Effect of Sinai San decoction on the development of non-alcoholic steatohepatitis in rats

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AIM: To explore the effect of Sinai san decoction on the development of non-alcoholic steatohepatitis induced by CCL₄ combined with a fat-rich diet in rats.

METHODS: Twenty-seven Sprague-Dawley rats were divided into three groups randomly: control group (n = 9), model group (n = 9) and treatment group (n = 9). The rats of model group and treatment group were given small dosage of CCL₄ combined with a fat-rich diet, and those of control group were given normal diet. After four weeks of fat-rich diet feeding, the rats of treatment group were given Sinai san decoction. The serum levels of aminotransferase and lipid were measured, and the pathology of livers was observed by HE staining after the rats were sacrificed at eight weeks.

RESULTS: The rats’ livers presented the pathology of steatosis and inflammation with higher serum levels of ALT and AST in the model group. In the treatment group the serum ALT and AST levels decreased significantly and were close to the control group. The hepatic inflammation scores also decreased markedly, but were still higher than those of control group. And the degree of hepatocyte steatosis was similar to that of model group.

CONCLUSION: Sinai san decoction may ameliorate the hepatic inflammation of rats with steatohepatitis induced by small dosage of CCL₄ combined with a fat-rich diet, but does not prevent the development of hepatocyte steatosis.

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Key words: Sinai san decoction; Fatty liver; Non-alcoholic steatohepatitis; Traditional Chinese Medicine

INTRODUCTION
Fatty liver is a relatively common incidental finding on imaging studies[1]. Although generally a benign condition, fat in the liver can be troubling for clinicians because it can cause persistently elevated liver enzyme levels. The finding of fatty liver may also indicate the presence of non-alcoholic steatohepatitis (NASH)[2,3]. NASH is a histologic diagnosis applied to a constellation of liver biopsy findings that appear similar to alcoholic liver disease but are found in the absence of alcohol abuse[4]. NASH is typically identified during the evaluation of elevated aminotransferase levels after exclusion of viral, metabolic, and other causes of liver disease[5]. About 15 to 40% of NASH patients develop hepatic fibrosis, a precursor to cirrhosis[6]. As the pathogenesis of this common liver disease is not better understood, there is complete absence of specific and effective treatments[6]. Clinical researches show that Sinai san has the effects of ameliorating the symptom of fatty liver patients. The specific purpose of this study was to determine if this recipe would protect against liver injury induced by small dosage of CCL₄ combined with a fat-rich diet in rats.

MATERIALS AND METHODS

Drugs and chemicals
Sinai san is composed of Chai Hu (Radix Bupleuri), Shao Yao (Radix Paeoniae), Zhi Shi (Fructus Aurantii Immaturus), and Gan Cao (Radix Glycyrrhizae), which were obtained from the Affiliated Hospital of Shaanxi College of TCM. According to the routine decoction methods, all the drugs were decocted in water twice, removing the sediments and condensing into 100% Sinai san decoction. CCL₄ (1,1,3,3-tetraethoxypropane) was purchased from Yixing Chemical Co (batch number 920801). Cholesterol was obtained from Beijing Aobo Biotech Co (batch number 024303).

Animals
Twenty-seven male Sprague-Dawley rats weighing approximately 160 g were provided by the Experimental Animal Center of Xi’an Jiaotong University. All the rats were kept in an air-conditioned room controlled at 23±1.8 and 55±5% humidity under a 12 h dark/12 h light cycle. Rats were fed a standard laboratory diet and were provided water ad libitum.
for a week. Rats were randomized into three groups: control group (n = 9), model group (n = 9) and treatment group (n = 9). The control group rats were fed a standard laboratory pelleted diet only. The model group and treatment group rats were administered 1 mL/kg of CCl₄ in a 40% corn oil solution only once by subcutaneous injection combined with a fat-rich diet consisting of (by weight) 2% cholesterol, 10% lard and 88% ground pellet diet [7]. All animals had free access to water and the experimental diet. Four weeks later, 100% pure Sinai san decoction and distilled water (0.1 mL/kg per d) were administered to the treatment group and model group rats respectively by oral gavage during the experimental period. Body weights were recorded every week. After eight weeks from the start of the study, rats were subjected to 12 h of fasting and then killed by removing the blood through the abdominal aorta under a slight ether anesthesia. Livers were also excised.

Serum lipid and enzyme activity measurements

The blood was collected through the abdominal aorta. Serum was assayed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by standard enzymatic procedures. The serum concentrations of triglycerides (TG) and total cholesterol (TC) were measured spectrophotometrically (Hitachi Clinical Spectrophotometer 7010 with X-Y Auto sampler) using test kits (Wako Pure Chemicals).

Histological analysis

The livers were quickly removed and weighed after killing the rats. A part of the largest liver lobe was fixed in a 40% corn oil neutral buffered formalin solution and embedded in paraffin for hematoxylin-eosin (HE) staining. The steatosis, inflammation, and necrosis in the hepatocytes were evaluated under light microscopy. The histological changes were graded according to the following criteria as described by Fan et al. The severity of steatosis was graded on the basis of the extent of parenchyma involved. Grade 1 (+): <33% of hepatocytes were involved. Grade 2 (++): 33% to 66% of hepatocytes were involved. Grade 3 (+++): >66% of hepatocytes were involved. Normal (-): no hepatocytes were involved. The hepatic inflammation scores were used to determine the hepatic necroinflammatory activity scored by the severity of portal inflammation (P), intralobular inflammation (I), piecemeal necrosis (PN) and bridging necrosis (BN). The score from 1 to 4 was in accordance with the severity of lesions and the total score was calculated as P+L+2 (PN+BN). Pathology was scored in a blinded manner by an expert in rodent liver pathology.

Statistical analysis

Data are represented as mean±SD. The level of significance for differences between the groups was tested using the Student’s t test and Ridit test. Differences of P<0.05 were considered significant.

RESULTS

Body weight and liver/body weight ratio

There was no death in the three groups. The final mean body weights of the rats were 335.4±43.4 g for the control group, 379.4±16.8 g for the model group and 341.3±32.8 g for the treatment group; there was no significant difference in body weight between the groups. However, the liver/body weight ratio was higher in the model group rats than that in the control group (P<0.01). The liver/body weight ratio of rats treated with Sinai san decreased significantly than that in the model group (P<0.05, Table 1).

| Groups          | n  | Body weight (g) | Liver/body weight ratio (%) |
|-----------------|----|----------------|-----------------------------|
| Control group   | 9  | 335.4±43.4      | 2.38±0.18                   |
| Model group     | 9  | 379.4±16.8      | 3.45±0.21                   |
| Treatment group | 9  | 341.3±32.8      | 2.99±0.12                   |

P<0.05 vs the model group; *P<0.01 vs the control group.

Serum lipid measurements

In the model group serum total cholesterol (TC) and triglyceride (TG) levels were significantly higher than that in the control group (P<0.01). There was no significant difference of the serum TC level on treatment with Sinai san. On the other hand, the serum TG level was significantly lower in the treatment group with Sinai san than that in the model group (P<0.05, Table 2).

| Groups          | n  | TG (mmol/L) | TC (mmol/L) |
|-----------------|----|-------------|-------------|
| Control group   | 9  | 0.77±0.17   | 0.38±0.09   |
| Model group     | 9  | 5.23±1.14a  | 4.46±1.21a  |
| Treatment group | 9  | 2.76±1.06b  | 3.34±1.02b  |

P<0.05 vs the model group; *P<0.01 vs the control group.

Enzyme activity measurements

In the model group serum ALT and AST levels were 72.92±14.22 U/L and 113.65±21.16 U/L respectively after 8 wk of high-fat diet, which increased significantly over the control group (39.01±5.56, 83.12±7.88). In contrast, Sinai san decreased serum ALT and AST levels significantly over the model group (P<0.05), and they were close to the control group. (Table 3).

| Groups          | n  | ALT (U/L) | AST (U/L) |
|-----------------|----|-----------|-----------|
| Control group   | 9  | 39.01±5.56 | 83.12±7.88 |
| Model group     | 9  | 72.92±14.22| 113.65±21.16|
| Treatment group | 9  | 50.80±11.76| 89.23±21.20|

P<0.05 vs the model group; *P<0.01 vs the control group.

Pathological changes

Gross appearance of the liver of the control group rats...
Table 4 Effects of Sinai san decoction on the degree of fatty changes and hepatic inflammation score (mean±SD)

| Groups            | n | Degree of Fatty Changes | Hepatic Inflammation Score |
|-------------------|---|-------------------------|----------------------------|
| Control group     | 9 | 0                       | 0.52±0.62                  |
| Model group       | 9 | 0                       | 4.96±1.82                  |
| Treatment group   | 9 | 0                       | 3.23±0.38<sup>b</sup>      |

<sup>a</sup>P<0.05 vs the model group; <sup>b</sup>P<0.01 vs the control group.

DISCUSSION

Fatty liver (hepatic steatosis) is a common clinical finding caused by the accumulation of triglyceride droplets within individual hepatocytes, which is characterized by steatosis, inflammation, necrosis, and ultimately fibrosis and cirrhosis<sup>3</sup>. Excess body weight is the primary predictor of fatty liver<sup>0,18</sup>. Other factors such as diabetes, medications, nutrient deficiencies, and genetic abnormalities in lipid metabolism also contribute to the development of hepatic steatosis. Risk factors for fatty liver generally overlap those for nonalcoholic steatohepatitis (NASH)<sup>11</sup>. Fatty liver is discovered in the setting of persistently elevated ALT levels or AST levels; a liver biopsy is often warranted to diagnose NASH<sup>45</sup>. NASH is a common cause of liver enzyme abnormalities and can lead to progressive liver disease and cirrhosis. Approximately 15% to 40% of NASH patients develop hepatic fibrosis, a precursor to cirrhosis<sup>13</sup>. It is crucial to prevent the progress of NASH. However, dietary fat enters the circulation as triglycerides and lipoprotein secretion in endoplasmic reticulum decreases.

The link between the accumulation of fat in the liver and development of NASH is the generation of free radicals. In the liver CCL<sub>4</sub> is decomposed as CCL<sub>4</sub>- via microsomal cytochrome P450 reductase and NADPH-dependent reductive pathways. The formation of CCL<sub>4</sub>- and CCL<sub>4</sub> directly attack the microsomal membrane of the liver cells and cause lipid peroxidation, which subsequently produces severe hepatocellular damage. The oxidation and disposal of fatty acid in mitochondrion and lipoprotein secretion in endoplasmic reticulum decreases. At the same time, the administered high-fat diet may cause fat to accumulate in the liver in the form of triglyceride and the development of cell injury, inflammation, and fibrosis. The present study demonstrates that in this model there are typically elevated aminotransferase levels and a liver biopsy characterized by steatosis, inflammation and necrosis. It is also shown that this model is consistent with clinical finding.

In this study, Sinai san significantly decreased liver/body weight ratio, serum ALT and AST levels, and markedly ameliorated the hepatic inflammation. It is suggested that Sinai san has the inhibitory effect on the development of steatohepatitis. It may result from the protection of Sinai san to liver cell, and reduction of persistent liver injury induced by CCl<sub>4</sub> combined with a fat-rich diet. However,
although the hepatic inflammation in the treatment group decreased, they were still higher than those of the control group. And the degree of hepatocyte steatosis was similar to that of model group. It was shown that Sinai san could ameliorate the hepatic inflammation of rats with steatohepatitis in this model, but did not prevent the development of hepatocyte steatosis. It is possible that the treatment with Sinai san was done when on persistently high-fat diet. So it only partly improves the state of illness, if the inducements of fatty liver are not removed. Moreover, the four weeks treatment duration is possibly too short to completely improve the pathologic changes. The clinical application of Sinai san should get rid of the inducements and pathogenesis, and be modified according to symptoms.

In summary, Sinai san decoction prevents liver injury, decreasing inflammation caused by small dosage of CCL4 combined with a fat-rich diet in the rats. Sinai san is already used in patients to treat chronic hepatitis and cholecystitis with little or no side effects. The results of this study give a possible new evidence for Sinai san in the clinical prevention/treatment of non-alcoholic steatohepatitis.

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