The inhibitory effects of Xiao-Gao-Jiang-Zhuo-containing serum on adipogenesis in 3T3-L1 preadipocytes

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Background. Obesity and related metabolic diseases are becoming a worldwide epidemic, leading to increased mortality and heavy medical costs. Our Chinese herbal formula Xiao-Gao-Jiang-Zhuo (XGJZ) has remarkable effects on curing obese patients in the clinic, but the cellular and molecular basis remains unknown. This study aimed to reveal the molecular mechanism involved in adipogenesis *in vitro*.

Methods. Chinese herbal formula XGJZ-containing serum was prepared from XGJZ-treated obesity model rats. The function of XGJZ-containing serum was validated in 3T3-L1 preadipocytes. Oil O staining was performed to determine intracellular lipid accumulation in differentiated 3T3-L1 cells. The expression of pro-adipogenic transcription factors was measured to further validate the adipogenesis of 3T3-L1 adipocytes. The contents of triglyceride (TG), free fatty acid (FFA), and glycerin, along with the activities of lipid metabolism-related enzymes (including FAT, FATP1, DGAT, GPAT, ATGL, and HSL) were measured to study the lipogenesis in 3T3-L1 adipocytes.

Results. XGJZ-containing serum inhibited 3T3-L1 differentiation, decreased intracellular lipid accumulation, and suppressed the expression of pro-adipogenic transcription factors in differentiated 3T3-L1 cells. The contents of TG, FFA, and glycerin were decreased when treated with XGJZ-containing serum, which also modulated lipid metabolism-related enzyme activities. The activities of fatty acid transporters (FAT, FATP1) and lipid mobilization enzymes (ATGL, HSL) were promoted, while activities of triglyceride biosynthesis enzymes (DGAT, GPAT) were attenuated in differentiated 3T3-L1 cells.

Conclusion. XGJZ-containing serum has inhibitory effects on adipogenesis in 3T3-L1 preadipocytes, affirming the effect of XGJZ in treating obesity. It provides evidence for the mechanism of obesity.

Key words: XGJZ; lipid accumulation; adipokines; 3T3-L1 preadipocytes

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INTRODUCTION

Obesity refers to a chronic disease in which the body controls the imbalance of body fat and energy metabolism, resulting in excessive accumulation and/or abnormal distribution of body fat. It is the result of the joint action of genetic factors and environmental factors[1]. Obesity is an important independent risk factor for many metabolic diseases, such as cardiovascular disease, hypertension, diabetes, and so on. It is closely related to the aggregation of these diseases[2]. Obesity has become a global condition that seriously affects over 2 billion people worldwide[3].

Adipogenesis refers to the development and accumulation of adipocytes in the form of adipose tissue in different parts of the body[4]. The main function of adipocytes is to store energy in the form of fat when energy intake exceeds consumption, and mobilize stored fuel when energy consumption exceeds intake. Adipocytes appear in late embryonic development and mature animals under the condition of promoting obesity[5]. The excessive accumulation of body fat is the result of continuous energy intake exceeding energy consumption, which will lead to obesity. This condition leads to hyperplasia and hypertrophy of tissue adipose cells[6].

Many transcription factors regulate the process of adipogenesis, including CCAAT/enhancer-binding protein α (C/EBPα), sterol regulatory element-binding protein 1 (SREBP1c), and peroxisome proliferator-activated receptor γ (PPAR γ) (ref.[7]). In addition, lipid-producing related enzymes, such as fatty acid translocase (FAT) and diglyceride acyltransferase (DGAT), play important roles in lipid accumulation during adipogenesis[8]. Adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) are the key enzymes for the release of fatty acids from TG stores during intracellular lipolysis[9,10].

Natural products have been studied to show inhibitory effects on lipid accumulation in adipocytes[11]. Based on clinical practice, our XGJZ formula is modified with rhubarb and Huanglian Xiexin Decoction and contains 10 kinds of herbs in all. The main natural products are ginger, pinellia ternata, Radix Codonopsis (known as Dangshen), Coptis chinensis (Huanglian in Chinese), *Scutellaria baicalensis* (Huangqin in Chinese), *Crataegus pinnatifida* (Chinese hawthorn), Monascus, Eupatorium fortunei

Turcz (Peilan in Chinese), and the maggots of Chrysomyis megacephala Fabricius (Wuguchong in Chinese), all are proven to be effective on health. The XGJZ Formula is based on the Xiaogao Jiangzhuo method, Xiaogao Jiangzhuo method and traditional Chinese medicines in the XGJZ Formula have shown clinical efficacy in treatment of obesity[12-16]. Rhubarb supplementation is sufficient to prevent metabolic disturbances caused by lipid-and-carbohydrates-rich diet associated with the interaction between Reg3γ and Akkermansia muciniphila[17]. Ginger can effectively reduce the harmful effects of obesity on blood lipids, without side effects[18]. Pinellia ternata was proved to be able to affect anti-obesity through thermogenesis and fatty acid oxidation[19]. Codonopsis lanceolata extract has been proved to prevent obesity in mice[20]. Coptis chinensis also has an anti-obesity effect and improves insulin resistance[21]. *Scutellaria baicalensis* has shown an anti-steatosis effect and ameliorates obesity[22]. The leaves, fruits, and seeds of hawthorn contain flavonoids, triterpene acids, sesquiterpenes, and other active substances, which are beneficial to metabolic syndrome through different mechanisms[23]. Monascus pigments have recently been widely used for commercial and academic purposes. The pigments and derivatives formed during the fermentation of Monascus have pharmaceutical and clinical properties that could combat common diseases, including type-2 diabetes, obesity, and even cancer[24]. Eupatorium fortunei Turcz and the maggots of Chrysomyis megacephala Fabricius contain antibacterial compounds[25,26].

In this study, we attempted to evaluate the direct effects of the XGJZ-containing serum on adipogenesis by using the murine 3T3-L1 preadipocyte cell line.

MATERIALS AND METHODS

Preparation of XGJZ formula

XGJZ formula 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 h, 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 h, and then filtered. The two filtrates were collected and frozen in a −80 °C refrigerator. After being frozen for 24 h, the filtrate was placed in a vacuum freeze dryer for drying to an extract.

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 supernatants 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 1200 m/z with a 70000 resolution for parent ions, and the resolution was 17500, and the energy gradient was 20/40/60 for daughter ions.

Animals and cells

Wistar male rats (weighing 180–220 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animal care and experiments were approved by the Animal Welfare Committee of China Academy of Chinese Medical Sciences (Beijing, China), and complied with the Protection Law of the Czech Republic 501/2020. These animals were raised in SPF breeding facilities maintained following national standards. The 3T3-L1 cells were purchased from the National Collection of Authenticated Cell Cultures (China) and were cryopreserved in laboratory refrigerators.

XGJZ-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 XGJZ was prepared according to the previous method[27]. 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 h before the last gavage and 1 h after the last gavage, blood was collected from the abdominal aorta and centrifuged at 400 × g for 15 min, and then serum was isolated and inactivated at 56 °C for nearly 30 min. Finally, the XGJZ-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 XGJZ-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 medium added with 0.5 mmol/L IBMX, 10 mg/L insulin, 1 μmol/L dexamethasone, and 100 ml/L FBS for 48 h, then cultured with high glucose DMEM containing 10 mg/L insulin and 100 ml/L FBS for another 48 h. 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.

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

Oil red O staining

3T3-L1 preadipocytes were induced to differentiation with different concentrations of XGJZ-containing serum and 5 μmol/L resveratrol-containing differentiation-inducing agent (10 mg/L insulin, 1 μmol/L dexamethasone, and 0.5 mmol/L IBMX). DMEM medium 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 considered to be 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-containing serum, or resveratrol). The cells were inoculated into 96 well culture plates. After 72 h of intervention with XGJZ-containing serum in different dose groups, each well was added with 10 μL of CCK-8 reagent. After incubation for 4 h, a microplate reader was used to detect the absorbance at 450 nm.

Western blotting

Differentiated 3T3-L1 cells were lysed in lysis buffer RIPA (Beyotime, China) and added with 1 mM PMSF. at 4°C for 10 min and then centrifuged at 400 × g for 15 min to obtain the supernatant. Protein concentration was quantified using a BCA kit (Beyotime, China). Total protein was separated in 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes, and then transferred onto polyvinylidene difluoride membranes. Following blocking with 5% non-fat milk in Tris-buffered saline containing Tween-20 (TBST) buffer at room temperature, the membranes were incubated with the primary antibodies at 4 °C overnight. The antibodies of PPARγ (1:5000, ab59256), C/EBPα (1:5000, ab40761), SREBP1 (1:5000, ab3259), and GAPDH (1:5000, ab125247) have been bought from Abcam. After that, the membranes were washed three times with TBST buffer for 10 min and incubated with the corresponding secondary antibody (1:10,000) at room temperature. Finally, the membrane was processed using an ECL kit for color reaction. Western blot results were normalized to those of GAPDH for semi-quantification.

Measurement of intracellular triglyceride (TG), FFA, and glycerol contents

Differentiated 3T3-L1 cells were induced as the following groups: Control group, Model group, high, medium, and low dose of XGJZ-containing serum groups, and resveratrol group. All groups except for the control group were induced by high concentration FFA (0.5 mmol/L palmitic acid). The 3T3-L1 preadipocytes were differentiated in the absence or presence of different concentrations of the XGJZ formula for the indicated times.

According to the manufacturer's instructions, triglyceride (TG) was quantitatively measured using a triglyceride determination kit (applygen, China). Then, TG content was normalized to 100 mg of protein and reported as TG mg per 100 mg of cell protein.

Free fatty acids (FFA) in cells were measured using a commercial kit (Solarbio Biotechnology Co., Ltd. Shanghai, China). The cells were digested, centrifuged and the cell particles were collected. The cell particles were then added with the reagent in the analysis kit and ultrasonically broken at 4 °C. After that, the solution was centrifuged and the supernatant was collected. The optical density of supernatants was measured at 550 nm with an automatic microplate reader (Thermo Fisher Scientific, USA), and the free fatty acid level was calculated according to the calculation formula provided by the commercial analysis kit. The protein concentration was determined using the double cinchonic acid (BCA) kit (beyotime, Shanghai, China). Free fatty acid levels were expressed as mM FFA/mg protein.

Glycerol was determined by the glycerol assay kit (Applygen Technologies Inc., China) according to the manufacturer's instructions. The total protein concentration was estimated by the BCA method. Lipolysis data were expressed as nM of glycerol/mg of protein.

Lipase activity assay

The substrate metabolism method was applied to detect the activities of key enzymes in TG metabolism according to previous studies[28,29]. Adipocytes were homogenized on ice for 30 min with 1mL of homogenization buffer and then centrifuged at 1000 × g for 10 min at 4 °C. The supernatants were used to detect the activity of lipases. The protein concentration of the supernatants was determined with a BCA Protein Assay Kit (Beyotime Biotechnology, China).

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 ± SEM of at least three independent experiments. One-way or two-way analysis of variance (ANOVA) followed by Tukey post hoc test was performed to calculate the statistical differences. Values of $P<0.05$ were considered to be statistically significant. Image J was used to carry out the semi-quantitative analysis. All P values in our study have been displayed in Supplementary Table 1. Raw data for Fig. 2–5 have been shown in Supplementary Table 2.

RESULTS

Composition of XGJZ

After UPLC-Q-TOF/MS analysis, 19 compounds were identified based on multi-stage mass spectrometry information and a high-resolution mass spectrometry database for natural products (Table 1, Fig. 1). 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 bisepoxide, Curcumin II, and Ovalitenin A were main compounds of GPT (Table 1).

XGJZ-containing serum inhibited 3T3-L1 differentiation

To explore whether XGJZ-containing serum can inhibit adipocyte maturation, differentiation of 3T3-L1 was induced to be differentiated for 8 days, and the intracellular storage of lipids was evaluated by performing Oil Red O staining on day 8. Treatment with the XGJZ-containing serum significantly reduced lipid accumulation both at the middle (P<0.05) and high dose (P<0.01), compared with the saline group. A high dose of XGJZ formula played a similar role with resveratrol, a representative drug to reduce triglyceride in the clinic (Fig. 2A, Supplementary Fig. 1). This result was further confirmed by measuring the Oil Red O absorbance at 490 nm (Fig. 2B). Besides, we examined whether XGJZ-containing serum reduced adipogenesis by suppressing cell viability during the differentiation process. Cell viability was assessed by performing a CCK-8 assay. Our results showed that XGJZ-containing serum did not reduce cell viability, further confirming its direct effect on adipogenesis (Fig. 2C).

XGJZ-containing serum suppressed the expression of pro-adipogenic transcription factors in differentiated 3T3-L1 cells

Next, the protein expression levels of the key regulatory transcription factors involved in adipogenesis, PPARγ, C/EBPα, and SREBP1, were detected. SREBP1 plays a role in lipid synthesis, insulin signaling, and lipid homeostasis. As shown in Fig. 3 and Supplementary Fig. 2, in mature adipocytes stimulated with different doses of XGJZ-containing serum, the protein expression levels of PPARγ, C/EBPα, and SREBP1 were all notably suppressed in comparison with non-XGJZ treated model cells. Besides, the XGJZ-containing serum suppressed the expression of pro-adipogenic transcription factors in a dose-dependent manner, and high-dose of XGJZ-containing serum acted a similar role as resveratrol.

XGJZ-containing serum decreased intracellular lipid accumulation in differentiated 3T3-L1 cells

To further validate the function of the XGJZ-containing serum in reducing the intracellular lipid accumulation, the cellular contents of triglycerides (TG), free fatty acid (FFA), and glycerin were measured. The contents of TG, FFA, and glycerin were all markedly increased in the model group compared with the control group, but significantly decreased in the XGJZ-containing serum treated group, especially in the high-dose of XGJZ-containing serum group, which showed a similar effect

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

NO.	Identification	Formula	Error (PPM)	m/z	RT [min]	Reference Ion
1	1-O-Arsonopentofuranose	C5H11AsO8	−3.32	272.9588	0.683	[M−H]−1
2	β-Alanine	C3H7NO2	−1.25	90.05484	0.867	[M+H]+1
3	D-(+)-Proline	C5H9NO2	−0.84	116.07051	0.909	[M+H]+1
4	L-(+)-Valine	C5H11NO2	−2.2	118.086	0.931	[M+H]+1
5	5-Formylfurfural	C6H4O3	−2.68	169.01391	1.257	[M+FA−H]−1
6	4-Hydroxyphenylacetic acid	C8H8O3	−2.01	197.04525	1.979	[M+FA−H]−1
7	Trans-3-Indoleacrylic acid	C11H9NO2	0.15	188.07063	2.926	[M+H]+1
8	4-Hydroxybenzoic acid	C7H6O3	−2.78	137.02403	3.198	[M−H]−1
9	Corymboside	C26H28O14	−1.2	565.1545	3.783	[M+H]+1
10	Salvianolic acid D	C20H18O10	3.36	417.08412	4.179	[M−H]−1
11	Phenyl hexopyranosiduronic acid	C20H26O10	−1.7	427.15915	4.838	[M+H]+1
12	Oxane-2-carboxylic acid	C22H20O11	−2.71	461.10661	5.331	[M+H]+1
13	Doxylamine	C17H22N2O	−2.41	271.17984	5.92	[M+H]+1
14	Formononetin	C16H12O4	−2.34	269.08024	6.118	[M+H]+1
15	5,2'-Dihydroxy-6,7,8,6'-tetramethoxyflavone	C19H18O8	−2.64	375.10649	6.648	[M+H]+1
16	Juvenile hormone III bisepoxide	C16H26O4	−2.44	283.1897	6.848	[M+H]+1
17	Curcumin II	C20H18O5	−1.7	361.10394	7.719	[M+Na]+1
18	Ovalitenin A	C18H14O3	−1.13	279.10126	8.185	[M+H]+1
19	Hexadecyllysophosphatidylcholine	C24H52NO6P	−1.85	482.35974	8.656	[M+H]+1

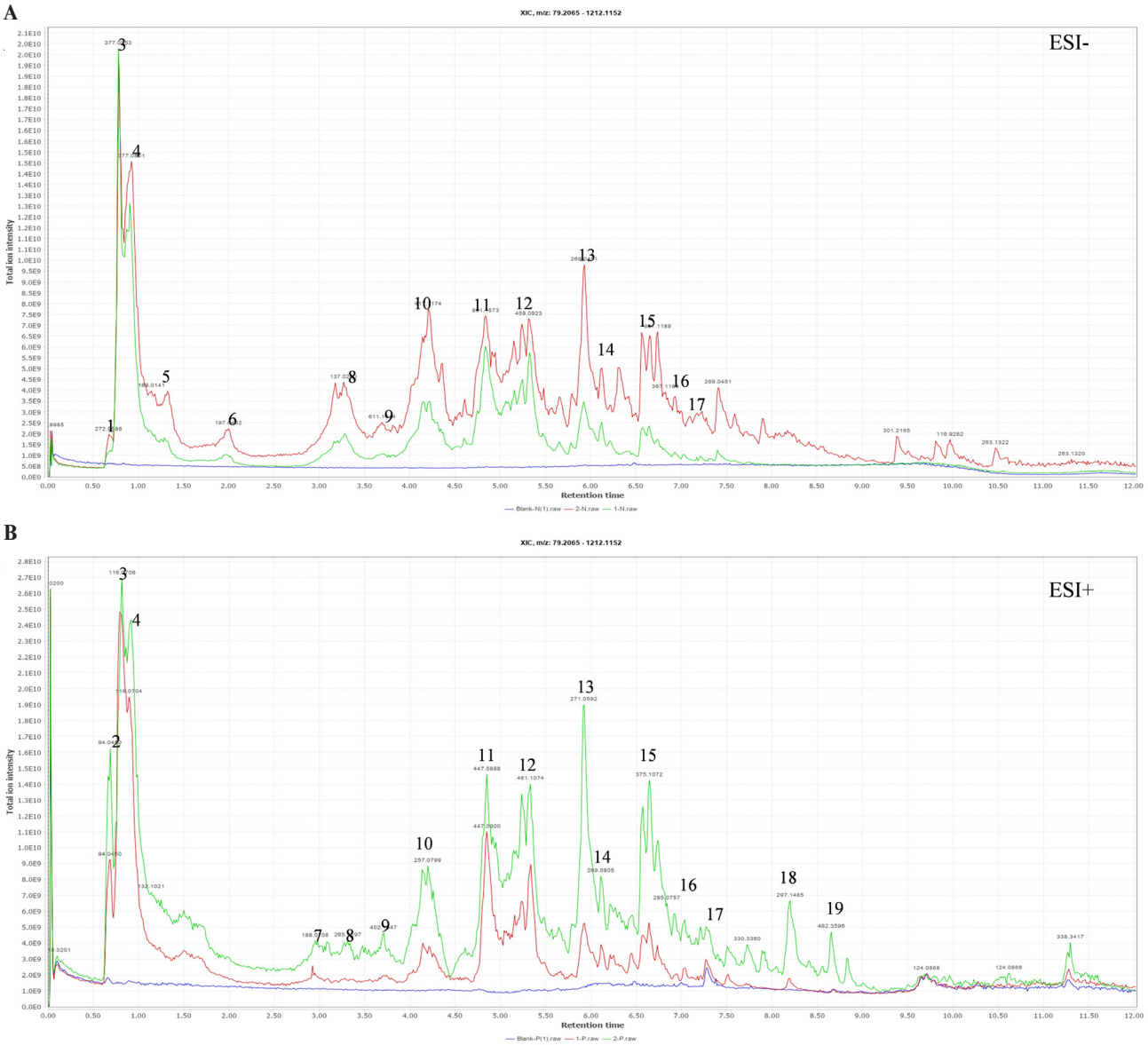

Fig. 1. The compositional analysis of XGJZ formula using UPLC-HRMS. **A.** Negative ion mode. **B.** Positive ion mode.

with resveratrol (Fig. 4A–C). To sum up, all these results emphasized the significant role of XGJZ-containing serum in reducing adipocyte differentiation, which then decreased adipose cell mass accumulation.

XGJZ-containing serum modulated lipid metabolism-related enzyme activities in differentiated 3T3-L1 cells

Next, critical lipid metabolism-related enzymes, including FAT, FATP-1, GPAT, DGAT, ATGL, and HSL were analyzed in differentiated 3T3-L1 cells treated with XGJZ-containing serum. As shown in Fig. 5A, the fatty acid transport enzyme activities of FAT and FATP-1 were significantly promoted compared with the model cells without XGJZ-containing serum treatment (P<0.001, high-dose group *vs*. model group; P<0.01, low-dose group *vs*. high-dose group). Meanwhile, the enzyme activities of critical lipolysis enzymes GPAT and DGAT were reduced in comparison with the model cells (Fig. 5B, P<0.01, high-dose group *vs*. model group; P<0.05, low-dose group *vs*. high-dose group). In contrast, the activities of lipid synthesis enzymes ATGL and HSL were highly increased compared with the model cells without XGJZ-containing serum treatment (Fig. 5C, P<0.01, high-dose group *vs*. model group; P<0.05, low-dose and middle-dose groups *vs*. high-dose group). A high dose of XGJZ-containing serum played a similar role with resveratrol in regulating the activities of the lipid metabolism-related enzymes. Our results showed that the XGJZ-containing serum inhibited lipid synthesis and increased lipolysis.

DISCUSSION

Several studies have reported that herbal medicine can ameliorate obesity by downregulation of adipogenesis and improvement of other metabolic disorders[30,31]. XGJZ is an herbal remedy to treat obesity and related metabolic

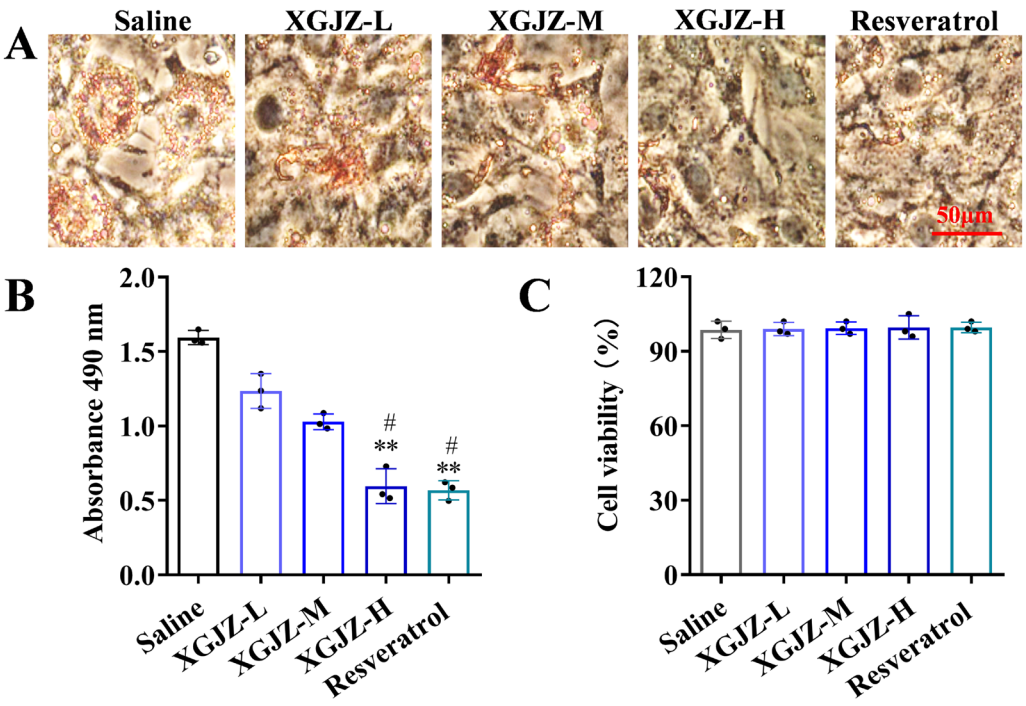

Fig. 2. Oil Red O staining and CCK-8 assay in 3T3-L1 adipocytes. **A.** Cells were seeded in a 24-well plate at a density of 1.6×10^4 cells per well. Differentiation was induced with Xiao-Gao-Jiang-Zhuo (XGJZ) – containing serum or resveratrol for 8 days (XGJZ-L: low dose of XGJZ-containing serum, XGJZ-M: middle dose of XGJZ-containing serum, XGJZ-L: high dose of XGJZ-containing serum). Then, cells were stained with oil red O, and the lipid droplet storage was visualized in optical microscopy and **B.** quantified by measuring absorbance at 490 nm. Scale bar = 50 μm. **C.** Cell viability was assessed by performing a CCK-8 assay. $^{**}P<0.01$, $^{***}P<0.001$ *vs.* the saline group, $^{\#}P<0.05$ $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs.* the XGJZ-L group.

diseases, such as diabetes and non-alcoholic fatty liver. In a clinical observation of 86 cases of obesity treated with the XGJZ formula, 73.26% of patients achieved a remarkable curative effect[12]. XGJZ formula can reduce liver lipid peroxidation to protect the liver; at the same time, this formula can improve the state of insulin resistance by increasing the expression of insulin receptor and insulin receptor substrate (IRS)-2 in the liver tissue[32].

In our present study, 3T3-L1 cells were used to examine the effects of the XGJZ-containing serum on adipogenesis and lipogenesis. We observed that the XGJZ-containing serum did not inhibit the cell viability, but significantly reduce lipid accumulation. XGJZ-containing serum also repressed the cellular contents of TG, FFA, and glycerin. At the same time, the XGJZ-containing serum regulated the lipid metabolism-related enzyme activities in differentiated 3T3-L1 cells and inhibited lipid synthesis and increased lipolysis. Besides, XGJZ-containing serum suppressed the expression of pro-adipogenic transcription factors in differentiated 3T3-L1 cells, and the protein expression levels of PPARγ, C/EBPα, and SREBP1 were remarkably reduced. XGJZ-containing serum suppressed the lipid accumulation in a dose-dependent manner, high-dose of XGJZ-containing serum acted a similar role with resveratrol.

The main transcriptional regulators of adipogenesis, including PPARγ, C/EBPα, and SREBP1, are necessary modulators of target gene expression involved in adipocyte differentiation at different stages[33]. C/EBPα plays an important role in the differentiation and development of adipocytes. It can activate the differentiation of many adipocytes and the transcription and expression of related genes, promote the formation of lipid droplets, and maintain activity in mature adipocytes[34]. Herein, XGJZ-containing serum reduced the level of C/EBPαin 3T3-L1 adipocytes, thus indicating that XGJZ-containing serum can induce fat loss by reducing the adipocyte differentiation in the adipose tissue. C/EBPα and PPARγ work together to establish the phenotype of mature adipocytes[35]. The members of the nuclear receptor PPAR family play a vital role in lipid metabolism, mainly controlling the storage and release of fat, regulating the body's energy balance and insulin resistance, and promoting the expression of adipocyte genes, in the early process of adipocyte differentiation[36]. PPARγ plays a key role in adipogenesis and is related to the pathology of many diseases. After PPARγ is activated, cells will show morphological changes (such as adipocytes becoming larger), fat accumulation, and insulin sensitivity. Studies have shown that PPARγ is both necessary and sufficient in promoting the differentiation of adipocytes[37]. XGJZ-containing serum also downregulated the level of PPARγ in 3T3-L1 adipocytes, indicating that XGJZ-containing serum can prevent the development of adipocytes by inhibiting PPARγ. The SREBP transcription factor family is an important transcription factor in mammals. It is mainly involved in regulating the biosynthesis and metabolism of endogenous cholesterol, fatty acids and phospholipids, and triglycerides[38].

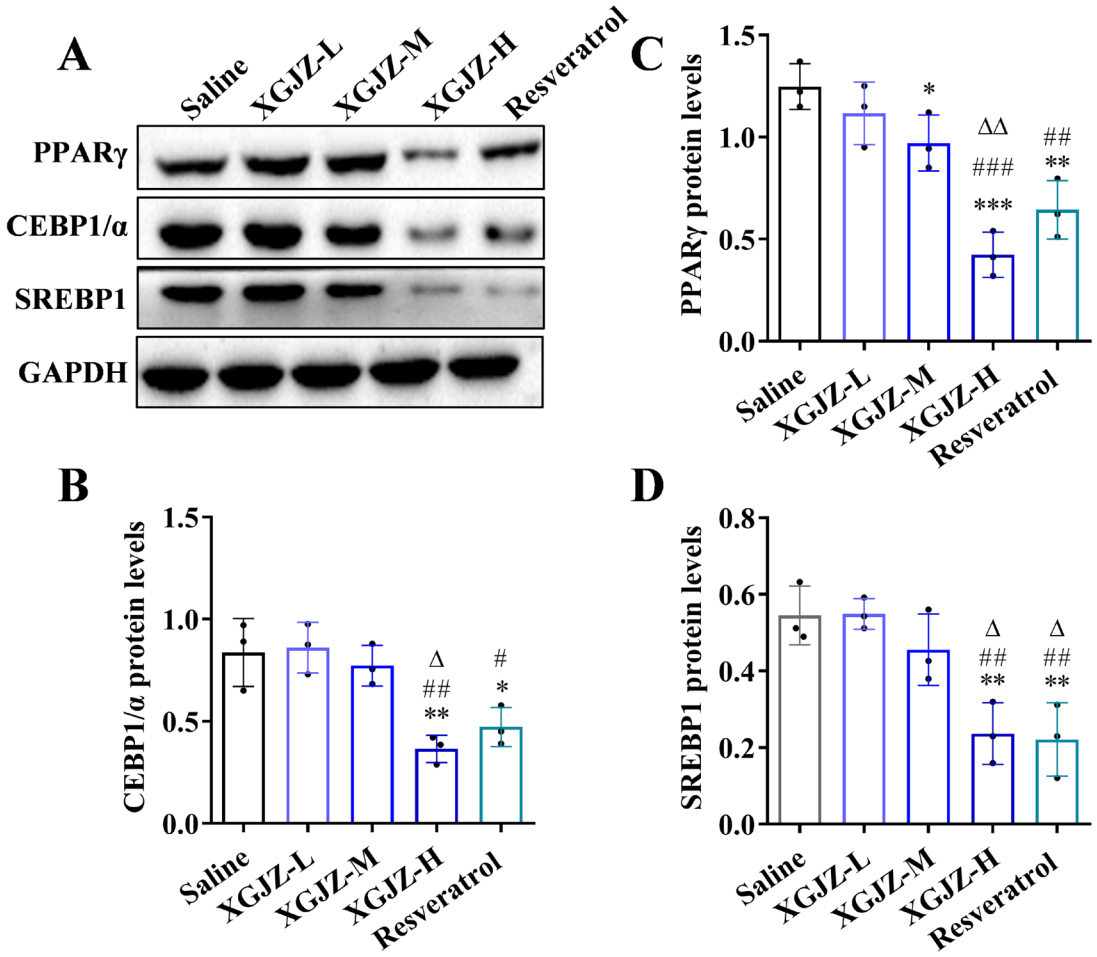

Fig. 3. Effects of XGJZ-containing serum on adipogenic transcriptional factors. Differentiation was induced with or without XGJZ for 8 days. Protein levels of C/EBPα, PPARγ, and SREBP1 were evaluated by western blot analysis. **P*<0.05, ***P*<0.01 *vs.* the saline group, #*P*<0.05, ##*P*<0.01, ###*P*<0.001 *vs.* the XGJZ-L group, Δ*P*<0.05 *vs*. the XGJZ-M group.

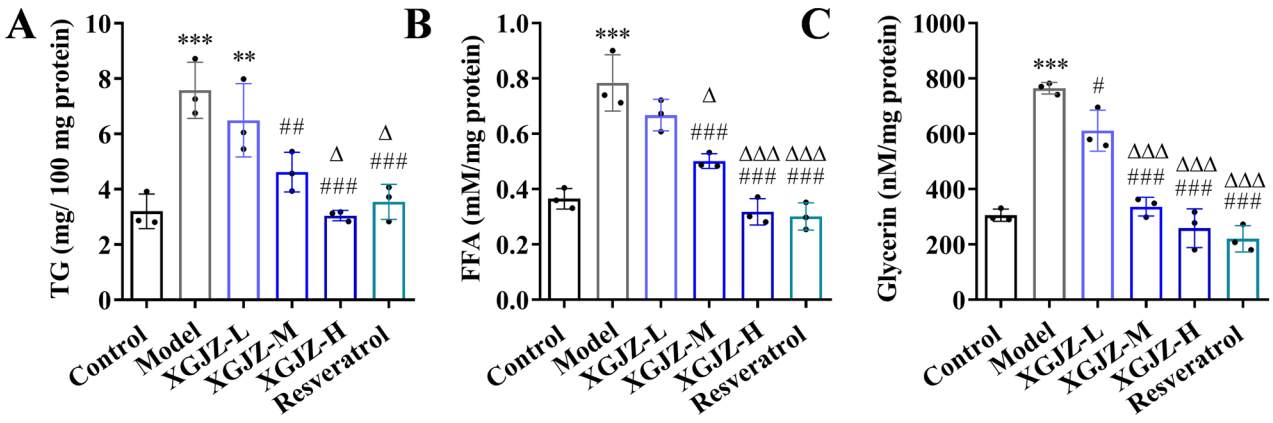

Fig. 4. Effects of XGJZ-containing serum on lipid metabolism in 3T3-L1 cells. **A.** Cellular TG contents were measured using a commercial triglycerides GPO-POD enzymatic assay kit. **B.** The free fatty acid in cells was measured with a commercial kit. **C.** glycerol was determined by a glycerol assay kit. ***P*<0.01, ****P*<0.001 *vs.* the control group, $^{\#}P$<0.05 $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 *vs*. the model group, Δ*P*<0.05, ΔΔΔ*P*<0.001 *vs*. the XGJZ-L group.

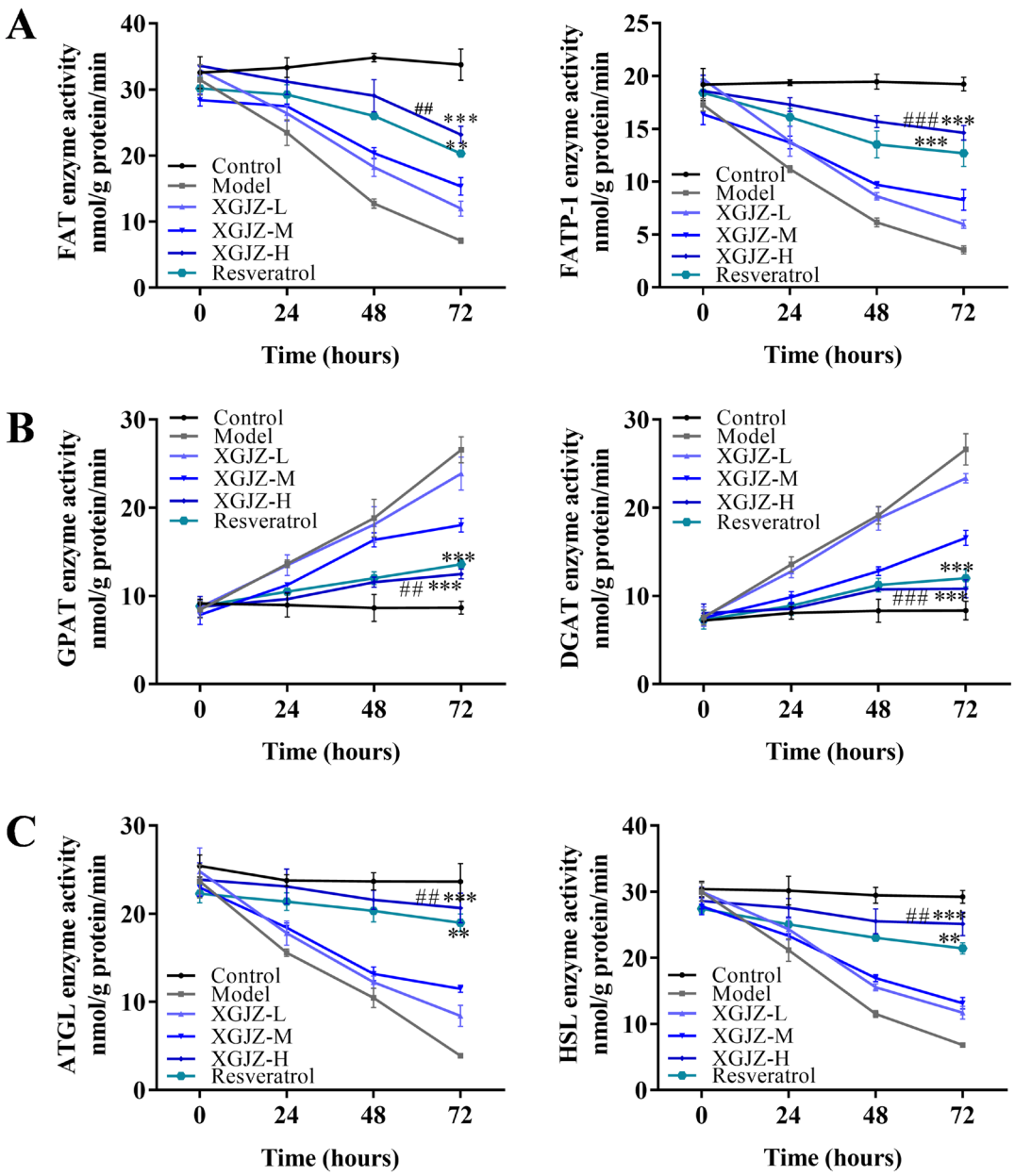

Fig. 5. TG metabolism related enzymes activities in 3T3-L1 cells. **A.** The activities of fatty acid transporters (FAT, FATP1), **B.** triglyceride biosynthesis enzymes (DGAT, GPAT), and lipid mobilization enzymes (ATGL, HSL) (C) were detected. $^{**}P<0.01$, $^{***}P<0.001$ *vs.* the model group, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs*. the XGJZ-L group.

Overexpression of the SREBP1 gene can cause lipid deposition and polymerization in non-fat tissues, which can cause tissue disease, on the other hand, the inhibition of SREBP1 expression can attenuate the differentiation of adipocytes. In addition, SREBP1 overexpression enhanced the activity of PPARγ (ref.[39]). Given that these are the major gene targets for the suppression of adipogenesis in differentiated cells and tissues, our results showed that XGJZ-containing serum decreased the protein expression of PPARγ, C/EBPα, and SREBP1, which are the major gene targets for the suppression of adipogenesis. Overall, the inhibitory effects of XGJZ-containing serum on lipid accumulation and adipogenesis may be mediated by the downregulation of the expression of adipogenesis-related genes. This study explains the antiadipogenic properties of XGJZ-containing serum.

In summary, this study provided evidence that XGJZ-containing serum inhibited adipogenesis of 3T3-L1 adipocytes by affecting C/EBPα, PPARγ, and SREBP1 and lipogenesis by modulating FAT, FATP1, DGAT, GPAT, ATGL, and HSL. Adipogenesis inhibition by XGJZ has been established in the clinic, and now we have presented the cellular and molecular explanations for XGJZ preventing obesity.

ABBREVIATIONS

ATGL: Adipose triglyceride lipase, DGAT: Diacylglycerol O-acyltransferase, FAT: Fatty acid translocase, FFA: Free fatty acid, FATP-1: Fatty acid transport protein 1, GPAT: Glyceryl triphosphate transferase, HSL: Hormone-sensitive lipase, TG: Triglyceride, XGJZ: Xiao-Gao-Jiang-Zhuo.

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Supplemental Material:

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