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Article

Xuezhikang alleviates lipid accumulation via AMPK activation in hepatocellular steatosis model

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Xuezhikang (XZK) is an extract of Chinese red yeast rice. It has multiple protective effects in cardiovascular systems. However, the underlying mechanism by which XZK affects free fatty acid (FFA)-induced lipogenesis in hepatocellular steatosis model is still unknown. In this study, the HepG2 cells were treated with palmitate acid (PA) to induce lipogenesis. Then the PA-induced HepG2 cells were treated with XZK. After 24 h treatment, we determined the intracellular triglyceride (TG) contents and average areas of lipid droplets. To study the involvement of AMPK signaling pathway, we pre-treated the PA-induced HepG2 cells with Compound C, an AMPK inhibitor, before XZK treatment. Expressions of p-AMPK and AMPK were determined by Western blot. The results showed that XZK decreased TG content and lipid accumulation in hepatocellular steatosis model. Compound C abolished the effects of XZK. These results demonstrated for the first time that XZK protects hepatocytes against lipid accumulation induced by free fatty acids. Its effects may be mediated by the activation of AMPK pathway.

Keywords: Xuezhikang (XZK). Hepatocellular steatosis model. AMPK. Lipid accumulation.

INTRODUCTION

In the past few decades, metabolic diseases have emerged as serious health problems in developed countries, part due to changes in life style (Allen *et al.*, 2018). Unnatural lipid accumulation in liver results in hepatic steatosis. The most common disease in liver in the developed countries is non-alcoholic fatty liver disease (NAFLD) (Jennison *et al.*, 2019). NAFLD begins with simple fat deposition and may progress to non-alcoholic steatohepatitis (NASH), fibrosis, severe cirrhosis and hepatocellular carcinoma (Carazo, Salmeron, 2014). Approximately 10% of NAFLD cases with benign steatosis develop to more severe NASH, which is manifested in the increase of inflammatory and apoptotic cells in liver that eventually triggers cirrhosis in about 25% of the cases (Kurbatova *et al.*, 2019). Increasing evidences suggest that ectopic fat in liver lead to lipid accumulation, which is characteristic of patients with NASH over simple steatosis and alcoholic steatohepatitis (Xiong *et al.*, 2015). The latter two diseases were mostly initiated by virus infection and alcoholic abuse. We have found many pharmacological therapies for NAFLD patients, such as thiazolidinedione, pioglitazone, rosiglitazone and metformin (Sumida, Yoneda, 2018). However, in the long-term treatment none of these drugs is proven to be fully effective and safe. There are a number of mechanisms responsible for the pathogenesis and progression of NASH.

AMP-activated protein kinase (AMPK) is a key regulator in energy circles and nutrient metabolisms. It has important implications for fat deposition and glucose homeostasis in cells and organs (Lin, Hardie, 2018; Wang *et al.,* 2018). In liver, activation of AMPK promotes fatty acid oxidation and inhibits cholesterol synthesis, fat production and triglyceride synthesis (Foretz, Even, Viollet, 2018).

Xuezhikang (XZK) is an extract from Chinese red yeast rice and contains several natural statins. For example, a 120mg XZK capsule contains ~1mg lovastatin (Heber *et al.,* 2001). XZK reduces the levels of triglyceride, total cholesterol and low-density lipoprotein in the plasma of coronary artery disease (CAD) patients (Jiang, 2011). As reported, it improves the parameters of arterial stiffness

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in patients with hypertension (Zheng *et al.,* 2017). XZK is associated with enhanced proliferation and adhesion capacity of endothelial progenitor cells derived from the peripheral blood of people with stable CAD (Kong *et al.,* 2008). In patients with type 2 diabetes, XZK has certain hypoglycemic effect (Li *et al.,* 2015).

In this study, we investigated the effects of XZK on Palmitate acid (PA)-induced HepG2 cells in an in-vitro model of hepatocellular steatosis. Our results demonstrated that XZK has anti-lipogenesis effect on hepatocytes and may prevent hepatocellular steatosis. These effects are likely mediated by AMPK activation.

MATERIAL AND METHODS

Preparation of XZK solution

XZK was purchased from Yiling Pharmaceutical Corporation (Shijiazhuang, China) and dissolved in sterile water. The remainder of the insoluble starch and fiber filtered by a 4.5mm filter. The concentration of the final stock solution was 1mg/mL. The stock solution was stored in -20 °C until use.

Cell culture

Human HepG2 cells were purchased from the Center of Cell Repository at the Wuhan University. Cells were maintained at 37ºC in a humidified incubator containing 5% CO_2 and cultured in DMEM (Dulbecco's modified Eagle Medium, Sigma Aldrich, St.Louis, Missouri, USA) medium supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY),100U/mL penicillin (GIBCO BRL, Grand Island, NY) and 100μg/mL streptomycin (GIBCO BRL, Grand Island, NY). Before intervention, cells at 70% confluence were starved in serum-free medium overnight. HepG2 cells were incubated with 0-0.25 mmol/L PA (Sigma Aldrich, St.Louis, Missouri, USA) for 24 h to induce lipid accumulation. The intervention reagent PA was prepared as previously described (Rojas *et al.,* 2014). Briefly, PA was dissolved in 50% (v/v) ethanol, diluted in serum-free DMEM containing 1% (w/v) BSA (Sigma Aldrich, St.Louis, Missouri, USA), and then incubated at 37ºC with shaking in a water bath for 2 h. The control medium containing ethanol and BSA was prepared in a similar fashion. To probe the mechanisms responsible for effects of XZK, PA-induced cells were pretreated with 20μmol/L AMPK inhibitor Compound C (Sigma Aldrich, St.Louis, Missouri, USA) for 2 h before the treatment with XZK.

Cell viability analysis

Cell viability was determined by MTT assay in 96-well cell culture plates. Briefly, HepG2 cells were treated with PA in concentration range of 0-0.25mmol/L. After the treatment, the culture medium was removed and 20μl TT reagent (5 mg.mL-1 in phosphate-buffered saline, Sigma Aldrich, St. Louis, Missouri, USA) at a concentration of 5mg/mL was added to each well. After 4 h incubation at 37ºC, MTT reagent was removed and 0.15ml DMSO (Sigma Aldrich, St.Louis, Missouri, USA) was added to each cell for 20min. The wells containing no cells but culture medium and MTT acted as blank, whereas the wells containing normal cells without XZK or vehicle were used as control. The absorbance of the converted dye was determined at 570nm.

Measurement of intracellular TG content

TG content in cells was measured using the commercial kits (Cayman Chemical Company, USA) according to manufacturer's instruction. Briefly, HepG2 cells were washed with PBS and lysed in RIPA buffer supplemented with a mix of protease and phosphatase inhibitors for 10 min at room temperature. Supernatant was collected, part of which was used to determine the protein concentration by BCA Protein Assay Kit (Pierce, USA).The rest was removed and heated to 70ºC for 10 min and centrifuged at 2000×g for 15 min. An aliquot of 10μl supernatant was incubated with 190μl work solution in the commercial kits for 20 min at 37ºC. The absorbance at 550nm measured with a microplate reader. With a standard curve, we determine the concentration of the TG content. The final TG content was reported as mg TG per mg protein.

Observation of lipid droplets

To observe the lipid droplets, 4 % glutaraldehyde (Sigma Aldrich, St. Louis, Missouri, USA) was slowly added into the collected cells to fix them, 1 % osmium acid (Sigma Aldrich, St.Louis, Missouri, USA) (phosphate) (pH 7.2-7.4) was subsequently added which were next dehydrated by alcohol and acetone. The epoxy resin was soaked, embedded and polymerized at 65ºC for 24 h. Finally the changes of lipid droplets in cells could be observed through citrate electron staining and transmission electron microscope in the prepared 50 nm ultrathin section.

Detection of the protein expression by Western blot

Expression of p-AMPK and AMPK in HepG2 cells were detected by Western blot. The culture medium was first removed and then the cells were washed with ice-cold PBS. After fully lysed and centrifuged at 12000×g for 15min at 4ºC, aliquots of supernatant were collected and analyzed by SDS-PAGE. Separated proteins were transferred to PVDF membranes. The membranes were blocked with non-fat milk at room temperature for 1 h, followed by incubation overnight at 4ºC with primary antibodies (Santa Cruz, CA, USA). The PVDF membrane was washed with PBS three times and incubated with IR Dye-conjugated second antibody (Santa Cruz, CA, USA) at room temperature for 1 h. Protein bands were visualized using an enhanced chemiluminescence kit (pierce, Rockford, IL). The final expression was quantified by densitometry.

Statistical analysis

All the reported values are obtained from at least three separate experiments and expressed as mean±SD Comparison of the means from different groups was performed by analysis of variance (ANOVA) with SPSS16.0 software. A value of $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Viability and lipid accumulation in PA-induced HepG2 cells

HepG2 cells were treated with PA in the concentration range of 0-0.25mmol/L. Cell viability was determined by MTT assay. No cytotoxicity was found at 0.15 mmol/L. However PA at higher concentrations up to 0.25mmol/L exhibited cytotoxic effects (Figure 1). Thus, the intracellular TG contents in HepG2 cells were determined after induction by 0.15mmol/L PA. The TG contents in HepG2 cells induced by 0.15mmol/L PA were significantly increased compared with the un-treated HepG2 cells ($P<0.05$) (Table I). The areas of lipid droplets in PA-induced HepG2 cells were also evaluated. The average areas of lipid droplets in PA-induced HepG2 cells were significantly larger than the un-treated group ($P<0.05$) (Figure 2). So in the next experiments, we chose 0.15mmol/L PA to induce steatosis model.

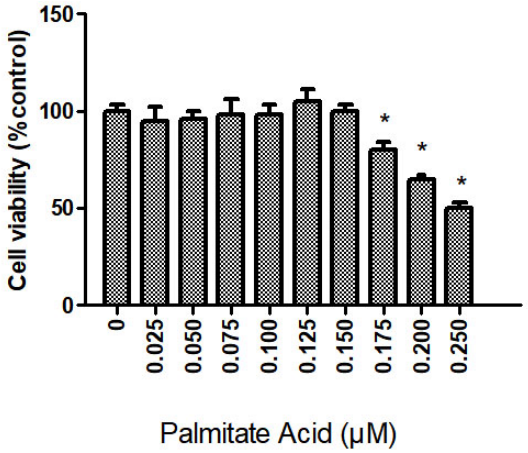

FIGURE 1 - Cytotoxicity of PA in HepG2 cells. HepG2 cells were incubated with various concentrations of XZK for up to 24 h and cell viability was determined by MTT assay. Results were expressed as the mean±SEM of three independent experiments (n=3).

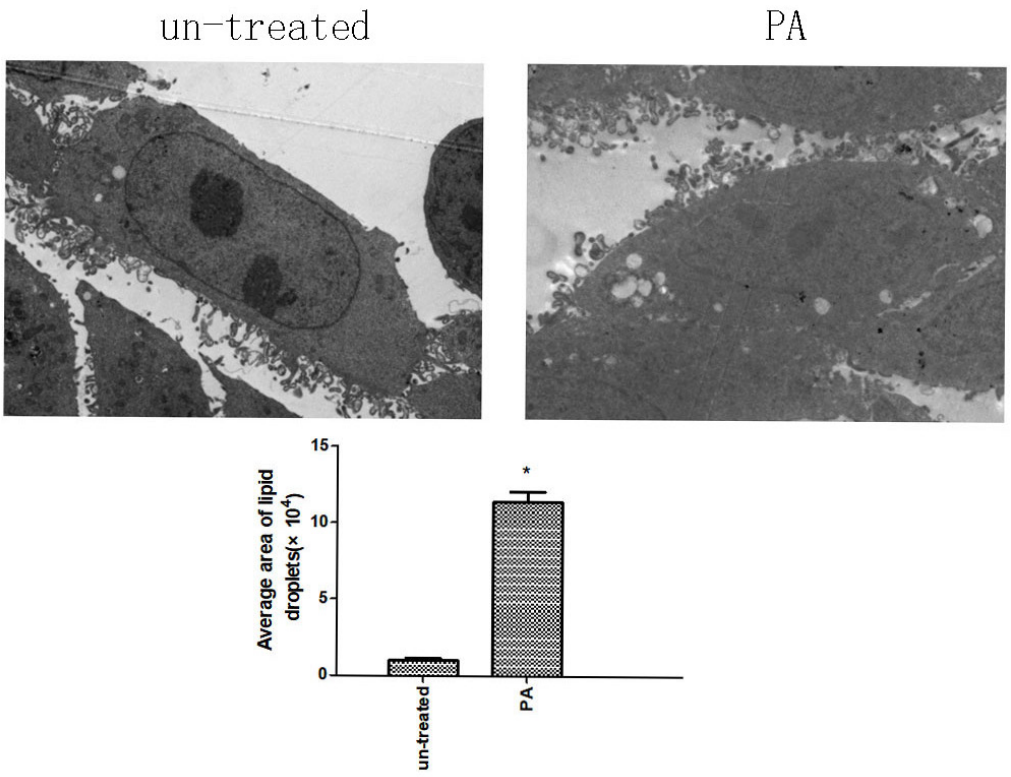

FIGURE 2 - Lipid droplets of HepG2 cells after 24 h induction with PA.

TABLE I - Intracellular TG content of HepG2 cells after 24 h induction with PA (n=4,$\bar{x}$±s)

Group	TG Content (umol/mg.prot)
Un-treated HepG2 cells	0.019±0.004
0.15 mmol/L PA-induced HepG2 cells	0.035±0.002#
Compared with un-treated HepG2 cells,#P<0.05	

XZK did not affect HepG2 cell viability

The cytotoxicity of XZK on HepG2 cells were tested – XZK did not exhibit any toxicity effect at the concentrations from 0- 800μg/ml (Figure 3).

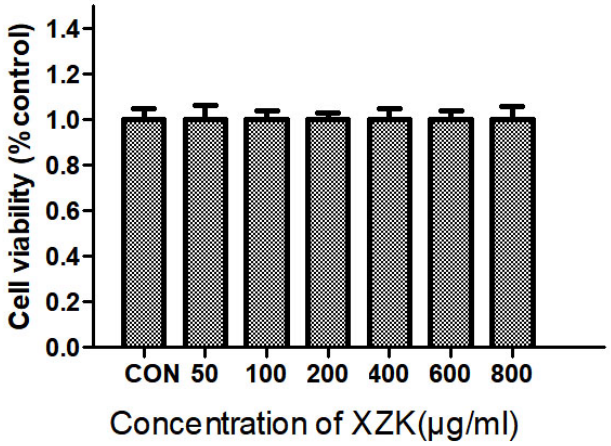

FIGURE 3 - The cytotoxicity of XZK in HepG2 cells using the MTT assay.

XZK reduced TG content in PA-induced HepG2 cells

At 250μg/ml XZK had no effect on intracellular TG content in PA-induced HepG2 cells after 24 h treatment. However, at 500μg/ml XZK significantly reduced the TG content following 24 h treatment ($P<0.05$). Compared with 500μg/ml XZK, a 24 h treatment with 750μg/ml XZK had further effect on TG content (Table II). These results showed that 24 h treatment with 500μg/ml XZK led to clearly reduction in intracellular TG content in PA-induced HepG2 cells.

TABLE II - TG content of intracellular HepG2 cells after 24 h intervention with XZK (n=4,$\bar{x}$±s)

Group	TG Content (umol/mg.prot)
Un-treated	0.017±0.002
PA	0.036±0.003$^{\#}$
PA+Vehicle	0.036±0.004$^{\#}$
PA+250μg/ml XZK	0.036±0.006$^{\#}$
PA+500μg/ml XZK	0.020±0.004*
PA+750μg/ml XZK	0.020±0.001*

Compared with the un-treated group,$^{\#}P<0.05$; Compared with the PA group,$^{*}P<0.05$

Effects of AMPK inhibitor Compound C on TG contents and lipid droplets in PA-induced HepG2 cells treated by XZK

In order to explore the role of AMPK signaling pathway in the inhibitory effect of XZK on lipid accumulation in PA-induced HepG2 cells, we pre-treated HepG2 cells with Compound C for 2 h and then treated the cells with 500μg/ml XZK for 24 h. As aforementioned, XZK significantly reduced the intracellular TG content in the PA-induced HepG2 cells. However, Compound C blocked the inhibitory effect of XZK ($P<0.05$) (Table III). This is clearly supported by evidence from transmission electron microscope. As shown in Figure 4, XZK significantly decreased the average areas of lipid droplets in the PA-induced HepG2 cells, whereas Compound C blocked the effect of XZK ($P<0.05$).

TABLE III - Intracellular TG content of HepG2 cells by pre-treatment of Compound C (n=4,$\bar{x}$±s)

Group	TG Concentration (umol/mg.prot)
Un-treated	0.016±0.002
PA	0.035±0.001$^{\#}$
PA+Vehicle	0.034±0.003$^{\#}$
PA+Compound C	0.040±0.005$^{\&}$
PA+XZK	0.017±0.003*
PA+XZK+Compound C	0.030±0.005$^{\&}$

Compared with the un-treated group,$^{\#}P<0.05$; Compared with the PA group,$^{*}P<0.05$; Compared with the PA+XZK group,$^{\&}P<0.05$

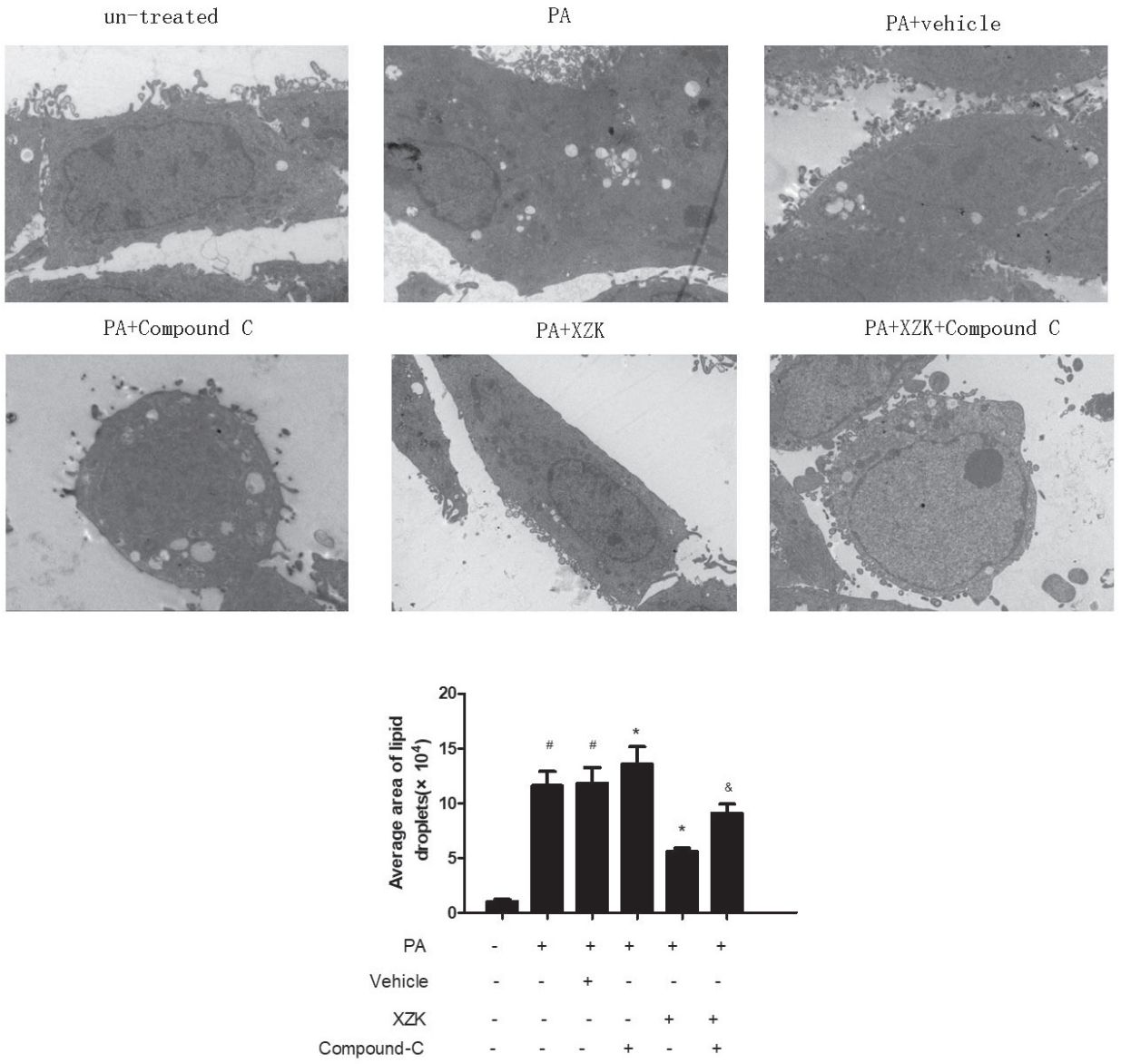

FIGURE 4 - Lipid droplets of HepG2 cells after signaling pathway inhibitor intervention. Representative images of lipid droplets were observed under transmission electron microscope. Area of lipid droplets were quantified by NIH Image J software. Results were expressed as mean±SEM from three independent experiments (n=3). Compared with the un-treated group,$^{\#}P<0.05$; Compared with the PA-treated group,$^{*}P<0.05$; Compared with the PA+XZK treated group,$^{\&}P<0.05$. Scale bar,50μm

XZK reduced lipid accumulation in PA-induced HepG2 cells through activation of AMPK

The p-AMPK/AMPK value in PA-induced cells was clearly decreased compared with the un-treated cells. As shown in Figure 5, treatment with 500μg/ml XZK for 24 h significantly increased p-AMPK/AMPK value which was prevented by Compound C ($P<0.05$).

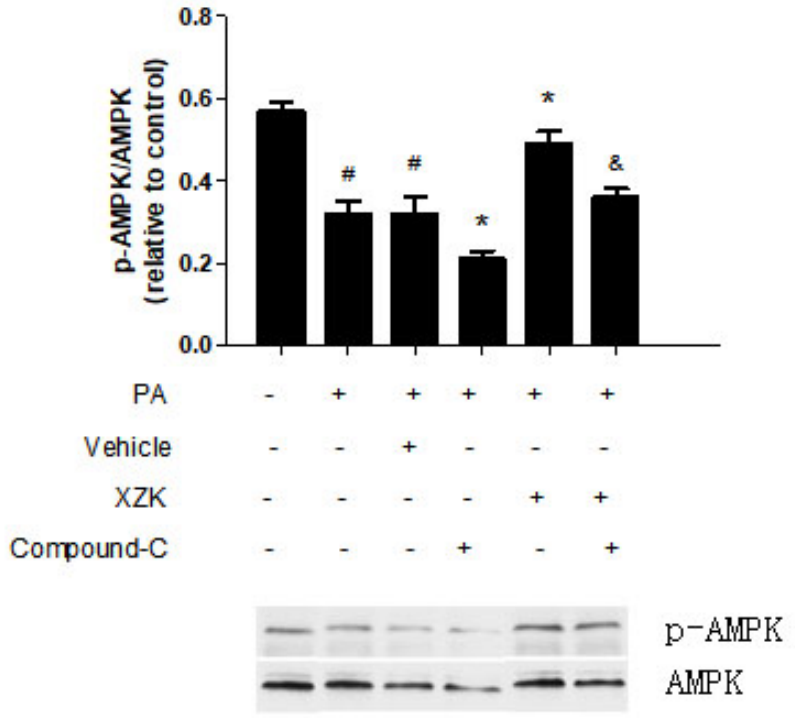

FIGURE 5 - The p-AMPK and AMPK protein expressions in HepG2 cells after signaling pathway inhibitor intervention detected by Western blot. Compared with the un-treated group,#P<0.05; Compared with the PA-treated group,*P<0.05; Compared with the PA+XZK treated group,&P<0.05.

XZK was fermented and refined from Chinese red yeast rice with modern biopharmaceutical methods (Zheng, Xiao, 2017). The main components of XZK include homologues of lovastatin, unsaturated fatty acids, sterols and low quantities of flavonoids. Compared with the chemically synthesized lovastatin, natural lovastatin in XZK had high solubility in the body (Chen *et al.,* 2012). In addition, other components of XZK could help reduce the first elimination of the active ingredient lovastatin acid and increase its bioavailability (Chen *et al.,* 2013). XZK was originally recommended for the treatment of dyslipidemia (Liu *et al.,* 2011). Hyperlipidemia was considered the major contributing factor to cardiovascular disease (CVD). In recent years, the benefits of XZK in cardiovascular system have been explored (Kong, Wang, 2008; Zheng, Xiao, 2017). Above all, the lipid-lowering effect of XZK was considered the most important one. The majority of cholesterol in the body is synthesized in liver. It was found that XZK decreased TG contents in hepatocytes. PPARα knockdown eliminated this effect. XZK treatment also contributed to the up-regulation of hepatic PPARα and apoA5 expressions. As a target gene of PPAR-α, apolipoprotein A5 (apoA5) was an important regulator of TG metabolism. Through this mechanism, XZK leads to reduction in the hepatic TG contents (Zhao *et al.,* 2017). The effects of different fractions from XZK extracts have been studied. The fraction enriched in isoflavones decreased the hepatic lipid concentration and increased the fecal concentration of bile acid. The fraction enriched in phytosterols decreased hepatic lipid content and increased the excretion of fecal lipids. These results showed that the two fractions of XZK extracts contribute to the cholesterol-lowering effect of XZK mainly by regulating bile acid and cholesterol homeostasis (Feng *et al.,* 2015). However, the effects of XZK on impaired hepatocytes by lipid overloading were still under investigation. In this study, HepG2 cells were induced with PA for 24 h before XZK treatment. The results showed that XZK decreased intracellular TG contents and the average areas of lipid droplets in PA-induced HepG2 cells. AMP-activated protein kinase (AMPK) plays critical roles in the regulation of lipid

metabolism by directly phosphorylating proteins or modulating gene transcription in pathways like synthesis, oxidation and lipolysis (Imai *et al.,* 2006; Jennison, Patel, 2019; Wang, Liu, 2018). Activation of AMPKα reduces TG accumulation in hepatocytes (Puljak *et al.,* 2008). The phosphorylation of SREBP1c at Ser372 by AMPK, leads to suppression of hepatic steatosis in diet-induced insulin-resistant mice (Li *et al.,* 2011). AMPK could suppress SREBP2 activity, repress the expression of its downstream targets, and thus control dyslipidemia (Jung *et al.,* 2011). Pre-treatment with AICAR, could decrease the incorporation of fatty acids into TG, resulting in inhibition of TG synthesis (Anthony, Gaidhu, Ceddia, 2009). In our study, the effects of XZK could be reversed by pre-treatment with AMPK inhibitor Compound C. This observation suggests that XZK attenuate lipid accumulation in PA-induced hepatocytes through AMPK-dependent mechanism.

CONCLUSION

Our findings provide *in vitro* evidence for the first time that XZK could attenuate PA-induced lipid accumulation in hepatocytes. This effect is likely mediated by AMPK activation. Our results also indicate that XZK ameliorates hepatic steatosis *in vitro*. These findings seem to suggest XZK as a promising therapeutic agent for the treatment of hepatic steatosis. The limitation of this work was that we investigated the effects of XZK only in HepG2 cell line. More studies are warranted to evaluate the efficacy of XZK in relevant animal models. Furthermore, the downstream targets of AMPK or other related signaling pathways by which XZK regulate lipid metabolisms remain to be investigated in future studies. With better understanding of the mechanism of XZK, XZK might be useful in prevention of lipogenesis in liver cells and NASH.

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