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Original Article

Oxymatrine attenuates lipid accumulation in HepG2 cells by inducing autophagy

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Abstract: Recent reports have revealed that Oxymatrine (OMT) can alleviate hepatosteatosis. The aim of this study was to explore the underlying molecular mechanisms of OMT in improving lipid accumulation in liver cells, and evaluate the impact of OMT on lipogenesis and autophagy in HepG2 cells exposed to fructose. The effects of OMT on hepatic steatosis were observed by oil red O staining and content detection of triglycerides (TG). The effects of OMT on autophagy were evaluated by MDC staining and an autophagy detection kit. Protein expression levels were examined by Western blot. After 48-hours of intervention with fructose, the TG content was significantly increased compared with the control group. Red staining areas by oil red O staining were increased significantly in the fructose group compared with the control group. After OMT intervention, lipid accumulation was decreased. Results of MDC staining and autophagy detection showed that OMT induced autophagy. Compared with the control group, the expression levels of LC3B, Beclin-1, and Atg7 were significantly decreased, and the expression levels of FAS, SCD1, p-Akt/Akt, and mTOR were increased in the fructose group. Compared with the fructose group, expression levels of FAS, SCD1, p-Akt/Akt, and mTOR were decreased, and the expression levels of LC3B, Beclin-1, and Atg7 were significantly increased in the OMT group. Inhibition of autophagy by beclin-1 siRNA increased expression of srebp-1, Fas, p-Akt/Akt, and mTOR, and expression of LC3B, Beclin-1, and Atg7 was decreased. OMT can thus inhibit lipid accumulation of HepG2 cells by activating autophagy through the Akt-mTOR signaling pathway.

Keywords: Oxymatrine, autophagy, fructose, lipid metabolism

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common type of liver disease worldwide, and is estimated to affect 30% of the population in the US [1]. NAFLD can progress into serious complications such as cirrhosis, hepatocellular carcinoma, and death. Numerous epidemiological studies have reported that NAFLD patients are associated with an increased risk of diabetes, metabolic syndrome, and cardiovascular diseases [2-4]. There are many factors that lead to development of fatty liver disease. High fructose diet is an important dietary factor that plays a significant role in the development of fatty liver [5]. Several studies have shown that exposure to high fructose caused lipid accumulation in HepG2 cells [6]. According to animal studies, mice have developed fatty liver after a few weeks of high-fructose diet [7, 8].

Several studies have demonstrated that autophagy is associated with NAFLD. Autophagy is a lysosomal pathway that degrades intracellular organelles and proteins to supply the cell with energy and maintain cellular homeostasis. Several studies have shown that autophagy could increase lipolysis [9, 10] and decrease lipogenesis [9, 11]. Atg7$^{-/-}$ mice with inhibition of autophagy led to marked increase in hepatic triglycerides (TG) and cholesterol content [12]. The study by Wang et al. showed that the signaling axis of CYP7A1-AKT-mTOR selectively induces hepatic autophagy and contributes to improvement of hepatocellular integrity and metabolic homeostasis [13]. These findings suggest that autophagy acts as a potential target for the treatment and protection of NAFLD through the Akt-mTOR pathway.

Lifestyle intervention is important for all patients irrespective of disease stage, but other therapies should be targeted to those most likely to benefit [14]. Oxymatrine (OMT) is a quinolizidine alkaloid extracted from the Chinese herb *Sophora flavescens* Ait, and pos-

sesses antioxidant, anti-inflammatory, anti-allergic, antiviral, anti-fibrotic and anti-apoptotic activities [15-18]. OMT has been used in the treatment of some inflammatory diseases. It has been reported that OMT attenuates hepatic steatosis in high-fructose-fed rats [19]. But the clear mechanism involved still remains unknown, and further investigation regarding the intervention effect of OMT on lipid metabolism in liver cells is required. Hence, the present study investigated the influence of OMT on lipid accumulation and autophagy in HepG2 cells and their possible underlying mechanisms.

Materials and methods

Cell line and groups

HepG2 cells were cultured in minimal essential medium (HyClone, Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% (v/v) fetal bovine serum (FBS, Sijiqing Tianhang Biotechnology Co. Hangzhou China), 1% (v/v) NAFF, 100 U/ml penicillin and 100 μg/ml streptomycin (Shijiazhuang, China) in 5% CO_2 humidified atmosphere. A fat deposition model in HepG2 cells was established by using high fructose (20 mmol/L). HepG2 cells were randomly divided into three groups: the control group (cultured with normal medium), the high-fructose group (cultured with 20 mM fructose for 48 h), and the OMT group (cultured with 20 mM fructose for 24 hours and 0.16 mg/mL OMT for 24 h, Sigma-Aldrich, St. Louis, MO, USA). After 24 hours of intervention, the cells were collected.

Oil red O staining

Cultured cells were washed twice with phosphate-buffered saline (PBS), and stained for 20-30 minutes with Oil Red O. The stained sections were imaged with an Olympus microscope.

Detection of TG

After different treatments, HepG2 cells were washed two to three times with PBS, and lysed on ice using radioimmunoprecipitation buffer for 30 minutes. After centrifugation at 2,000 rpm for 10 minutes at 4°C, supernatant was collected into a new tube. The protein concentration was determined by bicinchoninic acid assay method. TG levels were measured based on the enzymatic assay from Pulilai Bioengineering Institute (Changchun, China) according to the manufacturer's instructions.

MDC staining

Cultured cells were washed twice with PBS, and then were labeled with MDC (Sigma, St Louis, MO, United States) by incubating cells with 0.001 mmol/L MDC at 37° for 10~15 minutes. After incubation, cells were washed three times with PBS and immediately analyzed with a fluorescence microscopy (Olympus).

Measurement of autophagy activity

Autophagy activity was monitored by using the CYTO-ID™ Autophagy Detection Kit (Enzo Life Sciences, France). The kit could visualize autophagic accumulation and autophagic flux. First, the cells were grown on coverslips. When the cells reached 50%~70% confluence, the medium was carefully removed, and the cells were washed twice with 1X Assay buffer. Second, 100 μL of Microscopy Dual Detection Reagent was dispensed to cover each sample of monolayer cells. Third, the samples were protected from light and incubated for 30 minutes at 37°C. Last, the cells were carefully washed with 100 μL of 1X Assay Buffer. Excess buffer was removed and the coverslip was placed on microscope slide.

Transfection of beclin-1 siRNA

HepG2 cells were first cultured in high-fructose for 24 hours, then the cells were cultured in Opti-MEM (Invitrogen Life Technology) and transfected with *beclin-1* siRNA in reagent (Guangzhou RiboBio Co., Ltd.). At five hours after transfection, the culture medium was replaced with MEM supplemented with 10% FBS, and 0.16 mg/mL OMT was subsequently added, followed by incubation for 24 hours.

Western blot analysis

Cells were washed twice with ice-cold PBS and scraped using 1 ml lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM sodium orthovanadate, 50 mM NaF, 20 mM sodium pyrophosphate, 2 mmol/l PMSF, and protease inhibitors mixture], then rotated for 30 minutes at 4°C. Homogenates were centrifuged at 12,000 g for 10 minutes

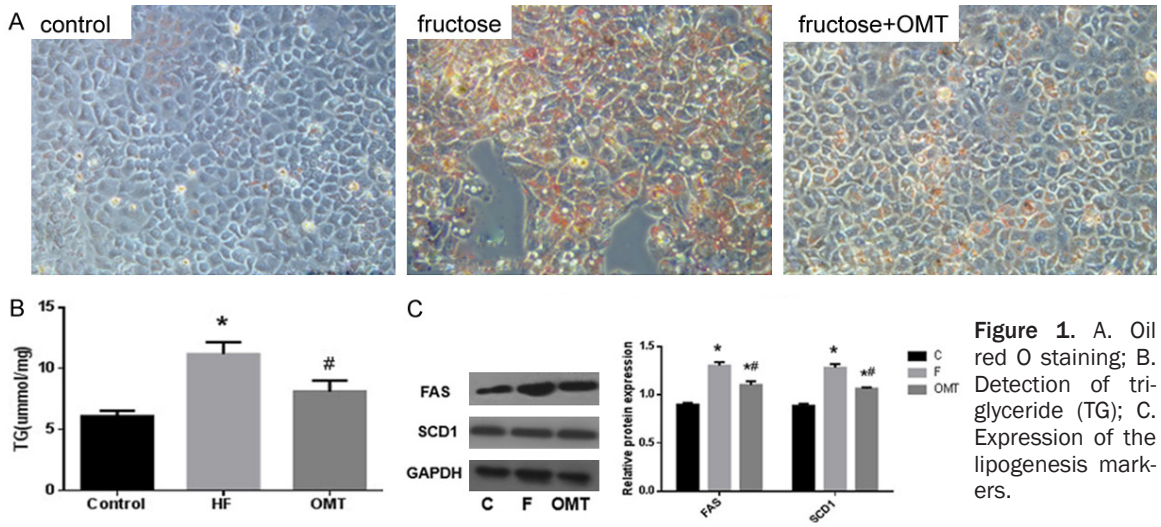

Figure 1. A. Oil red O staining; B. Detection of triglyceride (TG); C. Expression of the lipogenesis markers.

at 4°C. The supernatant was then obtained. Protein concentrations were determined via the BCA method and equivalent protein amounts (50 g) were loaded onto 10% acrylamide gel for SDS/PAGE and then transferred onto the PVDF membranes (Millipore, United States). Non-fat milk at 5% (w/v) in Tris buffered saline was used for blocking. Blots were incubated with primary antibodies overnight at 4°C, washed in TBST and incubated with secondary antibody (1:10,000, Sigma, goat anti-rabbit IgGHRP conjugated, goat anti-mouse IgG-HRP conjugated) for 1-2 hours and detected with an enhanced chemiluminescent detection system (Cwbiotech, Beijing, China). All immunoblots were developed with instant film and quantitated using ImageQuant system (UVP, United States).

Results

OMT alleviates lipid accumulation in HepG2 cells to explore high-fructose

As shown in **Figure 1A**, treatment with 20 mM fructose significantly increased the amount of red staining (lipid droplets). Compared with the high-fructose (HF) group, the OMT intervention group had decreased red staining. In **Figure 1B**, the TG content in HF group showed increased levels compared with the control group ($P < 0.05$), while significantly decreased in the OMT intervention group ($P < 0.05$). In **Figure 1C**, compared to the HF group, expression of FAS and SCD1 was lower in the control group and was decreased in the OMT intervention group (both $P < 0.05$).

OMT activates autophagy in HepG2 cells

Green fluorescence was used to determine the level of autophagy using the signal strength, indicating the level of autophagy activity. Compared to the control group, the green fluorescence was increased in the OMT intervention group (**Figure 2A**, **2B**). In **Figure 2C**, compared to the HF group, expression of Atg7, Beclin1, and LC3B was higher in the control group and similar results were obtained in the OMT intervention group (both $P < 0.05$).

Akt/mTOR pathway in OMT-induced autophagy in HepG2 cells to explore high-fructose

Figure 3 shows that the protein expression of phosphorylated-Akt (p-Akt) was significantly increased in the HF group ($P < 0.05$), and significantly decreased in the OMT intervention group ($P < 0.05$). The protein expression of mTOR was increased in the HF group ($P < 0.05$), but decreased in the OMT intervention ($P < 0.05$).

OMT alleviates lipid accumulation by inducing autophagy in fructose-induced HepG2 cells

To investigate whether OMT improved the hepatic steatosis via autophagy, a transfection technique was used to knockdown *beclin-1* gene in HepG2 cells. **Figure 4** shows that OMT indeed induced autophagy. In the OMT intervention group, the protein expression levels of Atg7, Beclin-1, and LC3B were increased compared to control group ($P < 0.05$), while the *beclin-1* siRNA intervention group showed

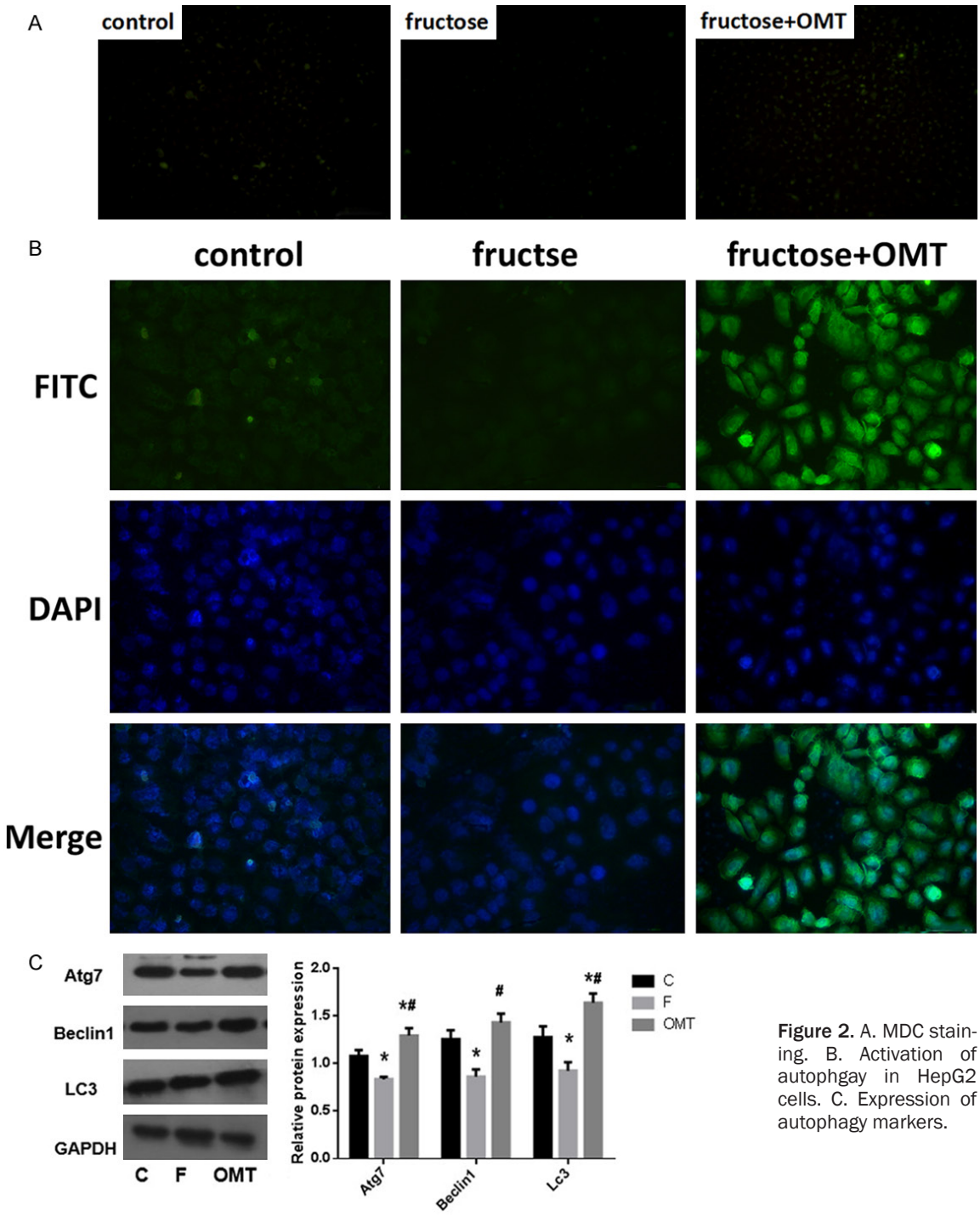

Figure 2. A. MDC staining. B. Activation of autophgay in HepG2 cells. C. Expression of autophagy markers.

decreased protein expression levels of Atg7, Beclin-1, and LC3B ($P < 0.01$; $P < 0.05$, respectively). OMT when added could not reverse the change.

The protein expression of srebp-1 in the HF group and *beclin-1* siRNA intervention group were up-regulated ($P < 0.01$; $P < 0.05$, respectively), but down-regulated with OMT intervention ($P < 0.01$; $P < 0.05$, respectively) compared to the control group (**Figure 5**). Compared with the control group, the protein expression of Fas also increased in the HF and the *beclin-1* siRNA intervention groups ($P < 0.01$; $P < 0.05$, respectively), and decreased with OMT intervention ($P < 0.01$; $P < 0.05$, respectively).

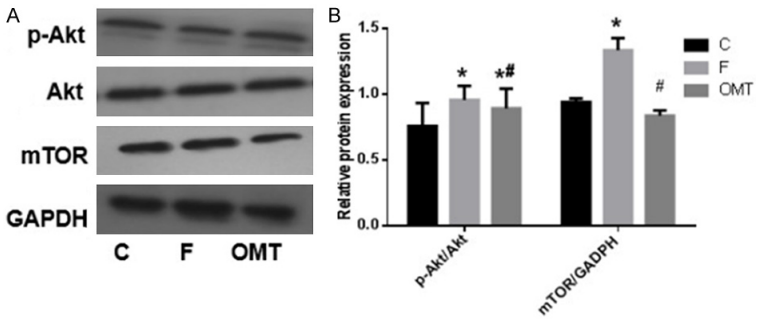

Figure 3. Activation of the Akt/mTOR signaling pathway.

Expression of *p*-Akt and mTOR in the HF group and *beclin-1* siRNA intervention group were increased ($P < 0.01$; $P < 0.05$, respectively), but decreased with OMT intervention ($P < 0.01$; $P < 0.05$, respectively), compared to the control group (**Figure 6**).

Discussion

Lipid accumulation is a characteristic of NAFLD, and is a product of metabolic imbalance. Recently studies have shown that oxymatrine, a natural herb-drug, could improve NAFLD. Our present study demonstrated that OMT reduced lipid accumulation in the steatotic HepG2 cell model. Autophagy is recognized as a new therapy target of NAFLD and our study provides potential mechanism that OMT improves lipid accumulation by enhancing autophagy.

The pathogenesis of NAFLD has not been clarified and the 'two hit theory' is widely accepted. Excess lipid accumulation in hepatocytes is recognized as the first hit. Fructose is one of major components of added sugar, It has been proved that overconsumption of fructose is one of the important dietary factors that lead to NAFLD [20]. Thus, in the present study, the effect of fructose on lipid metabolism and changes in autophagy in HepG2 cells was observed. Consistent with previous studies, high-fructose intervention induced lipid accumulation in liver cells [6]. Our study also shows an increased FAS and SCD-1 protein expressions induced by fructose incubation, indicating that fructose stimulates hepatic lipogenesis. Moreover, in the present study the effect of high-fructose incubation on autophagy in HepG2 cells was analyzed. Some studies have investigated the influence of fructose incubation on autophagy in liver cells. A previous study reported that expression of the LC3B protein was decreased in the liver of fructose-supplemented rats compared to the control group, resulting in hepatic steatosis in fructose-supplemented rats as assessed by biochemical and histological analysis [22]. So, high-fructose intervention reduced atuophagy and led to lipid accumulation, and was consistent with our study results. In our study, protein expression of autophagy markers Atg7, beclin-1, and LC3B was decreased in high-fructose medium, indicating that fructose suppressed autophagy in HepG2 cells.

OMT is one of the natural herb-drugs with antioxidant and anti-inflammatory effects [15, 16]. Our previous research showed that OMT reduced hepatic steatosis in high-fructose fed mice, however, there are still limited reports about the use of OMT in the protection against NAFLD. Furthermore, OMT intervention was found to reduce lipid deposition and decrease the level of TG in HepG2 cells. Few studies showed that OMT intervention could improve hepatic steatosis in high-fructose fed rats, and the possible mechanism consisted of down-regulation of srebp-1 and up-regulation of Pparα [19]. Other studies on high-fat diet and streptozotocin-induced diabetic rats showed that OMT affected the hypoglycemic and hypolipidemic phenotype [21]. Both these studies were animal experiments, and demonstrated that OMT intervention could improve hepatic steatosis. However, it remained unclear regarding the use of OMT in the improvement of lipid accumulation in liver cells. In our study, OMT treatment decreased expression of Fas, which is the key enzyme of lipogenesis. At the same time, OMT reduced the expression of srebp-1, which is an upstream transcriptional factor of lipogenesis. These findings indicate that the effect of OMT on lipid accumulation was partly done through down-regulation of *de novo* lipogenesis in HepG2 cells.

It has been found that autophagy may play a role in the development of hepatic steatosis. Macroautophagy (hereafter referred to as autophagy) is a process which cells are used to deliver cytoplasmic components to lysosomes

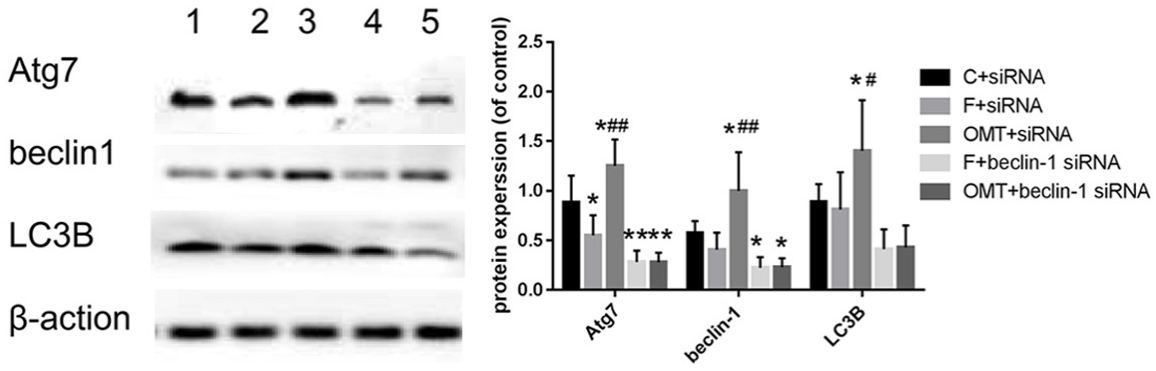

Figure 4. Expression of autophagy markers.

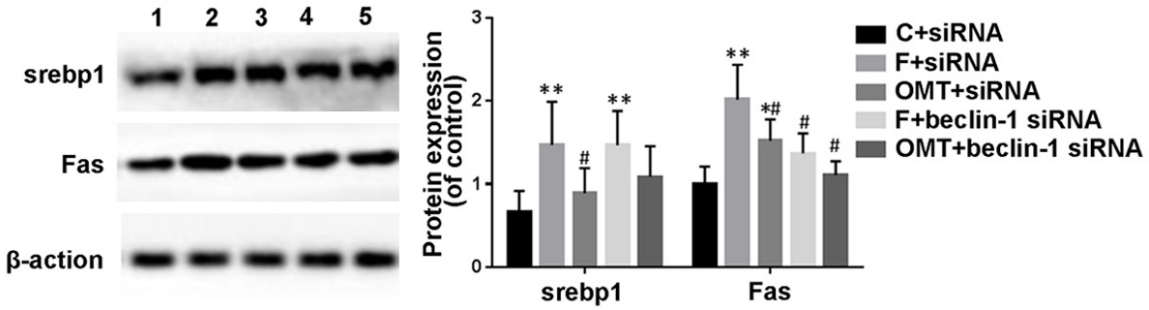

Figure 5. Expression of lipogenesis-related proteins.

for degradation. Singh et al. [23] first reported that autophagy was related to lipid metabolism, and they believed it to be a novel selective pathway for the lipid breakdown and called it as 'lipophagy'. In contrast, Shibata et al. [24] claimed that autophagy is necessary for the genesis of lipid droplets rather than participating in the breakdown of lipid droplets. After that, few studies confirmed these views. In high-fat diet fed mice, the intervention of 1,25 (OH) 2 D3 may ameliorate hepatic steatosis by inducing autophagy through up-regulating autophagy-related 16-like 1 (ATG16L1) [25]. In $Pld1^{-/-}$ mice, PLD1 elimination could cause autophagy defect, resulting in fatty liver [11]. Meanwhile, an *in vitro* study showed that GLP-1 analogue improved hepatic lipid accumulation by inducing autophagy in L-02 cells [26]. These studies suggested that autophagy could function as a protective mechanism in hepatic steatosis. In our study, further investigation on the role of autophagy in lipid accumulation on HepG2 cells and the intervention effects of OMT was achieved. As mentioned previously, lipogenesis was enhanced, while autophagy was suppressed in HepG2 cells. The results show that OMT could enhance autophagy in HepG2 cells, increasing expression of Atg7, beclin-1, and LC3B. In order to detect whether OMT alleviates lipid accumulation through inducing autophagy, beclin-1 siRNA was utilized for transfection to inhibit autophagy. Beclin-1, the orthologue of yeast Atg6, is one of the earliest mammalian proteins involved in regulating autophagy [27]. Beclin1 binds to class III phosphatidylinositol-3 kinase (PI3KC3)/vacuolar protein sorting 34 (Vps34), forming a core PI3-KC3 complex and mediates multiple vesicle-trafficking processes, including endocytosis and autophagy. It has been demonstrated that beclin-1 inhibition could enhance lipid accumulation. Rubicon, a beclin-1-interacting negative regulator for autophagosome-lysosome fusion studies, is up-regulated in NAFLD and is considered responsible for accelerating hepatocellular lipid accumulation and apoptosis, as well as for impairing autophagy maturation [28]. In our study, knockdown of beclin-1 gene increased the levels of TG, and increased the expression of Fas and srebp-1. Those results indicate that lipogenesis was increased with beclin-1 siRNA, whereas expression of Atg7 and LC3B

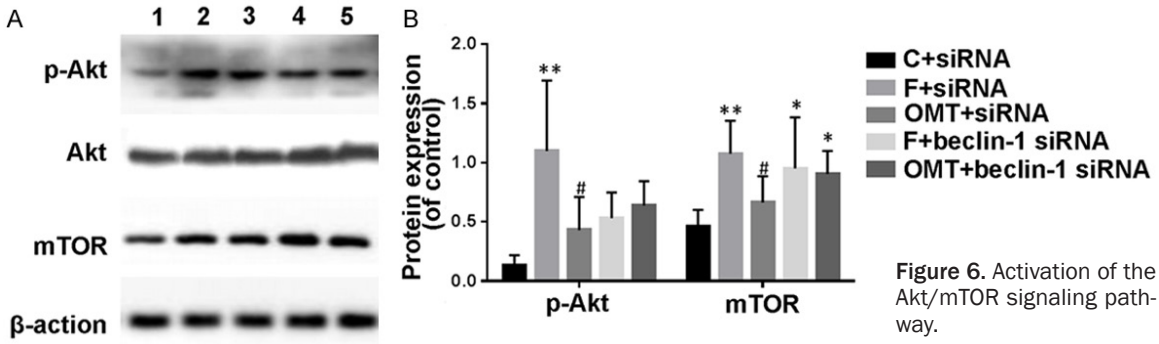

Figure 6. Activation of the Akt/mTOR signaling pathway.

was not enhanced with OMT when autophagy was inhibited. Therefore, Oxymatrine reduces lipid accumulation by affecting beclin-1 mediated autophagy. This might be the first indication that OMT improved lipid metabolism by inducing beclin-1 mediated autophagy.

Autophagy is regulated by a complex signaling network, involving PI3K-Akt-mTOR pathway [29]. PI3K and beclin-1 are downstream factors of Akt and mTOR, Some experimental models have revealed critical effects of Akt on lipogenesis regulation. Studies have reported that excessive activation of Akt in mice liver can accelerated fatty acid synthesis [30]. Meanwhile, Akt activation of mTOR contributed to the regulation of *de novo* lipogenesis. Through SREBP1 transcription upregulation, processing and nucleic accumulation, mTOR signaling senses the growth of nutrients and accelerates *de novo* lipogenesis [31]. During energy abundance in the fed state, lipogenesis may also be activated by mTOR, a pathway that has been recognized for controlling cell growth and metabolism in response to nutrients, growth factors, or energy states [32]. Akt plays a role in apoptosis through autophagy and apoptotic pathways due to their interconnection [33]. The mTOR pathway could inhibit autophagy induction [34]. The mTOR inhibited phosphorylation of autophagy/beclin 1 regulator 1 (AMBRA1) [35]. Several studies have shown that Akt-mTOR is one of the pathways related in autophagy [29, 35]. Hence, we explored whether OMT activated liver autophagy and lipid metabolism through Akt-mTOR pathway. In our study, expression of Akt and mTOR was increased with fructose culture. Our study also found that Akt and mTOR were activated when transfected with beclin-1 siRNA, while OMT decreased expression of Akt and mTOR. The results indicate that the mTOR-dependent pathway may participate in OMT-induced autophagy, and Akt may play a role in OMT-induced autophagy. Fructose may influence the Akt-mTOR pathway in altering lipid metabolism. Thus, OMT might enhance autophagy and decrease lipogenesis through AktmTOR.

In conclusion, the present study demonstrates that OMT alleviates lipid accumulation induced by high-fructose incubation in HepG2 cells. The underlying mechanism includes downregulation of the lipogenesis pathway by OMT intervention. Meanwhile, autophagy was also involved in alleviating the effects of OMT on lipid accumulation through Akt-mTOR pathway. Our results may improve the current understanding of the mechanisms involved in the positive effects of OMT on liver steatosis, and may provide evidence for clinical use of OMT in NAFLD.

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Disclosure of conflict of interest

None.

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