations was resected and the animal’s abdomen was sutured closed. Sham-operated rats served as controls. In these rats, the common bile duct was exposed, but no ligation or resection was performed. Each group contained seven animals. All experimental procedures were conducted in accordance with the guidelines for the use of experimental animals and were approved by the Institutional Review Committee on Animal Care and Use at the Experimental Animal Centre of Beijing University of Chinese Medicine (Certificate of Conformity: SCXK [jing] 2012-0001).

The rats were divided into three groups: sham group, common bile duct-ligated (CBDL) group, and GSFF group. SA-B and DG are present in GSFF at a 1:1 ratio. Before use, GSFF was first diluted with distilled water. After 2 weeks of ligation, GSFF group rats were administered GSFF (25 mg/kg/day) by gavage. The dose of GSFF depended on the results of the pharmacodynamic experiments.[10] The rats in the sham group and CBDL group were administered an equal volume of distilled water.

**Determination of hydroxyproline**

The hydroxyproline (Hyp) content of liver tissue was tested using a commercial kit (A030-2) purchased from the Nanjing Jiancheng Bioengineering Institute. The liver tissue samples were processed in accordance with the instructions.

**H and E staining and Masson staining**

The fixed liver tissues were dehydrated in a graded alcohol series and then embedded in paraffin. The sections were stained with H and E to observe the changes in the liver architecture changes and to assess the liver injury. Masson staining was used to assess the level of collagen deposition.

**Transmission electron microscopy**

The excised liver samples were cut into small pieces and immersed in excess volumes of 2.5% glutaraldehyde with 0.1 M phosphate buffer (pH 7.2). After fixation for 24 h, the samples were immersed in 2% osmium tetroxide (OsO₄) in 0.1 M cacodylate buffer (pH 7.2) for 2 h at 4°C, dehydrated, and embedded in epoxide resin (EPON 812). Ultrathin sections were stained with lead citrate and uranyl acetate, and examined using a JEM-1200EX transmission electron microscope. This procedure was conducted by the Electron Microscopy Laboratory of China-Japan Friendship Hospital.

**Scanning electron microscopy**

The benchmark used to confirm the differentiation of LSECs is the presence of fenestrae in sieve plates in electron microscopy. In this study, we therefore used scanning electron microscopy (SEM) to observe the change in LSECs under different conditions. The liver samples were fixed first in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.2) for 24 h at 4°C and then in 2% aqueous osmium tetroxide for 4 h. The samples were stored in desiccators until gold-palladium sputter coating for two 200-s intervals (Nano Structured Coating Co., Iran). The samples were analyzed by SEM in low vacuum.

**Statistical analysis**

All data were presented as the mean ± standard deviation. Statistical comparisons were performed using one-way ANOVA. P < 0.05 was considered to indicate statistical significance.

**Results**

**Effect of Gan Shen Fu Fang on hydroxyproline content of liver tissues**

Four weeks after bile duct ligation, the Hyp content of the CBDL group rats was significantly elevated compared with that of the sham group rats (P < 0.01). Treatment with GSFF reduced the Hyp content (P < 0.01) in comparison with the CBDL group [Figure 1].
Effect of Gan Shen Fu Fang on histological changes of liver tissue

The structure of the liver tissue was normal in sham group rats. Hepatocyte cords radiated around the central vein [Figure 2a] and only a small amount of collagen was present around the peripoortal area and central vein [Figure 3a]. After 4 weeks of ligation, diffuse proliferation of bile duct epithelial cells was obvious because of cholestasis. In addition, extensive hepatocyte necrosis and fibrous tissue deposition are other features of this animal model [Figure 2b]. The proliferated fibrotic tissue formed linkages [Figure 3b]. After treatment with GSFF, the extent of bile duct proliferation, hepatocyte necrosis, and proliferated fibrotic tissue decreased. However, focal fatty change was observed in hepatocytes [Figures 2c and 3c].

Effect of Gan Shen Fu Fang on ultrastructure of liver tissue

Normal hepatocytes have one or two large, round nuclei, rich with mitochondria, and a rough endoplasmic reticulum. There is no tight junction between LSECs [Figure 4a]. There were many microvilli in the Disse space. Four weeks after the ligation, some nuclei of the hepatocytes were shrunken. The fenestrae of LSECs gradually decreased and even disappeared. A subendothelial basement membrane was formed and the accumulation of fibrous tissue in LSECs was clear [Figure 4b]. After treatment with GSFF, hepatocellular necrosis was alleviated. The microvilli and fenestrae of the sinusoidal endothelial cells were visible [Figure 4c].

Effect of Gan Shen Fu Fang on fenestrae of liver sinusoidal endothelial cells

On the surface of the LSECs of rats in the sham group, we observed big and small fenestrae. Some fenestrae were clustered in the sieve plates. In addition, we clearly identified the microvilli through the orifices [Figure 5a]. After the development of liver cirrhosis, the fenestrae decreased and even disappeared [Figure 5b]. Proliferated and thick fibrous tissue was noted beneath the LSECs [Figure 5c]. The loss of fenestration and the development of an organized basement membrane indicated the capillarization of LSECs. GSFF promoted the restoration of fenestrae [Figure 5d]. The accumulation of fibrous tissue beneath LSECs significantly decreased.

DISCUSSION

In the progress of cirrhosis, it has been shown that different types of hepatic cells, including hepatocytes, HSCs, LSECs, and Kupffer cells, do not function alone. There is a complex crosstalk between these cells. Molecules produced during this crosstalk regulate cellular differentiation and activity both in healthy and diseased states. Due to the critical position of LSECs as an interface to blood components and their special morphological feature, LSECs are a vital link in cellular crosstalk. For example, it has been demonstrated that differentiated LSECs regulated HSC activation, not only via the release of soluble molecules but also through the release of exosomes. Therefore, the inhibition or reversal of HSC activation through the protection of LSECs has been proposed as an effective method to impede liver fibrosis progression.

In this study, we established a model of cirrhosis via common bile duct ligation in rats. This method is commonly used to induce liver fibrosis and cirrhosis. The histological changes indicated that the ligated rats develop liver fibrosis after 2 weeks and cirrhosis after 4 weeks. Therefore, to simulate clinical conditions, we treated the CBDL group rats after 2 weeks and terminated the treatment at 4 weeks; most patients begin to take medication when they are diagnosed with liver fibrosis.
Hyp is the main component of collagen. Therefore, the Hyp content is a partial reflection of the deposited collagen and level of cirrhosis. Our findings showed that GSFF significantly inhibited Hyp synthesis in CBDL models, which was consistent with our previous results. H and E staining showed that cholestasis, which resulted from the ligation, led to hepatocyte necrosis and proliferation of the bile duct cells. To repair the injury, a large amount of fibrous tissue proliferates, which increases the stiffness of the liver and the destruction of the normal architecture. GSFF reduced the infiltration of inflammatory cells and alleviated the proliferation of the bile duct cells.

To examine the protective effect of GSFF on LSECs, we observed the morphological changes of LSECs using transmission electron microscopy and SEM. We observed that there was no tight junction between LSECs, and rich microvilli were in the Disse space in normal condition. During the progression of cirrhosis, the fenestrae of LSECs decreased and even disappeared. Proliferated fibrous tissue occurred underneath LSECs, which indicated liver sinusoidal capillarization. GSFF restored the fenestrae of LSECs. Lu et al. also reported that SA-B alone improved the de-differentiation of LSECs induced by endothelin-1. Therefore, we concluded that GSFF protected injured LSECs in the common bile duct ligation model.

Marrone et al. reported that the amelioration of LSECs improves the HSC phenotype through a vascular endothelial growth factor-mediated mechanism. Xie et al. administered rats with thioacetamide for 6 weeks and co-administered a soluble guanylate cyclase (sGC) activator in the final 3 weeks. They found that the sGC activator had no direct effect on HSC activation in vivo, but induced the regression of existing fibrosis and prevented the progression of fibrosis through restoration of LSEC differentiation with the subsequent crosstalk between SEC and HSC. Their findings indicated that the restoration of LSEC differentiation promoted HSC quiescence and thereby accelerated regression and prevented the progression of fibrosis. There are some common features between our experiment and the work of Xie, even though we used a different animal model. In both studies, treatment was started after the rats developed liver fibrosis. Additionally, both GSFF and the sGC activator improved LSEC differentiation. Collectively, these results indicate that the protective effect of GSFF on LSECs may be a novel mechanism by which GSFF alleviates cirrhosis. To prove this hypothesis and elucidate the detailed mechanism, we will proceed with further in vivo and in vitro research.

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Conflicts of interest
There are no conflicts of interest.
Figure 5: Effect of Gan Shen Fu Fang on fenestrae of liver sinusoidal endothelial cells (× 20,000). (a) Liver sinusoidal endothelial cells of sham rats: The arrow shows big and small fenestrae in the surface of liver sinusoidal endothelial cells. The star represents numerous microvilli of hepatocytes. (b and c) Liver sinusoidal endothelial cells of common bile duct-ligated rats: Figure b shows lost fenestrae and capillarization of liver sinusoidal endothelial cells after 4 weeks of common bile duct-ligated and Figure c (arrow) shows the proliferated fibrous tissue. (d) Liver sinusoidal endothelial cells of Gan Shen Fu Fang rats: Restored fenestrae compared with common bile duct-ligated rats.

REFERENCES