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Xiao-Gao-Jiang-Zhuo-containing serum inhibits adipogenesis through SIRT1-IGF-1 crosstalk in 3T3-L1 preadipocytes

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Abstract: PPARγ, CEBP/α, and SREBP1C are the major transcriptional factors participating in adipogenesis and lipogenesis. SIRT1 and IGF-1 signaling pathways are important pathways involved in body endocrine and metabolism. Our unique Chinese herbal medicine Xiao-Gao-Jiang-Zhuo (XGJZ) has a remarkable clinical effect on obesity. However, the molecular basis remains unknown. XGJZ-containing serum was treated in the incubation of 3T3-L1 preadipocytes to observe its function in the 3T3-L1 cell differentiation. Oil Red O staining was used to monitor the lipid droplets accumulated after 8 days of incubation. RT-qPCR and western blotting were used to investigate the regulatory effects of XGJZ-containing serum on adipogenesis-related factors. The protein levels of main molecules in SIRT1 and IGF-1 signaling pathways were also detected by western blotting. XGJZ-containing serum notably suppressed the lipid accumulation in differentiated adipocytes through SIRT1/IGF-1 pathway. XGJZ-containing serum activated the SIRT1/IGF-1 pathway and reduced the expression levels of PPARγ, CEBP/α, and SREBP1C through this pathway. Additionally, XGJZ-containing serum enhanced the phosphorylation of ATGL and HSL and then induced lipolysis. XGJZ-containing serum has inhibitory effects on adipogenesis in 3T3-L1 preadipocytes through SIRT1/IGF-1 signaling pathway. Our study affirmed the effect of XGJZ-containing serum in the treatment of obesity. It provides a basis for the mechanism of obesity.

Keywords: XGJZ, adipogenesis, obesity, lipid metabolism, SIRT1, IGF-1

INTRODUCTION

Obesity is recognized as one of the world's largest chronic metabolic diseases, which is characterized by lipid metabolism disorder (Caballero, 2019). The imbalance of lipid metabolism in adipocytes leads to the increase of adipocyte volume and/or number and dysfunction, which is the cellular biological basis of the occurrence and development of obesity (Unger *et al*., 2010). Therefore, anti-obesity treatment must adjust the imbalance of lipid metabolism. Lipid homeostasis in adipocytes, which is mainly based on the proportional relationship between triglycerides and fatty acids, is maintained through the dynamic balance of lipid synthesis and lipid decomposition (Cisa-Wieczorek and Hernández-Alvarez, 2020). In this process, many proteases, receptors, and transporters are involved, which are regulated by some signal transduction pathways. Each signal pathway cross-talks through some specific proteins, forming a complex regulatory network (Stordeur *et al*., 2014). Therefore, the study of lipid metabolic instability from key signaling pathways and crosstalk between pathways has become a focus in the study of obesity mechanisms.

Insulin-like growth factor-1 (IGF-1) signaling pathway is closely related to cell proliferation, apoptosis, metabolism, and stress (Michaelsen *et al*., 2013). In adipocytes, insulin-like growth factor binding protein 1 (IGFBP1) is activated by insulin or IGF-1 to activate PI3K, which activates its activity through phosphorylated protein kinase AKT (Zhou *et al*., 2018). Activated protein kinase AKT phosphorylates forkhead protein box transcription factor 1 (FOXO1) and inhibits its activity, relieving PPARγ inhibition to promote adipocyte differentiation and adipogenesis. (Stitt *et al*., 2004).

Sirtuin 1 (SIRT1) signaling pathway, as a classical longevity factor, participates in gene silencing and recombination, maintains gene stability, and plays a key catalytic role in the process of transcriptional regulation (Ding *et al*., 2017). SIRT1 overexpression was induced by caloric restriction and PPARγ activity was inhibited after binding to nuclear receptor co-inhibitory protein (NCoR), thereby reducing the differentiation of precursor adipocytes into white fat, triggering lipolysis and reducing triglycerides accumulation (Picard *et al*., 2004).

SIRT1 and IGF-1 signaling pathways are important pathways involved in body endocrine and metabolism (Tran *et al*., 2014). The IGF-1 pathway is closely related to adipocyte differentiation and SIRT1's unique deacetylation can silence some transcription factor substrates (such as p53 and FOXO1). SIRT1 signaling pathway colludes with the IGF-1 pathway to jointly regulate cell differentiation and the transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and sterol regulatory element binding proteins (SREBP1), which transcriptionally regulate the expression of

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downstream target genes, key enzymes of triglycerides metabolism to regulate triglycerides metabolism and maintain lipid homeostasis.

The XGJZ-containing serum has a good theoretical basis and clinical efficacy, but its molecular mechanism for preventing obesity is not clear. This project intends to study the regulation mechanism of XGJZ-containing serum based on SIRT1 and IGF-1 signal pathways, to provide new methods and ideas for the treatment of obesity with traditional Chinese medicine.

MATERIALS AND METHODS

Preparation of XGJZ-containing serum

XGJZ-containing serum contained rhubarb, ginger, pinellia ternata, Radix Codonopsis, Coptis chinensis, *Scutellaria baicalensis*, *Crataegus pinnatifida*, Monascus, Eupatorium fortunei Turcz, and the maggots of Chrysomyis megacephala Fabricius. All 10 herbs were acquired and extracted from and prepared in our hospital. The herbs were boiled in water for 1 hour and the amount of water was 5 times the volume of the herbs. After being boiled and filtered, 6 times the volume of water of the herbs was added to the residue and boiled for another 1.5 hours and then filtered. The two filtrates were collected and frozen in an -80°C refrigerator. After being frozen for 24 hours, the filtrate was placed in a vacuum freeze dryer for drying to an extract.

Animals and cells

Wister male rats (weighing 180-220 g) were bought and raised in SPF breeding facilities maintained following national standards. The experimental protocol was approved by the Animal Welfare Committee of Guang'anmen Hospital, China Academy of Chinese Medical Sciences. The 3T3-L1 cells were purchased and cryopreserved in laboratory refrigerators.

Drug-containing serum preparation

After being fed for 12 weeks, rats whose body mass exceeded 20% of the average body mass of the conventional feeding group were selected as the model rats. Serum-containing drugs were prepared according to the previous method (Sadie-Van Gijsen, 2019). Each animal in the medium-dose group was given 2 mL each time (1 g crude drug per 1 mL of water decoction), twice a day for three consecutive days. The low-dose group was 1 mL, the high-dose group was 4 mL and the serum control group was given the same dose of saline. Fasting for 12 hours before the last gavage and 1 hour after the last gavage, blood was collected from the abdominal aorta and centrifuged at 400×g for 15 minutes and then serum was isolated and inactivated at 56°C for nearly 30 minutes. Finally, the drug-containing serum or the control serum was filtered and sterilized through a 0.22 μm microporous filter membrane and then stored at -20°C. The drug-containing serum and the control serum were used in our experiments for cell culture.

Adipocyte differentiation

3T3-L1 preadipocytes were resuscitated and cultured in a suitable medium (high sugar DMEM containing 100 mL/L FBS) under a condition of 37°C and 50 ml/L CO_2. After cell fusion for 2 days, the 3T3-L1 cells were cultured using high glucose DMEM added with 0.5 mmol/L IBMX, 10 mg/L insulin, 1 μmol/L dexamethasone and 100 ml/L FBS for 48 hours, then cultured with high glucose DMEM containing 10 mg/L insulin and 100 ml/L FBS for another 48 hours. The culture medium was changed once in 2 days. 90% ~ 95% of 3T3-L1 cells differentiated for 8-12 days showed adipocyte phenotype, which can be used in the following experiments.

Drug-containing serum was added to the culture medium during adipocyte differentiation. Differentiated 3T3-L1 cells were divided into the following five groups: saline group, XGJZ high, medium, and low dose formula groups, and EX527 group.

Oil red O staining

3T3-L1 preadipocytes were induced to differentiation with different concentrations of drug-containing serum and 5 μmol/L EX527 containing differentiation-inducing agent (10 mg/L insulin, 1 μmol/L dexamethasone, and 0.5 mmol/L IBMX). DMEM was added to the medium at the same time. Adherent cells were stained with oil-red O. Under the 200-fold field of an inverted microscope, all cells containing oil red O staining substance were differentiated into adipocytes. Each well had 2 fixed-size visual fields and each group had 3 multiple holes. The differentiated cells in each visual field were photographed, counted and compared. The oil red O staining substance in each well was extracted with isopropanol and OD was measured at 490 nm wavelength of enzyme-linked immunosorbent assay for semi-quantitative analysis.

CCK-8 assay

3T3-L1 cell viability was detected using a CCK-8 kit (in cells treated with serum isolated from the rats orally administrated with saline, low, middle and high doses of XGJZ, or EX527). The cells were inoculated into 96 well culture plates. After 72 hours of intervention with drug-containing serum in different dose groups, each well was added 10 μL of CCK-8 reagent. After incubation for 4 hours, the absorbance was detected at 450 nm.

Western blotting

Differentiated 3T3-L1 cells were washed with 1 mL of each tube twice in pre-cooled PBS at 4°C to wash off the culture medium and then lysed with radioimmune precipitation (RIPA) buffer containing protease inhibitor and placed on ice for 20 minutes. The protein content was

determined by adding a BCA solution. After denatured by boiling the samples for 5 minutes, the protein was separated by polyacrylamide gel and then fixed on a polyvinylidene fluoride (PVDF) membrane. After being washed with PBS solution, the membranes were blocked for 1 hour with 5% skimmed milk at room temperature. Subsequently, the PVDF membranes were incubated overnight at 4°C with a suitable dilution of primary antibodies and rinsed with PBST buffer three times for 10 minutes each time. Next, the membranes were incubated for 1 hour in horseradish peroxidase-labeled secondary antibodies diluted with blocking solution, shaken slowly and washed with PBST buffer three times. Finally, the pre-mixed ECL luminescent substrate was added to the membrane for color reaction.

Compositional analysis of XGJZ with UPLC-HRMS

The XGJZ medicine powder was extracted from water or alcohol for the compositional analysis. After filtration, the two types of supernatant were separately submitted to UPLC-Q-TOF MS (mass spectrometry). UPLC was performed on an Agilent 1100 HPLC system (Agilent 1290 Infinity LC) with an ACQUITY UPLC HSS T3 column (100 mm×2.1 mm, 1.8 μm; Waters, USA). The temperature was set at 40°C, the flow velocity was 0.3 mL/min. The mobile phase A consisted of H_2O and 0.1% formic acid, while the mobile phase B was acetonitrile. The water solution (5 μL) or ethanol solution (5 μL) underwent the following gradient elution: 0-1.0 min, 5% B; 1.0-9.0 min, 5~100% B; 9.0-12.0 min, 100% B; 12.0-12.1 min, 100~5% B; 12.1-15.0 min, 5% B. The conditions of Q-TOF were as follows: (1) for positive mode, sheath gas flow rate, aux gas flow rate, spray voltage, capillary temperature and heater temperature, were 40 arb, 10 arb, 3.50 V, 320°C and 300°C, respectively; (2) for negative mode, the above parameters were 38 arb, 10 arb, 2.80 V, 320°C and 300°C, respectively. The mass scan range was from 80 to 1200m/z with a 70000 resolution for parent ions, the resolution was 17500 and the energy gradient was 20/40/60 for daughter ions.

STATISTICAL ANALYSIS

GraphPad Prism 8 statistical software packages were used for data analysis and the results were from at least 3 independent experiments and presented as mean±SD. One-way analysis of variance (ANOVA) followed by the Tukey post hoc test was performed to calculate the statistical differences. Values of $P<0.05$ were statistically significant. Image J was used for the semi-quantitative analysis.

RESULTS

XGJZ-containing serum inhibited 3T3-L1 differentiation

To explore whether XGJZ-containing serum could inhibit adipocyte maturation, 3T3-L1 differentiation was evaluated by detecting the intracellular storage of lipids with Oil Red O staining after. Treatment with the XGJZ-containing serum significantly reduced lipid accumulation, especially with the high dose XGJZ-containing serum ($p<0.01$), compared with the saline group (fig. 1A). This result was further confirmed by measuring the Oil Red O absorbance at 490 nm (fig. 1B). Besides, we examined whether XGJZ-containing serum reduced adipogenesis by suppressing cell viability during the differentiation process. Our results indicated that XGJZ-containing serum did not reduce cell viability according to the results of CCK-8 assays. This result confirmed the direct effect of XGJZ-containing serum on adipogenesis (fig. 1C). A dose-dependent inhibition of 3T3-L1 differentiation was found in XGJZ-containing serum, the high-dose XGJZ-containing serum was used in our following experiments.

The Effects of XGJZ-containing serum are mediated through the SIRT1/IGF-1 pathway

To investigate the molecular mechanisms of XGJZ-containing serum. The protein levels of molecules of SIRT1 and IGF-1 pathways were detected. Both XGJZ and EX527 treatments during cellular differentiation raised the phosphorylation of both SIRT1 and FOXO1 ($P<0.05$) and significantly lowered the phosphorylation of PI3K and AKT and the protein expression level of IGFBP1, compared to saline group cells ($P<0.01$) (fig. 2). The results suggested that the antiadipogenic effect of XGJZ-containing serum might be mediated by the Sirt1/IGF-1 pathway.

XGJZ-containing serum blunted adipogenesis by controlling the SIRT1/IGF-1 pathway

Next, we assessed whether the anti-adipogenic effects of XGJZ-containing serum were mediated through the SIRT1/IGF-1 Pathway. In differentiated adipocytes, we observed that treatment with either XGJZ-containing serum or the SIRT1 inhibitor EX527 significantly reduced lipid accumulation by Oil Red O staining (fig. 3A-B). The cell viability of the four group cells was also detected to exclude the influence of cell viability (fig. 3C). The protein levels of molecules of SIRT1 and IGF-1 pathways, along with the key regulatory factors of adipogenesis (PPARγ, CEBP/α, and SREBP1C) were detected (fig. 3D). XGJZ and EX527 treatments during adipocytes cellular differentiation promoted the phosphorylation of both SIRT1 and FOXO1 ($P<0.05$) and remarkably decreased the phosphorylation of PI3K and AKT and the protein expression level of IGFBP1, compared to saline group cells ($P<0.01$) (fig. 3E-I). The protein levels of PPARγ, CEBP/α, and SREBP1C were all notably decreased in XGJZ and EX527 groups (fig. 3J-L). A graphical representation of the underlying mechanism was shown in fig. 4. XGJZ might contribute to the inhibition of adipocyte differentiation and adipogenesis through the crosstalk between SIRT1 and IGF-1 signaling pathways.

XGJZ-containing serum reduced intracellular lipid storage

In addition, we investigated lipolysis in this process. The phosphorylation of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) was examined with a western blot experiment. As shown in fig. 5A-C, XGJZ-containing serum and EX527 determined significant increases in ATGL at serine-406 (Ser406) and HSL at Ser565, which were markedly downregulated in the differentiated adipocytes. The mRNA level of uncoupling protein 1 (UCP1) was also detected to observe the effects of XGJZ-containing serum on adipose tissue browning. XGJZ-containing serum and EX527 potentiated the protein level of UCP1 (fig. 5D). Our above results stated that XGZJ-containing serum promoted lipolysis in differentiated 3T3-L1 adipocytes.

Composition of XGJZ

In order to further reveal the association between GPT and its anti-obesity effects, the main composition of XGJZ was analyzed. After UPLC-Q-TOF/MS was performed, 19 compounds were identified based on multi-stage mass spectrometry information and a high-resolution mass spectrometry database for natural products (table 1, fig. 6). Among these compounds, 1-O-Arsonopentofuranose, β-Alanine, 5-Formylfurfural, 4-Hydroxyphenylacetic acid, Trans-3-Indoleacrylic acid, 4-Hydroxybenzoic acid, Corymboside, Salvianolic acid D, Phenyl hexopyranosiduronic acid, Oxane-2-carboxylic acid, Doxylamine, Formononetin, Juvenile hormone III bis epoxide, Curcumin II and Ovalitenin A were some main compounds of GPT (table 1).

DISCUSSION

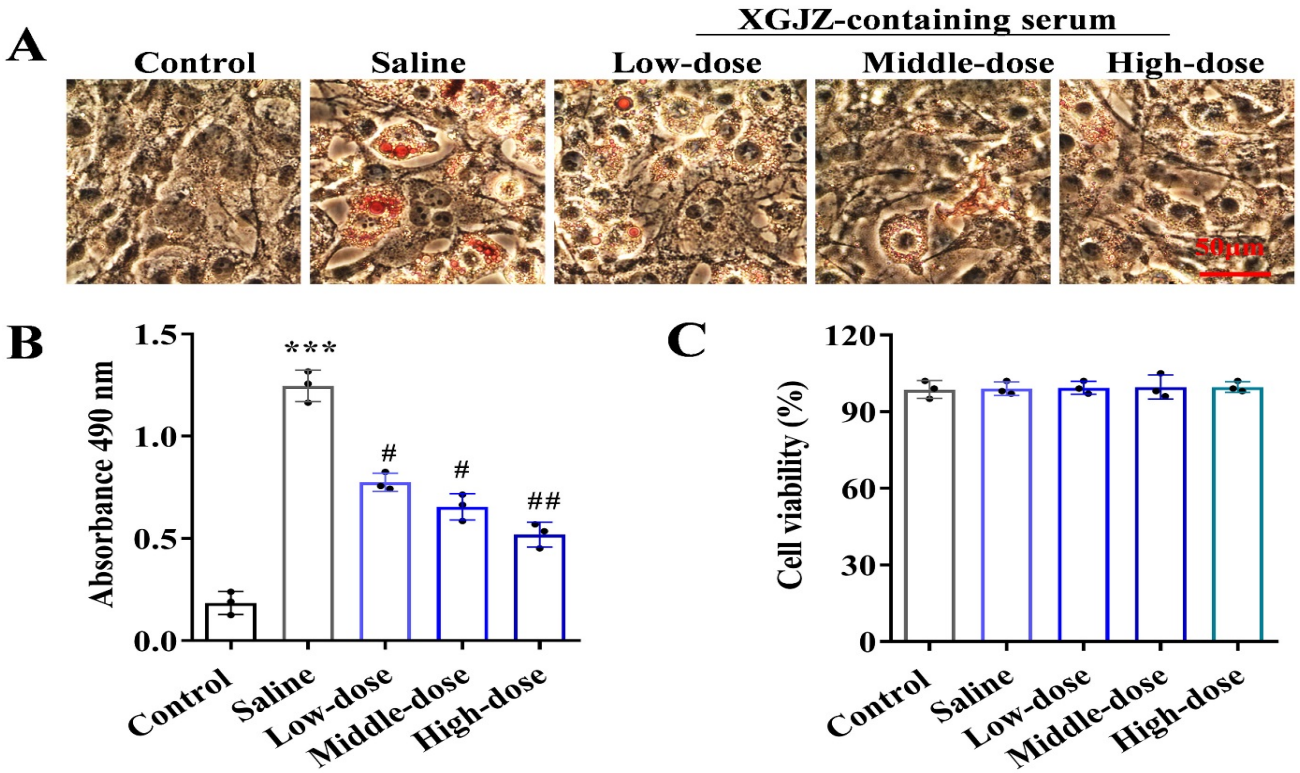
Obesity is a chronic nutritional imbalance disease, which has a significant negative impact on the quality of life. It imposes a huge mental and financial burden on society and individuals. Obesity can also cause a lot of chronic diseases. The number of obese people is increasing at an alarming rate and has become a global health problem. Finding effective drugs to prevent and treat obesity has become a hot topic.

Drug-containing serum refers to a non-monomer drug pharmacology research method that takes drugs to people or animals and then collects and separates the serum after a certain time to simulate and try to replace the drug itself for experimental research (Yu *et al*., 2020). This method of collecting serum to study the active components of drugs is called “serum pharmacology”. In recent years, with the wide application of traditional Chinese medicine in clinics and good curative effects in the treatment of many diseases, many scholars have begun to deeply study the effective components of single traditional Chinese medicine or compound traditional Chinese medicine (Cao *et al*., 2018). However, due to the difficulty of using traditional Chinese medicine to carry out *in vitro* pharmacological experiments, the experimental method of “drug-containing serum” has been used to study the pharmacology of traditional Chinese medicine (Qin *et al*., 2021).

In this study, we described the beneficial effect of XGJZ-containing serum on reducing adipogenesis of mouse preadipocyte 3T3-L1. In particular, the drug-containing serum of XGJZ could effectively regulate the expression of lipid metabolism genes through SIRT1/IGF-1 pathway. The drug-containing serum of a specific dose of XGJZ-containing serum down-regulated PPARγ, C/EBPα, and SREBP1C expression levels, which play important roles in 3T3-L1 preadipocyte differentiation, to inhibit the differentiation of 3T3-L1 preadipocytes and reduce the content of fat in cells. In addition, we demonstrated that treatment with XGJZ-containing serum regulated UCP1 expression levels associated with lipid metabolism, allowing the transformation of adipogenesis into adipose tissue browning. This may significantly reduce fat accumulation and help prevent fat dysfunction in the context of obesity.

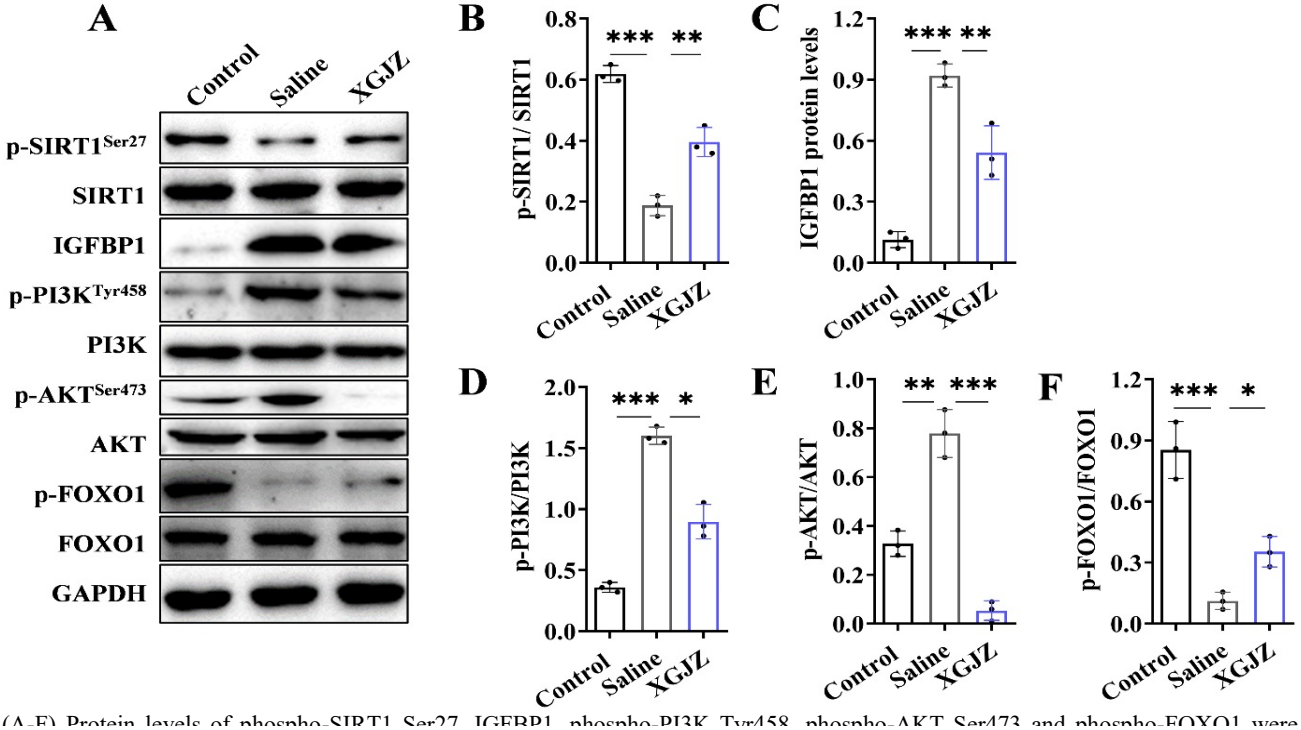
The happening of obesity is the result of both genetic and environmental factors. Insulin resistance plays a vital role in the pathogenesis of obesity (Huang *et al*., 2020). Glucose production and high levels of carbohydrate consumption increase the chances of insulin resistance, especially in the case of obesity (Hoffman *et al*., 2003). Therefore, maintaining blood glucose balance may be a strategy to preclude or cure diabetes and obesity. The initiation and inhibition of glucose production are complex due to many interference pathways. These pathways can be observed at the downstream level because they activate some transcription factors, including FOXO1. FOXO1 is important in regulating gluconeogenesis and glycogenolysis through insulin signal transduction (Lee and Dong, 2017). It is also important for preadipocytes to determine adipogenesis (Lin *et al*., 2020). The development of adipose tissue includes the enlargement, proliferation and differentiation of adipocytes. IGF-1 plays an essential role in the cell cycle and mitosis (Stephens, 2016). In this regard, IGF-1 may contribute to the development of obesity. Obesity can cause a high imbalance of IGF-1 system, resulting in hyperinsulinemia and the increase of free IGF-1 (AsghariHanjani and Vafa, 2019). The IGF-1-PI3K-AKT pathway is a typical pathway regulating FOXOs transcriptional activity (Zhou *et al*., 2018). In our present study, XGJZ-containing serum significantly reduced the protein expression level of IGFBP1 and phosphorylation of PI3K and AKT, compared to saline group cells.

Many pieces of evidence show that SIRT1 promotes fat mobilization in white adipose tissue and plays an important role in resisting obesity and maintaining metabolic homeostasis (Abduraman *et al*., 2021).

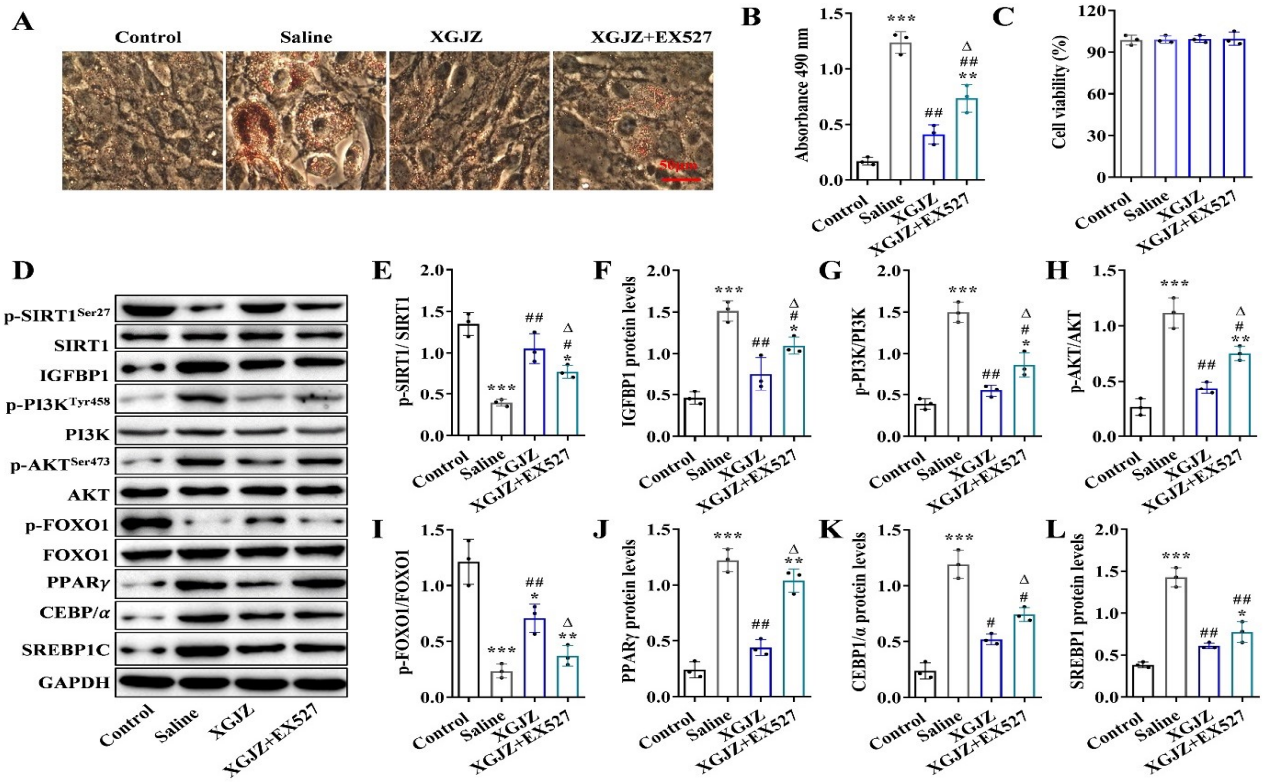


(A) Cells were incubated at a density of 1.6×10^4 cells/well in 24-well plates. Differentiated 3T3-L1 cells were treated with saline or different concentration of Xiao-Gao-Jiang-Zhuo (XGJZ) containing serum. After 8 days, cells were then stained with Oil Red O and (B) the lipid accumulation was assessed using the absorbance at OD 490 nm. Cells in the Control group were not differentiated. (C) Cell viability of 3T3-L1 cells was detected. ***p<0.001 *vs*. the control group, #p<0.05, ##p<0.01 *vs*. the saline group.

Fig. 1: Oil Red O staining and CCK-8 assay in 3T3-L1 adipocytes.



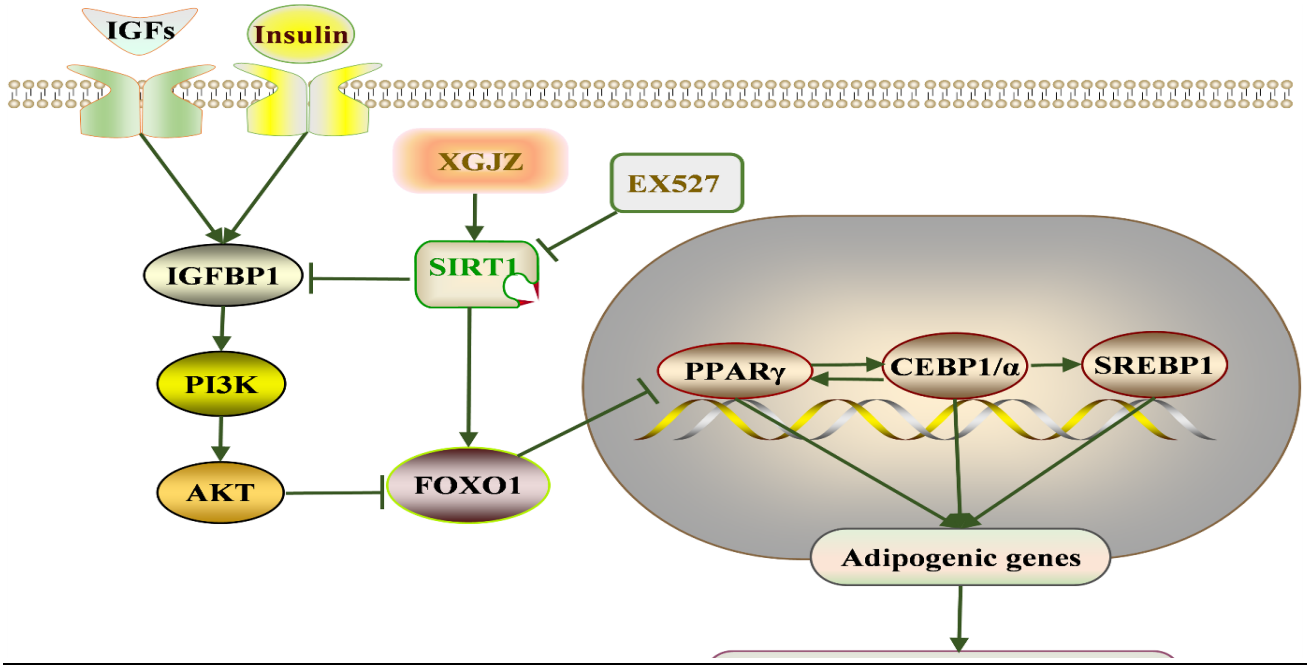
(A-F) Protein levels of phospho-SIRT1 Ser27, IGFBP1, phospho-PI3K Tyr458, phospho-AKT Ser473 and phospho-FOXO1 were



(A) Cells were incubated at a density of 1.6×10^4 cells per well in 24-well plates. Differentiated 3T3-L1 cells were then treated with high dose of XGJZ-containing serum or EX527. After 8 days' incubation, cells were then stained with Oil Red O and (B) the lipid accumulation was detected at OD 490 nm. (C) Cell viability of 3T3-L1 cells was assessed. (D-L) Protein levels of phospho-SIRT1 Ser27, IGFBP1, phospho-PI3K Tyr458, phospho-AKT Ser473 and phospho-FOXO1 were evaluated by western blot analysis.

∗p<0.05, ∗∗p<0.01, ∗∗∗p<0.001 *vs*. the control group, #p<0.05, ##p<0.01 *vs*. the saline group, Δp<0.05 *vs*. the XGJZ group.

Fig. 3: XGJZ-containing serum blunts adipogenesis by controlling the SIRT1/IGF-1 Pathway.



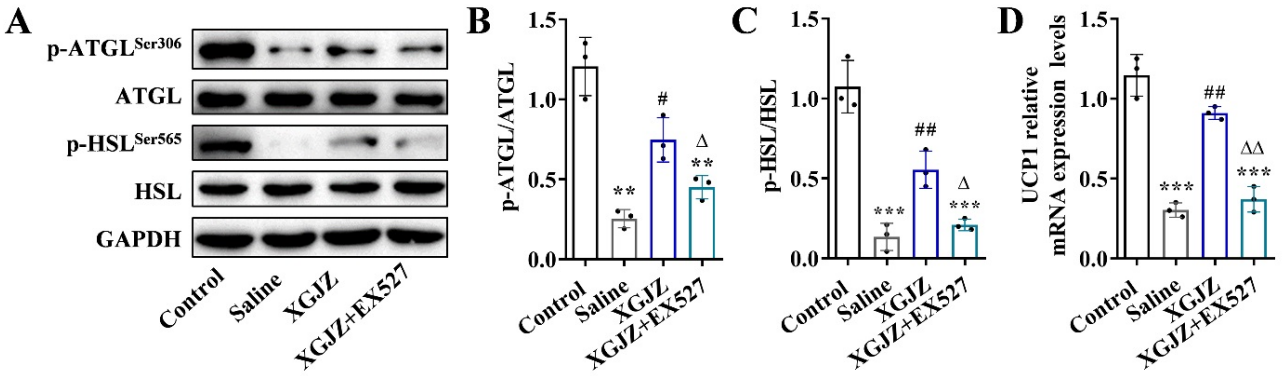


Fig. 5: XGJZ-containing serum induced lipolysis and triggered adipose tissue browning.
The 3T3-L1 Cells were incubated at a density of 8×10^4 cells per well in 6-well plates. Differentiation of 3T3-L1 Cells was induced with or without high dose of XGJZ up to day 8. (A-C) protein levels of phospho-ATGL Ser406 and phospho-HSL Ser565 were examined by western blotting. (D) The mRNA levels of UCP1 were measured by RT-qPCR. **p<0.01, ***p<0.001 *vs*. the control group, #p<0.05, ##p<0.01 *vs*. the saline group, Δp<0.05, ΔΔp<0.01 *vs*. the XGJZ group.

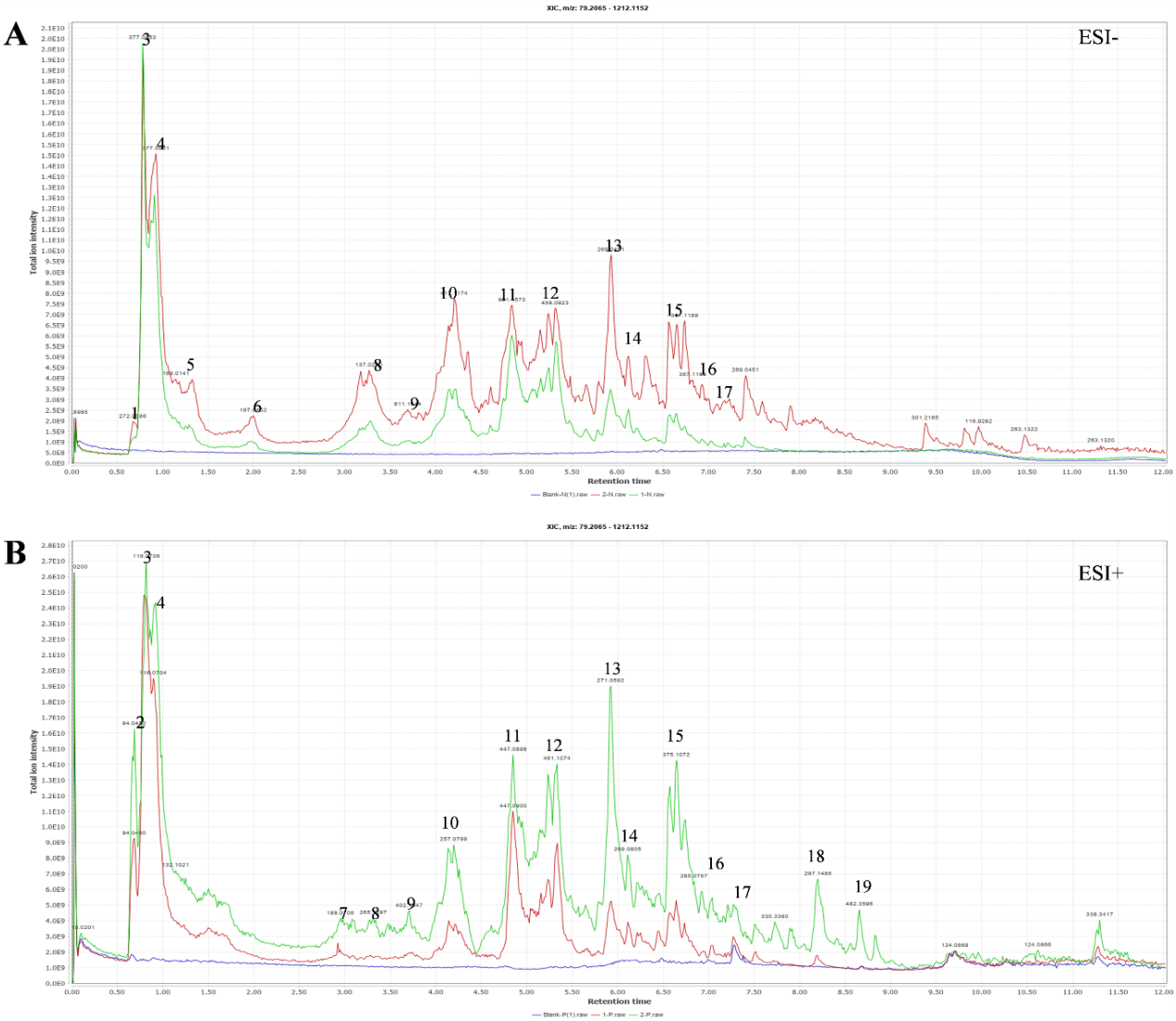


Table 1: Composition of XGJZ identified with UPLC-HRMS.

No.	Identification	Formula	Error (PPM)	m/z	RT [min]	Reference Ion
1	1-O-Arsonopentofuranose	C5 H11 As O8	-3.32	272.9588	0.683	[M-H]-1
2	β-Alanine	C3 H7 N O2	-1.25	90.05484	0.867	[M+H]+1
3	D-(+)-Proline	C5 H9 N O2	-0.84	116.07051	0.909	[M+H]+1
4	L-(+)-Valine	C5 H11 N O2	-2.2	118.086	0.931	[M+H]+1
5	5-Formylfurfural	C6 H4 O3	-2.68	169.01391	1.257	[M+FA-H]-1
6	4-Hydroxyphenylacetic acid	C8 H8 O3	-2.01	197.04525	1.979	[M+FA-H]-1
7	Trans-3-Indoleacrylic acid	C11 H9 N O2	0.15	188.07063	2.926	[M+H]+1
8	4-Hydroxybenzoic acid	C7 H6 O3	-2.78	137.02403	3.198	[M-H]-1
9	Corymboside	C26 H28 O14	-1.2	565.1545	3.783	[M+H]+1
10	Salvianolic acid D	C20 H18 O10	3.36	417.08412	4.179	[M-H]-1
11	Phenyl hexopyranosiduronic acid	C20 H26 O10	-1.7	427.15915	4.838	[M+H]+1
12	Oxane-2-carboxylic acid	C22 H20 O11	-2.71	461.10661	5.331	[M+H]+1
13	Doxylamine	C17 H22 N2 O	-2.41	271.17984	5.92	[M+H]+1
14	Formononetin	C16 H12 O4	-2.34	269.08024	6.118	[M+H]+1
15	5,2'-Dihydroxy-6,7,8,6'-tetramethoxyflavone	C19 H18 O8	-2.64	375.10649	6.648	[M+H]+1
16	Juvenile hormone III bisepoxide	C16 H26 O4	-2.44	283.1897	6.848	[M+H]+1
17	Curcumin II	C20 H18 O5	-1.7	361.10394	7.719	[M+Na]+1
18	Ovalitenin A	C18 H14 O3	-1.13	279.10126	8.185	[M+H]+1
19	Hexadecyllysophosphatidylcholine	C24 H52 N O6 P	-1.85	482.35974	8.656	[M+H]+1

SIRT1 has been supposed to be a potential marker for obesity (Salim *et al*., 2020). Obesity can cause the cleavage of SIRT1 protein in adipose tissue and aggravate the occurrence of systemic inflammation (Ong, 2019). In mature 3T3-L1 adipocytes, SIRT1 can deacetylate FOXO1 and promote FOXO1 and CEBP/α. It forms a complex and binds to the promoter region of the adiponectin gene to up-regulate adiponectin expression, thereby sensitizing insulin.

Our study showed that XGJZ-containing serum promoted the phosphorylation of SIRT1. Together with our previous results, that the protein expression level of IGFBP1 and the phosphorylation of PI3K and AKT could be reversed by the SIRT1 inhibitor EX527, we supposed that SIRT1 and the IGF pathway acted synergistically on FOXO1 and then regulated the fat metabolism.

To test this hypothesis, the protein levels of the key regulatory transcription factors for preadipocyte differentiation (PPARγ, CEBP/α, and SREBP1C) were detected. Overexpression of CEBP/α and PPARγ can induce and accelerate adipocyte differentiation, while SREBP1C is crucial in the early stage of preadipocyte differentiation (Sadie-Van Gijsen, 2019). CEBP/α and PPARγ were associated, they can activate transcription with each other, in the meantime, the expression of SREBP1C also depends on PPARγ, SREBP1C can be enhanced by PPARγ and increase the proportion of differentiated adipocytes (Kuri-Harcuch *et al*., 2019). Our results showed that XGJZ-containing serum could significantly suppress PPARγ, CEBP/α, and SREBP1C to inhibit the differentiation of 3T3-L1 preadipocytes.

ATGL and HSL are the key enzymes in animal fat catabolism (Lampidonis *et al*., 2011). The biological role of ATGL is to degrade triglycerides into diglycerides, phosphorylate and translocate at the same time, move from cytosol to lipid droplet surface and then decompose diglycerides into free fatty acids (Schreiber *et al*., 2019). To further explore the role of XGJZ in lipolysis, we evaluated the changes in phosphorylation levels of ATGL and HSL related to lipolysis. XGJZ-containing serum significantly enhanced the phosphorylation levels of ATGL and HSL. Ex527 could significantly counteract the effect of XGJZ-containing serum. At the same time, we also observed that XGJZ-containing serum promoted the increase of UCP1 expression. Ahmadian *et al.* also agreed that the activity of ATGL hydrolase in adipocytes may lead to the expression of UCP1 and promote adipose tissue browning (Ahmadian *et al*., 2011). UCP1 is a marker of brown adipose tissue (Chouchani *et al*., 2019).

To better understand the association between XGJZ and its anti-adipogenesis effects, the composition of XGJZ was detected. Among the identified 19 main compounds, some of them have been reported to have anti-adipogenesis effects. Curcumin II (demethoxycurcumin) exhibits greater differentiation suppression in 3T3-L1 adipocytes than curcumin (Alalaiwe *et al*., 2021). 4HPA can significantly reduce plasma triglycerides, cholesterol, free fatty acid and fasting blood glucose in mice (Singh *et al*., 2012). Formononetin has been proven to improve cholestasis through SIRT1 signaling (Yang *et al*., 2019). There are also several compounds that we have not yet found to directly inhibit the process of adipogenesis, but we have found that they may play a role in the treatment of other diseases by regulating the pathways involved in our study. For example, salvianolic acid D has been reported to inhibit oxidative stress and apoptosis through PI3K/AKT/mTOR pathway (Zhang *et al*., 2022). Skullcapflavone II (5,2'-Dihydroxy-6,7,8,6'-

tetramethoxyflavone) can regulate the PI3K/AKT signaling pathway against COVID-19 as reported (Feng *et al*., 2022). Studies have shown that some herbal formulations are more effective than the same dose of individual ingredients (Murugan *et al*., 2021). In addition, because of synergies, the isolated phytochemical may, in some cases, have to combine with other components of the mixture to be more effective (Andersen *et al*., 2010). These phytochemicals contained in our XGJZ herbal compound may play a synergistic role in the inhibition of adipogenesis, in which some mechanisms that were not discovered will be investigated in our subsequent studies.

CONCLUSIONS

In summary, we revealed the valuable effect of XGJZ-containing serum on diminishing adipogenesis of mouse preadipocyte 3T3-L1. In particular, the drug-containing serum of XGJZ could effectively regulate the expression of lipid metabolism genes through the crosstalk of SIRT1 and IGF-1 pathways.

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REFERENCES

Abduraman MA, Azizan NA, Teoh SH and Tan ML (2021). Ketogenesis and SIRT1 as a tool in managing obesity. *Obes Res Clin Pract,* **15**(1): 10-18.

Ahmadian M, Abbott Marcia J, Tang T, Hudak Carolyn S S, Kim Y, Bruss M, Hellerstein Marc K, Lee H, Samuel Varman T, Shulman Gerald I, Wang Y, Duncan Robin E, Kang C and Sul Hei S (2011). Desnutrin/ATGL Is regulated by AMPK and is required for a brown adipose phenotype. *Cell Metabolism,* **13**(6): 739-748.

Alalaiwe A, Fang JY, Lee HJ, Chiu CH and Hsu CY (2021). The demethoxy derivatives of curcumin exhibit greater differentiation suppression in 3t3-l1 adipocytes than curcumin: A mechanistic study of adipogenesis and molecular docking. *Biomolecules,* **11**(7) : 1025.

Andersen C, Rayalam S, Della-Fera MA and Baile CA (2010). Phytochemicals and adipogenesis. *Biofactors,* **36**(6): 415-422.

Asghari Hanjani N and Vafa M (2019). The role of IGF-1 in obesity, cardiovascular disease and cancer. *Med J Islam Repub Iran,* **33**: 56.

Caballero B (2019). Humans against Obesity: Who Will Win? *Adv Nutr,* **10**(suppl_1): S4-s9.

Cao Y, Guo Y, Wang Y, Cao Y, Zong R, Huang C and Liu J (2018). Drug-containing serum of Xinfeng capsules protect against H9C2 from death by enhancing miRNA-21 and inhibiting toll-like receptor 4/phosphorylated p-38 (p-p38)/p-p65 signaling pathway and proinflammatory cytokines expression. *J Tradit Chin Med,* **38**(3): 359-365.

Chouchani ET, Kazak L and Spiegelman BM (2019). New advances in adaptive thermogenesis: ucp1 and beyond. *Cell Metabolism,* **29**(1): 27-37.

Cisa-Wieczorek S and Hernández-Alvarez MI (2020). Deregulation of lipid homeostasis: A fact in the development of metabolic diseases. *Cells,* **9**(12): 2605.

Ding RB, Bao J and Deng CX (2017). Emerging roles of SIRT1 in fatty liver diseases. *Int. J Biol. Sci.,* **13**(7): 852-867.

Feng T, Zhang M, Xu Q, Song F, Wang L, Gai S, Tang H, Wang S, Zhou L and Li H (2022). Exploration of molecular targets and mechanisms of Chinese medicinal formula *Acacia catechu* -Scutellariae Radix in the treatment of COVID-19 by a systems pharmacology strategy. *Phytother Res,* **36**(11): 4210-4229.

Hoffman RM, Boston RC, Stefanovski D, Kronfeld DS and Harris PA (2003). Obesity and diet affect glucose dynamics and insulin sensitivity in Thoroughbred geldings. *J Anim Sci,* **81**(9): 2333-2342.

Huang Z, Huang L, Waters MJ and Chen C (2020). Insulin and growth hormone balance: Implications for obesity. *Trends Endocrinol Metab,* **31**(9): 642-654.

Kuri-Harcuch W, Velez-delValle C, Vazquez-Sandoval A, Hernández-Mosqueira C and Fernandez-Sanchez V (2019). A cellular perspective of adipogenesis transcriptional regulation. *J Cell Physiol.,* **234**(2): 1111-1129.

Lampidonis AD, Rogdakis E, Voutsinas GE and Stravopodis DJ (2011). The resurgence of Hormone-Sensitive Lipase (HSL) in mammalian lipolysis. *Gene,* **477**(1-2): 1-11.

Lee S and Dong HH (2017). FoxO integration of insulin signaling with glucose and lipid metabolism. *J Endocrinol,* **233**(2): R67-r79.

Lin W, Tang Y, Zhao Y, Zhao J, Zhang L, Wei W and Chen J (2020). MiR-144-3p targets foxo1 to reduce its regulation of adiponectin and promote adipogenesis. *Front Genet,* **11**: 603144.

Michaelsen KF, Larnkjaer A and Molgaard C (2013). Early diet, insulin-like growth factor-1, growth and later obesity. *World Rev Nutr Diet,* **106**: 113-118.

Murugan DD, Balan D and Wong PF (2021). Adipogenesis and therapeutic potentials of antiobesogenic phytochemicals: Insights from preclinical studies. *Phytother Res,* **35**(11): 5936-5960.

Ong ZY (2019). Breaking the intergenerational cycle of obesity with SIRT1. *J Physiol,* **597**(2): 369-370.

Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R, Leid M, McBurney M and Guarente L (2004). Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. *Nature,* **429**(6993): 771-776.

Qin MY, Huang SQ, Zou XQ, Zhong XB, Yang YF, Zhang YT, Mi ZC, Zhang YS and Huang ZG (2021). Drug-containing serum of rhubarb-astragalus capsule

inhibits the epithelial-mesenchymal transformation of HK-2 by downregulating TGF-β1/p38MAPK/Smad2/3 pathway. *J. Ethnopharmacol,* **280**: 114414.

Sadie-Van Gijsen H (2019). Adipocyte biology: It is time to upgrade to a new model. *J Cell Physiol.,* **234**(3): 2399-2425.

Salim S, Kartawidjajaputra F and Suwanto A (2020). SIRT1 as a Potential Biomarker for Obesity. *J Nutr Sci Vitaminol (Tokyo),* **66**(Supplement): S324-s328.

Schreiber R, Xie H and Schweiger M (2019). Of mice and men: The physiological role of adipose triglyceride lipase (ATGL). *Biochim Biophys Acta Mol Cell Biol Lipids,* **1864**(6): 880-899.

Singh AB, Khaliq T, Chaturvedi JP, Narender T and Srivastava AK (2012). Anti-diabetic and anti-oxidative effects of 4-hydroxypipecolic acid in C57BL/KsJ-db/db mice. *Hum Exp. Toxicol,* **31**(1): 57-65.

Stephens JM (2016). IGF1 and adipose tissue homeostasis. *Obesity (Silver Spring),* **24**(1): 10.

Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD and Glass DJ (2004). The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell,* **14**(3): 395-403.

Stordeur C, Puth K, Sáenz JP and Ernst R (2014). Crosstalk of lipid and protein homeostasis to maintain membrane function. *Biol Chem,* **395**(3): 313-326.

Tran D, Bergholz J, Zhang H, He H, Wang Y and Zhang Y, Li Q, Kirkland J and Xiao Z (2014). Insulin-like growth factor-1 regulates the SIRT1-p53 pathway in cellular senescence. *Aging Cell,* **13**(4): 669-678.

Unger RH, Clark GO, Scherer PE and Orci L (2010). Lipid homeostasis, lipotoxicity and the metabolic syndrome. *Biochim Biophys Acta,* **1801**(3): 209-214.

Yang S, Wei L, Xia R, Liu L, Chen Y, Zhang W, Li Q, Feng K, Yu M, Zhang W, Qu J, Xu S, Mao J and Fan G (2019). Formononetin ameliorates cholestasis by regulating hepatic SIRT1 and PPARα. *Biochem Biophys Res Commun,* **512**(4): 770-778.

Yu GS, Lin YB, Xu HB, Zhang SX, Li JH and Wang HY (2020). Effect of Bugu granules in a drug-containing serum on chondrocyte apoptosis and the Trx2 signaling pathway. *Z Rheumatol,* **79**(3): 304-311.

Zhang T, Liu W, Yang J, Xu H, Cao Y, Guo L, Sun J, Liang B, Du X, Chai L, Yuan Q and Hu L (2022). Components of Salvia miltiorrhiza and Panax notoginseng Protect Pericytes Against OGD/R-Induced Injury via Regulating the PI3K/AKT/mTOR and JNK/ERK/P38 Signaling Pathways. *J Mol Neurosci,* **72**(12): 2377-2388.

Zhou W, Rowitz BM and Dailey MJ (2018). Insulin/IGF-1 enhances intestinal epithelial crypt proliferation through PI3K/Akt and not ERK signaling in obese humans. *Exp Biol Med (Maywood),* **243**(11): 911-916.