The mechanism of jian-Gan-Xiao-Zhi decoction in IR adipocytes and its component analysis

Fang Fang

Nanjing University Medical School

WeiBo Wen (✉ WeiBoWen15@163.com)

Nanjing University Medical School

Xuehua Xie

Nanjing University Medical School

Ling Yang

Yunnan University of Traditional Chinese Medicine

Xu Zhang

First People's Hospital of Yunnan

jie Zhao

First People's Hospital of Yunnan

Research

Keywords: Jian-Gan-Xiao-Zhi decoction; insulin resistant; ROS; JNK; AMPK; LC-MS/MS.

Posted Date: June 29th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-18749/v3>

License: This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Background: Jian-Gan-Xiao-Zhi decoction (JGXZ) is a formula of traditional Chinese medicine to treat patients with NAFLD. Thus, the study aimed to analyze the mechanism of drug serum of JGXZ in adipocytes and detect the main components of JGXZ in rat serum.

Methods: The 3T3-L1 preadipocytes were used to establish IR adipocytes model. The lipid accumulation in adipocytes was detected by oil red O staining. After JGXZ treatment, glucose consumption, TC and TG were analyzed by corresponding kits. ROS levels were measured by flow cytometry. In addition, Western Blot was used to assess LKB1/AMPK and JNK/IRS/PI3k/AKT expressions. Except for that, the main components of JGXZ in rat serum samples were detected by LC-MS/MS method. A Phenomenex Luna C18 was used to perform chromatographic separation and detect the concentration of main components in JGXZ. The ion source was ESI. The mobile phase contained methanol and 0.1% formic acid solution.

Results: JGXZ significantly decreased glucose levels and adipogenesis, accompanied by decreased IR ($p < 0.01$). Besides, JGXZ markedly affected ROS, LKB1/AMPK and JNK/IRS/PI3k/AKT levels ($p < 0.01$). The R1, Rg1, paeoniflorin, Rb1, astragaloside and tanshinone could be significantly quantified.

Conclusions: Thus, JGXZ decreased glucose and lipid synthesis possibly via ROS/AMPK/JNK pathway. R1, Rg1, paeoniflorin, Rb1, astragaloside and tanshinone in JGXZ could play main roles in treating NAFLD, which could offer assistance for us to study the mechanism of JGXZ on treating NAFLD.

Background

Non-alcoholic fatty liver disease (NAFLD) is characterized by steatosis and adipose storage of liver parenchyma cells that is closely related to IR. Therefore, improvement of IR is one of the important therapeutic measures for treating NAFLD. Insulin sensitizers include thiazolidinediones-induced glucose uptake and lipid synthesis, which may increase weight. Thus, searching for drugs without induction of lipid synthesis is requisite. Adipose tissue is the main storage site for excess energy in the form of triglycerides, which provides up to 70% free fatty acids for synthesis of triglycerides in liver[1]. Under adipose tissue impairment, excess fatty acids are generated, accompanied by ectopic excessive lipid deposition, systemic insulin resistance and inflammation. In NAFLD, the number of fatty acids transported from adipose tissue to the triglyceride pool in the liver is more than that of fatty acids generated from DE novo synthesis of lipid in liver [2]. Lipid mobilization is strongly inhibited by insulin under normal physiological conditions. However, with the expansion of fat storage in the liver, free fatty acids are increased and the insulin signal is ameliorated, and then IR is accelerated. Adipose tissue is becoming a kind of mediator in nonalcoholic fatty liver disease and metabolic disease[3], and a vital target organ for insulin which stimulates glucose uptake and inhibits lipid lipolysis. IR is closely correlated with liver damage in NAFLD[4, 5]. Thus, it is an efficient way to recover the sensitivity of adipose to insulin for alleviating lipid toxicity of the liver.

Liver kinase B1 (LKB1) is involved in inhibition of lipid synthesis and promotion of glucose uptake via phosphorylating Thr-172 of AMPK which inactivates Acetyl-CoA Carboxylase, and via regulating 3-hydroxy-3-methyl glutaryl coenzyme A reductas[6]. A recent study has suggested that AMPK activation could improve metabolic imbalances caused by type 2 diabetes[7]. ROS could activate JNK pathway via various pathways, the activation of which further phosphorylates serine307 of IRS-1 to inhibit the transduction of insulin signal [8]. The phosphorylation of IRS-1 at serine307 site is the main mechanism in IR. Besides, insulin receptor substrate (IRS) could be phosphorylated by insulin receptor tyrosine kinase to mediate the transmission of insulin signal. Frequently, p-IRS

binds to the SH2 domain of PI3K regulatory subunit p85, which recruits and activates PI3K. Then, PI3K mediates the activation of AKT, which could regulate glycogen synthesis, gluconeogenesis and glucose absorption through phosphorylating corresponding substrates. Thus, ROS/JNK/IRS/PI3K/AKT affects the conversion of glucose to glycogen and protein, and absorption.

Jian-Gan-Xiao-Zhi decoction (JGXZ) is a formula of Traditional Chinese medicine (TCM) and applied for patients with NAFLD. Clinical researches have demonstrated that JGXZ has obvious effects in improving clinical symptoms and blood lipid level in NAFLD patients[9, 10]. JGXZ is made up of 12 medicine herbs, including salvia miltiorrhiza, zedoary turmeric, hawthorn, astragalus membranaceus, green peel, red peony root, turmeric, chrysanthemum, lotus leaf, licorice root and so on. Turmeric contains curcumin, zedoary turmeric oil and so on. Other several herbs mainly contain polysaccharide, flavonoids and glycosides. Previous animal experiment has indicated that JGXZ decreases IR and affects AMPK/JNK levels in non-alcoholic fatty liver rat model[11]. Although TCM, made up of many kinds of substances, is broadly used in clinic in China, the outcomes of patients are not effective. The low effect of some substances and the uncertainty of the active ingredients greatly limit its popularity. Besides, pharmacokinetic properties in TCM are usually poor. The long-term use or overdose of some TCM could cause certain toxicity reactions, limiting its clinical utilization to some extent[12].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed for qualitative and quantitative analysis of components in TCM including some components of lower content or not easily being separated[13, 14]. Therefore, it's important to determine the concentration of main components in serum after TCM administration for further analyzing active components and studying the specific mechanism. The study aimed to investigate the potential mechanism of JGXZ in adipocytes and provide the theoretical support for the clinical application of JGXZ. In addition, the concentration of main components in serum after TCM administration was detected by LC-MS/MS method.

Materials And Methods

Preparation of serum containing Jian-Gan-Xiao-Zhi (JGXZ) decoction

Sprague-Dawley (SD) rats were purchased from Chongqing shidongbiotechnology co. LTD (China), with age of 5~6 weeks old and weight of 180-200 kg. Jian-Gan-Xiao-Zhi-Fang (JGXZ) is consisted of Danshen 15g, notoginseng powder 6g, zedoary rhizome 15g, hawthorn 20g, astragalus 20g, qingpi 10g, red peony root 20g, turmeric 12g, alisma 15g, chrysanthemum 15g, lotus leaf 15g, liquorice 6g. SD rats were randomly divided into seven groups (10 in each group, half male and female), namely, JGXZ group (JGXZ-low dose: 5.43g/kg, JGXZ-medium dose: 10.86g/kg, JGXZ-high dose: 21.73g/kg), Metformin group (0.2mg/kg) and normal saline control group (5ml 0.9% NaCl). The different doses of JGXZ were calculated according to the table of conversion of equivalent doses of human and animal drugs[15].

The rats were administered orally two times for three days. One hour after the last dose, the blood was collected from abdominal aorta and centrifuged at 3000r/min for 15 min to collect serum. Afterwards, the serum was inactivated at 56°C for 30 min. DMEM was used to dissolve into 1.0 mg/mL after vacuum freeze-drying. After filtration and filtered to eliminate bacteria using 0.22µm microfiltration membrane. HPLC detected the main content in JGXZ containing curcumin, notoginseng, astragaloside, paeoniflorin, tanshinone and so on. After passing inspection, the serum was put into -80°C refrigerator for further experiment. The study was supported by according to the National Institutes of Health Regulations and approved by the Institutional Animal Care and Use Committee at The First Affiliated Hospital of Yunnan University of Chinese Medicine.

Cells

3T3-L1 preadipocytes were purchased by Kunming institute of animals, Chinese academy of sciences. The cells were cultured in DMEM high sugar medium containing 10% fetal Bovine Serum, and 1% Penicillin and streptomycin at 37°C, 5% CO₂. After cell adherence, the medium was replaced. The cells of logarithmic phase were inoculated on the culture plate, which then were induced for differentiation 48 h after cell contact inhibition. The medium containing 0.5mmol/l IBXM, 1.0μmol/L DEX, 10μg/ml INS replaced original medium and continued to culture cells for 48 h. Then, 10μg/ml INS medium continued to culture cells for 48 h. The medium was replaced by DMEM and observed after 7 d. After that, oil red O was used to stain fat in cells and determine whether cells were differentiated and mature (sigma, America). The cells were used for further study once more than 90% cells presented “ring” lipid droplet. The mature adipocytes were divided into control and model group. The control group was cultured in DMEM medium. The model group was provided with the medium containing 1μmol/L DEX to establish IR adipocyte model. IR adipocyte was cultured in DMEM medium containing 10% JGXZ (Serum containing drug 100 μL, 200μL, 300μL), Rosiglitazone or normal saline (NS) drug-containing serum. 48 h after JGXZ or Rosiglitazone treatment, glucose content kit (YanJin, Shanghai, China) was used to detect glucose levels in medium for evaluating insulin sensitivity of cells. The absorbance at 505nm was detected. The absorbances in blank, standard and measured tube were recorded as A1, A2 and A3 respectively. The glucose contents were calculated by the formula: glucose contents (μmol/mg prot) = (C standard×V1)×(A3-A1)÷(A2-A1)÷(V1×Cpr)=0.5×(A3-A1)÷(A2-A1)÷Cpr (V1 is sample volume, Cpr is protein concentrations, C standard is standard substance concentration).

CCK8 assay

IR cells were seeded into 96-well plate(1×10^5 /ml). The serum containing JGXZ was added according to different groups. After incubation of 12h, 48h or 72h, the cell supernatant was abandoned and 10μL CCK8 solution was added (Beyotime Biotechnology, Shanghai, China). The absorbance at 450nm was detected.

TC and TG contents

After JGXZ or Rosiglitazone treatment, the cells were washed with PBS twice and broken by ultrasound under the condition of ice bath. Then, the prepared cell homogenate was used to detect TC levels by TC kits according to the protocol, along with the detection of TG contents via TG kits according to the protocol (NanJing JianCheng, China).

The detection of ROS

The cell suspension was prepared, the concentration of which was 5×10^4 cells/well. The cell was seeded into 6-well plate and differentiation was induced. The cell suspension was prepared through digestion with pancreatin 48 h after JGXZ or Rosiglitazone treatment. Then, the cells were centrifuged at 1000r/min for 5 min. After using PBS to wash cells twice, the cell precipitation was collected by centrifugation. ROS Assay Stain Solution (eBioscience, America) of 100μL was added to each tube, and the blank group without dye was set as the control. The green fluorescence intensity of DCFH-DA was detected by flow cytometry after incubation at 37°C for 60 min.

Western blot

The cells were collected into the EP tube, which then was added by protein lysate. After centrifuging for 10 min at 12000r/min at 4°C for 15 min. The supernatant was collected and the protein concentration was detected by BCA method. The target protein was separated by using SDS-PAGE electrophoresis. Blots was blocked in skimmed milk powder solution at 37°C for 2 h. Afterwards, the blots were incubated with the primary antibody at 4°C overnight.

Next, the blots were washed and incubated with secondary antibody. The bands were colored with ECL solution for analysis.

LC-MS/MS method for analyzing the components of JGXZ

The 9 main components of JGXZ was determined in LC-MS/MS instrument (Triple Quad™ 5500, Applied Biosystem Sciex, America). Chromatographic separation was performed on a Phenomenex Luna C18 (150mm×2.0mm, 3μm), and conducted by the gradient program as shown in table1. The running time of each needle sample was 8 min and the column temperature was kept at 40°C. 0-4 min belonged to the negative ion detection mode. Analytes were detected with the following mass spectrometer source settings. ESI source; ion spray voltage: -4500V; temperature: 500°C; collision gas (N₂) pressure: 8psi; nebulizer gas (N₂) pressure: 50psi; heater gas (N₂) pressure: 50psi; curtain gas (N₂) pressure: 35psi; inlet potential (EP): -10V; the ion parameters used for quantitative analysis were respectively: notoginsenoside R1 m/z 977.3→931.4, DP: -134, CE: -42; Ginsenoside Rg1 m/z 845.3 DP: -166.3 CE: -44.3; Paeoniflorin m/z 525.1→449.2 DP: -63.5 CE: -21.1; Internal standard naringin m/z 579.2→271 DP: -214 CE: -44. The results of ion scan were shown in Figure4. 4-8min belonged to the positive ion detection mode. Analytes were detected with the following mass spectrometer source settings. ESI source; ion spray voltage: 5500V; temperature: 500°C; collision gas (N₂) pressure: 8psi; nebulizer gas (N₂) pressure: 50psi; heater gas (N₂) pressure: 35psi; curtain gas (N₂) pressure: 35psi; inlet potential (EP): 10V; the ion parameters used for quantitative analysis were respectively: ginsenosideRB1 m/z 1131.5→365.3, declustering potential (DP) 219.8, collision energy (CE): 76.9 Astragaloside m/z 785.6→143.1 DP: 123.2 CE: 16.8; Curcumin m/z 369.2→285.2, DP: 87, CE: 21.9; Entanshinone m/z 297.2→251.2 DP: 168 CE: 32.2; Tanshinone m/z 277.1→249.1 DP: 191.5 CE: 28.9; Tanshinone A m/z 295.2→277.2 DP: 174.7 CE: 26.4; Internal standard cannabidiol m/z 315.2→193.1 DP: 90.6 CE: 28.8. The results of ion scan were shown in Figure5.

Quantitative ranges and determination of nine compounds in serum

SD rats (n=6, half male and half female) were randomly divided into blank group (n=5) and JGXZ (n=1, marked as 3) group. The blood was collected by eye socket. The serum of 100μL was placed into a blank centrifuge tube as blank serum group. After vortex for 30 seconds, a mixture of methanol-acetonitrile (200μL) in 1:1 ratio was added into the tube. Following vortex for 1 min, the mixture was centrifuged for 5 min at 10000r/min and the supernatant of 100μL was collected, among which 5 μL was used for sample injection. Chromatographs were taken as shown in Figure 4. The mixture solution of reference substance (10μL, 1000ng/ml) was added into blank serum of 90μL. Then, sample injection of 5μL was obtained through same way. The dose equivalent to 32g/kg of JGXZ was given to rats by gavage twice a day in interval of 12 hours for three consecutive days. Then, the blood was collected through the abdominal aorta 1 h after the last JGX administration. The blood was stored at 4°C overnight. Afterwards, the serum was collected through centrifugation at 3000r/min for 15 min and inactivated at 56°C for 30 min, then filtered and sterilized with microporous membrane of 0.22 μm. The serum of 100μL was placed into a blank centrifuge tube, followed by the addition of the mixed internal standard solution of 10μL (containing naringin of 10000ng/ml, cannabidiol of 1000ng/ml). Similarly, sample injection of 5μL was obtained through same way. The quantitative ranges of 9 compounds in serum were as follows: notoginsenoside R1: 0.5~50 ng/mL; Ginsenoside Rg1: 1~100 ng/mL; Paeoniflorin: 5~500 ng/mL; Ginsenoside Rb1: 10~1000 ng/mL; Astragaloside : 0.1~10 ng/mL; Curcumin: 1~100 ng/mL; Cryptotanshinone: 1~100ng/mL; Tanshinone: 0.2~20 ng/mL; Tanshinone A: 2~200 ng/mL. The dose equivalent to 32g/kg of JGXZ was given to rats (n=12, half male and female) by gavage twice a day in interval of 12 hours for three consecutive days. Then, the blood was collected from orbital venous plexus of the rats 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 20 h after the last JGXZ administration.

The pharmacokinetic analysis of 9 main components

PKsolver 2.0 software was used to analyze the plasma drug concentration data of 9 components in JGXZ. The pharmacokinetic parameters such as $T_{1/2}$, T_{max} and C_{max} , AUC_{0-t} , were calculated according to non-compartment model (PKsolver 2.0 (Chinese Pharmaceutical University, Nanjing)).

Statistical analysis

The experimental data was analyzed using Graph Pad software. $P < 0.05$ was considered statistically significant.

Results

3T3-L1 IR adipocytes model was established

The 3T3-L1 preadipocytes were long fusiform and presented no lipid droplet (LD). After cell differentiation was induced, cells became larger and rounder with LD appearing in the cytoplasm. The small LDs appeared in the cytoplasm, which was the reason for the accumulation of lipid. With the extension of time in cell culture, the small LDs would merge into larger LDs. Nucleus and organelles of mature adipocytes were squeezed to the side of cells by larger LD. Besides, cells were round. In the seventh day, oil red O staining indicated that large amounts of LDs were stained red in cytoplasm, which formed “ring” structure around the nucleus. 95% cells differentiated into mature adipocytes according to random cell counting results (Figure1A). Then, we evaluated the effects of JGXZ on adipocytes viabilities through CCK8 assay. Comparing to control group, cell viabilities showed obvious decrease in 12h, 48h and 72h (Figure1B). Comparing to Model group, JGXZ of different dose markedly enhanced adipocytes viabilities. In addition, cell viabilities presented evident increase after JGXZ administration of 48h.

JGXZ markedly reduced lipid deposition in IR adipocytes

LD is the organelle closely related to lipid storage, metabolism and secretion, and consists of a single layer of phospholipid and a hydrophobic core composed of neutral lipids. The hydrophobic core mainly includes TG and cholesterol esters. The results showed that JGXZ significantly lowered the LD levels (Figure2A). The glucose contents in the model group were significantly reduced comparing with control group. It replied that cells became insulin-resistant (Figure2B). Furthermore, TG and TC were notably decreased by JGXZ treatment (Fig2C-D). Thus, JGXZ significantly reduced lipid deposition in IR adipocytes. In addition, high-dose JGXZ had similar effects to Rosiglitazone in decreasing lipid deposition.

JGXZ affected AMPK and ROS/JNK/IRS/PI3k/AKT levels

After medicine treatment, LKB1 and AMPK were significantly increased by JGXZ or Rosiglitazone. It might reply that JGXZ affected lipid deposition and IR partly via LKB1/AMPK. JGXZ of low concentration significantly increased ROS production in IR adipocytes, the effect of which was similar to Rosiglitazone treatment (Figure3A-B). ROS levels gradually recovered to the normal with the increase of the JGXZ concentration compared with control group. In addition, the effects of JGXZ with high concentration on AMPK/JNK/IRS/PI3K/AKT levels were similar to Rosiglitazone treatment in IR adipocytes (Fig3C). Moreover, the phosphorylation levels of the Ser-473 and Thr-308 residues were all obviously increased after JGXZ treatment. The complexity of JGXZ components might explain this phenomenon. Therefore, the results indicated that JGXZ reduced lipid deposition and IR partly through ROS/AMPK/JNK pathway.

Chromatograms of nine analytes

The chromatograms of nine analytes were shown in Figure 4 and 5. The blank serum in the blank group was used to perform chromatographic analysis under anion detection, as exhibited in Figure 4A. The chromatograms of serum containing nine analytes and JGXZ were separately presented in Figure 4B and C. The blank serum in the blank group was used to perform chromatographic analysis under cationic mode, as shown in Figure 5A. The chromatograms of serum containing nine analytes and JGXZ were separately shown in Figure 5B and C.

Content of nine components in JGXZ

The blood wasn't collected. The concentration of nine main compounds in serum containing JGXZ was determined 0.25, 0.5, 1, 2, 4, 6, 8, 10, 16, 20 h after the last JGXZ administration. According to the identification of the nine components in serum containing JGXZ in rats, Rb was the most abundant. However, the concentrations of curcumin, cryptotanshinone and tanshinone weren't significantly, as detected by LC-MS/MS. The pharmacokinetic parameters of six constituents of JGXZ, were shown in Table 2, and the curve of blood drug concentrations at different time points in vivo was shown in Figure 6. It could be seen from the table that these 6 analytes can be detected after 0.25h of gavage, indicating that these components have been rapidly absorbed in rats. The AUC_{0-t} value of Rg1, astragaloside and tanshinone was relatively lower, indicating that drug absorption is low and most of the content may be in the form of metabolism.

Discussion

Although numerous studies have demonstrated that ROS in mitochondria could not contribute to IR in adipocytes[16], there is still a study reporting that ROS inhibitors reduce IR caused by chronic insulin treatment[17]. In our study, JGXZ of high dose significantly reduced ROS levels, accompanied by decreased lipid disposition in adipocyte, the effects of which was reversible to Rosiglitazone. It indicated that except for decreasing IR and increasing glucose uptake, JGXZ also could decrease lipid synthesis possibly by decreasing ROS levels. We speculated that the complexity of JGXZ components was the main reason. A previous study has suggested that ROS, the main reason of IR in adipocytes, promotes lipid deposition in liver[18]. Furthermore, ROS has been proved to be closely related with decreased lipid accumulation and ROS suppression in mitochondria-mediated preadipocytes differentiation[19-21].

ROS is reported to partake in regulating insulin signal pathway and inducing JNK activation[17]. However, with the increasing concentration of JGXZ, ROS presented reversal trend with p-JNK levels in our study, which suggested that JNK pathway was also activated by other molecular apart from ROS. ROS is not only involved in promoting AMPK phosphorylation, but also a upstream signal involved in the activation of AMPK[22, 23]. Redox status can modulate AMPK effects and antioxidation-reduced lipid accumulation via activating AMPK in adipocyte[24]. However, the trend of AMPK was different from that of ROS after JGXZ treatment. Thus, JGXZ activated AMPK possibly not only through ROS mediation. More and more studies showed that AMPK activation could decrease lipid accumulation in adipocytes[19, 25]. JGXZ reduced lipid accumulation possibly partly via LKB1/AMPK pathway. Compelling evidence has suggested that AMPK activation could phosphorylate ACC, inhibit fatty acid synthesis and promote fatty acid oxidation[26, 27]. Besides, insulin signalling was significantly increased at IRS/AKT pathway by JGXZ, indicating that JGXZ recovered the sensitivity of adipocytes to insulin. In our study, JGXZ could significantly reduce DEX-induced IR in adipocytes and increase glucose uptake possibly through AMPK, ROS and JNK/IRS/PI3K/AKT pathway.

To evaluate blood concentration of JGXZ in rats, LC-MS/MS method was used. It was observed that the concentrations of R1, Rg1, paeoniflorin, Rb1, astragaloside and tanshinone in rat serum could be determined by LC-MS/MS method, implying that they could play important roles in IR adipocyte induced by DEX. Some researches have demonstrated that some active components in JGXZ has obvious effects on decreasing lipid levels in NAFLD. A study demonstrated that Rg1 and Rb1 were involved improving lipid levels and ameliorate inflammation in NAFLD mice[28]. Paeoniflorin has obvious effects on suppressing lipid ectopic deposition, enhancing insulin sensitivities and decreasing oxidation in NAFLD rats[29, 30]. Astragaloside has obvious anti-lipid accumulation effects through regulating AMPK signal in NAFLD cell models[31, 32]. Taken together, JGXZ possibly exerted significant effects on decreasing glucose and lipid disposition mainly through these components. Although Notoginsenoside, Ginsenoside, Curcumin and Tanshinone were unable to be detect based the present instrument, their roles were unignorable. It is widely believed that the therapeutic effects of herbal medicines are based on the synergistic effects of multiple components. It has been successfully used to determine the blood drug concentration of JGXZ in rats, helps to clarify the drug effect mechanism of JGXZ, and is of great significance for the rational use of JGXZ. The results were essential for JGXZ use in clinic and further studying the effects of these components in NAFLD.

Conclusions

JGXZ decreases glucose and lipid disposition via regulating ROS/LKB1/AMPK and JNK/IRS/PI3K/AKT pathway. R1, Rg1, paeoniflorin, Rb1, astragaloside and tanshinone in JGXZ play pivotal roles in NAFLD, which is important for us to understand the mechanism of JGXZ in NAFLD.

Abbreviations

JGXZ: Jian-Gan-Xiao-Zhi decoction. NAFLD: Non-alcoholic fatty liver disease. LKB1: Liver kinase B1. IRS: insulin receptor substrate. TCM: Traditional Chinese medicine. LC-MS/MS: Liquid chromatography-tandem mass spectrometry. SD: Sprague-Dawley. LD: lipid droplet. AMPK: Adenosine 5'-monophosphate (AMP)-activated protein kinase. JNK: c-Jun N-terminal kinase. PI3k: phosphatidylinositol 3-kinase. AKT: PKB Protein Kinase

Declarations

Author details

¹Nanjing University of Chinese Medicine, Nanjing, 210023, China. The First Affiliated Hospital of Yunnan University of Chinese Medicine, Kunming, 650021, China. ²Yunnan University of Chinese Medicine, Kunming, 650500, China. ³Department of Dermatology, The First Affiliated Hospital of Yunnan University of Chinese Medicine, Kunming, 650021, China. ⁴Department of Senile disease, The First Affiliated Hospital of Yunnan University of Chinese Medicine, Kunming, 650021, China. *WeiBoWen15@163.com. The First Affiliated Hospital of Yunnan University of Chinese Medicine, No.120 Guanghua Road, Kunming, China.

Ethics approval and consent to participate

The study was supported by according to the National Institutes of Health Regulations and approved by the Institutional Animal Care and Use Committee at The First Affiliated Hospital of Yunnan University of Chinese Medicine.

Consent for publication

Not applicable.

Availability of data and materials

The experimental data are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

Funding

This work was supported by the National Natural Science Foundation of China (The Study of Jiangan Xiaozhi Recipe Improving Non-alcoholic Fatty Liver Disease by Regulating Insulin Resistance on Basis of AMPK/ROS/JNK Signaling Pathway, 81560772).

Author Contributions

Fang fang, Xue-Hua Xie and Ling Yang performed the experiment, analyzed data, and drafted the paper. Xu Zhang and Jie Zhao performed the experiment Fang Fang and Wei-Bo Wen organized and designed the study. All authors read and approved of the final manuscript.

Acknowledgements

Not applicable.

References

- [1] Barrows BR and Parks EJ. Contributions of different fatty acid sources to very low-density lipoprotein-triacylglycerol in the fasted and fed states. *J Clin Endocrinol Metab* 2006; 91: 1446-1452.
- [2] Greenberg AS, Coleman RA, Kraemer FB, McManaman JL, Obin MS, Puri V, Yan QW, Miyoshi H and Mashek DG. The role of lipid droplets in metabolic disease in rodents and humans. *J Clin Invest* 2011; 121: 2102-2110.
- [3] Cusi K. Role of obesity and lipotoxicity in the development of nonalcoholic steatohepatitis: pathophysiology and clinical implications. *Gastroenterology* 2012; 142: 711-725.e716.
- [4] Chen G and Goeddel DV. TNF-R1 signaling: a beautiful pathway. *Science* 2002; 296: 1634-1635.
- [5] Hotamisligil GS and Spiegelman BM. Tumor necrosis factor alpha: a key component of the obesity-diabetes link. *Diabetes* 1994; 43: 1271-1278.
- [6] Wang F, Yang X, Lu Y, Li Z, Xu Y, Hu J, Liu J and Xiong W. The natural product antroalbol H promotes phosphorylation of liver kinase B1 (LKB1) at threonine 189 and thereby enhances cellular glucose uptake. *J Biol Chem* 2019; 294: 10415-10427.
- [7] Zhang BB, Zhou G and Li C. AMPK: an emerging drug target for diabetes and the metabolic syndrome. *Cell Metab* 2009; 9: 407-416.

- [8] Jaeschke A, Czech MP and Davis RJ. An essential role of the JIP1 scaffold protein for JNK activation in adipose tissue. *Genes Dev* 2004; 18: 1976-1980.
- [9] Weibo WEN, Mengxue JIN, Fang FANG. Clinical research of Jiangnan Xiaozhi Granule in treating nonalcoholic fatty liver disease of type of qi stagnancy and blood stasis. *China medicine herald*, 2014,11(27):78-82..
- [10] Meng Xue Jin, Weibo Wen. The clinical research about the treatment of nonalcoholic fatty liver. M. disease with the particles for Jiangnanxiaozi
- [11] Xue-Hua Xie J-BL, Fang Fang, Jie Zhao, Yong-Jun Cao, Huan-Tian Cui, Hong-Wu Wang, Zhai-Yi Zhang, Zhao-Hui Sun, Yuan Yin, Wei-Bo Wen. Jian-Gan-Xiao-Zhi decoction ameliorates high-fat high-carbohydrate diet-induced non-alcoholic fatty liver disease and insulin resistance by regulating the AMPK/JNK pathwayTraditional Medicine Research 1-12.
- [12] Su T, Cheng BC, Fu XQ, Li T, Guo H, Cao HH, Kwan HY, Tse AK, Yu H, Cao H and Yu ZL. Comparison of the toxicities, activities and chemical profiles of raw and processed *Xanthii Fructus*. *BMC Complement Altern Med* 2016; 16: 24.
- [13] Liang C, Yin J, Ma Y, Zhang X and Zhang L. Quantitative determination of characteristic components from compound of *Lysionotus pauciflorus* Maxim. by LC-MS/MS and its application to a pharmacokinetic study. *J Pharm Biomed Anal* 2020; 177: 112835.
- [14] Jie Y, Mei-Zhen S, Jing W, Yun-Fei L, Pei L, Lin Q and Zhaorigetu B. In vitro cytotoxicity and in vivo acute and chronic toxicity of *Xanthii Fructus* and its processed product. *BioMed research international* 2013; 2013:
- [15] Reagan-Shaw S, Nihal M and Ahmad N. Dose translation from animal to human studies revisited. *Faseb j* 2008; 22: 659-661.
- [16] Martin SD, Morrison S, Konstantopoulos N and McGee SL. Mitochondrial dysfunction has divergent, cell type-dependent effects on insulin action. *Mol Metab* 2014; 3: 408-418.
- [17] Ge X, Yu Q, Qi W, Shi X and Zhai Q. Chronic insulin treatment causes insulin resistance in 3T3-L1 adipocytes through oxidative stress. *Free Radic Res* 2008; 42: 582-591.
- [18] Jin Y, Tan Y, Chen L, Liu Y and Ren Z. Reactive Oxygen Species Induces Lipid Droplet Accumulation in HepG2 Cells by Increasing Perilipin 2 Expression. *Int J Mol Sci* 2018; 19:
- [19] Liu M, Zheng M, Cai D, Xie J, Jin Z, Liu H and Liu J. Zeaxanthin promotes mitochondrial biogenesis and adipocyte browning via AMPK α 1 activation. *Food Funct* 2019; 10: 2221-2233.
- [20] Li G, Luan G, He Y, Tie F, Wang Z, Suo Y, Ma C and Wang H. Polyphenol Stilbenes from Fenugreek (*Trigonella foenum-graecum* L.) Seeds Improve Insulin Sensitivity and Mitochondrial Function in 3T3-L1 Adipocytes. *Oxid Med Cell Longev* 2018; 2018: 7634362.
- [21] Liu GS, Chan EC, Higuchi M, Dusting GJ and Jiang F. Redox mechanisms in regulation of adipocyte differentiation: beyond a general stress response. *Cells* 2012; 1: 976-993.

- [22] Tian ZH, Weng JT, Shih LJ, Siao AC, Chan TY, Tsuei YW, Kuo YC, Wang TS and Kao YH. Arecoline inhibits the growth of 3T3-L1 preadipocytes via AMP-activated protein kinase and reactive oxygen species pathways. *PLoS One* 2018; 13: e0200508.
- [23] Hwang JT, Kim SH, Lee MS, Kim SH, Yang HJ, Kim MJ, Kim HS, Ha J, Kim MS and Kwon DY. Anti-obesity effects of ginsenoside Rh2 are associated with the activation of AMPK signaling pathway in 3T3-L1 adipocyte. *Biochem Biophys Res Commun* 2007; 364: 1002-1008.
- [24] Jager S, Handschin C, St-Pierre J and Spiegelman BM. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1 α . *Proc Natl Acad Sci U S A* 2007; 104: 12017-12022.
- [25] Herranz-Lopez M, Barrajon-Catalan E, Segura-Carretero A, Menendez JA, Joven J and Micol V. Lemon verbena (*Lippia citriodora*) polyphenols alleviate obesity-related disturbances in hypertrophic adipocytes through AMPK-dependent mechanisms. *Phytomedicine* 2015; 22: 605-614.
- [26] Peng IC, Chen Z, Sun W, Li YS, Marin TL, Hsu PH, Su MI, Cui X, Pan S, Lytle CY, Johnson DA, Blaesus F, Chatila T and Shyy JY. Glucagon regulates ACC activity in adipocytes through the CAMKK β /AMPK pathway. *Am J Physiol Endocrinol Metab* 2012; 302: E1560-1568.
- [27] Qin Y, Yang RY, Chen MG, Shen XL and Ying-Jie HU. Inhibitory effect of cajanonic acid A on lipogenesis and lipolysis in 3T3-L1 adipocytes. *Chinese Pharmacological Bulletin* 2016;
- [28] Kim JC, Jeon JY, Yang WS, Kim CH and Eom DW. Combined Amelioration of Ginsenoside (Rg1, Rb1, and Rg3)-enriched Korean Red Ginseng and Probiotic *Lactobacillus* on Non-alcoholic Fatty Liver Disease. *Curr Pharm Biotechnol* 2019; 20: 222-231.
- [29] Ma Z, Chu L, Liu H, Wang W, Li J, Yao W, Yi J and Gao Y. Beneficial effects of paeoniflorin on non-alcoholic fatty liver disease induced by high-fat diet in rats. *Sci Rep* 2017; 7: 44819.
- [30] Zhang L, Yang B and Yu B. Paeoniflorin Protects against Nonalcoholic Fatty Liver Disease Induced by a High-Fat Diet in Mice. *Biol Pharm Bull* 2015; 38: 1005-1011.
- [31] Wang C, Li Y, Hao M and Li W. Astragaloside IV Inhibits Triglyceride Accumulation in Insulin-Resistant HepG2 Cells via AMPK-Induced SREBP-1c Phosphorylation. *Front Pharmacol* 2018; 9: 345.
- [32] Zhou B, Zhou DL, Wei XH, Zhong RY, Xu J and Sun L. Astragaloside IV attenuates free fatty acid-induced ER stress and lipid accumulation in hepatocytes via AMPK activation. *Acta Pharmacol Sin* 2017; 38: 998-1008.

Tables

Table 1 Pharmacokinetic parameters of six constituents of JGXZ (n=12)

Components/parameters	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (μ g/mL)	AUC_{0-t} (μ g/mL*h)	$MRT_{0-inf-obs}$ (h)	CI/F-obs
R1	3.00	6	21.31	113.49	7.64	0.27
Rg1	8.36	6	4.58	44.28	10.81	0.61
paeoniflorin	3.65	1	118.73	505.16	5.90	0.06
Rb1	20.86	6	301.16	3734.38	29.78	0.004
astragaloside	8.55	4	2.03	22.32	15.48	1.042
tanshinoneⅡA	3.73	0.5	0.72	6.21	11.62	4.67

Note: $T_{1/2}$: Terminal half-life. C_{max} : Maximum observed concentration occurring at T_{max} . AUC_{0-t} : Area under the curve from the time of dosing to t_{last} . $MRT_{0-inf-obs}$: mean retention time. CI/F-obs: clean rate.

Table II Connetrations of 9 main contents in JGXZ in rat serum were detected by LC-MS/MS. (n=12, No. 5 rat died).

number	R1	Rg1	paeoniflorin	Rb1	astragaloside	curcumin	cryptotanshinone	tanshinoneⅡA	tanshinoneⅡ
1	1.77	0.556	45	144	0.344	ND	ND	0.211	ND
2	55.7	38.9	223	635	5.49	ND	ND	0.229	ND
3	2.6	1.41	85.8	153	0.446	ND	ND	0.11	ND
4	9.41	10.8	145	161	0.591	ND	ND	0.157	ND
6	2.53	1.89	94.3	202	0.663	ND	ND	0.219	ND
7	3.26	4.18	112	261	0.466	ND	ND	0.184	ND
8	1.02	1.4	63.6	152	0.403	ND	ND	0.147	ND
9	7.74	9.42	368	358	0.928	ND	ND	0.154	ND
10	4	7.33	161	193	0.61	ND	ND	0.183	ND
11	0.967	2.14	60.9	132	0.223	ND	ND	0.113	ND
12	1.56	2.93	51.7	126	0.354	ND	ND	0.15	ND

Figures

Figure 1

A Oil red staining showed preadipocytes differentiation. B CCK8 assay analyzed activities of adipocytes treated by different concentration of JGXZ through CCK8 assay analysis. * $p < 0.05$, *** $p < 0.001$.

Figure 2

A the effects of serum containing drug on lipid accumulation for IR adipocytes. B the effects of serum containing drug on glucose consumption for IR adipocytes. C the effects of serum containing drug on TC for IR adipocytes. D the effects of serum containing drug on TG for IR adipocytes. * $p < 0.05$

Figure 3

A-B ROS levels were detected by flow cytometry after adipocytes were treated with serum containing drug. C the effects of serum containing drug on LKB1/AMPK and JNK/IRS/PI3k/AKT levels for IR adipocytes. * $p < 0.05$

Figure 4

A Representative LC-MS negative ion chromatograms of blank plasma; B lank plasma spiked with nine compounds(ng/ml) and C a rat plasma sample after oral administration of JGXF.

Figure 5

A Representative LC-MS positive ion chromatograms of blank plasma; B blank plasma spiked with nine compounds(ng/ml) and C a rat plasma sample after oral administration of JGXF.

Figure 6

Dose-time profiles of the six components in rat plasma after oral administration of JGXZ.