

Original Article

PFP alleviates nonalcoholic steatohepatitis fatty liver in both Apo E^{-/-} mice and Changliver cell[S]

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Received April 14, 2016; Accepted April 10, 2017; Epub June 15, 2017; Published June 30, 2017

Abstract: High-calorie food leads to nonalcoholic fatty liver disease (NAFLD) through the dysregulation of genes involved in lipid metabolism, but the precise mechanism is still unknown. Pomegranate flowers are used to treat diabetes mellitus in traditional Uighur medicine. Here we sought to investigate the effect and mechanism of pomegranate flower polyphenols (PFP) on NAFLD Apo E^{-/-} mice induced by a high-fat diet (HFD) and whether PFP improves NAFLD through decreasing oxidative stress. PFP supplementation in mice significantly reduced the HFD-induced gains in body weight compared with the mice fed only with HFD. It also significantly reduced HFD-induced increases in serum lipids, including cholesterol and triglyceride. Consistent with the reduced liver weight, hepatic lipid accumulation, and the size of lipid droplets in the epididymal fat pads were also reduced by PFP supplementation. To further investigate how PFP may reduce obesity, we analyzed lipid metabolism-related genes in the liver. PFP supplementation altered expression profiles of several lipid metabolism-related genes, including ACC, AMPK, CPT-1 α , FAS, LDLR, Leptin, LXR, PON1, PPAR, SirT3, and SREBP, relative to those in HFD control mice. The expression patterns of these genes observed by quantitative reverse transcriptase-polymerase chain reaction and AMPK, SirT3, ACC2, and CPT-1A expression were confirmed by immunohistochemical assays. Collectively, our results indicate that PFP prevents HFD-induced obesity in Apo E^{-/-} mice, and its anti-obesity effects may be related to the regulation of lipogenesis at the level of transcription.

Keywords: PFP (pomegranate flower polyphone), nonalcoholic fatty liver disease, Apo E^{-/-} mice

Introduction

Nonalcoholic fatty liver disease (NAFLD), characterized by excessive accumulation of triglyceride (TG) in the hepatocytes, affects about one-third of adults and an increasing number of children worldwide [1, 2]. It is closely associated with obesity [3], insulin resistance [4], and type 2 diabetes [5]. NAFLD is a group of hepatic-related disorders with four different stages, differing from a typical disease. The earliest stage is hepatic steatosis is defined as the deposition of TG droplets in the cytoplasm in more than 5% of hepatocytes [6]. Part of hepatic steatosis can develop into NASH (nonalcoholic steatohepatitis), which is characterized by the presence of hepatocyte injury (hepatocyte ballooning and cell death). About one third of individuals with NASH can develop cirrhosis within 10 years [2]. In cirrhosis, most hepato-

cytes are replaced by type I collagen, resulting in partial loss of hepatic function and can ultimately progress to liver cancer [3]. Therefore, cirrhosis is thought of as an irreversible disease, and some researchers think that the pharmacotherapy effect is very modest. However, botanical drugs present a curative effect for NAFLD and NASH with relatively low poisonous effects. Recently, more and more botanical drugs are applied to treat NAFLD because they are rich in typical materials, such as plant polyphenols, which are abundant in vegetables and fruits and can reduce the risk factors associated with metabolic syndrome, such as insulin resistance, cholesterol abnormalities, obesity, and hypertension [7-9]. They also contain a number of phenolic hydroxyl groups that have strong antioxidant activity [10].

Table 1. With forward and reverse primer sequences

Target	OLigonuLeotide (5' to 3')	Tm/°C	bp
ACC2	F TCAGCCTACAAAACCGCCCA	150	
	R AAGGCCGTCCACGATGTAGG		
AMPK	F TTGAAACCTGAAAATGTCCTGCT	113	
	R GGTGAGCCACAACCTTGTCTT		
SirT3	F ACCCAGTGGCATTCCAGAC	123	
	R GGCTTGGGGTTGTGAAAGAAG		
CPT-1A	F TGCTGATGACGGCTATGGTG	115	
	R GTGCCTTCCAAAGCGATGAG		
β-Actin	F TGACGTGGACATCCGCAAAG	205	
	R CTGGAAGGTGGACAGCGAGG		

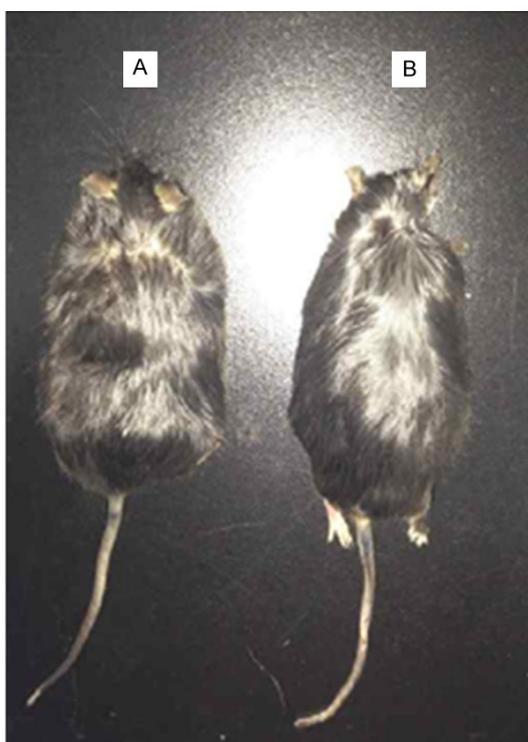


Figure 1. Effects of PFP on body weight of mice fed a high-fat diet. Male Apo E^{-/-} mice at 6 weeks of age received a regular HFD. After 4 weeks of feeding, mice on the HFD were treated for 8 weeks with PFP or vehicle (n=8 per group). A: Gross appearance of HFD-fed mice at the end of treatment with PFP (PFP + HFD) or vehicle (HFD). B: Changes of body weight were monitored during PFP treatment.

Pomegranate flower is a traditional Uighur herb that had been used to treat diabetes mellitus for centuries. Previous studies show that pomegranate flower possesses antioxidant, anti-inflammatory, antiviral, and antibacterial properties. Bagri et al. reported that *Punica grana-*

tum aqueous extract (PgAq) prevents hyperglycemia and hyperlipidemia in STZ-induced diabetic mice. The study by Celik et al. suggests that a *Punica granatum* (PG) beverage produced hepatic-protective and antioxidant properties against trichloroacetic acid (TCA)-exposure in mice. PG fruit juice flavonoids have also been found to prevent low-density lipoprotein (LDL) oxidation and hence are antiarthrogenic [11]. These studies indicate the PG may reduce the risk of NAFLD and have the potential of being anti-NAFLD, and we believe this evidence should be sustained through more experiments in vivo.

Therefore, we observed the effects of pomegranate flower polyphenol extracts on NAFLD apo E^{-/-} mice and sought to investigate how PFP regulates NAFLD induced by high-fat diet (HFD). Further to clarify the mechanism underlying the regulation of obesity, we analyzed hepatic expression of genes regulating lipid metabolism in HFD-fed mice with or without PFP using quantitative reverse transcriptase-polymerase chain reaction (PCR) and verified the results by immunohistochemical assays.

Methods and materials

Chemicals and reagents

Pomegranate flower polyphenols and total phenolics measurement: *P. granatum* flower polyphenol (PFP) was a commercial spray-dried ethanol extract without excipient, dried pomegranate flower powder was extracted with 70% ethanol for 20 hours at 95°C. The resulting extracts were filtered, concentrated under vacuum at 60°C, and stored at stored at 4°C until used. The extraction yield of PFP was 10.4% (g/100 g, w/w). Determination of total polyphenol content in pomegranate polyphenols was done with a Waters Symmetry Shield RP18 column (250 mm×4.6 mm×5 μm) by UV spectrophotometry. Methanol -0.05 phosphoric acid solution (5:95) was used as the mobile phase. Total phenol content in PFP was 40.13% (g/100 g, w/w).

Reagents: The kits for determination of glucose, triglyceride (TG), and total cholesterol (TC) low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-

Both Apo E^{-/-} mice and Changliver cell

Table 2. Apo E^{-/-} mice weight gain value (n=10)

	4 weeks before treatment (g)	8 weeks before treatment (g)	4 weeks after treatment (g)	8 weeks after treatment (g)
Normal group	5.81±1.27	1.92±0.36	1.69±0.33	1.12±0.48
Model group	8.87±1.64**	3.3±0.77*	2.14±0.5	2.62±0.56**
PFP group	6.65±1.27	3.07±0.59	1.18±0.25ΔΔ	0.89±0.35ΔΔ

Comparison with normal group: *P<0.05 **P<0.01; comparison with model group: ΔΔP<0.01.

C) were purchased commercially from Rongsheng Company in Shanghai, China. Tumor necrosis factor (TNF)-α and interleukin (IL-8) assay kits were from Shibayagi Co. Ltd., Gunma, Japan. All other chemicals were of the best grade commercially available, unless noted otherwise.

Animal and diets

Twenty male mice (Apo E^{-/-} mice) and 10 C57BL/6 mice 8-week-old with an average weight 18±2 g were purchased from Cavens Laboratory Animal Co. Ltd. (Changzhou, China). They were housed at 20±2°C in a daily light/dark cycle. All mice were fed normal diet adaptability and water ad libitum in stainless cages for one week before initiation of the experiment. Apo E^{-/-} mice received a high-fat diet (normal diet 81%, fat 7.5%, cholesterol 1% and bile acid sodium 0.3%, propylthiouracil 0.2%) for 8 weeks and randomly divided into 2 groups (n=8): the PFP group (treated with PFP 140 mg/kg/d) and model group (treated with distilled water, same dose). C57BL/6 mice as the normal control group were fed a regular rodent food (normal diet: 53% carbohydrate, 5% fat, 23% protein calories). Both the PFP group and model group were fed intragastrically once daily for 8 weeks, with dose adjustments weekly according to body weight.

Collection of blood and tissue samples

Blood was collected after 9-10 hours of fasting by an orbital cardiac puncture. Serum was separated by centrifugation at 1,100×g for 15 min. at 4°C, and stored at -20°C for subsequent analysis. After blood collection, organs, such as liver, kidney, and pancreas, were surgically removed. The samples were rinsed with a phosphate-buffered saline solution, wiped with a paper towel, weighed, and immediately frozen in liquid nitrogen and stored at -80°C until analyzed.

Plasma analysis

Plasma glucose, total cholesterol (TC), triglycerides (TG), and low-density lipid cholesterol (LDL-C) were measured using the commercial kits from Jiancheng (Nanjing, China) according to the manufacturer's instructions. Plasma IL-8 and TNF-α were measured by ELISA assay kit (R&D systems).

Histological analysis

After the mice were killed, the livers were removed and, subsequently, fixed in phosphate-buffered 10% formalin. The right lateral lobule of the liver was then divided into 2 sections at the long middle line, one of which was embedded in paraffin blocks and the other in O.C.T. compound. A section from each paraffin block was stained with hematoxylin and eosin (HE) to examine the pathologic structures of the liver, and serial cryosections were stained with Oil Red O to evaluate lipid droplets.

Oxidative stress assessment

Liver lipid content: Hepatic lipids were extracted according to the method of Folch [12] et al. The TG content was determined as described previously [13]. Briefly, lipid was extracted from frozen liver tissues (30 mg) by homogenization in 1 mL of 2:1 chloroform:methanol, followed by shaking at room temperature overnight and centrifugation at 3000 rpm for 10 min. Aliquots (400 μL) of the organic-extract lipid suspension were used for the measurement of triglyceride concentrations. Hepatic lipid content was defined as milligram of triglyceride per gram of the liver.

Real-time quantitative RT-PCR analysis: Approximately 100 mg of liver sample was homogenized in 1 mL of TRIzol reagent (Invitrogen) for total RNA isolation. qRT-PCR analysis for hepatic gene expression was conducted as previously described using SYBR Green procedure CFX 96 real-time PCR detection system (Bio-Rad). Primers were designed using Beacon Designer software (Premier Biosoft) with forward and reverse primer sequences (Table 1).

Both Apo E^{-/-} mice and Changliver cell

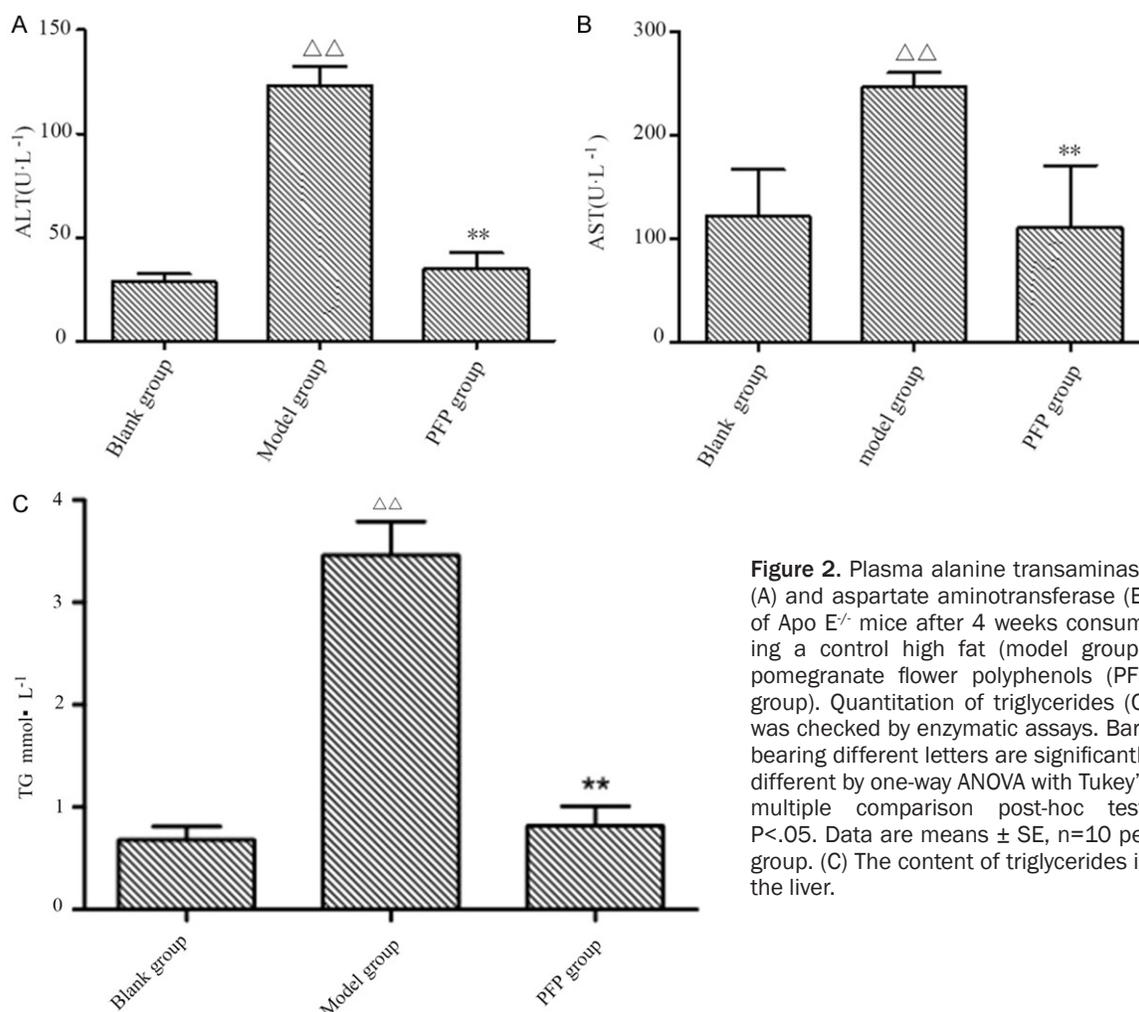


Figure 2. Plasma alanine transaminase (A) and aspartate aminotransferase (B) of Apo E^{-/-} mice after 4 weeks consuming a control high fat (model group), pomegranate flower polyphenols (PFP group). Quantitation of triglycerides (C) was checked by enzymatic assays. Bars bearing different letters are significantly different by one-way ANOVA with Tukey's multiple comparison post-hoc test, P<.05. Data are means ± SE, n=10 per group. (C) The content of triglycerides in the liver.

Table 3. Effect of PFP on the changes of TG, TC, LDL-C, HDLC-C, $\bar{x} \pm s$

	Normal group	Model group	PFP group
Serum parameters			
TG mmol·L ⁻¹	0.68±0.13	3.46±0.33 ^{**}	0.82±0.19 ^{ΔΔ}
TC/mmol·L ⁻¹	2.34±0.33	27.62±3.17 ^{**}	15.10±9.01 ^{ΔΔ}
LDL-C/mmol·L ⁻¹	0.28±0.06	9.64±2.27 ^{**}	6.70±4.12
HDLc/mmol·L ⁻¹	1.38±0.13	0.77±0.11 ^Δ	1.13±0.82
GLU	8.96±1.79	8.85±0.31	6.53±1.55
Hepatic parameters			
TC	2.37±0.37	27.54±3.92 ^{ΔΔ}	15.19±9.55 ^{**}
TG	0.68±0.15	3.47±0.43 ^{ΔΔ}	0.83±0.20 ^{**}
HDLC	1.39±0.15	0.77±0.14	1.13±0.87
LDLC	0.28±0.06	9.43±2.91 ^{ΔΔ}	6.80±4.35

^{**}P<0.05 vs normal group, ^{**}P<0.01 vs normal group, ^{ΔΔ}P<0.01 vs model group, ^ΔP<0.05.

Immunohistochemistry: Livers were processed with the Cell and Tissue Staining Kit (R&D

Systems) and protein from rat liver samples was extracted with RIPA buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP-40, 0.5% sodium deoxycholate, 150 mmol/L NaCl, 0.1% SDS, EDTA, etc.] containing protease and phosphatase inhibitors. Protein concentrations were measured using a BCA-100 Protein Quantitative Analysis Kit. After denaturation, protein samples were subjected to SDS-PAGE and blotted onto polyvinylidene difluoride (Millipore) membranes. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h and then incubated with primary antibodies against CPT-1α (Santa Cruz), AMPKα (Santa Cruz), ACC2 (Abcam), SirT3 (Abcam) overnight at 4°C. After three

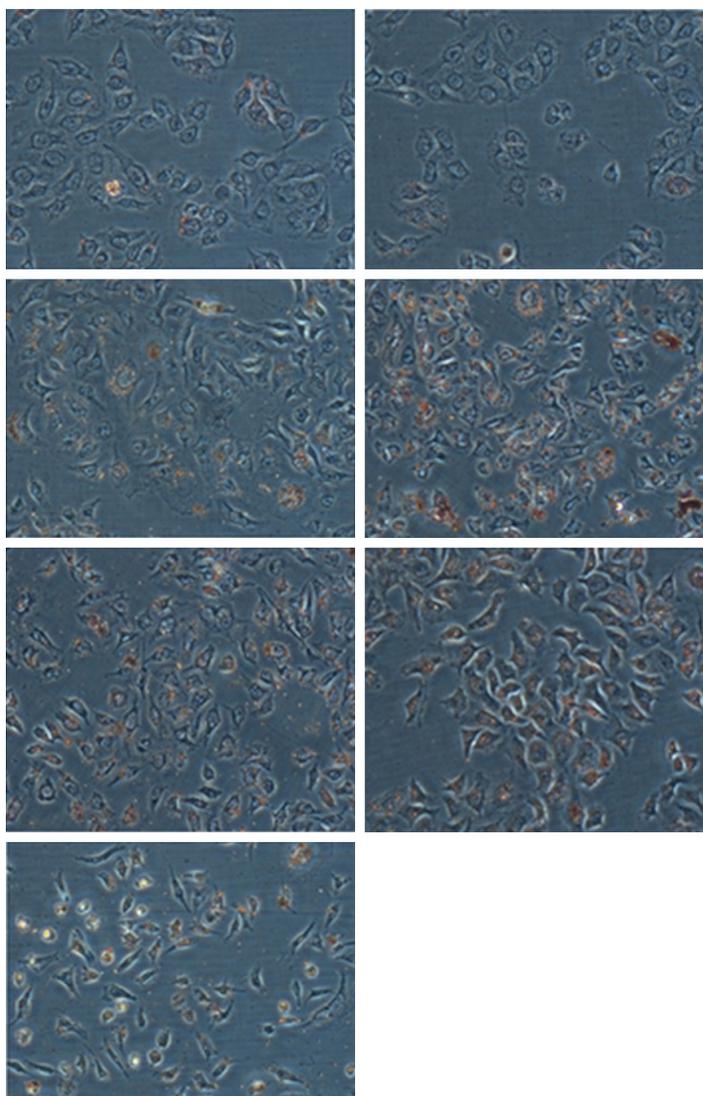


Figure 3. Oil Red O results of chang Liver cell intervened by different concentration OA for 24 ($\times 400$). The DMSO group was similar to Control group, in which cells showed clear outline and arranged as shuttle like shape and with lower accumulation of lipids. After intervention of 0.2~0.8 mM OA, we observed much orange mass suggesting the accumulation of lipid significantly increased than control group. And the cells numbers reduced under the similar visual field, and their volume significantly shrunk compared with Control group after treating with 1 mM OA, those indicated that the 1 mM OA might damage the cells. According to the results of Oil Red O stain and TG levels after intervention of OA, we selected the 0.2 mM OA to intervene the chang Liver cells for 24 h to prepare NAFLD model. Just shown in the picture.

washes in Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with hor-seradish peroxidase-conjugated secondary antibodies (anti-mouse or anti-rabbit IgG) for 1 h and visualized by ECL detection (Pierce Biotechnology, Rockford, IL). Quantitation was performed by Fujifilm Las-3000 Luminescent Image Analyzer.

Statistical analysis

The data are expressed as mean \pm standard deviation (SD). Statistical comparisons were carried out by one-way ANOVA with the Tukey post hoc test using the 11.5 version of SPSS software. The differences were considered statistically significant if the p values were less than 0.05.

Results

Effects of PFP on body weight, serum lipid profiles of HFD-fed mice

Mice fed a HFD tended to become obese (**Figure 1A**). After 11 weeks of PFP treatment, body weights of HFD mice were significantly reduced, reaching similar levels of ND-fed control animals ($P < 0.05$, **Figure 1B**). PFP did not result in dramatic changes in food intake, although slight differences were observed at weeks 10 and 13. Notably, PFP treatment for 16 weeks significantly lowered visceral fat mass by up to 42.8% ($P < 0.05$) and reduced the serum levels of TC and LDL-C (**Table 2**), whereas PFP did not significantly affect serum TG levels (HFD group, 1.02 ± 0.10 mmol/L, $n=8$; PFP + HFD group, 0.95 ± 0.12 mmol/L, $n=7$; $P=0.32$) or HDL-C levels. Further analysis of the lipid profiles at 4, 8, and 16 weeks after PFP treatment showed that PFP did not exhibit significant effects on serum TC and LDL-C until 16 weeks. These data suggest that PFP exerts antagonizing effects on HFD-induced obesity, and long-term PFP treatment can improve serum TC and LDL-C (**Table 2**).

PFP alleviates liver steatosis in HFD-fed Apo E^{-/-} mice

Histological analysis by HE or Sudan III staining of the liver sections showed derangement of liver cells and excessive lipid droplets in the hepatocytes of Apo E^{-/-} mice fed the HFD (**Figure 2A**), which were alleviated by PFP treatment.

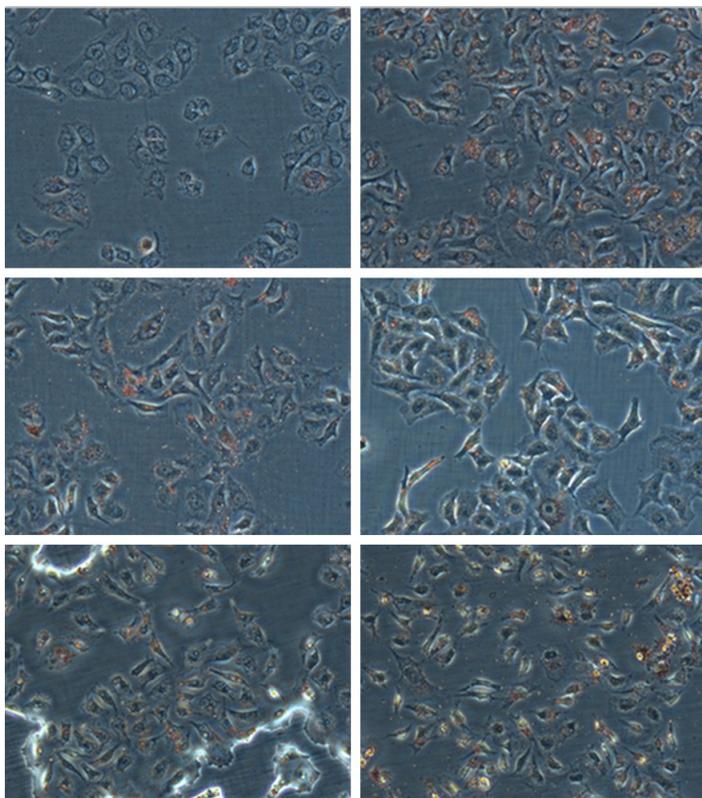


Figure 4. Oil Red O results of NAFLD cell model intervened by PFP ($\times 400$). NAFLD model were treated with different concentrates PFP for 24 h, and stained with Oil Red O and observed under microscope. Results showed that the cells showed clear outline in model group and DMSO group, while there was much stained accumulation of lipids in cells and their proportions of fatty degeneration dramatically higher than DMSO group. And the rate was much lower in the PFP 100, 200 $\mu\text{g}\cdot\text{mL}^{-1}$ groups where the orange lipid drops scattered in cells while cell outlines in two groups were fuzzier than DMSO group. Compared with model group, the PFP 50 $\mu\text{g}\cdot\text{mL}^{-1}$ group had lower accumulation of lipids and showed clear outline, suggesting that the 50 $\mu\text{g}\cdot\text{mL}^{-1}$ PFP could significantly regulate the cellular TG levels.

Table 4. Effects of PFP to SOD, GSH-PX and MDA level in NAFLD cell model ($\bar{x} \pm s$, n=6)

Group	SOD (U·mgprot ⁻¹)	GSH-PX (U·mgprot ⁻¹)	MDA (nmol·mgprot ⁻¹)
DMSO group	23.60 \pm 3.14	290.68 \pm 73.04	0.07 \pm 0.02
Model gorup	4.96 \pm 1.39 Δ	158.79 \pm 40.07 Δ	0.20 \pm 0.03 $\Delta\Delta$
PFP 25 $\mu\text{g}\cdot\text{mL}^{-1}$	34.85 \pm 5.93**	234.58 \pm 21.48	0.06 \pm 0.03**
PFP 50 $\mu\text{g}\cdot\text{mL}^{-1}$	33.79 \pm 14.13**	632.81 \pm 50.36**	0.11 \pm 0.04*
PFP 100 $\mu\text{g}\cdot\text{mL}^{-1}$	36.31 \pm 12.19**	389.22 \pm 38.24**	0.08 \pm 0.01**
PDP 200 $\mu\text{g}\cdot\text{mL}^{-1}$	36.12 \pm 17.97**	215.73 \pm 17.47	0.12 \pm 0.03

Comparison with DMSO group, $\Delta P < 0.05$, $\Delta\Delta P < 0.01$; comparison with model group, * $P < 0.05$, ** $P < 0.01$.

Consistently, treatment of HFD-fed rats with PFP for 16 weeks significantly lowered liver weight by 21% ($P < 0.05$, **Figure 2B**) and

decreased hepatic triglyceride contents by 76%, ($P < 0.01$, **Figure 2C**). Serum alanine transaminase (ALT) and aspartate aminotransferase (AST) levels were also reduced by PFP treatment (**Table 3**). Thus, PFP alleviates HFD-induced liver steatosis.

Oil Red O stain after intervention of different concentration OA

The different concentration OA was prepared using culture medium containing 0.48% DMSO to intervene Chang liver cells for 24 h. After Oil Red O stain, cells were observed and photographed, and results are shown in **Figure 3**. In the control group, the cells showed clear outlines and were arranged in a spindle, and several cells had spot lipid, showing orange fluorescence. The DMSO group was similar to the control group with little lipid storage, which suggested that DMSO had no significant effects on cellular lipid content. After intervention of 0.2-0.8 mM OA, the cell outline was clear, and there were no significant changes in cell arrangement and amounts compared with the control group at the same area under microscope. However, there were many orange spots, which suggested that lipid storage significantly increased compared to the control group. After intervention of 1 mM OA for 24 h, cell amounts decreased in the same area under microscope, and cells were smaller than normal ones, which suggested that this concentration of OA and intervention at 24 h might cause injury to cells. Based on the Oil Red O results and changes of TG levels after OA intervention, the 0.2 mM OA was used to prepare the Chang liver cell NAFLD model for 24 h in late experiments.

Oil Red O results of NAFLD cell model intervened by different concentration of PFP

After intervention by different concentrations of PFP, ellagic acid, and gallic acid at 24 h, the NAFLD model cells were colored by Oil Red O

Both Apo E^{-/-} mice and Changliver cell

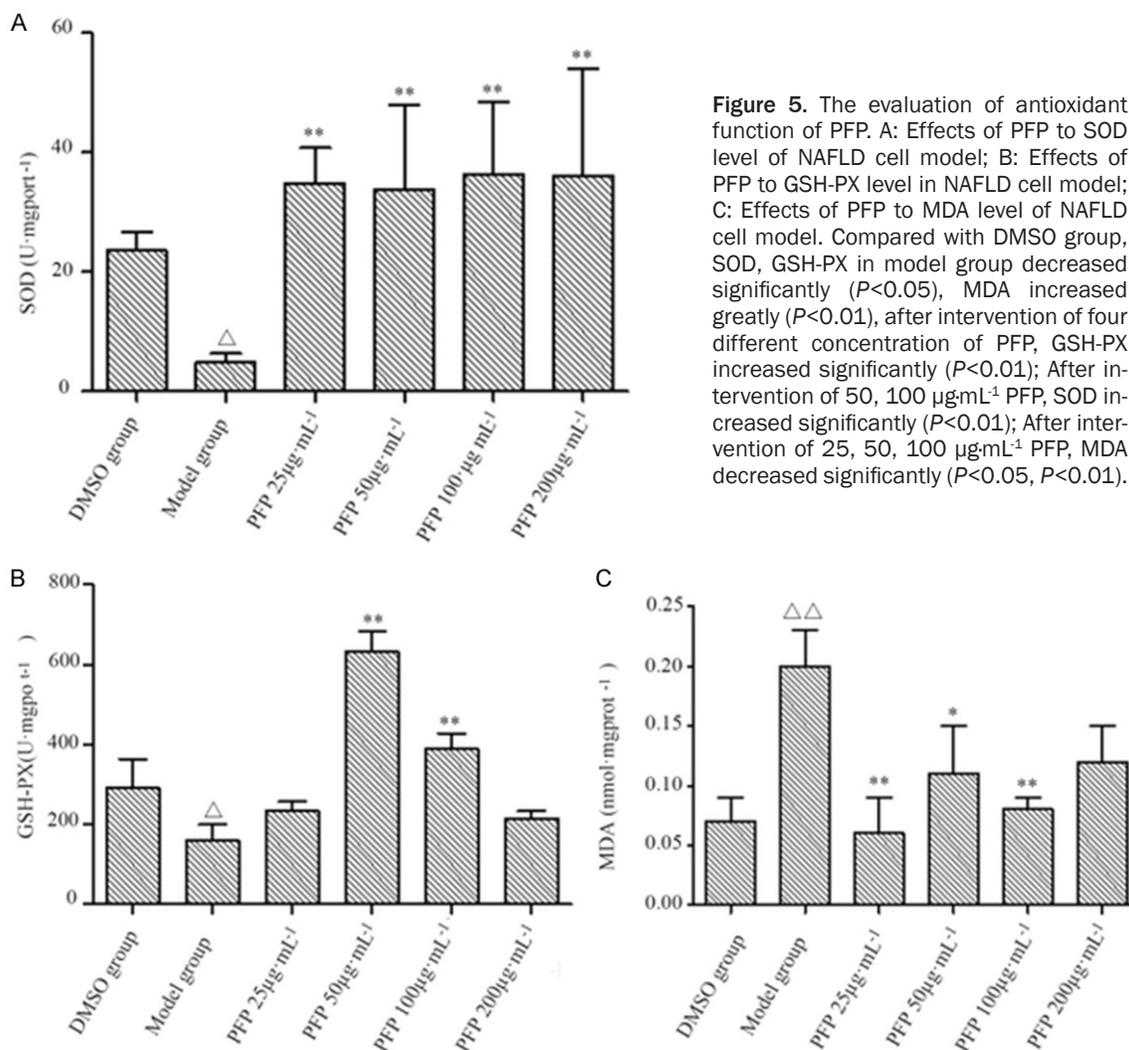


Figure 5. The evaluation of antioxidant function of PFP. A: Effects of PFP to SOD level of NAFLD cell model; B: Effects of PFP to GSH-PX level in NAFLD cell model; C: Effects of PFP to MDA level of NAFLD cell model. Compared with DMSO group, SOD, GSH-PX in model group decreased significantly ($P<0.05$), MDA increased greatly ($P<0.01$), after intervention of four different concentration of PFP, GSH-PX increased significantly ($P<0.01$); After intervention of 50, 100 $\mu\text{g}\cdot\text{mL}^{-1}$ PFP, SOD increased significantly ($P<0.01$); After intervention of 25, 50, 100 $\mu\text{g}\cdot\text{mL}^{-1}$ PFP, MDA decreased significantly ($P<0.05$, $P<0.01$).

Table 5. Effects of PFP to the TNF- α and IL-8 level of NAFLD cell model ($\bar{x}\pm s$, $n=3$)

Group	TNF- α ($\text{pg}\cdot\text{mL}^{-1}$)	IL-8 ($\text{pg}\cdot\text{mL}^{-1}$)
DMSO group	15.24 \pm 2.83	14.93 \pm 6.01
Model gorup	32.03 \pm 8.04 $\Delta\Delta$	41.79 \pm 13.88 $\Delta\Delta$
PFP 25 $\mu\text{g}\cdot\text{mL}^{-1}$	15.26 \pm 8.06**	2.91 \pm 1.01**
PFP 50 $\mu\text{g}\cdot\text{mL}^{-1}$	10.05 \pm 2.30**	1.8 \pm 0.65**
PFP 100 $\mu\text{g}\cdot\text{mL}^{-1}$	8.03 \pm 0.89**	5.54 \pm 3.64**
PFP 200 $\mu\text{g}\cdot\text{mL}^{-1}$	7.63 \pm 1.19**	7.95 \pm 1.76**

Comparison with DMSO group, $\Delta\Delta P<0.01$; comparison with model group, ** $P<0.01$.

and photographed under a microscope. The cells showed clearer outlines in model group than the DMSO group, and many stained lipid drops were found in the cells. Additionally, its lipid rate was significant higher than the DMSO group in the same area, in which the lipid rates of the PFP 100 $\mu\text{g}\cdot\text{mL}^{-1}$ and 200 $\mu\text{g}\cdot\text{mL}^{-1}$ group

were significantly lower than the model group. A few orange lipid drops were located in the model group cells, but cell outline was fuzzier than the DMSO group. Moreover, the lipid rate significantly decreased in the PFP 50 $\mu\text{g}\cdot\text{mL}^{-1}$ group compared to the model group, and the cell outline was clear, which suggested that 50 $\mu\text{g}\cdot\text{mL}^{-1}$ PFP could regulate the cellular TG content. Results are shown in **Figure 4**.

Effects of PFP to the SOD, MDA, and GSH-PX levels of NAFLD model cells

The SOD and GSH-PX levels significantly decreased and MDA level significantly increased. The differences compared with the DMSO group were statistically significant ($P<0.05$, $P<0.01$). After intervention of PFP, the SOD level was significantly improved in the PFP 25~200 $\mu\text{g}\cdot\text{mL}^{-1}$ group compared to the model group, and differences were statistically signifi-

Table 6. Effects of PFP to mRNA relative expression of cell lipid metabolizing enzymes of NAFLD cell model ($\bar{x} \pm s$, n=5)

Group	Sirt3	CPT-1A	ACC2	AMPK
DMSO group	1.31±0.30	1.31±0.47	0.41±0.04	1.16±0.10
Model gorup	0.78±0.12	0.40±0.05 $\Delta\Delta$	0.74±0.05 $\Delta\Delta$	0.69±0.10 $\Delta\Delta$
PFP 50 $\mu\text{g}\cdot\text{mL}^{-1}$	1.58±0.10*	0.71±0.22**	0.23±0.04**	0.47±0.24

Comparison with DMSO group, $\Delta\Delta P < 0.01$; comparison with model group, * $P < 0.05$, ** $P < 0.01$.

cant ($P < 0.01$). The GSH-PX levels were significantly improved in the PFP 50 $\mu\text{g}\cdot\text{mL}^{-1}$ and 100 $\mu\text{g}\cdot\text{mL}^{-1}$ groups compared with the model group, and the differences were statistically significant ($P < 0.01$). Lastly, the MDA level significantly decreased in the PFP 25~200 $\mu\text{g}\cdot\text{mL}^{-1}$ group compared with the model group, and the differences were statistical significant ($P < 0.05$, $P < 0.01$). Results are shown in **Table 4** and **Figure 5**.

Effects of PFP to TNF- α and IL-8 of NAFLD cell model

Compared with the DMSO group, the TNF- α level was significantly improved in the model group, and the differences were statistically significant ($P < 0.01$). After intervention of different concentrations of PFP, the TNF- α significantly decreased in NAFLD cells, and the differences were statistically significant compared with the model group ($P < 0.01$). Compared with the DMSO group, the IL-8 level significantly increased in the model group, and the differences were statistically significant ($P < 0.01$). After intervention of four different concentrations of PFP, the IL-8 level of NAFLD cells significantly decreased, and the differences compared with the model group were statistically significant ($P < 0.01$). Results are shown in **Tables 5, 6**, and **Figure 6**.

Discussion

The present study clearly shows that pomegranate flower polyphenols have an effect on the development of NAFLD in Apo E^{-/-} mice. The mechanisms responsible for PFP-induced improvements in lipid and antioxidant function have not been fully characterized. We hypothesized that a polyphenol-rich PFP would present modulate hepatic lipid metabolism and improved antioxidant function for Apo E^{-/-} mice fed high-fat, high-cholesterol diets.

The results of this study partially support this hypothesis, in that PFP modulated plasma cholesterol as well as hepatic triglyceride, cholesterol, or mRNA and protein levels of AMPK, Sirt3, ACC2, and CPT-1A in Apo E^{-/-} mice. PFP supplementation improved antioxidant function

in the plasma and liver of Apo E^{-/-} mice and reduced plasma ALT and AST. Histological analysis of livers shows that PFP attenuates obesity-induced liver steatosis and inflammation.

Collectively, our findings provide novel evidence that PFP exerts anti-inflammatory activities in Apo E^{-/-} mice and protects against pathogenic events contributing to liver injury associated with obesity-induced NASH.

We examined effects of PFP on the elevated serum lipid parameters in the HFD-fed mice. As shown in **Table 2**, the levels of total cholesterol, triglycerides, and free cholesterol were significantly decreased at week 12 of HFD + PFP feeding, whereas HDL cholesterol levels did not change significantly ($P < 0.05$). Furthermore, we measured serum TBARS and the total antioxidant status of each group. Obesity is associated with defective antioxidant status and oxidative stress, which is an important early instigator of obesity, and dietary antioxidants play a role in the reduction of oxidative stress [3, 5]. PFP supplementation also decreased the levels of serum TBARS and the total antioxidant status by 48.4% and 18.4%, respectively, suggesting that PFP supplementation effectively prevents increased oxidative stress induced by HFD consumption.

Pomegranate flower extracts inhibit lipopolysaccharide activation of NF κ B, suppressing overexpressed cardiac fibronectin and collagen I and III mRNAs in the heart of ZDF rats [14]. K. Zhe et al. investigated how pomegranate flower extracts ameliorate fatty liver in rats with type 2 diabetes and obesity by upregulation of hepatic expression of the PPAR and SCD-1-regulated genes responsible for fatty acid oxidation, resulting in a decrease in lipid accumulation in the liver. These findings are potentially important for supporting further study of the subject to demonstrate the effectiveness of

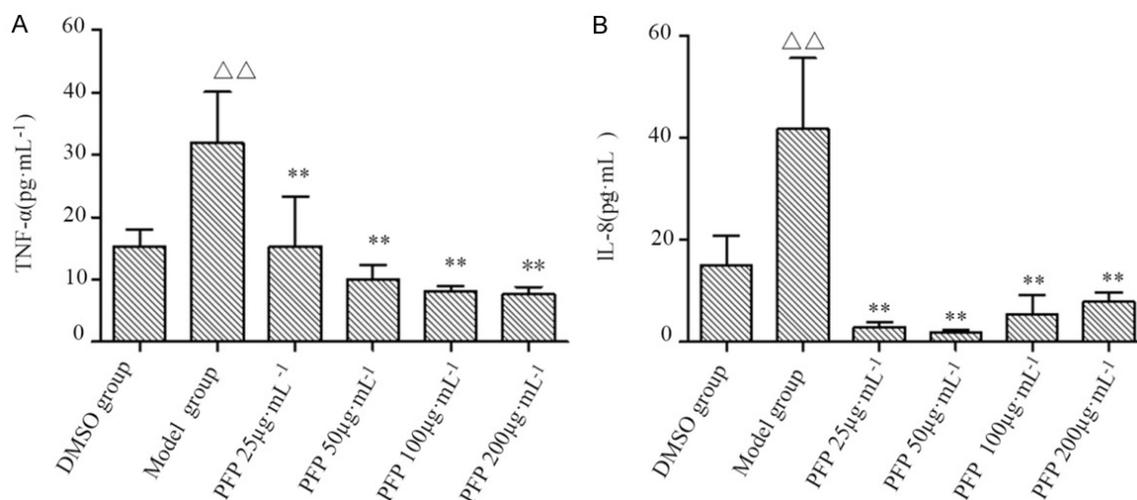


Figure 6. Effects on the inflammation. A: Effects of PFP to the TNF- α level of NAFLD cell model; B: Effects of PFP to the IL-8 level of NAFLD cell model; Compared with DMSO group, the TNF- α level was significantly improved in model group, and the differences were statistically significant ($P < 0.01$); after intervention of different concentration of PFP, the TNF- α significantly decreased in NAFLD cells, and the differences were statistically significant compared with model group ($P < 0.01$). Compared with DMSO group, the IL-8 level significantly increased in model group, and the differences were statistically significant ($P < 0.01$); and after intervention of four different concentration of PFP, the IL-8 level of NAFLD cells significantly decreased, and the differences compared with model group were statistically significant ($P < 0.01$). Compare with DMSO group, TNF- α , IL-8 in model group increased significantly ($P < 0.01$); after intervention of four different concentration of PFP, TNF- α , IL-8 level decreased significantly ($P < 0.01$).

PFP in the prevention and/or treatment of diabetes and obesity induced non-alcoholic fatty liver disease, as it may specifically affect the liver due to accumulation after absorption by the intestines and liver. Moreover, the treatment increases the expression of hepatic genes related to lipid metabolism and decreases the levels of plasma triglyceride and NEFA by attenuating the expression of hepatic PPAR α [1]. Here, we show that levels of total lipids and triglycerides in the liver were significantly decreased in the HFD + PFP-fed mice compared with the HFD control mice (**Figure 1B**). We observed a decreased number of lipid droplets in the hepatic tissue by Oil Red O staining when the HFD-fed mice were treated with PFP. Taken together, these results clearly demonstrate that PFP reduces fat accumulation in mice with HFD-induced obesity.

To investigate the molecular mechanism by which PFP regulates hepatosteatosis, we compared hepatic gene expression in HFD-fed or HFD + PFP-fed mice versus normal mice using quantitative reverse transcriptase-polymerase chain reaction and immunohistochemical assays, finding various genes of lipid metabolism were altered (data not shown). PFP pre-

vented the reduction of Sirt3 expression, in both mRNA and protein levels. SIRT is a NAD⁺-dependent protein deacetylase, which was first discovered in yeast and termed Silent Information Regulator 2 (SIR2) [5, 6]. SIRT3 reduces lipid accumulation via AMPK activation in human hepatic cells [15]. Our data also indicate that AMPK, ACC2, and CPT-1A are up or down regulated by PFP. Recent studies demonstrated that AMP-activated protein kinase (AMPK) was involved in the regulation of FFA metabolism [16]. AMPK is a heterotrimeric complex that serves as a sensor at the cellular energy level. AMPK is activated when the cellular energy store decreases, leading to a rise in cellular AMP level. Upon the activation of AMPK, the adenosine triphosphate (ATP)-utilizing pathway (such as FFA synthesis) is switched off and the ATP-producing pathway (such as FFA oxidation) is switched on. Two anti-diabetic drugs have been found that could activate AMPK and reduce liver fat accumulation [17, 18]. FFA synthesis is mediated by seven enzymes, where acetyl coenzyme A carboxylase (ACC) is the rate-limiting enzyme. ACC is phosphorylated by activated AMPK, which reduces malonyl-coenzyme A formation. CPT-1A is a rate-limiting enzyme in the process of

fatty acid oxidation. Malonyl CoA inhibits CPT-1A, and ACC2 activity reduction led to the reduction of malonyl CoA synthesis, and thus a weakening CPT-1A of inhibition. Fatty acid oxidation was, therefore, enhanced and there was improved fat degeneration [2]. In our study, PFP moderately increased the expression of Sirt3, AMPK, and CPT-1 compared with HFD control by both RT-PCR and immunohistochemical assays, suggesting that inhibitory effects of PFP on HFD-induced obesity may be partly caused by the recovery of normal expression of these genes. PFP also suppressed expression of ACC2 in the livers of the HFD-fed mice. The reduction of ACC2 expression brought about enhanced fatty acids.

Collectively, these data demonstrate that the reduction of hepatic lipid accumulation is induced by PFP treatment. We also confirmed the presence of regulatory proteins involved in lipid metabolism, such as Sirt3, AMPK, ACC2, and CPT-1, suggesting that changes in lipid metabolism-related gene expression also occurred.

Acknowledgements

Moderator: Yuan-Yuan Wei. National Natural Science Foundation of China NO: 81160546; National Natural Science Foundation of China NO: 81360699.

Disclosure of conflict of interest

None.

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References

- [1] Kobori M, Masumoto S, Akimoto Y and Oike H. Chronic dietary intake of quercetin alleviates hepatic fat accumulation associated with consumption of a Western-style diet in C57/BL6J mice. *Mol Nutr Food Res* 2011; 55: 530-540.
- [2] Cho YS, Lee JI, Shin D, Kim HT, Jung HY, Lee TG, Kang LW, Ahn YJ, Cho HS and Heo YS. Molecular mechanism for the regulation of human ACC2 through phosphorylation by AMPK. *Biochem Biophys Res Commun* 2010; 391: 187-192.
- [3] Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Maki-shima M, Matsuda M and Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 2004; 114: 1752-1761.
- [4] Valdecantos MP, Perez-Matute P and Martinez JA. [Obesity and oxidative stress: role of antioxidant supplementation]. *Rev Invest Clin* 2009; 61: 127-139.
- [5] Landry J, Sutton A, Tafrov ST, Heller RC, Stebbins J, Pillus L and Sternglanz R. The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proc Natl Acad Sci U S A* 2000; 97: 5807-5811.
- [6] Smith JS, Brachmann CB, Celic I, Kenna MA, Muhammad S, Starai VJ, Avalos JL, Escalante-Semerena JC, Grubmeyer C, Wolberger C and Boeke JD. A phylogenetically conserved NAD⁺-dependent protein deacetylase activity in the Sir2 protein family. *Proc Natl Acad Sci U S A* 2000; 97: 6658-6663.
- [7] Colak Y, Tuncer I, Senates E, Ozturk O, Doganay L and Yilmaz Y. Nonalcoholic fatty liver disease: a nutritional approach. *Metab Syndr Relat Disord* 2012; 10: 161-166.
- [8] Lidofsky SD. Nonalcoholic fatty liver disease: diagnosis and relation to metabolic syndrome and approach to treatment. *Curr Diab Rep* 2008; 8: 25-30.
- [9] Moscatiello S, Di Luzio R, Sasdelli AS and Marchesini G. Managing the combination of nonalcoholic fatty liver disease and metabolic syndrome. *Expert Opin Pharmacother* 2011; 12: 2657-2672.
- [10] Peres RG, Tonin FG, Tavares MF and Rodriguez-Amaya DB. HPLC-DAD-ESI/MS identification and quantification of phenolic compounds in Ilex paraguariensis beverages and on-line evaluation of individual antioxidant activity. *Molecules* 2013; 18: 3859-3871.
- [11] Ichikawa T, Kitajima S, Liang J, Koike T, Wang X, Sun H, Okazaki M, Morimoto M, Shikama H, Watanabe T, Yamada N and Fan J. Overexpression of lipoprotein lipase in transgenic rabbits leads to increased small dense LDL in plasma and promotes atherosclerosis. *Lab Invest* 2004; 84: 715-726.
- [12] Folch J and Schneider HA. An amino acid constituent of ox brain cephalin. *Journal of Biological Chemistry* 1940; 137: 51-62.
- [13] Chang X, Yan H, Fei J, Jiang M, Zhu H, Lu D and Gao X. Berberine reduces methylation of the MTTp promoter and alleviates fatty liver induced by a high-fat diet in rats. *J Lipid Res* 2010; 51: 2504-2515.
- [14] Huang TH, Yang QM, Li GQ, Yamahara J, Roufogalis BD and Li Y. Pomegranate flower extract diminishes cardiac fibrosis in Zucker diabetic fatty rats: modulation of cardiac endothelin-1 and nuclear factor-kappaB pathways. *J Cardiovasc Pharmacol* 2005; 46: 856-862.

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- [15] Tong S, Guo QF, Shu DX. SIRT3 reduces lipid accumulation via AMPK activation in human hepatic cells. *J Dig Dis* 2010; 11: 55-62.
- [16] Huang CC, Shi L, Lin CH, Kim AJ, Ko ML and Ko GY. A new role for AMP-activated protein kinase (AMPK) in the circadian regulation of I-type voltage-gated calcium channels (L-VGCCs) in late-stage embryonic retinal photoreceptors. *J Neurochem* 2015; 135: 727-741.
- [17] Rocco U and Ubaldo VC. Metformin in non-alcoholic steatohepatitis. *Lancet* 2002; 359: 355-356.
- [18] Fryer LG, Asha PP and David C. The Anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. *J Biol Chem* 2002; 277: 25226-25232.