Original Article

Protective effect of Sheng-jiang powder on nonalcoholic fatty liver disease induced by high fat diet through AMPK/mTOR signaling pathway in rats

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Abstract: Nonalcoholic fatty liver disease (NAFLD) has become a global public health problem, but its specific mechanism remains unclear and lacks effective therapeutic drugs. Sheng-jiang powder (SJP) has shown great potential for prevention and treatment of NAFLD in Chinese clinical reports, but the study of specific curative effects and mechanisms are still lacking. The purpose of this study was to observe the effect of SJP on high fat diet-induced rat NAFLD and to explore its underlining mechanisms. After 4 weeks of normal diet or high-fat diet, SD rats were randomly divided into the following four groups and each was given intragastric administration: Normal control group, NAFLD group, Sheng-jiang powder group and Fenofibrate group. All rats were sacrificed after 4 weeks’ gavage. Blood was used to detect serum triglyceride (TG), total cholesterol (TC), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Homogenized liver tissue was used to determine liver TG and TC contents. HE staining and oil red O staining were performed to show liver pathological changes. AMP-activated protein kinase alpha (AMPKα), p-AMPKα, mammalian target of rapamycin (mTOR) and p-mTOR protein expression in liver tissue were tested by western blot. The results showed that SJP significantly reduced the body weight, serum TG level, TG and TC content in liver of NAFLD rats, and markedly improved hepatic steatosis. SJP increased the expression of p-AMPKα protein and decreased the expression of p-mTOR protein. These results indicate that SJP can improve serum lipid and hepatic steatosis in rats with NAFLD induced by high-fat diet, and its effect may be achieved through regulating AMPK/mTOR signal pathway, inhibiting lipid synthesis and reducing hepatic lipid deposition.

Keywords: NAFLD, Sheng-jiang powder, AMPK/mTOR signal pathway, lipid synthesis

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a multifactor-induced complex clinical pathology syndrome, which is not an independent disease. According to its long-term pathological progress, it is generally divided into four types, of which type 1 (steatosis) and type 2 (lipid necrosis) are referred to as non-alcoholic fatty liver, while type 3 (steatohepatitis) and type 4 (steatohepatitis with fibrosis) are called non-alcoholic steatohepatitis [1-4]. With the increase of people having high-fat or high-sugar diet, the incidence of NAFLD is rising year by year [5]. A recent meta-analysis showed that the global prevalence of NAFLD has reached 25.24%, and the proportion of progression to fibrosis in non-alcoholic steatohepatitis is as high as 40.76% [6]. Although the early main manifestation of NAFLD is simple fatty liver, it could progress toward steatohepatitis, hepatic fibrosis, cirrhosis, and even hepatocellular carcinoma later without intervention [7-10]. Moreover, once entering the cirrhosis stage, the course of the disease will be irreversible, and liver transplantation will be required in the later stage. This undoubtedly causes serious clinical and economic burden. In addition, NAFLD can significantly increase the risk of metabolic complications such as type II diabetes mellitus [11-
14], obesity [15, 16], hyperlipidemia [17], and hypertension [6, 18]. These metabolic complications and NAFLD can promote mutually, and the metabolic conditions will deteriorate, causing low life quality and life expectancy of the patients. Due to the complicated etiology of NAFLD, its pathogenesis has not yet been fully elucidated and there are no specific drugs for the disease. Therefore, strengthening the prevention and treatment of NAFLD, seeking new ideas and new targets for treating the diseases, are very important to human life and health.

The current main treatment for NAFLD is symptomatic treatment. First, diet control [19] and proper exercise, but patients often have poor compliance, resulting in disease progression to liver fibrosis, liver cirrhosis, and even related hepatocellular carcinoma that will result in a very poor prognosis. Second, to the patients with severe hyperlipidemia, give lipid-lowering drugs, such as fibrates, but the majority of lipid-lowering drugs have severe long-term side effects, and the elimination of intrahepatic lipids is limited. The deposition of large amounts of triglyceride and other lipid components in hepatocytes is an early manifestation of NAFLD, and intervention in the early stage of the disease is an important step in preventing its progression to a more severe stage.

Compared with the current western medicine treatment of NAFLD, a large number of studies have shown that traditional Chinese medicine has a unique advantage of overall conditioning and fewer side effects. The traditional Chinese medicine compound “Sheng-jiang powder” (SJP) is a famous prescription highly praised by traditional Chinese medicine experts. It is derived from the famous Chinese medical book “Shang Han Wen Yi Tiao Bian”, and was composed of Bombyx batryticatus, periostracum cicada, curcuma longa, rhubarb. Bombyx batryticatus (1604017), periostracum cicada (1508058), curcuma longa (1609006) and rhubarb (1601078) were purchased from Neo-green pharmaceutical technology development Co., Ltd (Sichuan, China). According to clinical evidence, the research group adjusted the SJP prescription (decoction pieces) to: Bombyx batryticatus (24 g), periostracum cicada (12 g), curcuma longa (6 g), rhubarb (24 g). According to the dose conversion relationship between rats and humans, the decoction pieces dosage for rats were converted, and the amount of decoction pieces was converted into the corresponding dose of decoction-free dosage: Bombyx batryticatus (225 mg/kg/day), periostracum cicada (104 mg/kg/d), curcuma longa (375 mg/kg/d), rhubarb (333 mg/kg/d). The corresponding dose of drug was dissolved in distilled water. Fenofibrate was purchased from Abbott Pharmaceuticals Co., Ltd. (Chicago, USA). According to previous experience [26, 27], the dose of fenofibrate was 100 mg/kg/d. Fenofibrate was mixed as suspension with sodium carboxymethyl cellulose.

Materials and methods

Preparation of Sheng-jiang powder and fenofibrate

Sheng-jiang powder (SJP) was derived from the famous Chinese medical book “Shang Han Wen Yi Tiao Bian”, and was composed of Bombyx batryticatus, periostracum cicada, curcuma longa, rhubarb. Bombyx batryticatus (1604017), periostracum cicada (1508058), curcuma longa (1609006) and rhubarb (1601078) were purchased from Neo-green pharmaceutical technology development Co., Ltd (Sichuan, China). According to clinical evidence, the research group adjusted the SJP prescription (decoction pieces) to: Bombyx batryticatus (24 g), periostracum cicada (12 g), curcuma longa (6 g), rhubarb (24 g). According to the dose conversion relationship between rats and humans, the decoction pieces dosage for rats were converted, and the amount of decoction pieces was converted into the corresponding dose of decoction-free dosage: Bombyx batryticatus (225 mg/kg/day), periostracum cicada (104 mg/kg/d), curcuma longa (375 mg/kg/d), rhubarb (333 mg/kg/d). The corresponding dose of drug was dissolved in distilled water. Fenofibrate was purchased from Abbott Pharmaceuticals Co., Ltd. (Chicago, USA). According to previous experience [26, 27], the dose of fenofibrate was 100 mg/kg/d. Fenofibrate was mixed as suspension with sodium carboxymethyl cellulose.
Animals and treatment

Thirty-six male Sprague-Dawley rats (100-120 g) were purchased from Chengdu Dashuo Experimental Animal Co., Ltd (Chengdu, China) and housed in regular cages and allowed free access to food and water. The animals were situated in a temperature and humidity controlled room (21.0 ± 2.0°C and 65 ± 5% humidity, respectively) and maintained on a 12-h light/12-h dark cycle. After 1 week of acclimatization, twelve rats were randomly selected as normal control group (NOR) and given basal diets (Chengdu Dashuo Experimental Animal Co., Ltd, Chengdu, China), while the remainder were given a high-fat diet (79% basal diet + 10% egg yolk + 10% lard + 1% cholesterol, Chengdu Dashuo Experimental Animal Co., Ltd, Chengdu, China) as nonalcoholic fatty liver disease group (NAFLD). After 4 weeks, 6 basal diet rats and 6 high-fat diet rats were randomly selected and anesthetized with 1% sodium pentobarbital (3-5 ml/kg) by intraperitoneal injection, then the liver tissue was harvested and stained with Oil red O and HE. The pathological changes of liver tissue were observed under microscope to determine whether the rat model of NAFLD was established successfully.

After successful establishment of the model, the remaining basal diet rats were used as normal control group (NOR, basal diet, n = 6), and the remaining high-fat diet rats were randomly divided into NAFLD group (NAFLD, high-fat diet, n = 6), SJP group (SJP, high-fat diet plus SJP, n = 6), fenofibrate group (FNBT, high-fat diet plus fenofibrate, n = 6). The intervention started at the end of 4th week, SJP group was given SJP by gavage. The FNBT group was given fenofibrate, and the other two groups were given normal saline with a volume of 1 ml/100 g once per day. After continuous gavage for 4 weeks, all rats were anesthetized by intraperitoneal injection of 1% sodium pentobarbital (3-5 ml/kg), blood and liver tissues were used for follow-up measurements. All experiments were carried out according to the guidelines approved by the Animal Ethics Committee of Southwest Medical University.

Serum lipid and liver function determination

The collected blood was centrifuged and serum was collected. Serum triglyceride (TG), total cholesterol (TC), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured by an automatic biochemical analyzer.

Determination of triglyceride and total cholesterol in liver tissue

After the liver tissue was mixed with absolute ethanol (liver quality:absolute ethanol volume = 1:9) and thoroughly homogenized, the triglyceride and total cholesterol in liver tissue were measured using the triglyceride (TG) assay kit (Nanjing Jiancheng Institute of Biotechnology, A110-2 GPO-PAP Enzyme Method, Single Reagent Type, Nanjing, China) and total cholesterol (T-CHO) assay kits (Nanjing Jincheng Institute of Biotechnology, A111-2, COD-PAP Enzyme Method, Single Reagent Type; Nanjing, China) according to manufacturer's instructions.

Hematoxylin and eosin staining

The 4% phosphate-buffered paraformaldehyde-fixed small pieces of liver were routinely processed and were cut into 3 μm serial sections. The sections were dewaxed in xylene and rehydrated in ethanol gradients and then stained with hematoxylin-eosin (H&E, Beyotime, China) for histological evaluation. Lipid in hepatocytes was dissolved and fat vacuoles appeared during processing, so hematoxylin and eosin staining can indirectly reflect the degree of lipid accumulation and hepatic steatosis through fat vacuoles. Finally, sections were imaged using a light microscope (Eclipse 80i, Nikon, Japan) at 200× magnification.

Oil Red O staining

Some of fresh liver tissues were made of frozen sections (7 um) and stained with Oil Red O (Solarbio, China) for 20 min to further reveal hepatic steatosis. Lipids were stained to red by Oil Red O, and the red lipid drops in hepatocytes directly reveal the degree of lipid accumulation and hepatic steatosis. Images were taken ditto.

Immunohistochemistry

For immunohistochemistry (IHC), the steps of dewaxing and rehydration were similar to HE staining. IHC was performed by using rabbit SP kit (SP-9001, ZSGB-BIO, China). After goat
serum blocking, the slices were incubated with rabbit anti-rat antibody against p-AMPKα (1:100, Cell Signaling Technology, Danvers, USA) at 4°C overnight and then combined with a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit antibody). At last, the antibody binding was visualized using DAB kit (ZLI-9033, ZSGB-BIO, China). The purpose of immunohistochemistry is to show the quantity and distribution of p-AMPKα protein in liver. Images were taken at 200× magnification (ditto) to present the expression of p-AMPKα in hepatocytes.

Western blotting

Fifty milligrams of liver tissue were obtained from each rat, which were ground into liver homogenate. Equal amounts of protein lysates from rat liver were resolved in SDS-PAGE gel and transferred to PVDF membranes (Milipore, USA). After 1-h blocking with 5% nonfat dried milk, antibodies against AMPKα, p-AMPKα, mTOR and p-mTOR (1:1000, Cell Signaling Technology, Danvers, USA) were added to identify AMPKα, p-AMPKα, mTOR and p-mTOR, respectively, at 4°C overnight. Subsequently, the membranes were incubated with the corresponding secondary antibody (horseradish peroxidase-conjugated secondary anti-rabbit antibodies, 1:5000; ZSGB-Bio, Beijing, China) at room temperature for 1 h. Antibody binding was visualized using ChemiDoc XRS + system (Bio-Rad, USA). Gray intensity of western blot signal band was calculated with Image J 1.47 V software (NIH, USA).

Statistical analysis

Data analysis was performed using SPSS 21.0 statistical software. The experimental results were expressed as x ± s. An independent sample t-test was used for comparison between the two groups, while one-way analysis of variance (ANOVA) was used when groups are more than 2, and LSD was used for comparison between the two groups. P<0.05 was considered statistically significant.

Results

High-fat diet for 4 weeks leads to massive lipid deposition in rat hepatocytes

We weighed rats and performed liver HE staining and Oil Red O staining. From the comparison of body weight at the same time point, it can be seen that the body weight of NAFLD group slightly increased, but it was not statistically significant (Figure 1). According to the liver HE staining, white bubbles were found in the cytoplasm of high-fat diet induced rats (Figure 2). The white bubbles in cytoplasm were caused by the dissolution of accumulated lipid during paraffin embedding, which indirectly represents lipid deposition levels in pathologic liver. To further confirm the lipid deposition in NAFLD group, Oil Red O staining was performed (Figure 2). The results demonstrated that NAFLD group showed hepatocytes with lipid accumulation.

SJP decreases body weight and serum TG levels in NAFLD rats

We weighed rats after SJP and fenofibrate intervention and found that the weight gain of rats in the NAFLD group was faster than the other three groups. Compared with the other three groups, the body weight of NAFLD group increased significantly in the 3rd and 4th week after the intervention, indicating that body weight decreased significantly after SJP and fenofibrate intervention (Figure 3). We also detected serum triglyceride (TG), total cholesterol (TC), aspartate transaminase (AST) and alanine aminotransferase (ALT) levels of rats after gavage. Compared with the NOR group, the serum TG levels in the NAFLD group increased significantly, and the TG levels decreased significantly after intervention with SJP and fenofibrate (Figure 4A). There were no
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In order to study the effect of SJP on lipid content in rat liver tissue, we examined the content of TG and TC in liver tissue of NAFLD group. Compared with the NAFLD group, the liver TG and TC levels in the SJP group and the FNBT group were significantly lower, and the SJP group was the lowest.

**SJP improves hepatic steatosis in NAFLD rats**

In order to show the effect of SJP on hepatic steatosis in NAFLD rats, we performed liver HE staining and Oil Red O staining (Figure 6). From the HE staining and Oil Red O staining, it can be seen that there is a large amount of lipid accumulation in the cytoplasm of rat hepatocytes in the NAFLD group. Hepatic cytoplasmic lipids were rare in SJP rats and liver steatosis was significantly improved compared with NAFLD group. Hepatic steatosis in FNBT group also improved compared with NAFLD group, but the degree was less than SJP group.

**SJP increases the expression of p-AMPKα protein and decreases the expression of p-mTOR protein**

Immunohistochemistry (Figure 7) and Western blot (Figure 8A, 8B) showed that the expression of p-AMPKα protein in the liver of the rats of NAFLD group was significantly reduced, and the expression of p-AMPKα protein was significantly increased after intervention of SJP and fenofibrate. The expression of p-mTOR protein (Figure 8C, 8D) in the liver of NAFLD rats was significantly increased, and the expression of p-mTOR protein was significantly decreased after intervention of SJP. No significant recovery was observed after the intervention with fenofibrate.

**Discussion**

In this study, we found that after 8 weeks of high-fat diet, the body weight of the model group increased significantly. Serum TG and liver tissue TG, TC levels were significantly increased. Significant differences in TC, ALT and AST levels between groups (Figure 4B-D).

**SJP decreases the content of TG and TC in liver tissue of NAFLD rats**

In order to study the effect of SJP on lipid content in rat liver tissue, we examined the content of TG and TC in liver tissue (Figure 5). Compared with NOR group, the contents of TG and TC in liver tissue of NAFLD group were significantly increased. Compared with the NAFLD group, the liver TG and TC levels in the SJP group and the FNBT group were significantly lower, and the SJP group was the lowest.
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Increased, and a large number of lipids accumulated in the liver cells. After SJP intervention, rats' body weight and serum TG levels were down-regulated, liver tissue TG and TC contents were significantly decreased, and liver tissue pathological changes were significantly restored. The above results show that SJP can regulate blood lipids and improve hepatic steatosis in rats, which has a therapeutic effect on rat NAFLD.

According to traditional Chinese medicine theory, the basic pathogenesis of high-fat diet-induced NAFLD is a dysfunction of “Qi”. Spleen dysfunction leads to abnormal motion of “Qi”, further causing “Qi” stagnation, phlegm retention, and blood stasis, which finally induces NAFLD. SJP improves NAFLD by regulating functional activity of “Qi”, ascend lucidity and descend turbidity. Evidence showed that curcuma longa and rhubarb have a role in lowering blood lipids. Some researchers have found that SJP plus bupleurum root and citrus aurantium can reduce serum TG, TC, and LDL levels in hyperlipidemic rats, and regulate lipid metabolism disorders. NAFLD is closely linked with hyperlipidemia, suggesting that SJP may have a treatment effect on NAFLD by regulating lipid metabolism. The results of this study also showed that SJP can reduce serum TG levels, which again confirms that therapeutic effect of SJP on NAFLD is related to its regulation of lipid metabolism.

In this study, the established NAFLD model was in the early stage. At present, diet control and proper exercise are mainly suggested in the early stage of NAFLD. However, the compliance is not satisfactory, and the patients with severe hyperlipidemia are treated with lipid-lowering therapy. Fenofibrate is a common lipid-lowering drug, which mainly reduces serum triglyceride levels. Considering the main characteristics of NAFLD is the dysfunction of triglycerides metabolism, this study set the fenofibrate group as positive control group. The results of this study showed that after intervention with fenofibrate, rats' body weight, serum TG, liver tissue TG and TC, and liver pathological changes all recovered to varying degrees, but the decrease degree of TG and TC in liver tissue were not as significant as SJP group. Similarly, the pathological recovery of liver tissue is also not as obvious as SJP group. The above results suggest that fenofibrate has a significant effect in reducing blood lipids, but it is not ideal for the reduction of intrahepatic

Figure 4. SJP reduces serum TG levels in NAFLD rats significantly. A. The content of serum TG; B. The content of serum TC; C. The content of serum ALT; D. The content of serum AST. *P<0.05 versus NOR group, #P<0.05 versus NAFLD group.

Figure 5. SJP reduces liver TG and TC levels in NAFLD rats significantly. A. The content of liver TG; B. The content of liver TC. **P<0.01 versus NOR group; ###P<0.01 versus NAFLD group; ΔΔP<0.01 versus FNB group.
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As a traditional Chinese medicine compound, SJP may be better than fenofibrate in regulating the overall lipid metabolism in NAFLD.

The pathogenesis of NAFLD is not yet fully understood, but lipids such as triglycerides caused by various causes are deposited in large numbers of hepatocytes, resulting in steatosis of hepatocytes. This is one of the most widely recognized mechanisms [28-30]. AMP-activated protein kinase (AMPK) is the key to energy metabolism in the body [31-33]. It is activated by the low-energy state of the cell (phosphorylation). After activation, it triggers the downstream catalytic process to promote ATP production while inhibiting the anabolic metabolism of ATP, facilitating the restoration of cellular energy metabolism homeostasis. Energy metabolism and lipid metabolism are closely linked. Hong-Ai Zhang et al. [34] found in in vitro experiments that overexpression of activated AMPKα in hepatocytes caused a decrease of triglyceride levels in hepatocytes. Studies by Eunhui Seo et al. [35] showed that overexpression of activated AMPKα in the liver downregulated the expression of lipogenic genes in hyperlipidemia type 2 diabetic rat models, further reducing liver triglyceride levels, resulting in reduced hepatic steatosis in rats. This suggests that AMPKα may become a therapeutic target for NAFLD. In recent years, studies have shown that mTOR plays a key role in the promotion of lipid biosynthesis [36]. After mTOR is activated (phosphorylated), it regulates downstream molecules or helps regulate other related pathways to promote lipid biosynthesis [37, 38]. In addition, studies have shown that activation of

Figure 6. SJP improves hepatic steatosis in NAFLD rats (HE staining, Oil Red O staining, ×200). White vacuoles show lipids in HE staining, and red areas show lipids in Oil Red O staining (as shown by arrows).

Figure 7. SJP increases p-AMPKα protein expression in NAFLD rats (immunohistochemistry, ×200). A. NOR group; B. NAFLD group; C. SJP group; D. FNBT group. Brown-yellow shows positive expression.
AMPK has an inhibitory effect on p-mTOR [39]. Therefore, this study focuses on the above pathogenesis and possible therapeutic targets, exploring the mechanisms of SJP’s treatment effect on NAFLD. The results of this study showed that the expression of p-AMPKα protein in the liver of NAFLD rats was significantly lower than that in normal rats, and the expression of p-mTOR protein was significantly increased. After SJP intervention, the expression of p-AMPKα protein was significantly increased, and the expression of p-mTOR protein was significantly decreased. This suggests that SJP may treat rat NAFLD by regulating the AMPK/mTOR signaling pathway, inhibiting lipid synthesis, and reducing hepatic lipid deposition.

In this study, we also found that after the intervention of fenofibrate, the expression of p-AMPKα in liver recovered, but p-mTOR did not fall back. This suggests that fenofibrate treatment of NAFLD may be related to the up-regulation of p-AMPKα, but p-mTOR is not much affected.

In conclusion, this study shows that SJP can improve the lipid and hepatic lipid accumulation of rat NAFLD induced by high-fat diet, and its therapeutic effect may be achieved by regulating the AMPK/mTOR signaling pathway. In this study, the mechanisms of SJP in the treatment of NAFLD is preliminarily explored, and the mechanisms of SJP will be further confirmed and thoroughly studied at the cellular level through the AMPK/mTOR pathway inhibitors and other methods in the following studies. In addition, we will look for effective ingredients to further enhance and optimize the prescription so as to provide new methods and new targets for clinical treatment of NAFLD.

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Disclosure of conflict of interest

None.
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