A study on the mechanism of adipokine in non-alcoholic fatty liver in rats treated by four herbs decoction

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Abstract
The objective of the study is to determine adipokine-associated mechanism of efficacy of Si He Decoction (SHD) for treating non-alcoholic fatty liver disease (NAFLD). Forty-five Sprague-Dawley (SD) rats were randomly divided into control group, model group, SHD low-dose group, SHD middle-dose group, and SHD high-dose group. Except control group, others were fed with a high-fat diet for 12 weeks to establish model. Then, H&E and oil red O staining were performed, and enzyme-linked immunosorbent assay (ELISA) was used to detect expression level of adipokine-associated molecules. H&E and oil red O staining results revealed that SHD treatment for NAFLD could effectively improve liver pathological conditions compared to that in model group, and the best efficacy was observed in SHD high-dose group. Compared to model group, SHD treatment could effectively downregulate expression level of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), and upregulate expression level of visfatin, adiponectin (APN), leptin (LEP), and resistin in NAFLD rats. SHD can improve NAFLD through multiple means of targeting adipokines.

Keywords
adipokine, clearing heat and expelling dampness and remove blood stasis, insulin resistance, non-alcoholic fatty liver disease, Si He Decoction

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Introduction
Non-alcoholic fatty liver disease (NAFLD) is a clinicopathological syndrome that can develop into advanced liver disease. NAFLD contains a wide range of liver injury–related diseases, such as from a single fatty liver to liver fibrosis and cirrhosis in the late stage.1 With the change of people’s lifestyle and eating habits, the incidence of NAFLD has increased year by year, and the disease has become a common cause of chronic liver disease.2 Furthermore, the prevalence of NAFLD in China was approximately 15%.3 A study revealed that NAFLD is closely correlated to metabolic syndromes such as obesity and insulin resistance (IR),4 and these may be caused by inflammatory responses within liver parenchyma cells, which are induced by reactive oxygen species on the basis of the first attack.5 A study concluded that adipose tissue is one of the endocrine organs that can secrete many kinds of adipocyte factors, including leptin (LEP), adiponectin (APN), and resistin, and plays an important role in energy metabolism.6 However, its pathogenesis has not been fully understood at present, and no effective treatments and drugs have been developed.7 Traditional Chinese medicine...
(TCM) treatment for NAFLD has many advantages in terms of overall and individualized efficacies. In this study, NAFLD model rats were treated with heat-clearing, damp-clearing, and stasis-dissipating methods to observe its therapeutic effect and further explore the mechanism of adipokines involved in its therapeutic effect. The details are reported as follows.

Materials and methods

Experimental animals

A total of 45 clean-grade healthy male Sprague-Dawley (SD) rats (specific pathogen-free (SPF), mean body weight = 180 ± 10 g) were used in this study. These rats were purchased from Beijing VitalRiver Laboratory Animal Technology Co., Ltd. (License No. SCXK (Beijing) 2012-0001), and were raised in the Animal Experimental Center, Dongzhimen Hospital, Beijing University of Chinese Medicine. Prior to these experiments, these SD rats were fed in the experimental center for 1 week to adapt to the environment.

High-fat diet

The SPF class high-fat diet was purchased from Beijing Keaoxieli Feed Co., Ltd. (License No. SCXK (Beijing) 2009-0012). The high-fat diet (10 kg per package) comprised of 88% common feeds, 2% cholesterol, and 10% lard, which was sterilized with cobalt 60 and stored at room temperature.

Traditional Chinese medicine

The TCM prescription, Si He Decoction (SHD), was purchased from the pharmacy of Wangjing Hospital of the Chinese Academy of Traditional Chinese Medicine Sciences, which was prepared into the decoction in the herb decocting room, and was tested for volume, specific gravity, and hygiology. Liquid medicine of 1 mL was equivalent to 2 g of crude drug. The ingredients of the SHD were as follows: 10 g of Bupleurum, 10 g of Fructus Aurantii, 30 g of oriental wormwood, 9 g of gardenia, 6 g of prepared rhubarb, 30 g of oriental water plantain rhizome, 15 g of fried Atractylodes macrocephala, 9 g of Pinellia, 9 g of Magnolia officinalis, 20 g of coix seed, 6 g of round cardamom seeds, 15 g of talc, 12 g of tangerine peel, 9 g of chuanxiong rhizome, 12 g of nutgrass galingale rhizome, 20 g of fried Radix Paeoniae Alba, and 6 g of licorice.

Main reagents

Formaldehyde (Sinopharm Chemical Reagent Co., Ltd.), 10% chloral hydrate, xylene, eosin and hematoxylin (Beijing Chemical Reagent Co., Ltd.), oil red O (Sigma), and saline (Beijing Double-Crane Pharmaceutical Co., Ltd.) were used. Furthermore, enzyme-linked immunosorbent assay (ELISA) kit for rat visfatin (blue base), rat APN, and LEP, ELISA kit for rat resistin (Senxiong Technologies), and ELISA kits for rat tumor necrosis factor-alpha (TNF-α) and rat interleukin-6 (IL-6) (Beijing Huanya Tektronix Biomedical Technology Co. Ltd.) were also used.

Main instruments

Main instruments used were automatic paraffin embedding machine, ultra-thin semiautomatic microtome, freezing microtome (Leica), a microscope and its corresponding photography system (Olympus), a –80°C cryogenic refrigerator (Sanyo), electronic balance (Shanghai Second Balance Instrument Factory), a fully automatic multifunctional microplate reader (ThermoFisher), a constant-temperature incubator (Tianjin TAISITE Instrument), and a shaking table (QILINBEIER).

Experimental methods

The experimental protocol strictly followed the basic experimental principles (randomization, control, and repeatability) and was approved by the Ethics Committee of Dongzhimen Hospital of Beijing University of Chinese Medicine.

Animal grouping and NAFLD model establishment

The SD rats were fed in the experimental center for 1 week, and grouped using the random number table method: control group (n=9), randomly assigned; experimental group (n=36), fed with high-fat diet for 12 weeks to establish the NAFLD model. At the 7th week of induction, rats for modeling in the experimental group were randomly divided into two groups using random number table method: model group (n=9) and treatment
group (n=27). Rats in the treatment group were further divided into three sub-groups: SHD low-dose group (10 mL/kg, n=9), SHD medium-dose group (20 mL/kg, n=9), and SHD high-dose group (30 mL/kg, n=9). Rats in the model and treatment groups were given sufficient high-fat diet daily and were allowed to eat freely ad libitum. For the SHD high-dose group, the drug was administered by gavage twice a day in the morning and evening (3 mL each time). For other SHD groups, the drug was administered by gavage in the morning. Rats in the control and model groups were given 10 mL/kg of normal saline by intragastric administration for control. Rats in all groups were given by intragastrical infusion once a day for 6 weeks. After the end of treatment, rats were fasted, but were provided with water, for 24h. Then, blood was sampled from the abdominal aorta, and tissues were obtained for subsequent experiments.

Pathological observation of rat liver tissues

After 6 weeks of treatment and fasting with drinking water for 24h, three rats were randomly selected from each group and given euthanasia. Then, the livers were rapidly harvested, placed on ice, and washed with normal saline. Then, half of the right lobe of the liver was obtained, wrapped with a tinfoil, and quickly sent to the Pathology Department of our hospital for frozen section, followed by oil red O staining. Subsequently, the remaining half of the right liver was fixed with 4% paraformaldehyde, embedded in paraffin, routinely sliced, and underwent H&E staining to observe the pathological changes in the liver.

Detection of visfatin, APN, LEP, resistin, TNF-α, and IL-6 levels by ELISA

The animal experiment method was the same as that in “Experimental animals” section. After 6 weeks of treatment and fasting with drinking water for 24h, liver samples were obtained from rats in each group, washed with normal saline, and weighed. These samples were added with 10 times the volume of phosphate buffered saline (PBS), grounded, centrifuged, and the supernatant was obtained. Double-antibody sandwich ELISA was performed, and specific steps were carried out according to the operating instructions on the box of the reagent.

Statistics analysis

Data were analyzed using statistical software SPSS13.0. The results were presented as mean ± standard deviation (x±SD). The normal distribution and homogeneity of variance of the data were first evaluated. Measurement data were evaluated using t test or analysis of variance, and count data were evaluated using chi-square test or non-parametric test. The P-value of <0.05 was considered statistically significant.

Results

Performance characteristics of rats

The NAFLD rats presented weight loss, inactivity, hypotrichotrophy, grouping, bad appetite, and lassitude. The NAFLD rats showed all the above signs; these signs are similar to the symptoms of TCM NAFLD pattern in human. These conditions were alleviated in SHD group.

Observation of pathology in the liver of rats

H&E staining results

Control group (Figure 1(a)): Liver tissues had intact and clear structures, liver cells were regularly arranged, the nuclear structure of liver cells was intact, the cytoplasm was pink stained, and no inflammation was found.

Model group (Figure 1(b)): Severe fat accumulation was found in liver tissue, the structure was disordered, swelling and deformation of liver cells was observed, a large amount of lipid droplets were found in the cytoplasm, the nuclei were deviated, the infiltration of a large amount of inflammatory cells was observed, and necrotic foci were found in lobules.

SHD low-dose group (Figure 1(c)): Mild fat lesions were found in liver tissue, small lipid droplets were found in liver cells, part of the liver cells were swollen, the infiltration of a small amount of inflammatory cells was found, and necrotic foci were occasionally found in the lobules.

SHD medium-dose group (Figure 1(d)): Fat accumulation in liver tissue decreased, hepatic lobule structures were relatively intact, the structures of liver cells were relatively clear, no
necrotic foci were found, and the infiltration of a small amount of inflammatory cells was found.

SHD high-dose group (Figure 1(e)): The fatty degeneration of liver cells was not obvious, hepatic lobule structures were relatively intact, the structures of liver cells were relatively clear, no necrotic foci were found, and the infiltration of a small amount of inflammatory cells was found (Figure 1).

The SHD high-dose group was close to the control group in H&E staining results.

**Oil red O staining results**

Control group (Figure 2(a)): No red stained fat drop was found.

Model group (Figure 2(b)): The samples presented with flaky scarlet staining, a large amount of lipid droplets were distributed in the cytoplasm, and the number of cells with lipid droplets/the number of total cells in hepatic lobules was 70%–90%.

SHD low-dose group (Figure 2(c)): The scarlet stained areas decreased, and the number of cells with lipid droplets/the number of total cells in hepatic lobules was 35%–50%.

SHD medium-dose group (Figure 2(d)): The red stained areas significantly decreased, and the number of cells with lipid droplets/the number of total cells in hepatic lobules was approximately 27%–45%.

SHD high-dose group (Figure 2(e)): The red stained areas significantly decreased or were not found, and the number of cells with lipid droplets/the number of total cells in hepatic lobules was approximately 18%–32%. The results of the oil red O staining of liver tissues from rats in each group are shown in Figure 2.

The SHD high-dose group was close to the control group in Oil red O staining results.

**The expression of adipokines in the liver of rats in each group**

Data in each group were tested for normal distribution and homogeneity of variance. These data were found to be normally distributed, and the variance was homogeneous. Then, these data were compared using univariate analysis of variance.
Comparison of expression levels of visfatin

The level of visfatin in the liver of rats was higher in the SHD high-dose, SHD medium-dose, and SHD low-dose groups than that in the control group ($P < 0.05$). Furthermore, the differences in optic density (OD) value and level of visfatin between the SHD high-dose group and the control group, between the SHD medium-dose group and the control group, and between the SHD low-dose group and the control group were statistically significant ($P < 0.05$). The difference in OD value for visfatin in the liver of rats between the SHD medium-dose group and the model group, and between the SHD high-dose group and the model group were statistically significant ($P < 0.05$). The level of visfatin in the liver of rats was higher in the SHD medium-dose group than in the model group, and the differences were statistically significant ($P < 0.05$). It is indicate that SHD can significantly increase the content of visfatin in NAFLD rats ($P < 0.05$, Table 1).

Comparison of APN expression levels

The OD value and APN level in the liver of rats were higher in the SHD high-dose, SHD medium-dose, and SHD low-dose groups than those in the control group, and all differences were statistically significant ($P < 0.05$). The OD value and APN level in the liver of rats were lower in the SHD low-dose group than in the model group, while the OD value and APN level in the liver of rats were higher in the SHD high-dose group than in the model group, and the differences were statistically significant ($P < 0.01$, Table 1). The SHD low-dose could regulate the expression level of APN better, and it made the expression level of APN close to the control group.

Comparison of LEP expression levels

The OD value and LEP level in the liver of rats were higher in the SHD high-dose and model groups than that in the control group ($P < 0.05$). The OD value in the liver of rats was higher in the SHD medium-dose group than in the control group ($P < 0.01$). The difference in OD value for LEP in the liver of rats between the SHD medium-dose group and the model group were statistically significant ($P < 0.01$). The level of LEP in the liver of rats was lower in the SHD high-dose, SHD medium-dose, and SHD low-dose groups than that in the model group, and the differences were
| Groups               | n  | Visfatin Concentration (ng/mL) | APN OD value | LEP Concentration (µg/mL) | Resistin OD value | TNF-α Concentration (µg/mL) | IL-6 OD value | Concentration (pg/mL) |
|----------------------|----|-------------------------------|-------------|---------------------------|-----------------|-----------------------------|-------------|---------------------|
| Control group        | 9  | 1.243 ± 0.035                 | 1.638 ± 0.192 | 0.372 ± 0.049             | 10.946 ± 2.353  | 8.197 ± 0.815               | 2.889 ± 0.026 | 92.441 ± 18.886     |
| Model group          | 7  | 1.199 ± 0.093                 | 1.914 ± 0.585 | 0.527 ± 0.278*            | 18.870 ± 3.025* | 18.870 ± 3.025*             | 142.085 ± 39.741 | 10.946 ± 2.353*     |
| SHD low-dose group   | 9  | 1.160 ± 0.086*                | 2.116 ± 0.212*| 0.444 ± 0.249*            | 14.487 ± 1.263* | 11.757 ± 2.434***           | 170.402 ± 39.405*| 11.025 ± 1.287*     |
| SHD middle-dose group| 9  | 1.128 ± 0.075*, ***           | 2.332 ± 0.473*, ***| 0.535 ± 0.061***          | 19.299 ± 3.251* | 11.015 ± 2.041***           | 131.820 ± 44.033*, ***| 11.025 ± 1.287*     |
| SHD high-dose group  | 9  | 1.133 ± 0.048*, ***           | 2.287 ± 0.308*| 0.676 ± 0.050*, ***       | 27.334 ± 3.162*, ***| 12.810 ± 1.287*            | 131.820 ± 44.033*, ***| 11.025 ± 1.287*     |

Compared with the control group, *P < 0.05, **P < 0.01; compared with the model group, ***P < 0.005, ****P < 0.001. APN: adiponectin; LEP: leptin; TNF-α: tumor necrosis factor-alpha; IL-6: interleukin-6; OD: optic density; SHD: Si He Decoc-
statistically significant ($P < 0.01$, Table 1). The SHD low-dose and SHD medium-dose could regulate the expression level of LEP better, and it made the expression level of LEP close to the control group. The effect of SHD medium-dose is the best.

**Comparison of resistin expression levels**

The OD value and resistin level in the liver of rats were higher in the SHD high-dose, SHD medium-dose, and SHD low-dose groups than that in the control group, and the differences were statistically significant ($P < 0.01$). The OD value and resistin level in the liver of rats were higher in the SHD high-dose group and SHD medium-dose group than in the model group, and all differences were statistically significant ($P < 0.05$, Table 1). This indicates that SHD can significantly increase the content of resistin expression levels in NAFLD rats.

**Comparison of TNF-α expression levels**

The OD value and TNF-α level in the liver of rats were higher in the model group than in the control group, and the differences were statistically significant ($P < 0.01$). The OD value and TNF-α level in the liver of rats were lower in the SHD low-dose, SHD medium-dose, and SHD high-dose groups than that in the model group, and the differences were statistically significant ($P < 0.01$, Table 1). The SHD low-dose, SHD medium-dose, and SHD high-dose groups could regulate the expression level of TNF-α better, and it made the expression level of TNF-α close to the control group. The effect of SHD medium-dose is the best.

**Comparison of IL-6 expression levels**

The OD value and IL-6 level in the liver of rats were higher in the SHD high-dose, SHD medium-dose, and SHD low-dose groups than that in the control group, and the differences were statistically significant ($P < 0.01$). The OD value and IL-6 level in the liver of rats were lower in the SHD low-dose, SHD medium-dose, and SHD high-dose groups than that in the model group, and the differences were statistically significant ($P < 0.01$, Table 1). The SHD low-dose, SHD medium-dose, and SHD high-dose groups could regulate the expression level of IL-6 better, and it made the expression level of IL-6 close to the control group. The effect of SHD medium-dose is the best.

**Discussion**

According to clinical characteristics, NAFLD belongs to the category of “hypochondriac pain,” “accumulation,” “lump at the left hypochondrium,” and “phlegmatic mass” in TCM. On this basis, we designed the prescription of SHD, and satisfactory results have been achieved in clinical practice. In the present study, we first established the NAFLD model by feeding SD rats with high-fat diet and applied SHD to treat NAFLD. After the end of treatment, the curative effect was observed by liver tissue sections. The results reveal the NAFLD model to be successfully established, which is consistent with the results of previous studies. The treatment with SHD could improve the pathology of liver in the H&E and oil red O staining, and rats in the SHD high-dose had the best improvement effect.

NAFLD lesions are mainly located in the hepatic lobule region, and fatty degeneration and accumulation are its pathological characteristics. Many studies have revealed that the occurrence and development of NAFLD are closely correlated to the mechanism of IR. The chronic inflammatory state caused by a large amount of adipokines secreted by adipose tissue plays a core role in IR, lipid metabolism disorders, and obesity. The expression levels of adipokine-related molecule visfatin, APN, LEP, resistin, TNF-α, and IL-6 in the liver of rats in all groups were detected by ELISA to explore the potential adipokine mechanism underlying the efficacy of SHD in the treatment of NAFLD. ELISA results of the present study also revealed that NAFLD could promote the expression of APN, LEP, TNF-α, and IL-6 in the liver, while the treatment of SHD could downregulate the expression of TNF-α and IL-6 in the liver of NAFLD rats, and upregulate the expression of visfatin, APN, LEP, and resistin in the liver of NAFLD rats. Among these rats, the upregulation of APN, LEP, and resistin in the liver of rats in the SHD high-dose group was the most significant, the upregulation of visfatin in the liver of rats in the SHD medium-dose group was particularly significant, and the expression of APN and LEP was downregulated in the liver of rats in the SHD low-dose group. Thus, it can be concluded that SHD can improve NAFLD by regulating adipokines in a network and multi-target manner. This provides an experimental basis for the multi-target treatment of SHD for NAFLD.
Declaration of conflicting interests
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References
1. Angulo P (2002) Nonalcoholic fatty liver disease. *New England Journal of Medicine* 346: 1221–1231.
2. Forbes S, Taylor-Robinson SD, Patel N, et al. (2011) Increased prevalence of non-alcoholic fatty liver disease in European women with a history of gestational diabetes. *Diabetologia* 54(3): 641–647.
3. Fan JG and Farrell GC (2009) Epidemiology of non-alcoholic fatty liver disease in China. *Journal of Hepatology* 50(1): 204–210.
4. Bhala N and George J (2015) The burden of non-alcoholic fatty liver disease (NAFLD) in the Asia Pacific Region. *Current Hepatology Reports* 14: 77–86.
5. El-Haggar SM and Mostafa TM (2015) Comparative clinical study between the effect of fenofibrate alone and its combination with pentoxifylline on biochemical parameters and liver stiffness in patients with non-alcoholic fatty liver disease. *Hepatology International* 9: 471–479.
6. Yu J, Marsh S, Hu J, et al. (2016) The pathogenesis of non-alcoholic fatty liver disease: Interplay between diet, gut microbiota, and genetic background. *Gastroenterology Research and Practice* 2016: 2862173.
7. Lisboa QC, Costa SM and Couto CA (2016) Current management of non-alcoholic fatty liver disease. *Revista da Associacao Medica Brasileira (1992)* 62(9): 872–878.
8. Zhang LD *Investigation of Transection of Non-Alcoholic Fatty Liver and Mechanism of Adipocytokines Intervened by Clearing Heat and Removing Dampness and Blood Stasis Therapy*. Beijing, China: Beijing University of Chinese Medicine, 2014 (in Chinese).
9. Qiu GQ, Ye F, Liu Y, et al. (2013) The experimental study on the effects of Xiaochaihu Soup on non-alcoholic rat fatty liver. *Journal of Xi’an Jiaotong University (Medical Sciences)* 3: 400–402.
10. Patell R, Dosi R, Joshi H, et al. (2014) Non-alcoholic fatty liver disease (NAFLD) in obesity. *Journal of Clinical and Diagnostic Research* 8(1): 62–66.
11. Antuna-Puente B, Feve B, Fellahi S, et al. (2008) Adipokines: The missing link between insulin resistance and obesity. *Diabetes & Metabolism* 34(1): 2–11.