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Original Article

Yi-Gan-Jie-Du-Fang ameliorates non-alcoholic fatty liver disease in rats via regulating PPAR-α, UCP2, SIRT-1 and SREBP-1

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Abstract: Non-alcoholic fatty liver disease (NASH) could cause serious liver damage and abnormal liver function. *Yi-Gan-Jie-Du-Fang* (YJF) is an empirical Chinese formula in our hospital for treating NASH. This paper aimed to investigate the therapeutic effects of YJF on NASH and the potential mechanism. The NASH rat model was established by feeding rats with high fat diet for 16 weeks. The YJF (150, 300 and 600 mg/kg) was administered orally for 16 weeks. Our results revealed that YJF significantly decreased the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), triglyceride (TG) and high-density lipoprotein (HDL-C) and alleviated hepatic steatosis in liver tissues of NASH rats. Results of the real-time PCR and western blotting assays indicated that YJF significantly down-regulated the mRNA and protein levels of UCP2, SERBP-1 and Caspase-3 in the liver tissues of NASH rats, whereas up-regulated the mRNA and protein levels of PPAR-α and SIRT1. Collectively, our present investigation revealed that YJF could ameliorate fatty liver in NASH rats via regulating PPAR-α, UCP2, SIRT-1 and SREBP-1.

Keywords: *Yi-Gan-Jie-Du-Fang*, fatty liver, non-alcoholic fatty liver disease, PPAR-α, UCP2, SIRT-1, SREBP-1

Introduction

Non-alcoholic fatty liver disease (NASH), a kind of liver inflammatory disease, causes damages and abnormal functions of liver [1, 2]. It has been reported that NASH has a prevalence rate of 10%-30% in the world, especially for the obesity patients [3, 4]. Importantly, increasing investigations have revealed that incidence of NASH in children is also increasing nowadays [5]. It is well known that NASH is primarily caused by the accumulating fat in liver despite few alcohol intakes. In addition, NASH could be induced by various reasons such as obesity, pathoglycemia, and hyperlipemia, *etc* [6]. For most NASH patients, it might cause no obvious clinical symptoms in the early stage; however, NASH could also result in decreased liver function, hepatic fibrosis and cirrhosis, and NASH is also considered as a potential hazard for hepatocellular carcinoma [7, 8]. Thus, it is urgent for finding more effective drugs to treat NASH.

Yi-Gan-Jie-Du-Fang (YJF), an empirical Chinese formula, is used for treating NASH in our hospital for decades [9]. In Chinese traditional medicinal theory, YJF possesses the efficiencies of resolving phlegm, removing blood stasis and detoxification. YJF is composed of 9 traditional Chinese medicines (TCM) (**Table 1**), including Sargassum, Curcumae Radix, Cassiae Semen, Curcumae Longae Rhizoma, Sedi Herba, Gentianae Radix et Rhizoma, Ginseng Folium, Eupolyphaga Steleophaga and Bupleuri Radix. However, there is no experimental report regarding the therapeutic effects of YJF on NASH so far, and the related pharmacological mechanism is still not clear. Thus, in our present research, we established a rat NASH model to evaluate the therapeutic effects of YJF on NASH and explore the potential molecular mechanisms.

Materials and methods

Animals

Male Wistar rats (140 g ± 20 g, 5 weeks old) were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). All the rats

Table 1. Composition of the Yi-Gan-Jie-Du-Fang

Name	Plant	Part	Content (g)
Sargassum	*Sargassum pallidum*	Alginite	30
Curcumae Radix	*Curcuma wenyujin*	Radix	15
Cassiae Semen	*Cassia obtusifolia*	Semen	30
Curcumae Longae Rhizoma	*Curcuma longa*	Radix	15
Sedi Herba	*Sedum sarmentosum*	Whole herba	30
Gentianae Radix et Rhizoma	*Gentiana manshurica*	Radix	10
Ginseng Folium	*Panax ginseng*	Folium	15
Eupolyphaga Steleophaga	*Eupolyhaga sinensis*	Whole worm	10
Bupleuri Radix	*Bupleurum chinense*	Radix	10

Table 2. Primers used in real-time Fluorogenic PCR assays

Genes		Sequences	Size
PPAR-α	Forward	5'-CGGAATTTGCCAAGGCTATC-3'	129 bps
	Reverse	5'-GCATCCCGTCTTTGTTCATC-3'	
SIRT 1	Forward	5'-AGACCAGTAGCACTAATTCC-3'	122 bps
	Reverse	5'-ATTATGACATCGCAGTCTCC-3'	
SREBF-1	Forward	5'-CGCTACCGTTCCTCTATCAATG-3'	136 bps
	Reverse	5'-TCTGGTTGCTGTGCTGTAAG-3'	
UCP2	Forward	5'-TGTGGTAAAGGTCCGCTTCC-3'	147 bps
	Reverse	5'-GCATTTCGGGCAACATTGGG-3'	
GAPDH	Forward	5'-GTCGGTGTGAACGGATTTG-3'	181 bps
	Reverse	5'-TCCCATTCTCAGCCTTGAC-3'	

were kept in temperature-controlled house (25 ± 1°C) with free assess to food and water. All animal protocols were strictly in accordance with the international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals, and approved by the Animal Experimentation Ethics Committee of our hospital.

Chemicals and reagents

Trizol reagents were purchased from the Invitrogen Co. (Carlsbad, California, CA, USA). Primary antibodies of peroxisome proliferator-activated receptors (PPAR)-α, uncoupling protein (UCP) 2, sirtuin (SIRT)-1 and Caspase-3 were purchased from the Abcam Biotech. (Cambridge, MA, USA). Primary antibodies of sterol regulatory element binding protein (SREBP)-1 and GAPDH were purchased from the Cell Signaling Technology Inc. (Beverly, MA, USA). Hematoxylin-eosin (H&E) staining kit was purchased from the Beyotime Biothech. (Shanghai, China).

Experimental protocols and animal grouping

A total of 50 rats were randomly divided into 5 groups (n = 10): control group, model group and 3 YJF treatment groups (150, 300 and 600 mg/kg). Rats in model group and YJE treatment groups (150, 300 and 600 mg/kg) were prepared by feeding with high fat diet *ad libitum* (standard chow diet containing extra 10% lard oil and 2% cholesterol) for 16 weeks, while the control rats were fed a standard chow diet [10]. From the first day of high fat diet treatment, rats in control and model groups were administered orally with normal saline (10 ml/kg), and rats in the 3 YJE treatment groups were administered orally with YJF (150, 300 and 600 mg/kg/day). Blood samples were collected using orbital blood sampling at 8 and 16 weeks after the experiment. Rats were sacrificed at the end of the experiment, and the liver tissues were collected.

Determination of the concentration of serum indices

All the blood samples were centrifuged at 18,000× *g* for 15 min under 4°C to obtain the serum samples. Then, the automatic biochemistry analyzer (HITACHI 7170S, Japan) was used to determine the concentrations of alanine transaminase (ALT) and aspartate transaminase (AST), total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C).

Histopathological examinations

Histopathological examinations were carried out according to the previous report with minor modification [11]. Briefly, liver tissues were fixed in 10% neutral buffered formalin, subsequently embedded in paraffin, and sectioned at

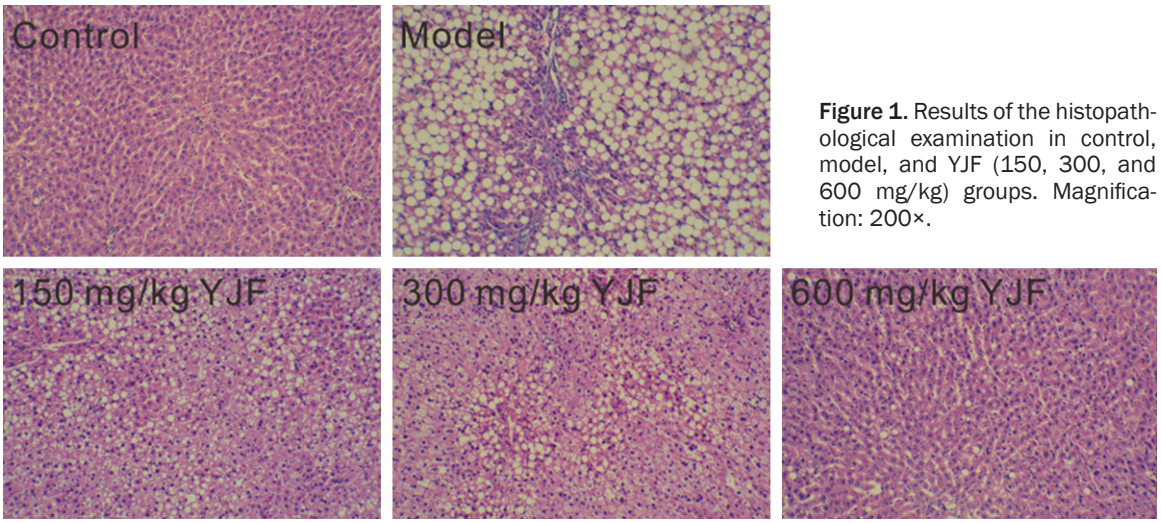

Figure 1. Results of the histopathological examination in control, model, and YJF (150, 300, and 600 mg/kg) groups. Magnification: 200×.

5 μm. After de-paraffinizing, the liver tissue sections were stained with hematoxylin-eosin (H&E). Finally, histopathological changes of liver tissues were examined using an optical microscope (Olympus, Japan).

Quantitative real-time PCR

Total RNA of the liver tissue was extracted using Trizol reagent, and used for cDNA synthesis of PPAR-α, UCP2, SIRT-1 and SREBP-1 by reverse transcription using quantitative real-time PCR (ABI-7300, USA) with SYBR Green reagents (Thermo, USA). All primers for the real-time PCR were designed by Primer 5.0 and synthesized by JRDun Biotech. (Shanghai, China) (**Table 2**). Amplification condition was conducted as: 95°C for 10 min, 40 cycle × (95°C for 15 s, 60°C for 45 s), 95°C for 15 s, 60°C for 60 s, 95°C for 15 s, and 60°C for 15 s. The relative mRNA expressions were evaluated by $2^{-\Delta\Delta Ct}$ relative quantitative analysis in each sample.

Western blotting assay

Liver tissues were homogenized and then total protein of the liver tissues was extracted. An equal amount of protein (35 μg) was loaded to SDS-PAGE and subsequently transferred to a PVDF membrane. Then, the primary antibodies of PPAR-α, UCP2, SIRT-1, SREBP-1, Caspase-3 and GAPDH were used to determine the expression of corresponding proteins, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. Finally, the protein bands were detected by an ECL-detecting kit (Beyotime Biothech, Shanghai, China). GAPDH was used as the internal reference to normalize the protein loading.

Statistical analysis

Data are expressed as mean ± SD. ANOVA was carried out to compare the difference between two groups using SPSS 18.0 software (SPSS Inc., USA) and *P* value less than 0.05 was recognized as statistically significant.

Results

Results of the histopathological examination of liver tissues

Histopathological changes of the liver tissues were showed in **Figure 1**. In control rats, hepatocytes were normal arranged and no obvious degeneration and necrosis was observed. On the contrary, severe fatty degeneration was observed in liver tissues of NASH model rats compared to the control rats. In YJF (150, 300 and 600 mg/kg) treated rats, the fatty degeneration of the liver tissues induced by high fat diet were gradually alleviated with the increasing dose of YJF.

Effects of YJF on AST, ALT, TC, TG, HDL-C, LDL-C in serum

Serum indices were also determined at 8 and 16 weeks after treatment. As shown in **Figure 2**, compared to control rats, after treatment with high fat diet, the serum levels of AST, ALT, TC, TG and HDL-C of NASH model rats were significantly increased at both 8 and 16 weeks. In

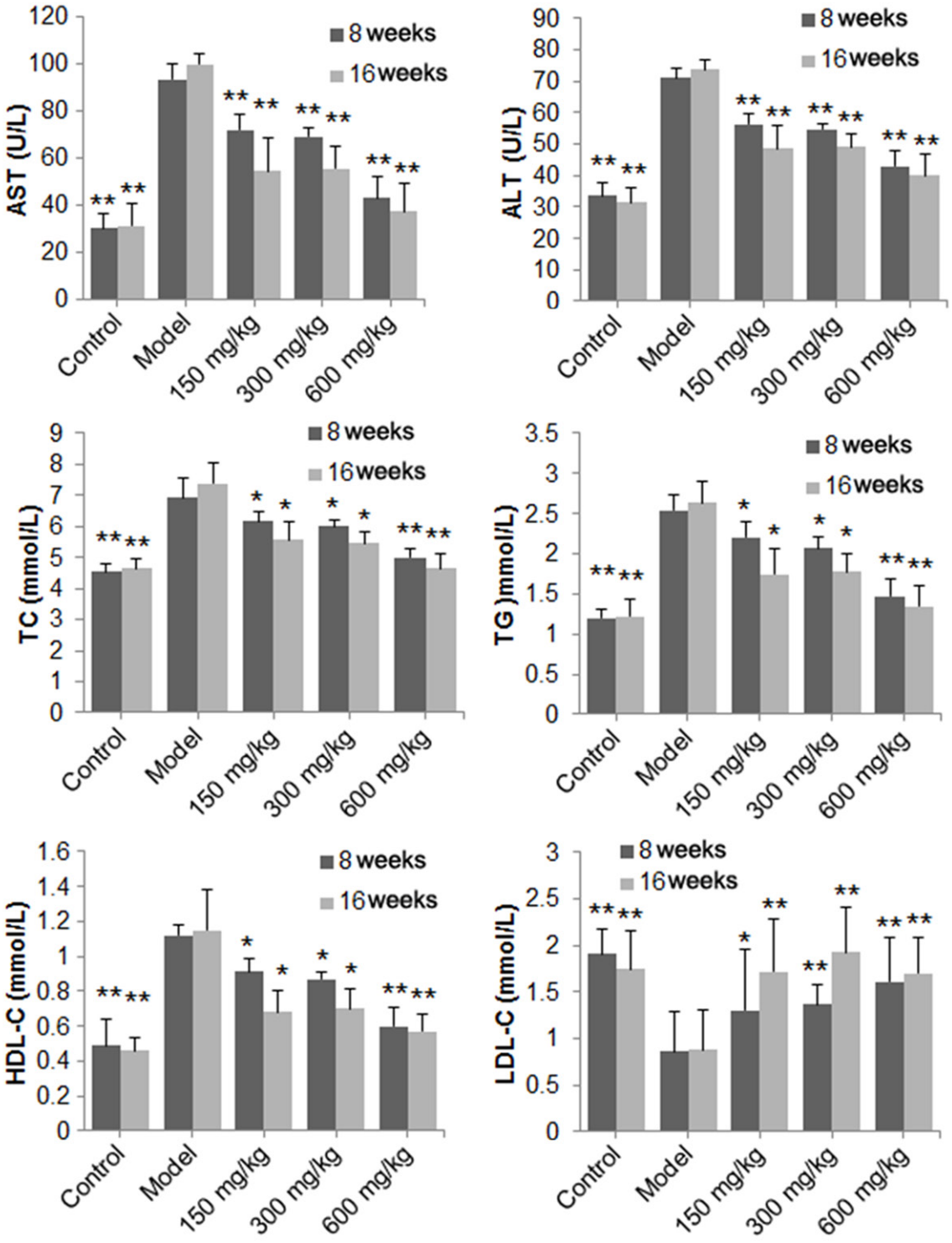

Figure 2. Effects of YJF on AST, ALT, TC, TG, HDL-C, LDL-C in serum. *P < 0.05, **P < 0.01, compared with model rats.

addition, the serum LDL-C level was significantly decreased in model rats at both 8 and 16 weeks. These data indicated that the liver functions of model rats were notably weakened. Interestingly, YJF (150, 300 and 600 mg/kg) treatment could significantly decrease the

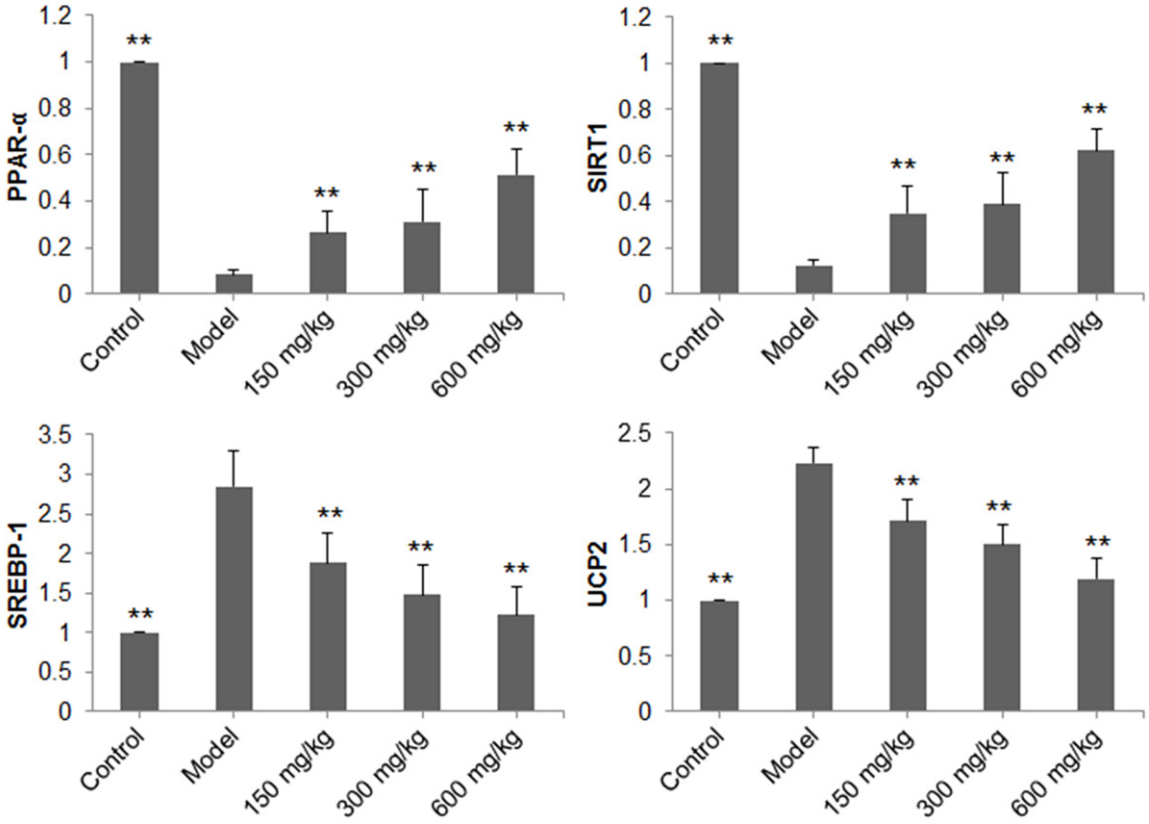

Figure 3. Effects of YJF on mRNA levels of PPAR-α, SIRT1, SREBP-1 and UCP2 in liver tissue. *P < 0.05, **P < 0.01, compared with model rats.

serum levels of AST, ALT, TC, TG and HDL-C of NASH rats at both 8 and 16 weeks, whereas increase the serum level of LDL-C, in a dose-dependent manner.

Effects of YJF on mRNA/protein expressions of PPAR-α, UCP2, SIRT-1, SREBP-1 and caspase-3 in liver tissues

To investigate the possible mechanism of YJF, we determined the expression of PPAR-α, UCP2, SIRT-1, SREBP-1 and Caspase-3 in liver tissues. As indicated in **Figure 3**, after treatment with high fat diet for 16 weeks, mRNA levels of PPAR-α and SIRT-1 were down-regulated sharply, whereas the SREBP-1 and UCP2 were significantly up-regulated, compared with control rats. Importantly, YJF (150, 300 and 600 mg/kg) could remarkably up-regulate PPAR-α and SIRT-1 dose-dependently, and significantly down-regulate the SREBP-1 and UCP2 dose-dependently, compared with model rats.

Similar to the mRNA levels, the YJF (150, 300 and 600 mg/kg) could also significantly up-regulate PPAR-α and SIRT-1, whereas down-regulate the SREBP-1 and UCP2, compared with model rats. Besides, our results also revealed that YJF (150, 300 and 600 mg/kg) could decrease the Caspase-3 in liver tissues (**Figure 4**).

Discussion

Currently, the available drugs for treatment of NASH include hepatocyte protective agents, lipid-lowering drugs and antioxidants, such as vitamins, lecithin, ursodesoxycholic acid, and silymarin, *etc* [12]. However, therapeutic effects of these drugs mentioned above are not reliable, and some of them even have potential hepatotoxicity. It is really a pity that no specific drug has been approved and firmly recommended for treating NASH by regulatory agencies so far. In our present research, we demonstrated that YJF has the potential therapeutic effects on NASH for the first time, and we also firstly investigated the possible pharmacological mechanisms of YJF.

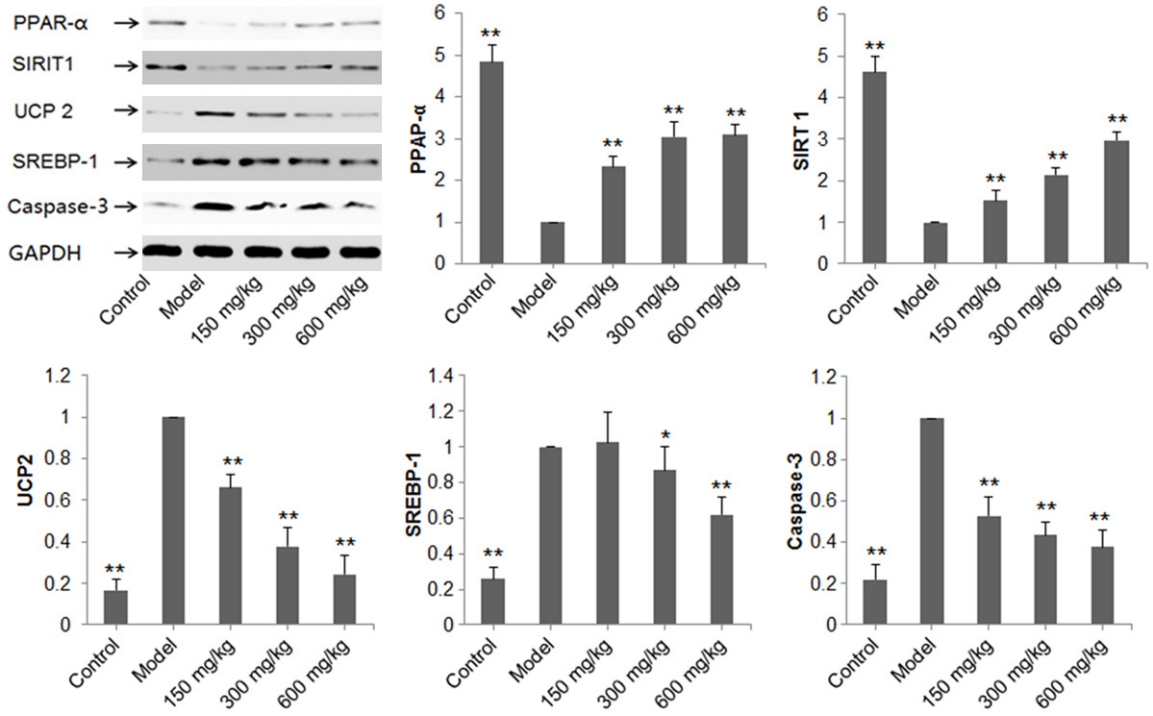

Figure 4. Effects of YJF on protein levels of PPAR-α, SIRT1, SREBP-1 and UCP2 in liver tissue. *P < 0.05, **P < 0.01, compared with model rats.

It is reported that most of the NASH patients have severe lipid metabolism disorder [13, 14]. In our results, YJF could notable down-regulate the serum levels of TC, TG and HDL-C. In addition, YJF could also decrease the serum levels of AST and ALT, indicating that YJF has the potential hepatoprotective effects. All these results above suggested that YJF could be beneficial for treating NASH. Besides, the improving effects of YJF on NASH rats could be also confirmed based on the results of histopathological examination.

The pathogenesis of NASH is complex, and the "two-hits" theory suggested by Day & James might be a relative accepted pathogenesis of NASH [15]. The "first-hit" is mainly regarding the insulin resistance and lipidosis in hepatocytes induced by lipid metabolism disorder [16]. The "second hit" mainly involves in oxidative stress [17], and UCP is one of the most important elements. UCPs are carrier proteins in the mitochondrial inner membrane, and belong to the mitochondrial electron transporter superfamily [18]. UCP2 is commonly low expressed in hepatocytes and only expressed in Kupffer cells [19]. UCP2 has important physiological effects in fatty acid metabolism, and could be activated by the compounds of free fatty acid (FFA) and PPAR-α. Previous research reported that UCP2 could also regulate the oxidation of free fatty acid based on reducing cell oxidation efficiency, resulting in lipopexia and cell steatosis [19, 20]. Previous reports revealed that PPAR-α is low expressed in hepatic steatosis patients, and PPAR-α agonists have been used to alleviate the hepatic steatosis [21, 22]. Furthermore, down-regulating SERBP could be beneficial for alleviating hepatic steatosis [22, 23]. Besides, SIRT1 has been reported to be positive on NASH patients based on its regulatory effects on deacetylation and inhibitory effects on the transcriptional activity of UCP2 [24]. Oxidative stress in liver tissues could induce over-expressed caspase-3 and hepatocyte apoptosis. Caspase-3 plays important roles in the development of NASH, and over-expressed caspase-3 could aggravate the damage of liver tissues [25, 26]. In our present work, we found that YJF could significantly down-regulate the UCP2, SERBP-1 and Caspase-3 in liver tissues of NASH rats, whereas up-regulate the PPAR-α and SIRT1. These

results mentioned above might be related to the potential molecular mechanisms of YJF.

In conclusion, this present research revealed that *Yi-Gan-Jie-Du-Fang* could ameliorate fatty liver in NASH rats via regulating PPAR-α, UCP2, SIRT-1 and SREBP-1.

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Disclosure of conflict of interest

None.

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References

[1] Hur JH, Park SY, Dall'Armi C, Lee JS, Di Paolo G, Lee HY, Yoon MS, Min DS and Choi CS. Phospholipase D1 deficiency in mice causes nonalcoholic fatty liver disease via an autophagy defect. Sci Rep 2016; 6: 39170.

[2] Jang MK, Nam JS, Kim JH, Yun YR, Han CW, Kim BJ, Jeong HS, Ha KT and Jung MH. Schisandra chinensis extract ameliorates nonalcoholic fatty liver via inhibition of endoplasmic reticulum stress. J Ethnopharmacol 2016; 185: 96-104.

[3] Farrell GC, Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. Hepatology 2006; 43: 99-112.

[4] de Alwis NM, Day CP. Non-alcoholic fatty liver disease: the mist gradually clears. J Hepatol 2008; 48 Suppl 1: S104-12.

[5] Chitturi S, Farrell GC. Etiopathogenesis of nonalcoholic steatohepatitis. Semin Liver Dis 2001; 21: 27-42.

[6] Reid AE. Nonalcoholic steatohepatitis. Gastroenterology 2001; 121: 710-23.

[7] Li BH, He FP, Yang X, Chen YW and Fan JG. Steatosis induced CCL5 contributes to early-stage liver fibrosis in nonalcoholic fatty liver disease progress. Transl Res 2017; 180: 103-117.

[8] Ludiwig J, McGill DB, Lindor KD. Nonalcoholic steatohepatitis. J Gastroenterol Hepatol 1997; 129: 398-403.

[9] Wang YP, Zheng X, Zhang ZY, ZHu Y, Zhuang MZ, Han J, Zhou F, Guo ZW, Zhang D and Y W. Clinical observation of "Yigan Jiedu Decoction" in treating nonalcoholic steatohepatitis of phlegm and blood-stasis accumulation. Shanghai J TCM 2012; 46: 25-27.

[10] Jiang Y, Chen L, Wang H, Narisi B and Chen B. Li-Gan-Shi-Liu-Ba-Wei-San improves non-alcoholic fatty liver disease through enhancing lipid oxidation and alleviating oxidation stress. J Ethnopharmacol 2015; 176: 499-507.

[11] Peng W, Qiu XQ, Shu ZH, Liu QC, Hu MB, Han T, Rahman K, Qin LP and Zheng CJ. Hepatoprotective activity of total iridoid glycosides isolated from Paederia scandens (lour.) Merr. var. tomentosa. J Ethnopharmacol 2015; 174: 317-321.

[12] Toplak H, Stauber R and Sourij H. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease: guidelines, clinical reality and health economic aspects. Diabetologia 2016; 59: 1148-9.

[13] Liu L, Yang M, Lin X, Li Y, Liu C, Yang Y, Yamahara J, Wang J and Li Y. Modulation of hepatic sterol regulatory element-binding protein-1c-mediated gene expression contributes to Salacia oblonga root-elicited improvement of fructose-induced fatty liver in rats. J Ethnopharmacol 2013; 150: 1045-1052.

[14] Xiao J, Xing F, Huo J, Fung ML, Liong EC, Ching YP, Xu A, Chang RCC, So KF and Tipoe GL. Lycium barbarum polysaccharides therapeutically improve hepatic functions in non-alcoholic steatohepatitis rats and cellular steatosis model. Sci Rep 2014; 4: 5587.

[15] Day CP and James OF. Steatohepatitis-a tale of two "hits"? Gastroenterology 1998; 114: 842-845.

[16] Carazo A, Leon J, Casado J, Gila A, Delgado S, Martín A, Sanjuan L, Caballero T, Muñoz JA, Quiles R, Ruiz-Extremera A, Alcázar LM, Salmerón J. Hepatic expression of adiponectin receptors increases with non-alcoholic fatty liver disease progression in morbid obesity in correlation with glutathione peroxidase 1. Obes Surg 2011; 21: 492-500.

[17] Chen ZW, Chen Ly, Dai HL, Chen JH, Fang LZ. Relationship between alanine aminotransferase levels and metabolic syndrome in nonalcoholic fatty liver disease. J Zhejiang Univ Sci B 2008; 9: 616-22.

[18] Ricquier D and Bouillaud F. The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. Biochem J 2000; 345 Pt 2: 161-79.

[19] Boss O, Hagen T, Lowell BB. Uncoupling proteins 2 and 3: potential regulators of mitochondrial energy metabolism. Diabetes 2000; 49: 143-56.

[20] Li LX, Skorpen F, Egeberg K, Jorgensen IH, Jorgensen IH and Grill V. Uncoupling protein-2 participates in cellular defense against oxida-

tive stress in clonal beta-cells. Biochem Biophys Res Commun 2001; 282: 273-7.
[21] Xu KZ, Zhu C, Kim MS, Yamahara J and Li Y. Pomegranate flower ameliorates fatty liver in an animal model of type 2 diabetes and obesity. J Ethnopharmacol 2009; 123: 280-287.
[22] Ziamajidi N, Khaghani S, Hassanzadeh G, Vardasbi S, Ahmadian S, Nowrouzi A, Ghaffari SM and Abdirad A. Amelioration by chicory seed extract of diabetes-and oleic acid-induced non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH) via modulation of PPARα and SREBP-1. Food Chem Toxicol 2013; 58: 198-209.
[23] Wang S, Li X, Guo H, Yuan Z, Wang T, Zhang L and Jiang Z. Emodin alleviates hepatic steatosis by inhibiting sterol regulatory element binding protein 1 activity by way of the calcium/calmodulin-dependent kinase kinase-AMP-activated protein kinase-mechanistic target of rapamycin-p70 ribosomal S6 kinase signaling pathway. Hepatol Res 2016; [Epub ahead of print].
[24] Colak Y, Yesil A, Mutlu HH, Caklili OT, Ulasoglu C, Senates E, Takir M, Kostek O, Yilmaz Y, Yilmaz Enc F, Tasan G and Tuncer I. A potential treatment of non-alcoholic fatty liver disease with SIRT1 activators. J Gastrointestin Liver Dis 2014; 23: 311-9.
[25] Feldstein AE, Canbay A, Angulo P, Taniai M, Taniai M, Burgart LJ, Lindor KD, Gores GJ. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. Gastroenterology 2003; 125: 437-43.
[26] Ribeiro PS, Cortez-Pinto H, Sola S, Castro RE, Ramalho RM, Baptista A, Moura MC, Camilo ME, Rodrigues CM. Hepatocyte apoptosis, expression of death receptors, and activation of NF-kappaB in the liver of nonalcoholic and alcoholic steatohepatitis patients. Am J Gastroenterol 2004; 99: 1708-17.