Research Article

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Study on the Mechanism of Shugan Xiaozhi Fang on Cells with Non-alcoholic Fatty Liver Disease

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Abstract: Cells with non-alcoholic fatty liver disease (NAFLD) were studied to determine the mechanism of liver deficiency via the AdipoR2-PPARα pathway. NAFLD cells were randomly divided into a normal control group, blank control group, model group, low dose group, medium dose group, and high dose group. The NAFLD models were established by incubating the cells with linoleic acid (LA) and palmitic acid (PA) (2:1) for 24 h. The test groups were incubated with different doses of Shugan Xiaozhi Fang extract. The pathological changes in cells that accumulated lipids were detected by Oil Red O staining. Malondialdehyde (MDA) and triglyceride (TG) levels were measured. The apoptosis of cells was evaluated by flow cytometry. The levels of AdipoR2, PPARα, CD36, acyl-CoA mRNA, and protein were confirmed by RT-PCR and Western blot. The results of the Oil Red O staining demonstrated that the NAFLD cell model was successfully established. Compared with the model group, the levels of TG and MDA in the groups that received low, medium, and high doses of Shugan Xiaozhi were significantly lower ($P < 0.01$), and a dose effect was evident. In addition, the expression of AdipoR2, PPARα, CD36, acyl-CoA protein, and mRNA in the Shugan Xiaozhi-treated groups was upregulated. Furthermore, the levels of AdipoR2, PPAR, CD36, acyl-CoA protein, and mRNA in all drug treatment groups that were extracted from L-02 normal human hepatocytes were

significantly upregulated ($P < 0.01$). Moreover, the factor pattern of HepG2 human liver carcinoma cells was similar to that of L-02. The levels of AdipoR, CD36, acyl-CoA, and AdipoR mRNA in the HepG2 low group were increased ($P < 0.05$). AdipoR, PPAR, CD36, and acyl-CoA protein levels and AdipoR mRNA expression were significantly increased in the intermediate dose group and high dose group ($P < 0.01$). Shugan Xiaozhi Fang attenuates hepatic lipid deposition in NAFLD induced by incubating with LA and PA for 24 h, which is associated with the activation of the AdipoR2-PPARα pathway.

Keywords: Shugan Xiaozhi Fang; Non-alcoholic Fatty Liver disease; AdipoR2-PPARα pathway; Lipid metabolism.

1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is severely debilitating the health of people in China with increasing frequency. Currently, a two-hit hypothesis has been widely accepted, which proposes that an increase in free fatty acid and accumulation of fat in the liver are responsible for the pathogenesis of NAFLD [1, 2]{Day, 1998 #1}.

At present, the research on the treatment of NALFD with traditional Chinese medicine mostly starts with dampness, phlegm and heat, and seldom considers qi depression (it is a physical state characterized by introversion and instability, melancholy and fragility, sensitivity and suspicion, which is caused by long-term emotional disorder and stagnation of qi) as an important pathogenic factor of NALFD [3]. This study is based on clinical findings. It is found that the disease is mainly caused by emotional discomfort, liver failure, spleen failure, phlegm-dampness heat, or blood stasis and other pathological factors accumulated in the liver. Finally, “depression”, “heat” and “blood stasis” are intertwined, and “liver stagnation and spleen deficiency” are the main clinical syndromes [3].

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Shugan Xiaozhi Fang has been found to significantly improve the clinical manifestations and laboratory indices of NALFD patients; however, the mechanism is still not clear. Based on a previous study, the influences of Shugan Xiaozhi Fang on the expression of lipid metabolic factors and relevant receptors in hepatocytes with steatosis were observed, and the effects and possible metabolic interaction with NAFLD cells were explored, which provides a theoretical basis for the mechanism of action of Shugan Xiaozhi Fang [3].

2 Materials and Methods

2.1 Preparation of Serum Containing Drugs

Sprague-Dawley rats were acclimated for 1 week, and randomly divided into a blank group, and Shugan Xiaozhi Fang (Guangdong Institute of Traditional Chinese Medicine Engineering Technology, Guangdong, China) high, medium, and low dose groups ($n=3$) according to body weight. The rats in the high, medium, and low dose groups received Shugan Xiaozhi Fang at doses of 20, 15, and 10 g/kg by gavage twice daily for 3 consecutive days, and those in the blank group were given distilled water (10 mL/kg) instead. On Day 4, the rats were given the corresponding drugs in the morning, and after 2 h, the rats received a second dose of Shugan Xiaozhi Fang that was a repeat of the first morning dose. Blood samples were collected from the abdominal aorta after 1 h, maintained at room temperature for 3–4 h, and then centrifuged at 3000 rpm/min under 4°C. The serum was collected, inactivated at 56°C in a water bath, filtered with a 0.22- μ m membrane, and stored at -70°C.

2.2 Cell culture

L-O2 (human fetal hepatocyte cells) and HepG2 (human liver carcinoma cells) cells (ATCC, VA, USA) were cultured with Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, NY, USA) and divided into a normal group, fenofibrate group (25 μ M), blank control group, model group, low dose group, medium dose group, and high dose group (Serum Containing Drugs in high, middle and low dose groups were derived from section 2.1). The cells in the normal control group were cultured with medium only, and those in the blank control group were cultured with medium containing 0.3% skim bovine serum

albumin (BSA) (Sigma-Aldrich, MO, USA). The cells in fenofibrate group (the drug control group) were cultured with medium containing 25 μ M fenofibrate. Cells in all groups were cultured for 48 hours. For the model and drug treatment groups, after the medium was discarded, a lipid deposition inducer was then added and reacted for 24 h.

2.3 Model establishment and identification

The L-O2 and HepG2 cells were cultured with (RPMI)-1640 medium (Gibco, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, NY, USA). When the growth of the cells was in the logarithmic stage, the cells were subcultured and divided into a normal group and model group. After being cultured for 24 h, the control group was incubated with RPMI-1640 medium containing 1% BSA, and the model group was incubated with linoleic acid/palmitic acid solution (LA:PA=2:1) for 24 h. Triglyceride (TG) and malondialdehyde (MDA) levels were measured and calculated according to the following equation:

$$\text{TG or MDA level (mmol/L)} = (\text{sample OD-blank OD}) / (\text{standard OD-blank OD}) \times \text{concentration of standard sample.}$$

The wavelengths for OD measurement are 450nm and 532nm respectively.

2.4 Oil Red O staining

Hepatocytes were grown on cover glasses. In the model group, the cells were cultured with high-fat medium (containing 200 μ M oleic acid and 100 μ M palmitic acid) for 24 h. The cells in the control group were cultured with medium containing 0.3% skim BSA for 24 h. The cover glasses were washed with sterile PBS, and fixed with 4% formaldehyde for 10 min. Afterwards, they were adequately washed, rinsed with 60% isopropanol, stained with Oil Red O (Beyotime Biotechnology, Shanghai, China) for 10 min, differentiated by 60% isopropanol until the mesenchyme became clear, and then washed with distilled water. After the cell nuclei were stained with hematoxylin, the cover glasses were adequately washed with distilled water again, and sealed with glycerin and gelatin. The lipid droplet distribution in the liver cells was observed by microscope.

2.5 Detection for Annexin V-FITC and PI apoptosis

The suspended cells were collected by centrifugation (2000 rpm, 5 min), and then digested with trypsin without EDTA. The cells were washed with phosphate-buffered saline (PBS) (Beyotime Biotechnology, Shanghai, China) twice, and collected by centrifugation (2000 rpm, 5 min). The cells (1.5×10^5) were then suspended in 500 μ L of binding buffer, mixed with 5 μ L of Annexin V-FITC, and 5 μ L of propidium iodide for 5-15 min at room temperature avoiding light, and finally were analyzed using flow cytometry.

2.6 Detection of dipoR2, PPAR α , CD36, and acyl-CoA mRNA levels

The cell precipitate was collected and digested with 0.8 mL of TRIzol reagent (Invitrogen, CA, USA). The homogenate was maintained at room temperature for 5-10 min, and a 0.2-fold volume of chloroform was added. After mixing for 15 s, the homogenate was centrifuged at $12000 \times g$ for 10 min at 4°C. The supernatant was transferred to a clear tube, and a 0.5-fold volume of isopropanol was added. After mixing, the solution was maintained at -20°C for 10 min and then centrifuged at $12000 \times g$ for 10 min under 4°C to separate the precipitate. The RNA precipitate was washed with 1 mL of 75% ethanol, and centrifuged at $7500 \times g$ for 5 min at 4°C to discard the supernatant. After the RNA precipitate was appropriately dried, RNase-free water was added to dissolve the RNA. The RNA was centrifuged at 2000 rpm for 20 s. The integrity of the RNA was determined by agarose gel electrophoresis.

Total RNA was reversely transcribed using a reverse transcription kit (Takara, Dalian, China) to obtain cDNA. A fluorescent quantitation PCR reaction system (Takara, Dalian, China) was used: cDNA 1 μ L, upstream primer 100 nM, downstream primer 100 nM, 2 $\times$ SYBR Green PCR Master mix 12.5 μ L, and then the volume was brought to 25 μ L with ddH₂O. Reaction conditions: 94°C for 3 min, 94°C for 30 s, 59°C for 30 s, 72°C for 45 s, 40 cycles total, 72°C for 5 min, and then the reactions were stored at 4°C.

2.7 Detection of AdipoR2, PPAR α , CD36, and acyl-CoA protein levels

The cells were collected, mixed with 1 mL of lysis buffer, blown, placed on ice for 10-20 min, blown for 5-20 min, and transferred into a 1.5-mL tube. The cells were ultrasonicated 3 times (3 s each time), and centrifuged

at 9000 rpm for 10 min. The obtained supernatant was transferred into a 1.5-mL tube, and stored at -20°C. The protein concentration was measured by bicinchoninic acid (BCA). After transformation, the membrane was soaked in 5% skim milk at 37°C for 2 h, and then washed with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) 2-3 times. Primary antibodies were added (goat anti-AdipoR2 antibody, mouse anti-PPAR α antibody, rabbit anti-CD36 antibody, rabbit acyl-CoA antibody, and mouse anti- β -actin antibody) (Abcam, MA, USA), and then the membrane was incubated at room temperature for 2 h. The membrane was washed with TBST 4 times, 5-10 min/time. Secondary antibody was added, and then the membrane was incubated at room temperature for 2 h. The membrane was washed with TBST 4 times, 5-10 min/time, exposed in a dark room, and developed using X-ray film.

2.8 Statistical analysis

SPSS 17.0 software was used to analyze the data. The measurement data are expressed as the mean $\pm$ SD. One-way analysis of variance (ANOVA) was used to analyze the comparisons within group. When the variance was equal, least squares difference (LSD) was used to perform comparison between groups, and Dunnett's T3 test was used when the variance was not equal. $P < 0.05$ was termed as statistical difference.

The research related to animal use has been complied with all the relevant national regulations and institutional policies, and has been approved by the Animal experiment center of Guangzhou university of Chinese medicine review board committee.

3 Results

3.1 Oil Red O staining

Pathological changes of Oil Red O staining in L-O2 human fetal hepatocyte cells and HepG2 human liver carcinoma cells were observed under a microscope. As illustrated in Figures 1 and 2, the cell nuclei of hepatocytes in the normal group (Figure 1A, 2A) and Fenofibrate group (Figure 1B, 2B) were blue. Orange lipid droplets were not observed, and the cell structure was normal. The hepatocytes in the model group (Figure 1C, 2C) were affected with orange lipid droplets of different sizes and diffuse orange lipid droplet fusion. Most of the cell nuclei were pushed to one

Figure 1: Oil Red O staining of the HepG2 cells in each group. A: Normal group; B: Fenofibrate group; C: model group; D: low dose group; E: medium dose group; F: high dose group. The hepatocytes in the model group were affected with orange lipid droplets of different sizes and diffuse orange lipid droplet fusion (scale bar 100 µm).

Figure 2: Oil Red O staining of L-02 cells in each group. A: Normal group; B: Fenofibrate group; C: Model group; D: Low dose group; E: Medium dose group; F: High dose group. The hepatocytes in the model group were affected with orange lipid droplets of different sizes and diffuse orange lipid droplet fusion (scale bar 100 µm).

Figure 3: TG and MDA levels in the L-O2 and HepG2 cells treated with different doses. A.TG levels in cells treated with different doses. B. MDA levels in cells treated with different doses. 1: Normal group; 2: blank control group; 3: Fenofibrate group; 4: model group; 5: low dose group; 6: medium dose group; 7: high dose group. * $p < 0.05$, ** $p < 0.01$ vs the normal group. # $p < 0.05$, ## $p < 0.01$ vs the model group.

side, and the cell structure was disordered. Compared with the model group (Figure 1D, 2D), the cells in the low dose group exhibited reduced lipid droplet deposition, and the deposition in the medium (Figure 1E, 2E) and high dose groups (Figure 1F, 2F) was significantly decreased.

3.2 Changes in the blood fat and liver fat levels

Compared with the normal group, the TG and MDA levels in the L-O2 and HepG2 cells of the model group were significantly increased ($P < 0.01$). Compared with the model group, those in the 3 dose groups were significantly decreased ($P < 0.01$), and were increased with the decrease in the dose (Figure 3). Fenofibrate, the approved chemotherapeutic agent for NAFLD, was used in

Table 1: List of PCR primers.

基因	序列(5'-3')	产物/bp
AdipoR2	F:CCCTCTGCAAGAGAAGGTGG	247
	R:AATGCCCAGCACACAGATGA	
PPARα	F:GCGAACGATTCGACTCAAGC	118
	R:CATCCCGACAGAAAGGCACT	
CD36	F:CGCTGAGGACAACACAGTCT	113
	R:CTGCCACAGCCAGATTGAGA	
acyl-CoA	F:ATGGAGCCTGAAGAGTTCGG	258
	R:TACCTCGGGATGACTGAGCA	
Beta-actin	F:CTCCATCCTGGCCTCGCTGT	268
	R:GCTGTCACCTTCACCGTTCC

Figure 4: Influence of dose on apoptosis. The apoptosis of L-O2 and HepG2 cells treated with Shugan Xiaozhi Fang at different doses for 24 h was detected using flow cytometry. 1: Normal group; 2: blank control group; 3: Fenofibrate group 4: model group; 5: low dose group; 6: medium dose group; 7: high dose group. * $p < 0.05$, ** $p < 0.01$ vs the normal group. # $p < 0.05$ ## $p < 0.01$ vs the model group.

the study and compared with the high dose group. There is no significant difference ($P > 0.05$).

3.3 Apoptosis

The apoptosis of L-O2 and HepG2 cells treated with Shugan Xiaozhi Fang at different doses for 24 h was detected using flow cytometry. Compared with the normal group, the apoptosis rate in the model group was significantly increased ($P < 0.01$). In the L-O2 cells, the apoptosis rate in the low dose group was significantly lower than that in the model group, and that in the medium and high dose groups was significantly decreased ($P < 0.01$). In HepG2 cells, the apoptosis rate in the 3 dose groups was significantly lower than that in the model group ($P < 0.01$). Compared with the normal group, the apoptosis rate in the drug control group wasn't significantly increased ($P > 0.05$). The results indicated that Shugan Xiaozhi Fang effectively inhibited the apoptosis of non-alcoholic fatty hepatocytes and was non-toxic to normal cells, as shown in Figure 4.

3.4 Change in AdipoR, PPAR α , CD36, and acyl-CoA mRNA expression

Compared with the normal group, AdipoR, PPAR α , CD36, and acyl-CoA mRNA expression in the L-O2 cell model

group was downregulated ($P < 0.05$ or $P < 0.01$). Compared with the model group, the expression levels of AdipoR, PPAR α , CD36, and acyl-CoA in the medium and high dose groups were significantly increased ($P < 0.01$). Compared with the model group, the expression levels of AdipoR, PPAR α , CD36, and acyl-CoA in HepG2 cells in the medium and high dose groups were significantly increased ($P < 0.01$) (Figure 5).

3.5 Changes in AdipoR, PPAR α , CD36, and acyl-CoA protein expression

Compared with the normal group, AdipoR, PPAR α and CD36 protein expression levels in the L-O2 model group were significantly decreased ($P < 0.01$). The acyl-CoA protein was downregulated without statistical significance ($P > 0.05$). Compared with the model group, the expression levels of AdipoR, PPAR α , CD36, and acyl-CoA in L-O2 cells in the 3 dose groups were significantly increased ($P < 0.01$) (Figure 6).

Compared with the normal group, AdipoR, PPAR α , CD36, and acyl-CoA protein expression levels in the HepG2 cell model group were significantly decreased ($P < 0.05$ or $P < 0.01$). Compared with the model group, AdipoR, CD36 and acyl-CoA proteins in the low dose group were upregulated ($P < 0.05$), and AdipoR, PPAR α , CD36, and acyl-CoA protein expression levels were all significantly increased ($P < 0.01$) (Figure 6).

Figure 5: AdipoR2, PPAR α , CD36, and acyl-CoA mRNA expression. 1: Normal group; 2: blank control group; 3: model group; 4: low dose group; 5: medium dose group; 6: high dose group. * $p < 0.05$, ** $p < 0.01$ vs the normal group. # $p < 0.05$, ## $p < 0.01$ vs the model group.

The results show that Shogun Xiaozhi Fang was proven to significantly reduce serum TG and MDA levels (Figure 7).

4 Discussion

NAFLD has been considered as the main reason for chronic liver disease [4, 5]. NAFLD is a metabolic stress that damages the liver, and is closely related to insulin resistance (IR) and genetic susceptibility, including nonalcoholic simple fatty liver, non-alcoholic steatohepatitis, and relevant cirrhosis and hepatocellular carcinoma. Currently, there is no consensus for the treatment of NAFLD. Most of the interventions for NAFLD involve improving the IR.

Adiponectin is one of the widely used intervention targets for NAFLD. It is a protein with 30 kDa, and specifically secreted in adipose tissue, possessing anti-inflammatory, anti-diabetic, and anti-atherosclerotic characteristics [6]. The serum adiponectin level has been

proven to be reduced under obesity, IR, NAFLD, and type 2 diabetes mellitus (T2DM) [7, 8]. Thus, adiponectin deficiency-type mice represent IR and diabetes [9]. Contrarily, transgenic overexpression of adiponectin or injection of recombinant adiponectin can prevent the occurrence of diabetes and hyperlipidaemia [10-13]. Thus, adiponectin plays a very important role in the development of diabetes.

The biological function of adiponectin not only depends on the serum circulation concentration of hormone, but also depends on the expression level and function of the specific receptor (including AdipoR1 and AdipoR2). AdipoR1 and AdipoR2 are widely expressed in each organ and tissue, and are highly expressed in the skeletal muscle and liver, respectively [14]. It has been reported that a deficiency of adiponectin receptors will lead to hyperglycemia and hyperinsulinemia [15, 16]. The triglyceride content in mice with AdipoR1/AdipoR2 knockout is increased, and systemic inflammation, oxidative stress, insulin resistance, and glucose tolerance

Figure 6: Changes in AdipoR, PPARα, CD36, and acyl-CoA protein expression. 1: Normal group; 2: blank control group; 3: model group; 4: low dose group; 5: medium dose group; 6: high dose group. * $p < 0.05$, ** $p < 0.01$ vs the normal group. # $p < 0.05$, ## $p < 0.01$ vs the model group.

are aggravated [17]. After AdipoR1/AdipoR2 is activated, fatty acid oxidation in the liver and skeletal muscle is increased, as well as the generation of lactic acid in skeletal muscle. Hepatic gluconeogenesis is reduced, glucose uptake of cells is increased, and inflammation and oxidative stress are inhibited [14].

As a main medium for adiponectin metabolism, AdipoR1 and AdipoR2 play very important roles in transmitting adiponectin signal. In the HepG2 PA cell model, MiR-375 regulates the expression of IL-6, TNF-α, leptin, and adiponectin by mediating AdipoR2 [18]. In skeletal muscle, globular and full-length adiponectin activates AMPK, and stimulates the phosphorylation of ACC, oxidation of fatty acid, and uptake of glucose [13].

Adiponectin can activate PPARα and stimulate fatty acid oxidation to reduce the TG content [14]. AdipoR2 plays a very important role in regulating glucose, lipid metabolism, and NAFLD inflammation, which is related to the activation of the PPARα signaling pathway and inhibition of inflammation [17, 19].

As a critical transcription factor of glycolipid metabolism, PPARα participates in lipid metabolism, energy balance, and expression of inflammatory genes. It is the main regulator of β-oxidation in the liver (mitochondrion and peroxidase) and ω-oxidation in microsomes. In the liver, adiponectin can upregulate the expression of the PPARα target gene and regulate fatty acid metabolism, such as induction of lipid transporter

Figure 7: A schematic diagram of mechanism of Shogun Xiaozhi Fang for reducing serum TG and MDA levels.. Shugan Xiaozhi Fang activated the AdipoR2-PPAR α -CD36/acyl-CoA signaling pathway. In the liver, Shugan Xiaozhi Fang can upregulate the expression of the PPAR α target gene and regulate fatty acid metabolism, such as induction of lipid transporter CD36 gene expression, as well as lipid uptake and transport. It can also upregulate acyl-CoA gene expression and fatty acid oxidation.

CD36 gene expression, as well as lipid uptake and transport. It can also upregulate acyl-CoA gene expression and fatty acid oxidation. Adiponectin improves insulin sensitivity and glucose uptake by regulating PPAR, and alleviates NAFLD development by the PPAR receptor [20–22]. Wogonin inhibits hepatocyte lipotoxicity by activating PPAR α and increasing AdipoR2 expression [23]. Thus, an increase in the activity of adiponectin signaling pathway AdipoR2-PPAR α -CD36/acyl-CoA is an effective method for treating glycolipid metabolic disorder diseases.

Currently, traditional Chinese medicine treats NAFLD by mainly focusing on dampness, phlegm, and heat, but often ignore that qi depression is the main pathogenic factor of non-alcoholic steatohepatitis (NASH). Based on clinical investigation, we found that the disease is mainly caused by accumulation of emotional upset, liver dysfunction, phlegm-damp retention, or blood stasis in the liver. It leads to interaction between depression, heat, and stasis, and subsequent formation by arthralgia blocked in the liver venation. Liver depression and spleen deficiency are the main clinical syndrome types. From the qi depression, we propose that qi depression-phlegm-damp retention-stasis is the pathogenesis of NAFLD.

Shugan Xiaozhi Fang was developed from an established prescription, and its formula has been submitted for a Chinese patent (application number: 201610520975.6). Shugan Xiaozhi Fang has been proven to significantly reduce blood fat, free fatty acid levels, and hemorheological indices in non-alcoholic fatty liver disease model rats. The intervention could upregulate expression of PPAR α mRNA and L-FABP mRNA, and alleviate the steatosis and inflammation in the liver [3].

In this study, Shugan Xiaozhi Fang was proven to significantly reduce serum TG and MDA levels in the model group, and improve steatosis and apoptosis, which indicates that it possesses the ability to ameliorate NAFLD. We also found that AdipoR2, PPAR α , CD36, and acyl-CoA protein and mRNA levels in the model group were downregulated, indicating that AdipoR2-PPAR α -CD36/acyl-CoA (the adiponectin signaling pathway) was inhibited. After the intervention by Shugan Xiaozhi Fang, the AdipoR2, PPAR α , CD36, acyl-CoA protein, and mRNA levels in cells were upregulated, suggesting that Shugan Xiaozhi Fang activated the AdipoR2-PPAR α -CD36/acyl-CoA signaling pathway. Thus, this intervention may be one of the effective treatments for glycolipid metabolic disorder diseases such as NAFLD.

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