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• *BRIEF REPORTS* •

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

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Abstract

AIM: To explore the effect of Sinai san decoction on the development of non-alcoholic steatohepatitis induced by CCL_4 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 CCL_4 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 CCL_4 combined with a fat-rich diet, but does not prevent the development of hepatocyte steatosis.

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

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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[3]. 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 CCL_4 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 above were decocted in water twice, removing the sediments and condensing into 100% Sinai san decoction. CCL_4 (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 CCl_4 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 g/L 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 (L), 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).

Table 1 Effects of Sinai san decoction on the body weight and liver/body weight ratio (mean±SD)

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[b]
Treatment group	9	341.3±32.8	2.99±0.12[a]

[a]P<0.05 *vs* the model group; [b]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, Table2).

Table 2 Effects of Sinai san decoction on the level of serum lipid (mean±SD)

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

[a]P<0.05 *vs* the model group; [b]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).

Table 3 Effects of Sinai san decoction on the level of serum enzyme activity (mean±SD)

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[b]	113.65±21.16[b]
Treatment group	9	50.80±11.76[a]	89.23±21.20[a]

[a]P<0.05 *vs* the model group; [b]P<0.01 *vs* the control group.

Pathological changes

Gross appearance of the liver of the control group rats

displayed normal configuration; however, in the model group and treatment group the livers showed some expansion, along with a full, oiled surface and a slight yellowish color change. Under light microscopy, in the control group, there were no obvious pathological changes. In the model group, the fat deposition was marked, with small (smaller than liver cells) and large (larger than liver cells) fatty droplets mainly in the central vein area. Fat accumulation caused ballooning of hepatocytes and narrowing of the sinusoidal space. Focal neutrophilic infiltrates can be found in areas of focal necrosis; mononuclear cell infiltration is a component of the inflammation. As expected, CCL_4 combined with a fat-rich diet for 8 wk caused severe fatty infiltration, inflammation, and focal necrosis. In the treatment group, the fat deposition was also marked, with small fatty droplets outnumbering large fatty droplets. Fatty degeneration was less severe in the treatment group than in the model group, but there was no significance for statistical analysis. However, the number of inflammatory and necrotic foci decreased in the treatment group. The hepatic inflammation score from rats treated with Sinai san was significantly lower than that in the model group ($P<0.05$, Table 4).

Table 4 Effects of Sinai san decotion on the degree of fatty changes and hepatic inflammation score (mean±SD)

Groups	*n*	Degree of Fatty Changes				Hepatic Inflammation Score
		-	+	++	+++	
Control group	9	9	0	0	0	0.52±0.62
Model group	9	0	0	4	5	4.96±1.82[b]
Treatment group	9	0	0	6	3	3.23±0.38[ab]

[a]$P<0.05$ *vs* the model group; [b]$P<0.01$ *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[8]. Excess body weight is the primary predictor of fatty liver[9,10]. 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)[11]. Fatty liver is discovered in the setting of persistently elevated ALT levels or AST levels; a liver biopsy is often warranted to diagnose NASH[8]. 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[3]. It is crucial to prevent the progress of NASH. However, dietary fat enters the circulation as triglycerides incorporated in chylomicrons and is not an immediate source of fat in the liver. The link between the accumulation of fat in the liver and development of cell injury, inflammation, and fibrosis that characterize NASH remains to be firmly established, and so there is absence of specific and effective treatments. At present, Dongbaogantai is a medicine in generally treating fatty liver, but Zhu *et al* reported that it had no effect on NASH in rats fed with a fat-rich diet. Clinical investigation showed that Sinai san might improve the symptom of fatty liver patients. Therefore, it is of interest to identify therapies that target NASH formation and prevent liver injury that can be used safely in humans.

Sinai san takes its source at *Shanghan Lun* (*Treatise on Febrile Disease*), which is composed of Chai Hu (Radix Bupleuri), Shao Yao (Radix Paeoniae), Zhi Shi (Fructus Aurantii Immaturus), and Gan Cao (Radix Glycyrrhizae). It is a common recipe in the treatment of liver diseases. Originally this recipe is used to treat cold limbs caused by inward shift of pathogenic heat and accumulation and obstruction of Yang-qi. Physicians of later generations have expanded its application and used it to deal with various syndromes due to disharmony between the liver and spleen. Chai Hu is a monarch drug with the action of releasing the stagnated liver-qi. Shao Yao functions as a minister drug to preserve yin with astringent and nourish blood and the liver. Zhi Shi can regulate liver-qi to remove stagnation and regulate qi movement of the liver and spleen when in compatibility with Chai Hu, acting as adjuvant drug. Gan Cao serves as a guiding drug, which can relieve spasm in combination with Shao Yao, and regulate the middle-energizer in combination with Zhi Shi, and coordinate the properties of other drugs. The Chai Hu and Shao Yao in compatibility are meant to regulate the liver, while the Zhi Shi and Gan Cao to regulate the spleen. And all together constitute the result of regulating the middle-energizer as well as the flow of qi. Researches have shown that Sinai san alleviate experimental liver injury, which has protective effects on hepatocyte membrane and affect immunocyte activation[12]. At present, although there are lots of researches on Sinai san to improve liver injury, the experimental study of Sinai san treating NASH is not reported. The NASH model of small dosage of CCL_4 combined with a fat-rich diet was selected for the study. CCL_4 is a well-known hepatotoxic chemical. The main cause of liver damage by CCL_4 is the generation of free radicals. In the liver CCL_4 is decomposed as CCL_3^- and CL^- via microsomal cytochrome *P*450 reductase and NADPH-dependent reductive pathways. The formation of CCL_3^- and CL^- 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 levers, 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_4 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 CCL_4 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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