Original Article

Acupuncture and Herbal Medicine

OPEN

Novel insights into the effect of Xiaoyao san on corticosterone-induced hepatic steatosis: inhibition of glucocorticoid receptor/perilipin-2 signaling pathway

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Abstract

Objective: Xiaoyao san (XYS) is a classic traditional Chinese medicinal formula. It has been clinically administered to regulate liver function. However, its mechanisms in glucocorticoid-induced hepatic steatosis are unknown. This study aimed to investigate whether XYS protects against corticosterone (CORT)-induced hepatic steatosis, and to explore its mechanism.

Methods: High-fat diet mice induced with hepatic steatosis by 2 mg/kg CORT were administered 2.56 g/kg or 5.12 g/kg XYS daily for 7 weeks. The effects of XYS on hepatic steatosis in mice were evaluated by H&E and Oil Red O staining and by measuring their plasma lipids (triglyceride, total cholesterol, and free fatty acids). The mechanism of XYS against hepatic steatosis was investigated by network pharmacology, immunohistochemistry, western blotting, and gain-of-function/loss-of-function experiments.

Results: XYS alleviated CORT-induced steatosis, decreased plasma lipids, and inhibited glucocorticoid receptor (GR) activation in the liver. Network pharmacology data indicated that XYS may have mitigated hepatic steatosis *via* GR which mediated adipose differentiation-related protein (ADFP). Gain-of-function/loss-of-function experiments *in vitro* confirmed that GR positively regulated ADFP expression.

Conclusions: XYS ameliorated CORT-induced hepatic steatosis by downregulating the GR/ADFP axis and inhibiting lipid metabolism. Our studies implicate that XYS is promising as a therapy for CORT-induced hepatic steatosis, and lay the foundation for designing novel prophylactic and therapeutic strategies on CORT-induced hepatic steatosis.

Keywords: Adipose differentiation-related protein, Glucocorticoid receptor, Hepatic steatosis, Network pharmacology, Xiaoyao san

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common metabolic liver disease in the world[1], and nonalcoholic hepatic steatosis (NASH) is a serious consequence of NAFLD[2]. It is reported that the prevalence of NASH is estimated to be approximately 3% to 6% in the US population, and more than 20% of patients with NASH progress to cirrhosis, end-stage liver disease, and even death with time[3]. Hepatic steatosis is also detected in 20% of all hypercortisolism patients[4–5] who have presented with excessive glucocorticoid secretion or have been subjected to glucocorticoid therapy[6–7]. Hypercortisolism-related hepatic steatosis is attributed mainly to hyperactivation of the glucocorticoid receptor (GR) *via* the hypothalamic-pituitary adrenocortical axis[8–9] and is regulated by a GR-dependent pathway[9–10]. GR activation inhibits fatty acid β-oxidation through the peroxisome proliferator-activated receptor pathway[11]. Heat shock cognate protein 70 (HSP 70) is a GR chaperonin protein that binds perilipin-2 (adipose differentiation related protein, ADFP) which stabilizes lipid droplets[12]. The foregoing evidence indicates that interference with GR activation and its downstream pathway could ameliorate hypercortisolism-related hepatic steatosis. This condition may potentially develop into steatohepatitis or cirrhosis[13–14] when therapy is delayed. Sterol regulatory element-binding proteins-1 inhibitor, acetyl-coenzyme A hydroxylase inhibitor, stearoyl-coenzyme A desaturase 1 inhibitor, peroxisomal proliferation-activated receptor activator, and so on have been widely applied in clinics for prevention and therapy hepatic steatosis[15]. However, the GR antagonists are still few applied for hepatic steatosis.

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Acupuncture and Herbal Medicine (2022) 2:1

Received 15 May 2021 / Accepted 2 December 2021

http://dx.doi.org/10.1097/HM9.0000000000000011

Even though mifepristone (RU-486), a well-known GR antagonist, can reverse the negative effects of GR including hepatic steatosis, it is still limited in clinic application because of hypovolemia, hypotension, and infertility[16]. Hence, a new safe and efficacious therapy against glucocorticoid-induced hepatic steatosis is urgently required. Therefore, in this study, we devote ourselves to discovering an effective and safe therapy against glucocorticoid-induced hepatic steatosis.

Xiaoyao san (XYS) is a classical traditional Chinese medicine (TCM) prescription. It is recorded in *Prescriptions of the Bureau of Taiping People's Welfare* (*Tài Píng Huì Mín Hé Jì Jú Fāng*) from the Song dynasty and has been administered to alleviate depression and liver diseases for >800 years. It has had obvious efficacy and induced few side effects[17]. XYS consists of *Bupleuri Radix, Angelicae Sinensis Radix, Paeoniae Radix Alba, Atractylodis Macrocephalae Rhizoma, Poria, Glycyrrhizae Radix et Rhizoma, Menthae Haplocalycis Herba,* and *Zingiberis Rhizoma Recens* in a 5:5:5:5:5:4:1:5 weight ratio according to the Xiaoyao wan (condensed pill) entry in the "Chinese Pharmacopoeia 2020 Edition". The active ingredients in XYS were confirmed to have anti-neuritis[18], anti-hepatitis[19–20], hepatoprotective[21–22], and antihepatotoxic[23] effects. However, the modes of action of XYS against glucocorticoid-induced hepatic steatosis are unknown. In this study, we aimed to investigate whether XYS protects against corticosterone (CORT)-induced hepatic steatosis, and to explore its mechanism. Several reports indicated that XYS restores serum CORT levels, increases energy metabolism, and reduces lipid accumulation[24–25]. Glycyrrhizin in XYS lowers HSP 90 levels and attenuates GR ligand affinity[26]. Moreover, aikosaponin D in XYS regulates GR translocation[27]. GR potentially regulates ADFP by binding HSP 70[12]. Therefore, we hypothesized that XYS protection against glucocorticoid-induced hepatic steatosis is mediated by GR and ADFP.

Materials and methods

Materials

XYS was prepared by Jiuzhitang Co. Ltd., Changsha, China, in accordance with the formulation process described in the "Chinese Pharmacopoeia 2020 Edition". A 0.42 extraction ratio of XYS was used in this study. XYS extractions were analyzed by liquid chromatography-mass spectrometry (LC–MS, TM Q ExactiveTM Orbitrap LC-MS/MS system, Thermo Scientific, Waltham, MA, USA) and quantified by ultra-high performance liquid chromatography (UHPLC) at 280 nm[28] (Supplemental Digital Content: XYS extraction was analyzed by UPLC, http://links.lww.com/AHM/A0). In brief, the mobile phases comprised eluent A (0.1% formic acid) and eluent B (methanol). The gradient flow was as follows: 0 to 5 min, 10% to 30% B; 5 to 12 min, 30% to 35% B; 12 to 20 min, 35% to 90% B. The analysis was performed at a flow rate of 0.3 mL/min with ACQUITY UPLCR HSS T_3 column 1.8 μm, 2.1 mm × 100 mm (Waters, Milford, MA, USA). UHPLC separations were conducted by Dionex Ultimate UHPLC 3000 system (Thermo Scientific, Waltham, MA, USA). The injection volume was 4 μL. The spectrum only identified and recorded the sample components within the first 20 min. CORT and RU-486 were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Animal experiments

Male C57BL/6J mice aged 3 weeks were purchased from Guangdong Provincial Medical Laboratory Animal Center, Guangdong, China and managed in accordance to protocols approved by the Institutional Animal Care and Use Committee of Jinan University, Guangzhou, China (Approval number: 20130904001). All mice were housed at (22 ± 2)°C under a 12/12 h light/dark cycle and randomly assigned to six groups ($n = 6$). Normal diet (D12450B; 3.84 kcal/g; 10% kcal from fat consisting of 25 g/kg soybean oil and 20 g/kg lard) and high-fat diet (HFD) (D12492; 5.24 kcal/g; 60% kcal from fat consisting of 25 g/kg soybean oil and 245 g/kg lard) were purchased from Research Diet, New Brunswick, NJ, USA. All mice except those administered the normal diet (ND) received a HFD for 14 weeks to induce hepatic steatosis[29–30]. From the 8th week, the HFD-fed mice were subcutaneously injected with 2 mg/kg CORT (Sigma-Aldrich Corp., St. Louis, MO, USA) once daily for 7 weeks to promote glucocorticoid-induced hepatic steatosis[31–32]. The daily XYS dosage was determined based on an average adult human body weight of 70 kg, a conversion factor of 9.1 between humans and mice, and a 0.42 extraction rate of XYS. To reflect the protective mechanism of XYS against CORT-induced hepatic steatosis, the low and high experimental XYS dosages (2.56 g/kg and 5.12 g/kg, respectively) were set to fourfold and eightfold the clinical dosage reported having satisfactory efficacy[17,33–34]. XYS and the GR antagonist RU-486 (12.5 mg/kg; positive control; Sigma-Aldrich Corp., St. Louis, MO, USA) were intragastrically administered and subcutaneously injected, respectively, to the CORT-treated mice once daily for 7 weeks.

The mice were sacrificed under ether anesthesia at the end of the experiment. Plasma was isolated from blood samples treated with heparin sodium. Plasma triglyceride (TG), total cholesterol (TC), and free fatty acid (FFA) levels were determined with commercial kits (Nanjing Jiancheng Bioengineering Co. Ltd., Nanjing, China). The livers were promptly excised for the subsequent experiments.

Histomorphology, immunohistochemistry (IHC), and immunofluorescence

For histomorphology, the liver tissues were fixed in 4% (v/v) paraformaldehyde, embedded in paraffin, cut into 5 μm slices by microtome (RM2235, Leica, Wetzlar, Germany), and stained with H&E and Oil Red O staining according to the manufacturer's instructions. Tissue images (200×) were viewed and recorded with the M8 microscope and scanner (Precipoint M8, Freising, Germany).

For IHC, the sections were incubated with primary antibodies against ADFP (NB110–40877; Novus Biologicals, Littleton, CO, USA) and GR (#12041; Cell Signaling Technology, Danvers, MA, USA). A 3,3′-diaminobenzidine detection kit (Gene Technology, Shanghai, China) was used according to the manufacturer's instructions. Tissue images (400×) were viewed and recorded with the M8 microscope and scanner (Precipoint M8, Freising, Germany).

For immunofluorescence, the liver sections were incubated with primary antibody against ADFP at 4°C overnight, incubated with secondary antibody conjugated to Alexa Fluor 488 at 37°C for 1 h, and stained with 4′,6-diamidino-2-phenylindole at 25°C for 10 min.

Immuno-fluorescence images (400×) were viewed and recorded with a Zeiss LSM510 Meta Duo Scan laser scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

Lipid droplet extraction

Lipid droplets were extracted according to the methods previously reported[35–36]. In brief, liver tissues were gently ground with phosphate-buffered saline in a Dunes homogenizer (Wheaton, Millville, NJ, USA) and the supernatants were collected by centrifugation (12,000 × *g*, 4°C, 10 min). Then 3 mL supernatant was mixed with 3 mL of 1.08 M sucrose and the mixture was gently layered onto a gradient separation solution consisting of equal volumes of 270 mM sucrose, 135 mM sucrose, and Tris-EDTA-EGTA solution. The gradient solution samples were separated by ultra-high-speed refrigerated centrifugation (150,000 × *g*, 4°C, 60 min). The milky white upper fraction contained the lipid droplets and was collected.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from the liver samples with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and reverse-transcribed to cDNA with a reverse transcription kit (Transgen Biotech, Guangzhou, China). The cDNA samples were combined with specific primers (Table 1) and SYBR qPCR Master Mix (Transgen Biotech, Guangzhou, China). The mixture was amplified on a CFX Connect™ RT-qPCR system. The relative mRNA expression of each gene was normalized with 18 s by the $2^{-\Delta\Delta Ct}$ method[37].

Western blotting

Total protein from the liver tissues and cells was extracted by lysis buffer and quantified with a Pierce® bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Franklin, MA, USA). Ten-microgram protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Target proteins were measured by incubating them with primary antibodies including anti-ADFP, anti-GR, and anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Transgen Biotech, Guangzhou, China). The target proteins were then incubated with secondary antibodies and the immunoblots were visualized with Pierce® enhanced chemiluminescence western blotting substrate (Thermo Fisher Scientific, Franklin, MA, USA). GAPDH expression was the quantitative reference. The expression of proteins was quantified by ImageJ software (version 1.46, NIH, Bethesda, MD, USA).

Table 1

Gene primer sequences

Gene	Forward (5′–3′)	Reverse (5′–3′)
18s	ACGGCTACCACATCC	CAGACTTGCCCTCCA
Plin1	TACCTAGCTGCTTTCTCGGTGTTAC	GGGCTTCTTTGGTGCTGTTGT
Plin2	GGAGTGGAAGAGAAGCATCG	CAACACAGTGGGACTCATGG
Plin3	TGCTGGGTGATGTCCCTGAAC	GCCTGATGGAATCCGTGAAAG
Plin4	GTGCTGGCTCATTC	TGGCTTGTCCTACC
Plin5	ACGAGGTGGTGGGTGA	GAAGGGAACAGAAGCC

Prediction of potential signaling pathways of XYS

Six constituent herbs in XYS were submitted to BATMAN-TCM (http://bionet.ncpsb.org/batman-tcm) to discover their therapeutic mechanisms. After the ingredients in the herbs were identified, the candidate targets were calculated by the similarity-based target prediction method[38]. Putative targets were predicted because the score cutoff was >30. XYS regulated GR and ADFP expression. Hence, a subsequent functional analysis calculated the intersections of GR, ADFP, the XYS compounds, and the putative XYS targets in DAVID 6.7 (https://david.ncifcrf.gov) and the STRING database (https://string-db.org/) and indicated the KEGG pathways. The herb-compound-target-pathway network was constructed with Cytoscape 3.7.2.

Plasmid and siRNA transfection

HepG2 cells (American Type Culture Collection, Rockville, MD, USA) were incubated in Roswell Park Memorial Institute 1640 medium with fetal bovine serum. HepG2 cells were transfected with NR3C1 plasmid to overexpress cellular GR. The overexpression-NR3C1 plasmid and the vector (NC) were purchased from Beijing TransGen Biotech Co. Ltd. (Beijing, China). Si-NR3C1 primers were used to knock down NR3C1 (Guangzhou Ribobio Co. Ltd., Guangzhou, China) by targeting the NR3C1 sequence CAACGGTGGCAATGTGAAA. Negative control siRNA primers (si-NC) were purchased from Guangzhou Ribobio Co. Ltd. and used as normal controls. HepG2 cells were transfected with plasmids, siRNAs, and 0.5% Lipofectamine™ LTX (Invitrogen, Carlsbad, CA, USA).

Statistical Analysis

Data are mean ± standard deviation (SD) and were plotted with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Statistically significant differences between treatment means were analyzed by one-way analysis of variance. $P < 0.05$ represented significant differences among groups.

Results

XYS ameliorated hepatic steatosis in CORT-induced fat liver disease (FLD) mice

The peaks indicated in Supplemental Digital Content (XYS extraction was analyzed by UPLC, http://links.lww.com/AHM/A0) were (1) paeoniflorin, (2) ferulic acid, and (3) liquiritin. H&E and Oil Red O staining in Figure 1A and 1B showed that both 2.56 g/kg and 5.12 g/kg XYS reduced the number of fat vacuoles in the livers of the CORT-induced FLD mice. XYS also lowered the levels of plasma lipids including TG, TC, and FFA in a dose-dependent manner in CORT-treated FLD mice (Figure 1C-1E, $P < 0.05$). The GR antagonist RU-486 reversed hepatic steatosis symptoms and lowered the plasma lipid levels in CORT-induced FLD mice (Figure 1, $P < 0.01$).

XYS inhibited GR activation in CORT-induced FLD mice

The effects of XYS on hepatic GR expression in CORT-treated FLD mice were shown in Figure 2. The IHC results in Figure 2A show that XYS decreased nuclear GR translocation in CORT-induced FLD mice in a manner similar to that of the GR antagonist RU-486.

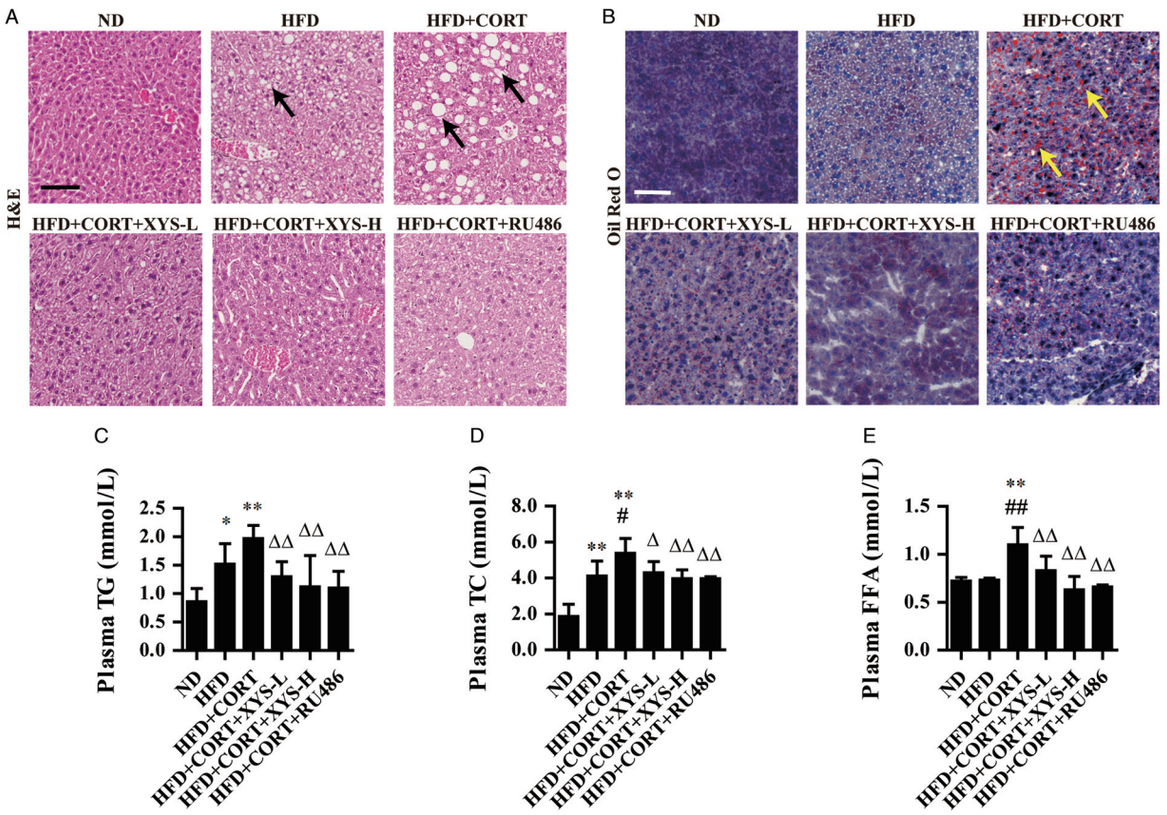

Figure 1. XYS ameliorated hepatic steatosis in CORT-induced FLD mice. HFD mice were used to assess the impact of XYS (2.56 g/kg and 5.12 g/kg daily for 7 weeks) on CORT (2 mg/kg)-induced hepatic steatosis. (A) H&E staining and (B) Oil Red O staining were used to observe morphological changes in liver sections ($n = 3$; 200 ×, bar = 50 μm). Dark arrows indicate fat vacuoles and yellow arrows indicate lipid droplets. (C–E) TG ($n = 4$), TC ($n = 4$), and FFA ($n = 5$) levels were measured. Data are mean ± SD. Significant differences between means were determined by one-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* ND group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs.* HFD group; $^{\Delta}P < 0.05$ and $^{\Delta\Delta}P < 0.01$ *vs.* CORT group. ANOVA: analysis of variance; CORT: corticosterone; FFA: free fatty acid; FLD: fatty liver disease; HFD: high-fat diet; ND: normal diet; SD: standard deviation; TC: total cholesterol; TG: triglyceride; XYS-L: Xiaoyao san low doage; XYS-H: Xiaoyao san high dosage; RU486: mifepristone.

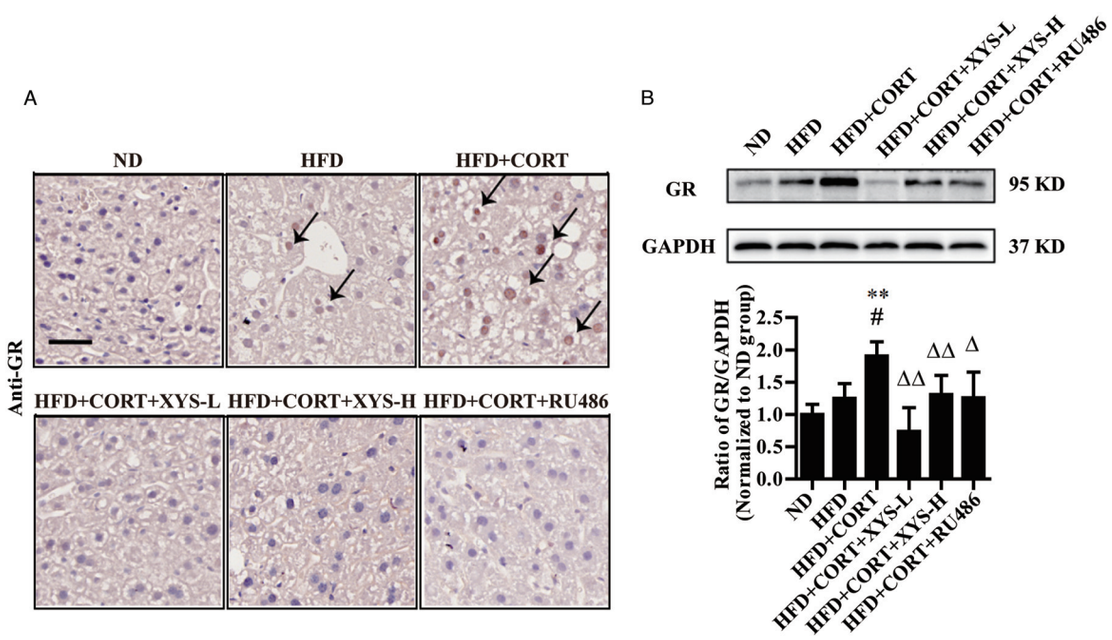

Figure 2. XYS inhibited GR activation in CORT-induced FLD mice. (A) Nuclear GR translocation in livers was detected by IHC. Positive brown staining is marked with dark arrows ($n = 3$; 400 ×, bar = 25 μm). (B) GR protein expression in the total lysate was determined by western blotting and quantified with ImageJ (version 1.46, NIH, Bethesda, MD, USA) ($n = 3$). Data are mean ± SD. Significant differences between means were determined by one-way ANOVA. $^{**}P < 0.01$ *vs.* ND group; $^{\#\#}P < 0.05$ *vs.* HFD group; $^{\Delta}P < 0.05$ and $^{\Delta\Delta}P < 0.01$ *vs.* CORT group. ANOVA: analysis of variance; CORT: corticosterone; GR: glucocorticoid receptor; HFD: high-fat diet; FLD: fatty liver disease; IHC: immunohistochemistry; ND: normal diet; SD: standard deviation; XYS-L: Xiaoyao san low dosage; XYS-H: Xiaoyao san high dosage; RU486: mifepristone.

Furthermore, XYS inhibited GR protein expression in CORT-induced FLD mice (Figure 2B, $P < 0.01$).

Potential signaling pathways of XYS

Figure 3 shows that the altered signaling pathways involved endocrine resistance, estrogen, glucagon, PPAR, and thyroid hormone. Several active ingredients in XYS may potentially target GR and modulate the foregoing signaling pathways. The active ingredients in XYS also regulated ADFP and linked it to the altered signaling pathways (Figure 3).

XYS reduced ADFP recruitment and expression around the lipid droplets in the livers of CORT-induced FLD mice

Figure 4A shows that XYS only downregulated Plin2 mRNA ($P < 0.01$). Western blotting revealed that XYS downregulated ADFP expression in total liver lysates (Figure 4B, $P < 0.05$). Results from IHC and confocal microscopy in Figure 4C–4E show that XYS significantly inhibited ADFP recruitment around the lipid droplets in CORT-induced FLD mice. Moreover, ADFP was downregulated in the lipid droplet fractions of CORT-induced FLD mice treated with XYS (Figure 4F). The foregoing observations corroborate the results obtained for the GR antagonist RU-486.

GR positively regulated ADFP expression in vitro

Results from the loss-of-function experiment on HepG2 cells *in vitro* showed that GR protein depletion significantly downregulated ADFP protein in HepG2 cells (Figure 5A). Furthermore, data from the gain-of-function experiment in GR plasmids transfected HepG2

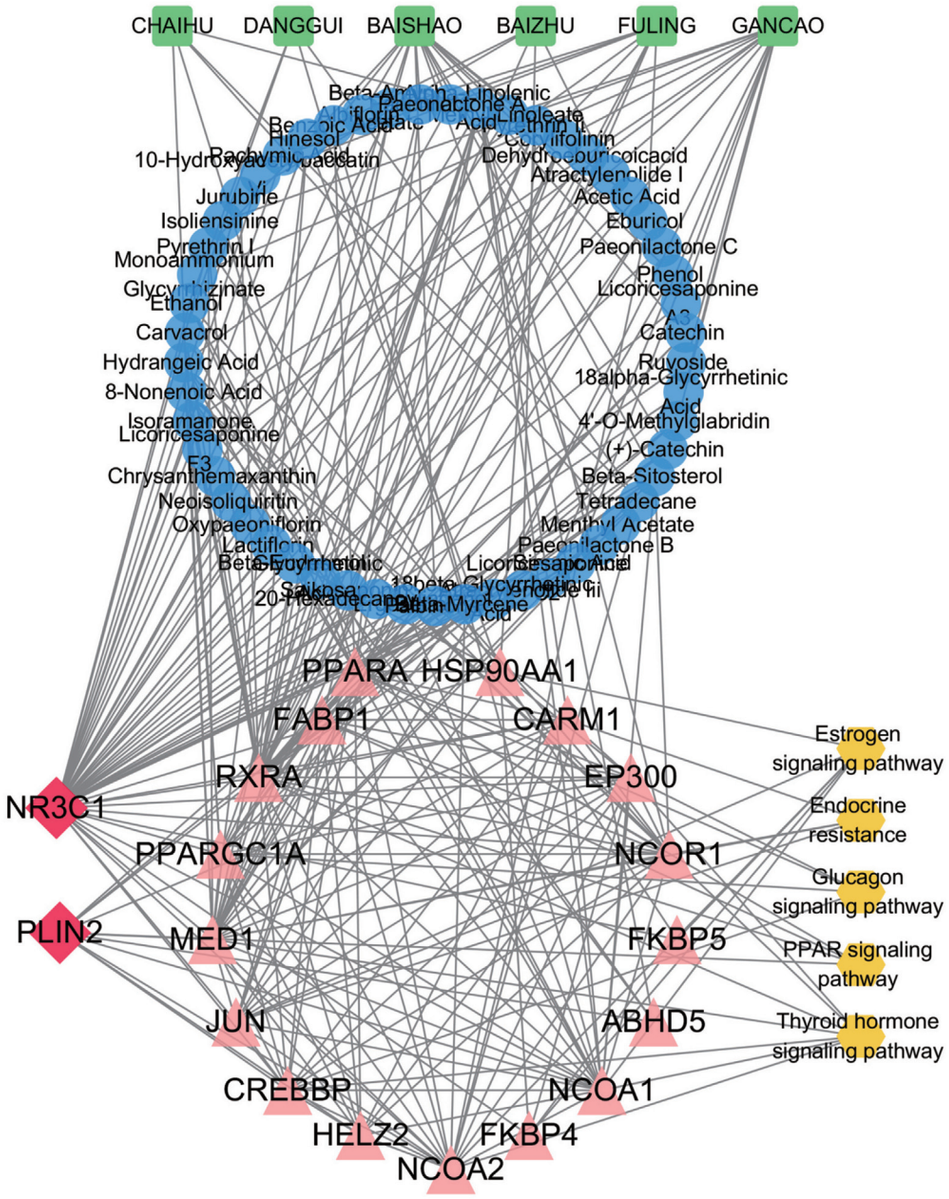

Figure 3. Potential signaling pathways of XYS. Rectangles represented XYS herbs. Circles represent relevant ingredients in XYS herbs. Triangles represent potential XYS targets regulating NR3C1 and PLIN2 (diamonds). Hexagons represent probable KEGG pathways linked to XYS ingredients. XYS: Xiaoyao san.

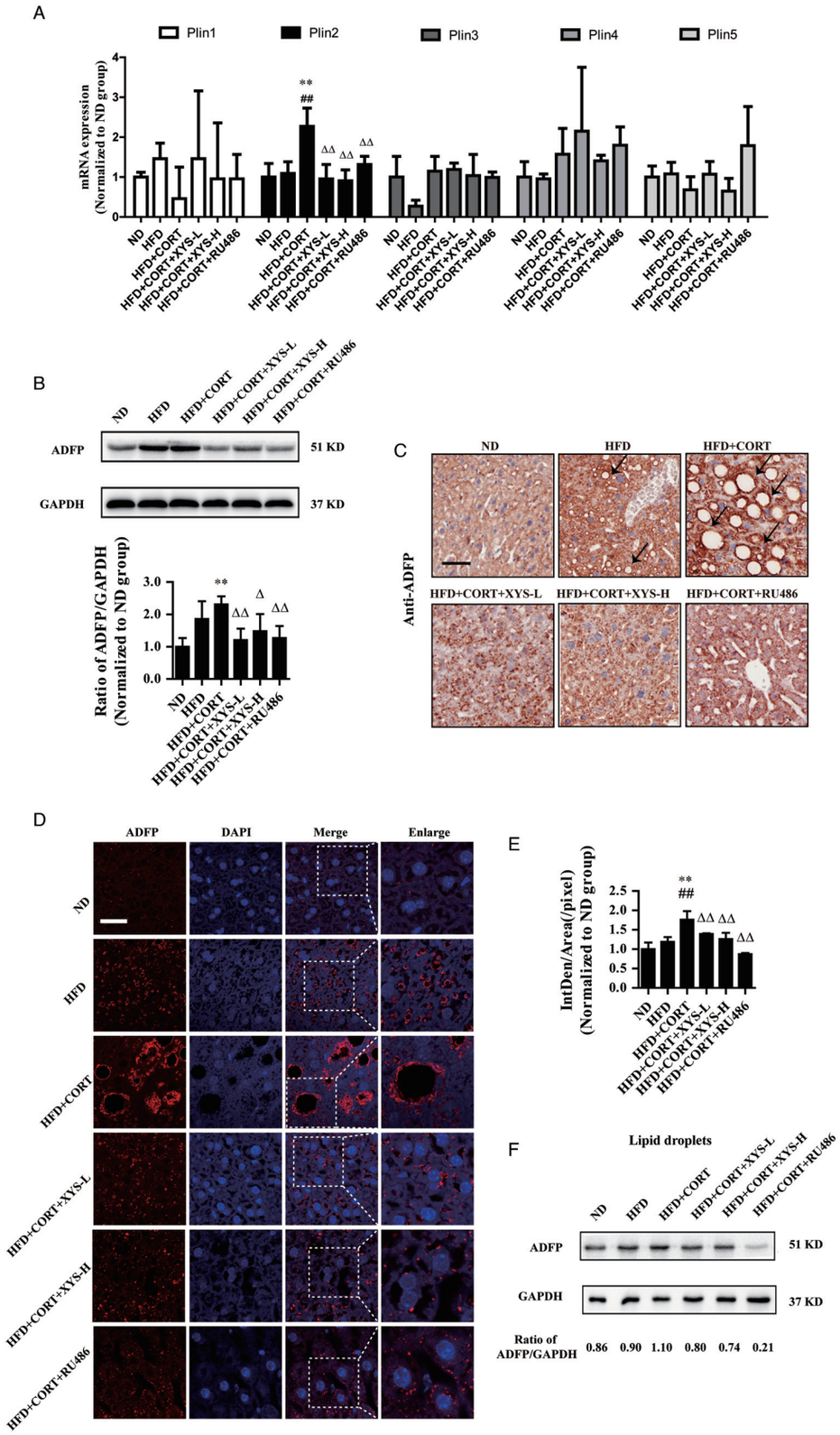

Figure 4. XYS reduced ADFP recruitment and expression around lipid droplets in livers of CORT-induced FLD mice. (A) mRNA and (B) protein expression levels of ADFP were detected by RT-qPCR and western blotting, respectively, and quantified with ImageJ (version 1.46, NIH, Bethesda, MD, USA) ($n=3$). (C) IHC data indicated that ADFP was stained brown. Here, ADFP is labeled with dark arrows ($n=3$; 400×, bar = 25 μm). (D) ADFP around lipid droplets was investigated by immunofluorescence and representative images were viewed and recorded by confocal microscopy (400×, bar = 10 μm). (E) Positive fluorescence densities (IntDen/Aear(/pixel)) were quantified with ImageJ (version 1.46, NIH, Bethesda, MD, USA) ($n=3$). (F) After the lipid droplet membranes were extracted, the ADFP expression in the lipid droplet lysate was determined by western blotting. Data are mean ± SD. Significant,differences between means were determined by one-way ANOVA. $^{**}P<0.01$ *vs*. ND group; $^{\#\#}P<0.01$ *vs*. HFD group; $^{\triangle}P<0.05$ and $^{\triangle\triangle}P<0.01$ *vs*. CORT group. ADFP: adipose differentiation-related protein; CORT: corticosterone; FLD: fatty liver disease; IHC: immunohistochemistry; ND: normal diet; SD: standard deviation; XYS-L: Xiaoyao san low dosage; XYS-H: Xiaoyao san high dosage; RU486: mifepristone; Plin1: perilipin 1; Plin2: perilipin 2; Plin3: perilipin 3; Plin4: perilipin 4; Plin5: perilipin 5; HFD: high-fat diet.

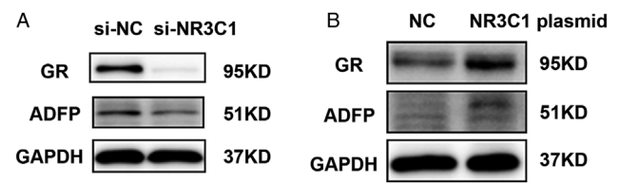

Figure 5. GR positively regulated ADFP expression in vitro. (A and B) HepG2 cells were transfected with si-NR3C1 or NR3C1 overexpression plasmid to knock down or overexpress GR, respectively. GR and ADFP expression levels were evaluated by western blotting. ADFP: adipose differentiation-related protein; GR: glucocorticoid receptor; NR3C1: nuclear receptor subfamily 3 group C member 1.

cells showed that GR overexpression upregulated the ADFP protein (Figure 5B).

Discussion

XYS has been clinically administered for Liver-*Qi* Stagnation and Spleen Deficiency Syndrome, and depression[39]. Its putative mode of action is serum glucocorticoid level reduction[40]. TCM theory states that XYS has efficacy on the symptoms of energy metabolism disorders such as hypercortisolism-related hepatic steatosis. However, the associated mechanisms of XYS against glucocorticoid-induced FLD are unclear.

Current therapeutic approaches for the management of hepatic steatosis include preventing lipid droplet formation and enlargement[39], improving lipid oxidation[41], and decreasing dietary fat intake[42]. However, reducing fat consumption was relatively less efficacious at ameliorating hepatic steatosis in obese patients with hypercortisolism[4–5]. The present study revealed that XYS protected against the development of glucocorticoid-induced hepatic steatosis in CORT-induced FLD mice by mitigating lipid droplets. The results of this study indicated that XYS activates fat turnover and metabolism. We found that XYS inhibited GR activation in CORT-induced hepatic steatosis by decreasing hepatic GR expression and nuclear translocation. Network pharmacology data disclosed that several active components in XFS targeted GR-dependent endocrine resistance and PPAR signaling pathways. The liver-specific lipid droplet membrane protein ADFP is associated with lipid droplet formation and growth in FLD[43]. Lipolysis was preceded by chaperone-mediated ADFP autophagy[12,44]. This mechanism shrank lipid droplets. Network pharmacology data revealed that ADFP is involved in XYS-mediated, GR-dependent pathways. Our data further demonstrated that XYS downregulates hepatic ADFP and decreases ADFP recruitment around lipid droplets. We also used loss-of-function and gain-of-function experiments to investigate the relationship between GR and ADFP protein and discovered that GR positively regulated ADFP protein expression. Hence, we identified a new GR/ADFP signaling pathway contributing to the mechanism by which XYS ameliorates hepatic steatosis.

The present study elucidated the effects of XYS on glucocorticoid-induced hepatic steatosis. XYS inhibits ADPF expression by decreasing hepatic GR activation and expression (Figure 6). GR/ADFP signaling pathway

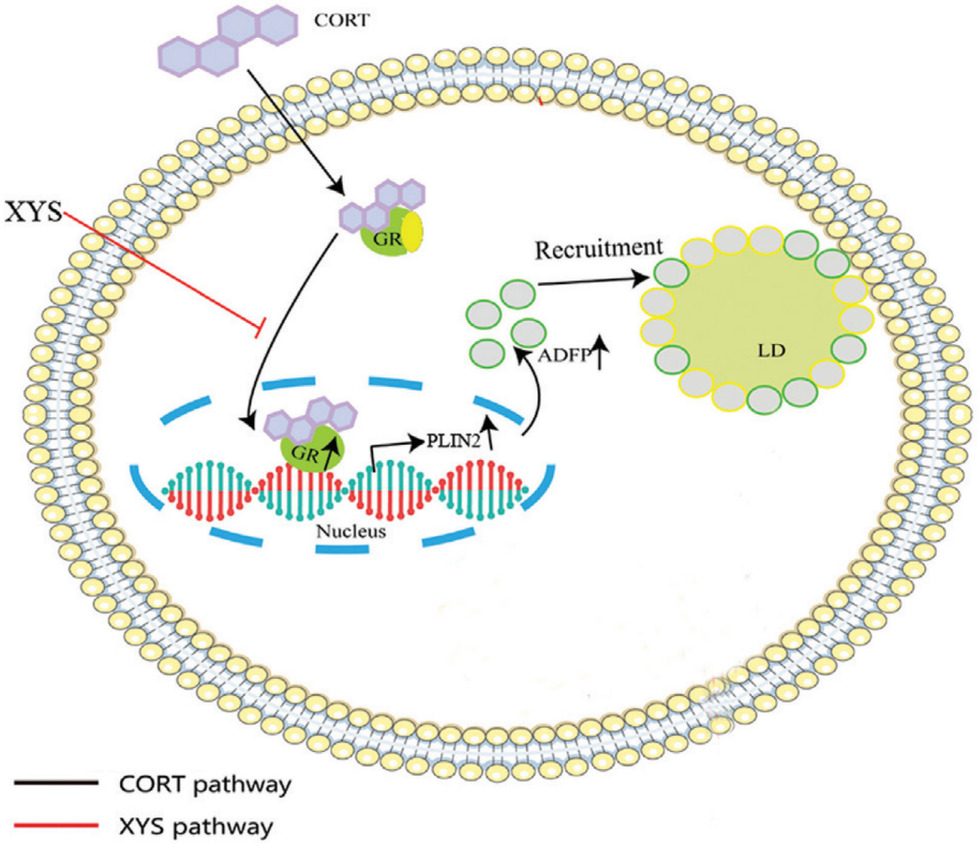

Figure 6. Schematic diagram. XYS ameliorated glucocorticoid-induced hepatic steatosis by downregulating the GR/ADFP signaling pathway. ADFP: adipose differentiation-related protein; GR: glucocorticoid receptor; XYS: Xiaoyao san.

downregulation constitutes a part of the mechanism by which XYS protects against glucocorticoid-induced hepatic steatosis. This discovery lays the foundation for the design and validation of novel prophylactic and therapeutic strategies for this medical condition. The mechanism of XYS is involved in "multi-targets and multi-systems"[45], the in-depth mechanism on XYS protecting against glucocorticoid-induced hepatic steatosis is limited in this study. In the future study, the active compound in XYS will be screened and will confirm its in-depth mechanism against glucocorticoid-induced hepatic steatosis.

Conclusions

XYS ameliorated CORT-induced hepatic steatosis by downregulating the GR/ADFP axis and inhibiting lipid metabolism. Our studies implicate that XYS is promising as a therapy for CORT-induced hepatic steatosis, and lay the foundation for designing novel prophylactic and therapeutic strategies on CORT-induced hepatic steatosis.

Conflict of interest statement

The authors declare no conflict of interest.

Funding

This work was supported in part by grants from the National Natural Science Foundation of China (Nos. 81630104 and 81622050).

Author contributions

Thanks for the design of Lian Gong, Guo-En Wang, Hiroshi Kurihara, Rong-Rong He and Jia-Xu Chen, writing draft of Lian Gong, Guo-En Wang and Yan-Ping Wu, the performance of Lian Gong, Qing-Yu Ma, Wen-Zhi Hao, Min-Hua Xian, the data analysis of Guo-En Wang, Qing-Yu Ma, Wen-Zhi Hao and Min-Hua Xian.

Ethical approval of studies and informed consent

The experimental rats were overseen and approved by the Institutional Animal Care and Use Committee of Jinan University, Guangzhou, China (Approval number: 20130904001).

Acknowledgments

The authors thank Chang Yan of Jinan University for assistance with scientific English in this manuscript.

References

[1] Younossi Z, Anstee QM, Marietti M, et al. Global burden of nafld and nash: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol* 2018;15(1):11–20.
[2] Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of nonalcoholic fatty liver disease: practice guidance from the American Association for the Study of Liver Diseases. *Hepatology (Baltimore MD)* 2018;67(1):328–357.
[3] Sheka AC, Adeyi O, Thompson J, et al. Nonalcoholic steatohepatitis: a review. *JAMA* 2020;323(12):1175–1183.
[4] Tarantino G, Finelli C. Pathogenesis of hepatic steatosis: the link between hypercortisolism and non-alcoholic fatty liver disease. *World J Gastroenterol* 2013;19(40):6735–6743.
[5] Rockall AG, Sohaib SA, Evans D, et al. Hepatic steatosis in Cushing's syndrome: a radiological assessment using computed tomography. *Eur J Endocrinol* 2003;149(6):543–548.
[6] Jung UJ, Choi MS. Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. *Int J Mol Sci* 2014;15(4):6184–6223.
[7] Fardet L, Kassar A, Cabane J, et al. Corticosteroid-induced adverse events in adults: frequency, screening and prevention. *Drug Saf* 2007;30(10):861–881.
[8] Pasquali R, Vicennati V, Cacciari M, et al. The hypothalamic-pituitary-adrenal axis activity in obesity and the metabolic syndrome. *Ann N Y Acad Sci* 2010;1083(1):111–128.
[9] Liu YZ, Wang YX, Jiang CL. Inflammation: the common pathway of stress-related diseases. *Front Human Neurosci* 2017;11:316.
[10] Fries GR, Gassen NC, Rein T. The fkbp51 glucocorticoid receptor co-chaperone: regulation, function, and implications in health and disease. *Int J Mol Sci* 2017;18(12):2614.
[11] Marino JS, Stechschulte LA, Stec DE, et al. Glucocorticoid receptor β induces hepatic steatosis by augmenting inflammation and inhibition of the peroxisome proliferator-activated receptor (ppar) α. *J Biol Chem* 2016;291(50):25776–25788.
[12] Kaushik S, Cuervo AM. Degradation of lipid droplet-associated proteins by chaperone-mediated autophagy facilitates lipolysis. *Nat Cell Biol* 2015;17(6):759–770.
[13] Day CP. Genes or environment to determine alcoholic liver disease and non-alcoholic fatty liver disease. *Liver Int* 2006;26(9):1021–1028.
[14] Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002;346(16):1221–1231.
[15] Greuter T, Malhi H, Gores GJ, et al. Therapeutic opportunities for alcoholic steatohepatitis and nonalcoholic steatohepatitis: exploiting similarities and differences in pathogenesis. *JCI Insight* 2017;2(17):e95354.
[16] Feiteiro J, Mariana M, Verde I, et al. Genomic and nongenomic effects of mifepristone at the cardiovascular level: a review. *Reprod Sci* 2017;24(7):976–988.
[17] Wang M, Huang W, Gao T, et al. Effects of xiao yao san on interferon-α-induced depression in mice. *Brain Res Bull* 2018;139:197–202.
[18] Zheng X, Cheng Y, Chen Y, et al. Ferulic acid improves depressive-like behavior in prenatally-stressed offspring rats *via* antiinflammatory activity and HPA axis. *Int J Mol Sci* 2019;20(3):493.
[19] Wei Z, Wang J, Shi M, et al. Saikosaponin A inhibits LPS-induced inflammatory response by inducing liver x receptor alpha activation in primary mouse macrophages. *Oncotarget* 2016;7(31):48995–49007.
[20] Xie T, Li K, Gong X, et al. Paeoniflorin protects against liver ischemia/reperfusion injury in mice *via* inhibiting HMGB1-TLR4 signaling pathway. *Phytother Res* 2018;32(11):2247–2255.
[21] Jung JC, Lee YH, Kim SH, et al. Hepatoprotective effect of licorice, the root of Glycyrrhiza uralensis Fischer, in alcohol-induced fatty liver disease. *BMC Complement Altern Med* 2016;16:19.
[22] Huo X, Yang S, Sun X, et al. Protective effect of glycyrrhizic acid on alcoholic liver injury in rats by modulating lipid metabolism. *Molecules (Basel, Switzerland)* 2018;23(7):1623.
[23] Orazizadeh M, Fakhredini F, Mansouri E, et al. Effect of glycyrrhizic acid on titanium dioxide nanoparticles-induced hepatotoxicity in rats. *Chem Biol Interact* 2014;220:214–221.
[24] Luo HG, Chen JX, Zhang Q, et al. Comparative study on effects of xiaoyao powder and its modified prescription on plasma metabolomics of rats with chronic immobilization stress. *Chin J Integr Med* 2013;19(8):610–615.
[25] Gao XX, Cui J, Zheng XY, et al. An investigation of the antidepressant action of xiaoyaosan in rats using ultra performance liquid chromatography-mass spectrometry combined with metabo- nomics. *Phytother Res* 2013;27(7):1074–1085.
[26] Yoh T, Nakashima T, Sumida Y, et al. Effects of glycyrrhizin on glucocorticoid signaling pathway in hepatocytes. *Dig Dis Sci* 2002;47(8):1775–1781.
[27] Li ZY, Jiang YM, Liu YM, et al. Saikosaponin D acts against corticosterone-induced apoptosis *via* regulation of mitochondrial GR translocation and a GR-dependent pathway. *Prog Neuro-psycho- pharmacol Biol Psychiatry* 2014;53:80–89.
[28] Zhang H, Chen S, Qin F, et al. Simultaneous determination of 12 chemical constituents in the traditional Chinese medicinal prescription Xiao-Yao-San-Jia-Wei by HPLC coupled with photodiode array detection. *J Pharm Biomed Anal* 2008;48(5):1462–1466.
[29] Zhang L, Dasuri K, Fernandez-Kim SO, et al. Adipose-specific ablation of Nrf2 transiently delayed high-fat diet-induced obesity

by altering glucose, lipid and energy metabolism of male mice. *Am J Transl Res* 2016;8(12):5309–5319.
[30] Jung HY, Kim B, Ryu HG, et al. Amodiaquine improves insulin resistance and lipid metabolism in diabetic model mice. *Diabetes Obes Metab* 2018;20(7):1688–1701.
[31] Woods CP, Hazlehurst JM, Tomlinson JW. Glucocorticoids and non-alcoholic fatty liver disease. *J Steroid Biochem Mol Biol* 2015;154:94–103.
[32] D'Souza AM, Beaudry JL, Szigiato AA, et al. Consumption of a high-fat diet rapidly exacerbates the development of fatty liver disease that occurs with chronically elevated glucocorticoids. *Am J Physiol Gastrointest Liver Physiol* 2012;302(8):G850–G863.
[33] Ding F, Wu J, Liu C, et al. Effect of xiaoyaosan on colon morphology and intestinal permeability in rats with chronic unpredictable mild stress. *Front Pharmacol* 2020;11:1069.
[34] Li P, Tang XD, Cai ZX, et al. Cnp signal pathway up-regulated in rectum of depressed rats and the interventional effect of xiaoyaosan. *World J Gastroenterol* 2015;21(5):1518–1530.
[35] Kuo T, Chen TC, Lee RA, et al. Pik3r1 is required for glucocorticoid-induced perilipin 1 phosphorylation in lipid droplet for adipocyte lipolysis. *Diabetes* 2017;66(6):1601–1610.
[36] Blouin CM, Le Lay S, Eberl A, et al. Lipid droplet analysis in caveolin-deficient adipocytes: alterations in surface phospholipid composition and maturation defects. *J Lipid Res* 2010;51(5):945–956.
[37] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-Delta Delta C(T)) method. *Methods* 2001;25(4):402–408.
[38] Ji E, Wang T, Guo F, et al. Xiaoerfupi alleviates the symptoms of functional dyspepsia by regulating the HTR3A and c-FOS. *Biomed Pharmacother* 2019;120:109442.
[39] Tian JS, Peng GJ, Wu YF, et al. A GC-MS urinary quantitative metabolomics analysis in depressed patients treated with TCM formula of xiaoyaosan. *J Chromatogr B Analyt Technol Biomed Life Sci* 2016;1026:227–235.
[40] Zhu X, Xia O, Han W, et al. Xiao Yao San improves depressive-like behavior in rats through modulation of β-arrestin 2-mediated pathways in hippocampus. *Evid Based Complement Alternat Med* 2014;2014:902516.
[41] Shepherd SO, Cocks M, Tipton KD, et al. Sprint interval and traditional endurance training increase net intramuscular triglyceridebreakdown and expression of perilipin 2 and 5. *J Physiol* 2013;591(3):657–675.
[42] Conte M, Franceschi C, Sandri M, et al. Perilipin 2 and age-related metabolic diseases: a new perspective. *Trends Endocrinol Metab* 2016;27(12):893–903.
[43] McManaman JL, Bales ES, Orlicky DJ, et al. Perilipin-2-null mice are protected against diet-induced obesity, adipose inflammation, and fatty liver disease. *J Lipid Res* 2013;54(5):1346–1359.
[44] Pawella LM, Hashani M, Eiteneuer E, et al. Perilipin discerns chronic from acute hepatocellular steatosis. *J Hepatol* 2014;60 (3):633–642.
[45] Pennington K, Föcking M, McManus CA, et al. A proteomic investigation of similarities between conventional and herbal antidepressant treatments. *J Psychopharmacol* 2009;23(5): 520–530.

How to cite this article: Gong L, Wang GE, Ma QY, Hao WZ, Xian MH, Wu YP, Kurihara H, He RR, Chen JX. Novel insights into the effect of Xiaoyao san on corticosterone-induced hepatic steatosis: inhibition of glucocorticoid receptor/perilipin-2 signaling pathway. Acupunct Herb Med 2022;2(1):49–57. doi: 10.1097/HM9.0000000000000011