Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;49:1870-1884
DOI: 10.1159/000493650
Published online: 21 September, 2018
Accepted: 12 September, 2018

Original Paper

Fisetin Protects Against Hepatic Steatosis Through Regulation of the Sirt1/AMPK and Fatty Acid β-Oxidation Signaling Pathway in High-Fat Diet-Induced Obese Mice

Chian-Jiun Liou[a,b] Ciao-Han Wei[c] Ya-Ling Chen[b,d] Ching-Yi Cheng[c,e,f] Chia-Ling Wang[c] Wen-Chung Huang[b,c]

[a]Department of Nursing, Research Center for Chinese Herbal Medicine, Chang Gung University of Science and Technology, Taoyuan, [b]Division of Allergy, Asthma, and Rheumatology, Department of Pediatrics, Chang Gung Memorial Hospital, Linkou, [c]Graduate Institute of Health Industry Technology, Research Center for Food and Cosmetic Safety, Research Center for Chinese Herbal Medicine, College of Human Ecology, Chang Gung University of Science and Technology, Taoyuan, [d]Department of Nutrition and Health Sciences, Chang Gung University of Science and Technology, Taoyuan, [e]Department of Ophthalmology, Chang Gung Memorial Hospital, Linkou, [f]Department of Traditional Chinese Medicine, Chang Gung Memorial Hospital, Keelung, Taiwan

Key Words
AMPK • CPT-1 • Fisetin • FL83B • Lipolysis • Nonalcoholic fatty liver disease

Abstract
Background/Aims: Fisetin is a naturally abundant flavonoid isolated from various fruits and vegetables that was recently identified to have potential biological functions in improving allergic airway inflammation, as well as anti-oxidative and anti-tumor properties. Fisetin has also been demonstrated to have anti-obesity properties in mice. However, the effect of fisetin on nonalcoholic fatty liver disease (NAFLD) is still elusive. Thus, the present study evaluated whether fisetin improves hepatic steatosis in high-fat diet (HFD)-induced obese mice and regulates lipid metabolism of FL83B hepatocytes *in vitro*. ***Methods:*** NAFLD was induced by HFD in male C57BL/6 mice. The mice were then injected intraperitoneally with fisetin for 10 weeks. In another experiment, FL83B cells were challenged with oleic acid to induce lipid accumulation and treated with various concentrations of fisetin. ***Results:*** NAFLD mice treated with fisetin had decreased body weight and epididymal adipose tissue weight compared to NAFLD mice. Fisetin treatment also reduced liver lipid droplet and hepatocyte steatosis, alleviated serum free fatty acid, and leptin concentrations, significantly decreased fatty acid synthase, and significantly increased phosphorylation of AMPKα and the production of sirt-1 and carnitine palmitoyltransferase I in the liver tissue. *In vitro*, fisetin decreased lipid

C.-J. Liou and C.-H. Wei contributed equally to this work.

Wen-Chung Huang, PhD
Graduate Institute of Health Industry Technology, Chang Gung University of Science and Technology
No. 261, Wenhua 1st Rd., Guishan Dist., Taoyuan City 33303 (Taiwan)
Tel. 886-3-2118999, Fax 886-3-2118293, E-Mail wchuang@mail.cgust.edu.tw

accumulation and increased lipolysis and β-oxidation in hepatocytes. ***Conclusion:*** This study suggests that fisetin is a potential novel treatment for alleviating hepatic lipid metabolism and improving NAFLD in mice via activation of the sirt1/AMPK and β-oxidation pathway.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common liver disease typically characterized by excessive lipid accumulation and metabolic disorder in hepatocytes [1]. The initial symptoms of NAFLD generally implicate the progress of simple fatty liver, which gradually develops into increasingly severe forms of liver disease, including nonalcoholic steatohepatitis, liver fibrosis, cirrhosis, and hepatocellular carcinoma [2]. Obesity would increase the risk of chronic diseases, including type 2 diabetes, hypertension, stroke, and cancer [3]. In addition, excess triglycerides (TGs) stored in hepatocytes may cause hepatic steatosis and interfere with the metabolic function of the liver [4]. Therefore, obesity could cause hepatic steatosis, which an important risk factor for the development of NAFLD.

Sterol regulatory element binding protein 1c (SREBP-1c) is an essential transcription factor in the regulation of lipid synthesis and maintenance of cellular homeostasis [5]. Previous studies have found that peroxisome proliferator–activated receptor (PPAR) and

CCAAT/enhancer-binding protein (C/EBP) are also important transcription factors for controlled lipid biosynthesis and can switch on fatty acid synthase (FAS) gene expression to synthesize fatty acid chains [6]. Therefore, the activation of lipid transcription factors would accelerate liver lipid accumulation and result in fatty liver disease.

AMPK is a heterotrimeric enzyme that is composed by AMPKα (a catalytic subunit), AMPKβ (a scaffolding subunit), and AMPKγ (a regulatory subunit) [7]. Recent studies have found that AMPK regulates energy balance [8]. When the liver, muscle, and adipose tissue accumulate excessive energy, these tissues activate the AMPK pathway to regulate lipid and glucose metabolism [9]. AMPK activity could induce ACC phosphorylation and decrease ACC activity for suppressed lipid biosynthesis [10, 11]. Thus, phosphorylation of AMPK can not only maintain energy balance, but also inhibit the formation of TGs to reduce lipid accumulation in the liver. In addition, sirt1 plays a role in regulating AMPK activity to enhance AMPK phosphorylation in adipocyte and hepatocytes [12]. Sirt1-deficient mice lack AMPK activity, but SREBP-1c expression is promoted in mice with induced obesity and hepatic steatosis compared to wild-type HFD-induced obese mice [13]. Animal studies have found that metformin is an antidiabetic drug that can activate AMPK and reduce hepatic steatosis by increasing fatty acid β-oxidation and reducing SREBP-1c expression [14, 15]. Therefore, sirt1/AMPK pathway activity potentially inhibits lipid accumulation in hepatocytes and improves hepatic steatosis.

Interestingly, accelerating lipid decomposition is an important strategy for improving NAFLD [6]. In the liver, excessive lipid biosynthesis is mainly a phenomenon of excessive energy accumulation. Exercise or calorie restriction can increase lipolysis to consume liver TGs and eliminate liver steatosis [16, 17]. Many studies have suggested that some vegetable and fruit extracts could increase TG metabolism in NAFLD therapy [18-20]. Omega-3 fatty acids (DHA and EPA), medium-chain fatty acids, *Lactobacillus plantarum*, and mulberry anthocyanins also reduce TG levels in the liver [21-23]. Furthermore, hepatocytes activate adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) to decompose TGs and form glycerol and free fatty acids in the citric cycle for energy production [24]. However, free fatty acids will stimulate liver macrophages and cause inflammatory responses, and activated macrophages release inflammatory mediators to induce insulin resistance in hepatocytes [25]. Notably, excess TGs in the liver are broken down, and released free fatty acids must be decomposed to produce energy via fatty acid β-oxidation and reduce the damage to liver and adipose tissue.

Fisetin is a flavonoid isolated from various fruits and vegetables [26] that can suppress inflammatory effects, with strong anti-oxidant and anti-tumor effects [27, 28]. Previous studies have found that fisetin can enhance the levels of adiponectin in 3T3-L1 adipocytes by enhancing sirt1 expression, and inhibit mTORC1 signaling to block differentiation of 3T3-L1 preadipocytes [29, 30]. Fisetin can also regulate fatty acid synthase (FAS), ATP citrate lyase, and glucose 6-phosphatase gene expression and enhance glucose transporter type 4 gene expression in hepatocytes compared to HFD-fed mice [30], but how fisetin regulates lipid accumulation in the liver and improves NAFLD is not clear. In the present study, we evaluated whether fisetin regulates lipid metabolism in FL83B hepatocytes *in vitro*. We also investigated whether fisetin modulates adipogenesis and lipolysis in steatotic hepatocytes, and improves NAFLD in HFD-induced obese mice.

Materials and Methods

Animals and administration of fisetin

Four-week-old male C57BL/6 mice were procured from the National Laboratory Animal Center, Taipei, Taiwan. All experimental animals were approved and supervised by the Laboratory Animal Care Committee of Chang Gung University of Science and Technology (IACUC approval numbers 2016-002 and 2017-007). The mice were housed in an air conditioned room at a constant temperature of 23 ± 2°C, and maintained on a normal chow diet with clean water ad libitum for 1 week before being randomly divided into three groups of 12 (Fig. 1A): normal mice maintained with normal chow diet (11.4% fat), HFD-fed mice maintained with HFD (60% fat), and F20 mice maintained with HFD and administered 20 mg/kg fisetin (purity ≥98%, St. Louis, MO, USA) dissolved in DMSO. The HFD and normal mice administered DMSO as control by intraperitoneal injection twice a week for last 10 weeks. F20 groups were maintained on HFD for 4 weeks and then treated with 20 mg/kg fisetin by intraperitoneal injection twice a week for last 10 weeks. Food intake was defined as weight of consumed food (g) x calorie of diet per day, and the diet intake of mouse was monitored per day and body weight recorded weekly [31]. We also calculated the food efficiency ratio as defined by weight gain (g)/food intake (g) x 100 [32].

Biochemical analysis

After sacrificing the mice, serum was collected and assayed by a biochemistry analyzer (DRI-CHEM NX500, Fuji, Tokyo, Japan). We investigated the levels of total cholesterol (TC), high-density lipoprotein (HDL), and total TGs according to the manufacturer's instructions. Free fatty acid was measured using the free fatty acid quantitation kit (Sigma) and the manufacturer's protocol. Serum treated with the assay reagent and the levels of free fatty acids were determined by the absorbance at 570 nm using a microplate reader (Multiskan FC, Thermo, Waltham, MA, USA).

Histological analysis

Liver and adipose tissue was fixed in 10% formalin and embedded with paraffin before staining with hematoxylin and eosin (HE) solution as previously described [33, 34]. Periodic acid-Schiff (PAS) stain using the periodic acid-Schiff staining system (Sigma) indicated glycogen accumulation in liver tissue as described previously [35]. All biopsy specimens were assayed using a light microscope (Olympus, Tokyo, Japan).

Immunohistochemistry (IHC)

The liver was fixed and embedded with paraffin and the tissue sliced into 6-μm sections. Each slide was incubated with CPT-1 or sirt1 antibody (1:50) overnight, washed, and incubated with HRP anti-rabbit secondary antibody. Finally, the slide was treated with DAB substrate and CPT-1 or sirt1 expression observed with a light microscope.

Western blot analysis

Protein bands were separated on 8–10% SDS–PAGE gels and transferred to polyvinylidene difluoride (PVDF) membrane for probing with primary antibodies overnight. The membrane was washed and incubated with secondary antibodies and specific protein signals detected with Luminol/Enhancer solution (Millipore, Billerica, MA, USA) by the BioSpectrum 600 system (UVP, Upland, CA, USA). Primary antibodies included acetyl CoA carboxylase-1 (ACC-1), phosphorylated-ACC-1 (pACC-1), ATGL, HSL, phosphorylated HSL (pHSL), C/EBPα, C/EBPβ, PPAR-α and PPAR-γ (Abcam, Cambridge, MA, USA), phosphorylated AMPKα (pAMPKα), AMPKα, FAS, carnitine palmitoyltransferase 1 (CPT-1), CPT2, sirt1, SREBP-1c (Cell Signaling Technology, MA, USA), and β-actin (Sigma).

Cell culture and induced fatty liver cells

Normal mouse liver cell line FL83B was purchased from the Bioresource Collection and Research Center (BCRC, Taiwan). Cells cultured in F12 medium (Invitrogen-Gibco™, Paisley, Scotland) contained 10% FBS at 37°C in a 5% CO_2 atmosphere. FL83B cells were incubated with 0.5 mM oleic acid to induce lipid accumulation in hepatocytes for 48 h, then treated with fisetin (0-100 μM) for 24 h to analyze the experimental results.

Cell viability assay

Fisetin was dissolved in DMSO and ≤0.1% DMSO was used in all cell experiments. FL83B cells were incubated with fisetin for 24 h and treated with MTT solution (Sigma) for 4 h. The culture plate was then treated with isopropanol and absorbance measured at 570 nm to evaluate cell viability using a spectrophotometer (Multiskan FC).

Oil red O staining

FL83B cells were seeded in 6-well plate and incubated with 0.5 mM oleic acid for 48 h before being treated with fisetin (0- 100 μM) for 24 h. FL83B cells were fixed with formalin and oil droplets stained using oil red O solution (Sigma) as described previously [36]. The oil droplets in hepatocytes were observed using an inverted microscope (Olympus).

Hepatic lipid accumulation and lipoperoxidation

FL83B cells were seeded in culture plates and incubated with 0.5 mM oleic acid for 48 h before being treated with fisetin (0- 100 μM) for 24 h. FL83B cells were fixed with formalin, and stained with BODIPY 581/591 C11 and BODIPY 493/503 (Invitrogen, Carlsbad, CA, USA) to investigate lipoperoxidation and lipid accumulation, respectively. Cell nuclei were stained with DAPI and all results observed with a fluorescence microscope (Olympus).

Hepatic fatty acid uptake

FL83B cells were incubated with 0.5 mM oleic acid for 48 h and then treated with fisetin for 24 h before staining with BODIPY FL C12 fluorescent probe to evaluate fatty acid uptake by fluorescence microscopy (Olympus).

Statistical analysis

Statistical analyses included one-way analysis of variance (ANOVA) and a Dunnett post hoc test. Results are expressed as mean ± SEM. *P*-values < 0.05 were considered significant.

Results

Fisetin reduced HFD-induced obesity in mice

Visual observation revealed that HFD mice had greater body weight than normal mice. Interestingly, F20 mice had significantly reduced body weight compared to HFD mice in the last weeks of the experiment (Fig. 1B). We also found that F20 mice did not have altered food intake, but inhibited food efficiency ratio compared to HFD mice (Fig. 1C-D). The last week of the experiment, F20 mice had significantly reduced body weight compared to HFD

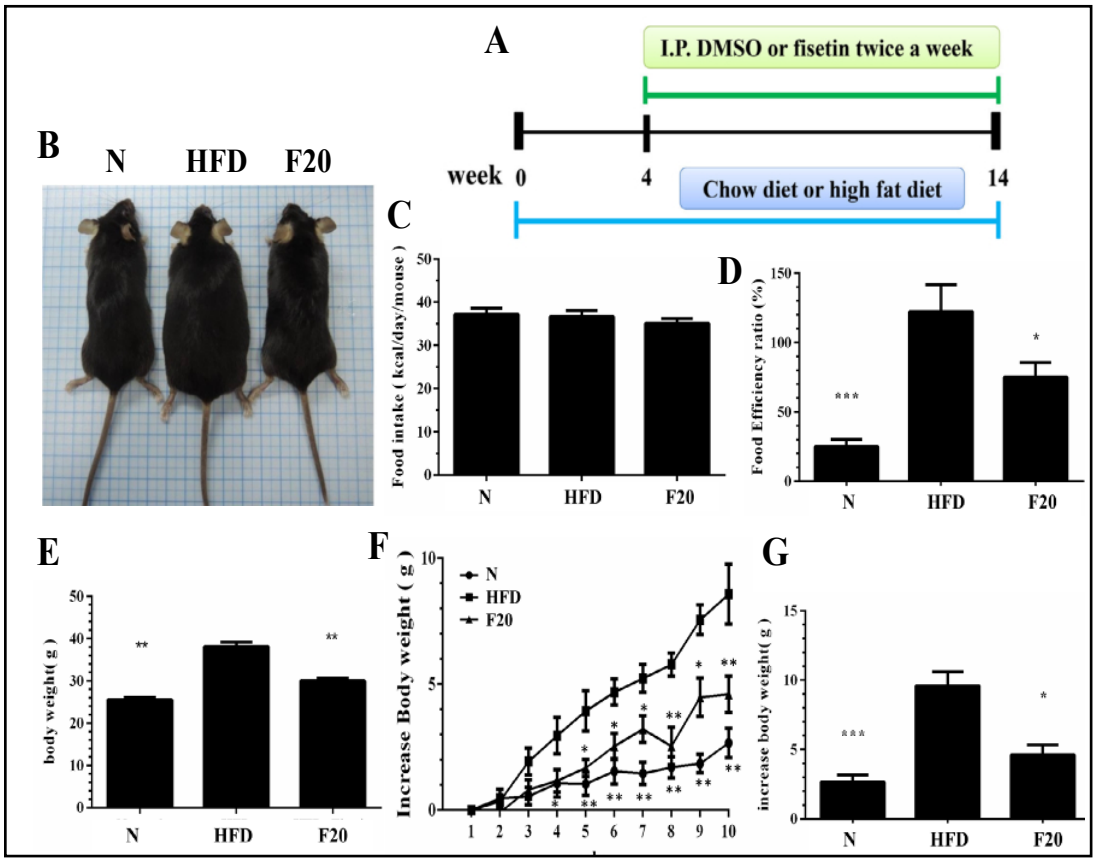

Fig. 1. Fisetin reduced body weight in HFD-induced obese mice. (A) Male mice were fed a HFD (containing 60% fat) for 14 weeks, and administered DMSO, 20 mg/kg fisetin (F20) by intraperitoneal injection (I.P.) twice a week from Week 4 to Week 14. (B) The appearance of the animal, (C) food intake, (D) food efficiency ratio, and (E) body weight. (F) Weight gain was measured when fisetin was administered by intraperitoneal injection for 10 weeks. (G) Weight gain was measured in the last week. Data are presented as mean ± SEM; n = 12. *P<0.05, **P<0.01 compared to mice with HFD-induced obesity.

mice (34.5±1.41 g vs. 39.4±1.52 g; Fig. 1E). As fisetin was given for 10 weeks, body weight in F20 mice was significantly decreased compared to HFD mice (Fig. 1F). Weight gain was measured in the last week, and F20 mice were significantly decreased weight gain compared to HFD mice (F20: 4.62±1.25 g vs. HFD: 9.55±1.38 g; Fig. 1G).

Fisetin attenuated liver steatosis in obese mice

Grossly, the livers of the normal group were dark brown/red, and the livers of the HFD group were yellowish and lacked luster. The F20 group had recovered the dark brown/red color of the liver (Fig. 2A). Interestingly, fisetin did not eliminate the liver weight and relative weight of liver/body weight compared to obese mice (Fig. 2B-C). However, histologically, many lipid droplets and fat vacuoles were observed in HFD mice, and fisetin significantly reduced the number of lipid droplets and fat vacuoles in F20 mice (Fig. 2D-E). Glycogen accumulation in hepatocytes was evaluated by PAS staining, which showed that the HFD group had significantly reduced glycogen accumulation. Fisetin elevated glycogen accumulation in F20 mice compared to HFD mice (Fig. 2F).

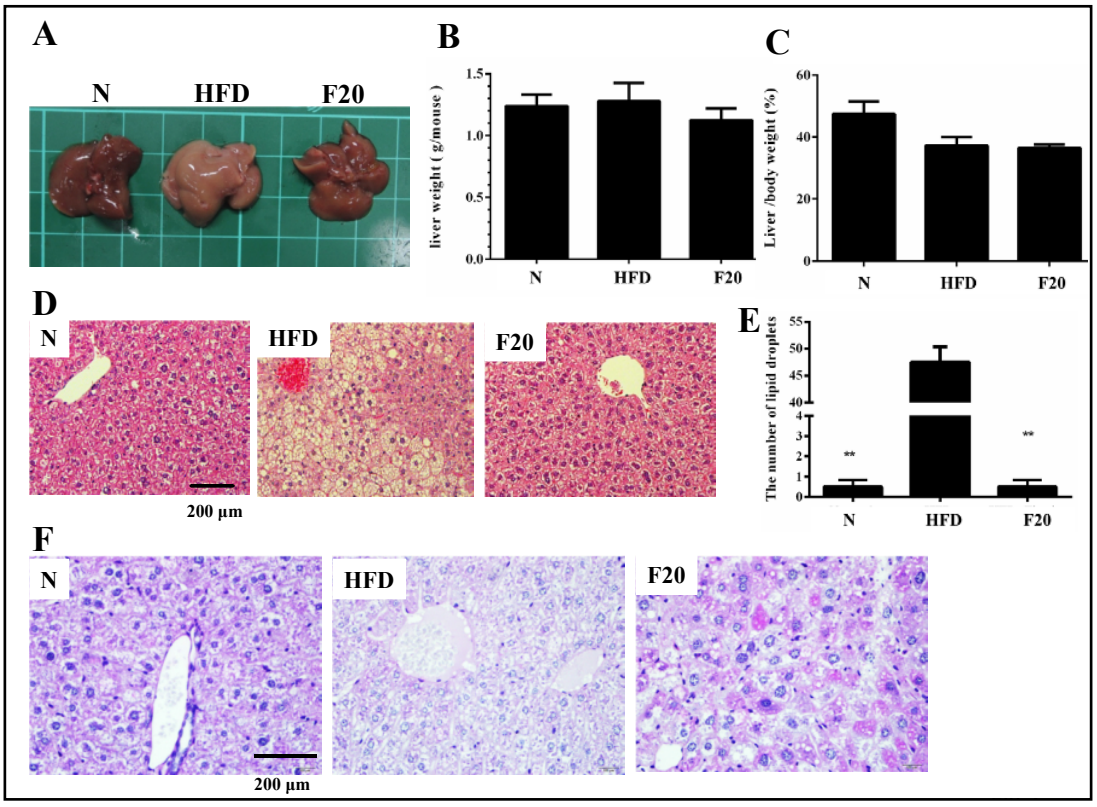

Fig. 2. Fisetin ameliorated hepatic steatosis in HFD-induced obese mice. (A) The appearance of the liver, (B) liver weight, and (C) relative weight of the liver. (D) HE staining of liver tissues (200× magnification). (E) Calculated number of lipid droplets in liver tissue (400× magnification). Data are presented as mean ± SEM; n = 12. **P<0.01 compared to mice with HFD-induced obesity. (F) PAS stain demonstrating the glycogen distribution in the liver.

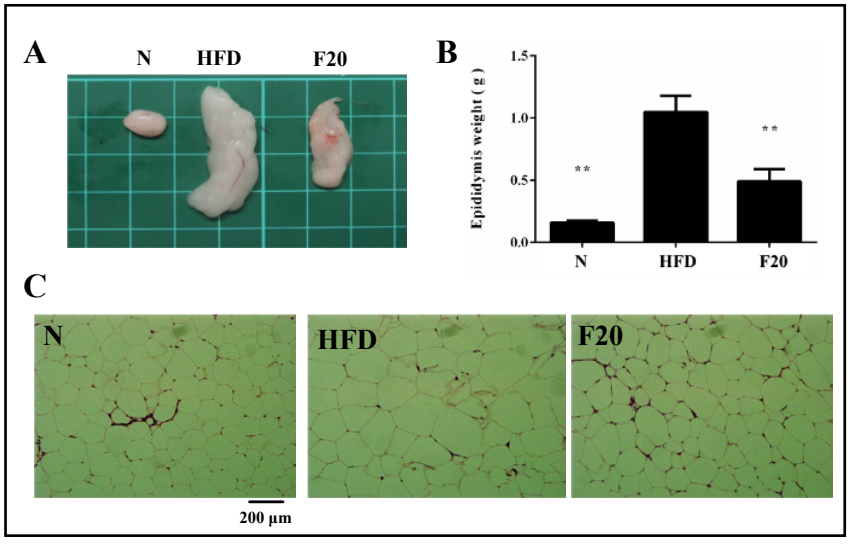

Fig. 3. Fisetin reduced the epididymal tissue weight in HFD-induced obese mice. (A) Appearance and (B) weight of epididymal adipose tissue. (C) HE staining of epididymal adipose tissue (200× magnification). Data are presented as mean ± SEM; n = 12. **P<0.01 compared to mice with HFD-induced obesity.

Fisetin attenuated the weight of adipose tissue in obese mice

Fisetin significantly reduced the epididymal adipose tissue weight compared to HFD mice (Fig. 3A-B). Histological staining and analysis demonstrated that fisetin reduced adipocyte size compared to HFD mice (Fig. 3C).

Effects of fisetin on serum lipid metabolism

Serum analysis showed that fisetin did not significantly decrease TC, TG, and HDL levels in HFD mice (Fig. 4A-C). However, fisetin significantly decreased free fatty acid levels compared to the HFD group (Fig. 4D). Fisetin also significantly suppressed serum leptin levels compared to HFD mice (Fig. 4E).

Fisetin regulated adipogenesis in liver tissue

Western blot detecting specific liver proteins showed that fisetin significantly suppressed transcription factor expression associated with adipogenesis, including SREBP-1c, C/EBPα, C/EBPα, and PPAR-γ, compared to the HFD group. However, fisetin increased PPARα expression and suppressed FAS expression compared to the HFD group (Fig. 5A).

Fisetin regulated the AMPK/sirt1 pathway and lipolysis in liver tissue

In liver tissue, fisetin significantly enhanced sirt1, pAMPKα, and pACC-1 expression compared to the HFD group (Fig. 5B). In lipolysis, fisetin could also promote ATGL and the phosphorylation of HSL expression compared to the HFD group (Fig. 5C). Fisetin increased CPT-1, but not CPT2, expression in the fatty acid β-oxidation pathway compared to the HFD group (Fig. 5C). Using IHC, we examined stained liver tissue slides to observe the distribution

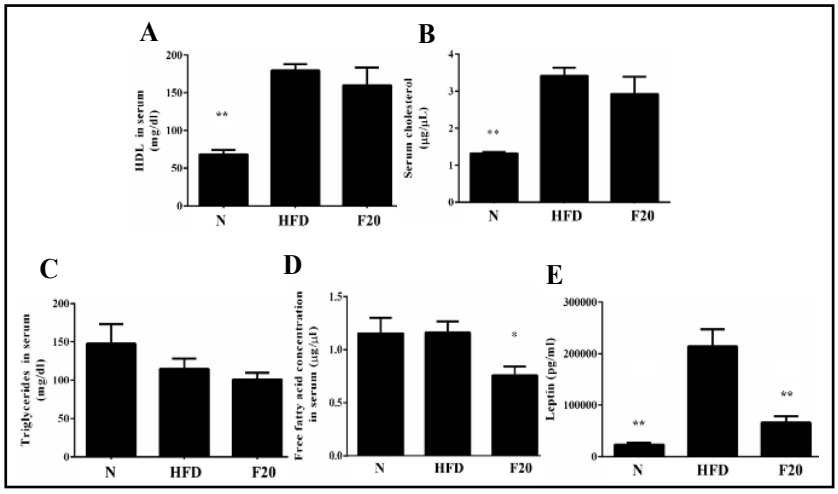

Fig. 4. Effects of fisetin on serum biochemical analysis. (A) HDL, (B) TC, (C) TG, (D) free fatty acids, (E) leptin. Data are presented as mean ± SEM; n = 12. *P<0.05, **P<0.01 compared to mice with HFD-induced obesity.

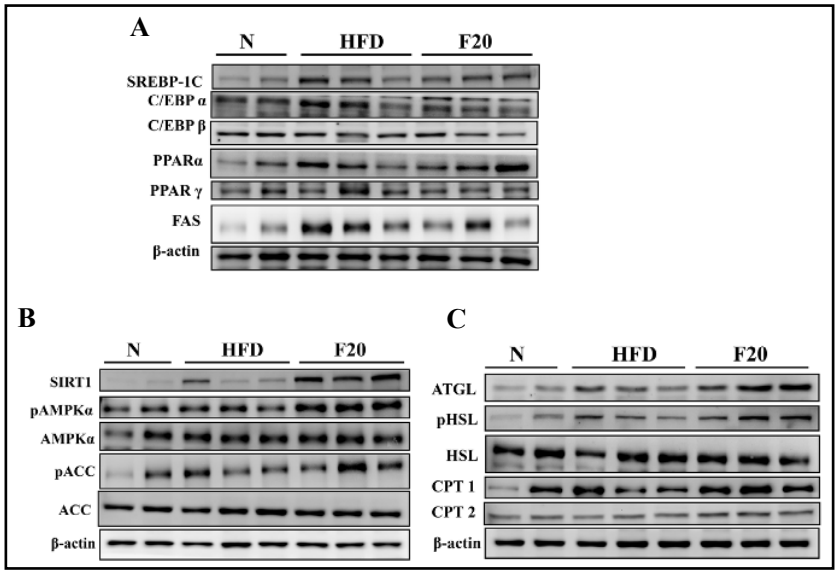

Fig. 5. Effects of fisetin on lipid metabolism in mouse liver tissue. (A) Transcription factors associated with adipogenesis and lipogenesis, (B) the sirt-1/AMPK pathway, and (C) lipolysis and β-oxidation were detected by Western blot. Three independent experiments were analyzed using β-actin as an internal control.

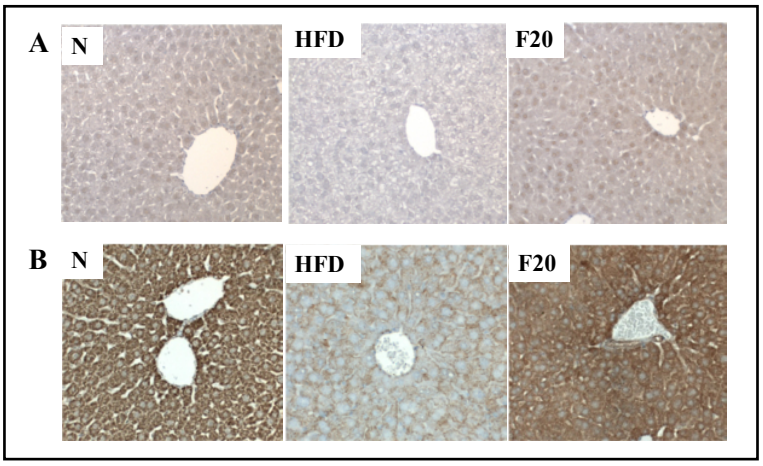

Fig. 6. Fisetin modulated sirt-1 and CPT-1 expression in the liver. (A) sirt1 and (B) CPT-1 expression was analyzed by immunohistochemistry and labeled as a brown color drop. Three independent experiments were analyzed .

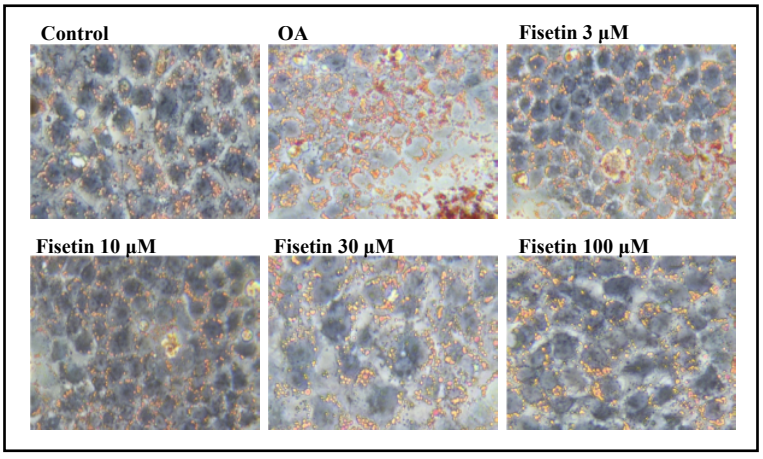

Fig. 7. Fisetin reduced lipid accumulation in FL83B cells. FL83B cells were treated with 0.5 mM oleic acid (OA) at 37°C for 48 h to induce lipid accumulation in hepatocytes, followed by fisetin (3–100 μM) for 24 h. Oil Red O stain shows lipid accumulation. Three independent experiments were analyzed.

of sirt1 and CPT-1 protein, finding a large amount of sirt1 and CPT-1 in the normal group, but sirt1 and CPT-1 was significantly decreased in the liver tissue of HFD mice. Notably, fisetin significantly recovered sirt1 and CPT-1 expression in the liver tissue compared to the HFD group (Fig. 6).

Cell viability of fisetin in FL83B cells

We investigated whether fisetin can modulate lipid metabolism in hepatocytes *in vitro*. First, we used the MTT assay to investigate the cytotoxicity of fisetin in FL83B hepatocytes. We found no cell cytotoxicity at fisetin concentrations ≤100 μM (data not shown), and 3–100 μM fisetin was assayed in all cell experiments.

Fisetin regulated lipid accumulation and lipoperoxidation in FL83B cells

Fisetin alleviated lipid droplets induced by oleic acid treatment (Fig. 7). The fluorescent dye BODIPY 493/503 confirmed that fisetin treatment decreased lipid accumulation (Fig. 8A). Furthermore, BODIPY 581/591 C11 detected hepatic lipoperoxidation, and the immunofluorescent images demonstrated that fisetin significantly decreased lipoperoxidation compared to oleic acid-induced hepatocytes (Fig. 8B). Next, fatty acid uptake was detected using the BODIPY FL C12 fluorescent probe at 0 and 24 h. Oleic acid promoted fatty acid uptake in hepatocytes after 24 h. Fisetin clearly suppressed fatty acid uptake compared to the oleic acid group (Fig. 9).

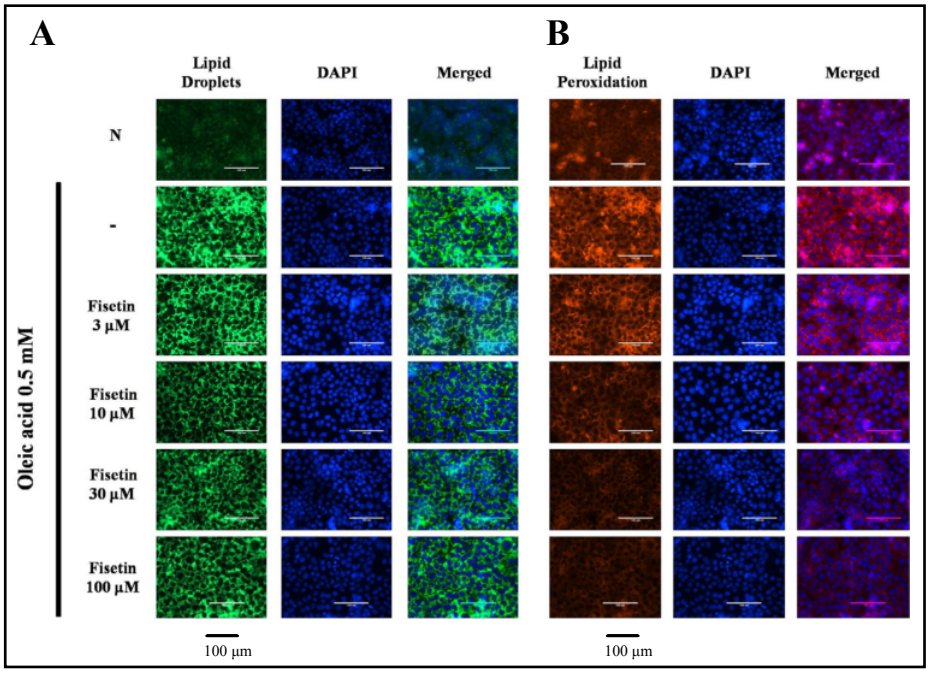

Fig. 8. Fisetin reduced lipid accumulation in FL83B cells. FL83B cells were treated with 0.5 mM oleic acid (OA) at 37°C for 48 h to induce lipid accumulation in hepatocytes, followed by fisetin (3–100 μM) for 24 h. The fluorescent dyes BODIPY 493/503 (green) and BODIPY 581/591 C11 (red) were used to detect hepatic lipid droplets and lipoperoxidation, respectively, under a fluorescent microscope. Three independent experiments were analyzed. Nuclei were stained with DAPI (blue).

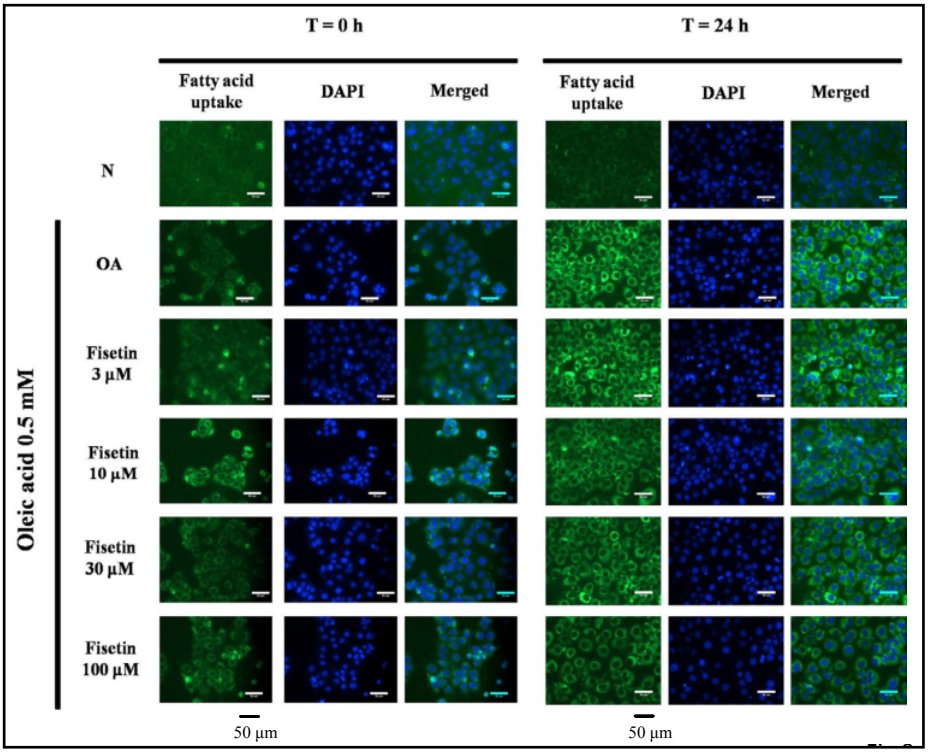

Fig. 9. Fisetin reduced fatty acid uptake into FL83B cells. FL83B cells were treated with 0.5 mM oleic acid (OA) and fisetin (3–30 μM) and observed by fluorescence microscopy. BODIPY FL C12 (green) was used to detect fatty acid uptake by hepatocytes at 0 and 24 h. Three independent experiments were analyzed. Nuclei were stained with DAPI (blue).

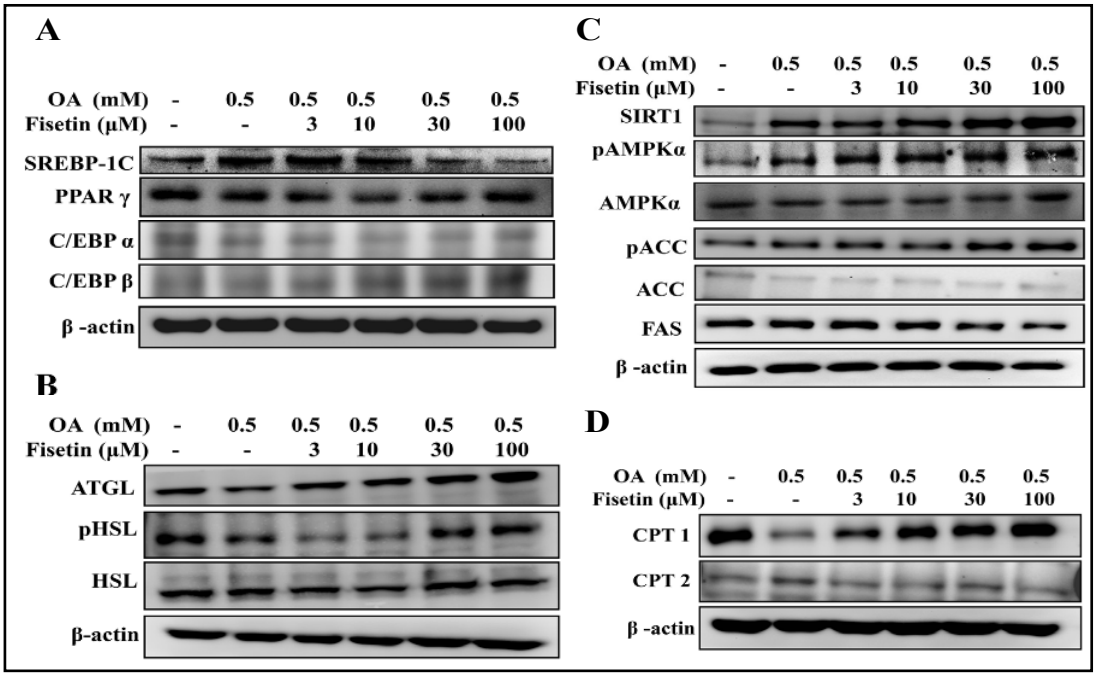

Fig. 10. Effects of fisetin on lipid metabolism in FL83B cells. FL83B cells were treated with 0.5 mM oleic acid (OA) for 48 h to induce lipid accumulation in hepatocytes, followed by fisetin (3–100 μM) 24 h. (A) Transcription factors associated with adipogenesis and lipogenesis proteins, (B) lipolysis, (C) the AMPK/Sirt-1 pathway, and (D) β-oxidation were detected by Western blot. Three independent experiments were analyzed using β-actin as an internal control.

Effect of fisetin on lipid metabolism in hepatocytes

Western blot showed that fisetin reduced SREBP-1c, PPAR-γ, and C/EBPα expression, but not C/EBPβ expression, compared to oleic acid (Fig. 10A). Fisetin also increased ATGL and pHSL expression in a concentration-dependent manner compared to oleic acid (Fig. 10B). Furthermore, fisetin significantly promoted sirt1, phosphorylation of AMPK, and phosphorylation of ACC, and decreased FAS production compared to oleic acid (Fig. 10C). Finally, fisetin increased CPT-1, but not CPT2, expression (Fig. 10D).

Discussion

Obesity is an important factor in many serious chronic diseases, and studies have shown that obesity is a high risk group for hypertension, coronary atherosclerosis, type 2 diabetes, and cancer [1, 37]. Being overweight and obesity do not only increase adipocyte size and hyperplasia, but in these states the liver tissue accumulates excessive lipid droplets, causing hepatic steatosis, the early stage of NAFLD [3, 37]. Therefore, in the hepatic steatosis stage, treating and maintaining liver health and function, or attenuating the deterioration of liver steatosis, would create an opportunity to prevent and attenuate the development of NAFLD. In liver tissue, excessive lipid accumulation would interfere with the normal physiological function of hepatocytes, causing lipid and carbohydrate metabolism abnormalities, liver cell inflammation, peroxidation, and insulin resistance [1, 3]. These symptoms may not only cause diabetes, but can also promote chronic liver fibrosis and liver cancer [2]. A recent study pointed out that new therapy targets of hepatic steatosis mainly block lipid generation and accumulation in the liver, leading to the decomposition of fat oil droplets, enhancing the β-oxidation metabolism of fatty acids, and maintaining mitochondrial function [14, 38, 39].

In recent years, some plant compounds have been found to improve NAFLD [19, 40-42]. In obese mice, resveratrol can improve body weight and reduce hepatic steatosis by

enhancing sirt1 and AMPK activity [38]. Luteolin could attenuate NAFLD by blocking the expression of liver X receptor and SERBP-1c [43]. Quercetin has also been confirmed to alleviate oxidized LDL levels in the liver and decrease hepatic steatosis in HFD-induced obese mice [44]. In this current study, fisetin effectively alleviated body weight and epididymal adipose tissue weight. However, fisetin did not decrease the liver weight. Interestingly, fisetin could suppress lipid accumulation and fat vacuoles in the livers of HFD-fed mice. Fisetin also inhibited lipogenesis, regulated the sirt1/AMPK pathways, and enhanced β-oxidation to reduce the development of hepatic steatosis in HFD-induced obese mice. Therefore, fisetin has the potential to ameliorate obesity-induced NAFLD.

AMPK is an important enzyme in sensing and regulating cellular and whole-body energy balance, and sitr-1 has a regulatory effect on lipid metabolism by activating AMPK expression for the maintenance and regulation of energy metabolism [45]. Phosphorylation of Thr172 in AMPKα is required for kinase activity and enhanced more 100-fold activation [46]. AMPKα activation can inhibit lipid biosynthesis, accelerating the decomposition of lipid to reducing liver fat accumulation [10]. Sirt1 is an NAD-dependent deacetylase and has been reported to have many beneficial effects for controlled glucose homeostasis, lipid metabolism, and insulin resistance in the liver tissue of obese mice [12]. In our current experiment, we found that HFD obese mice treated with fisetin express increased level of sirt1 and pAMPKα in liver tissue, and FL83B hepatocyte experiments demonstrated that fisetin significantly promotes sirt1 and pAMPKα expression *in vitro*. Resveratrol, a sirt1 agonist, could significantly increase sirt1 and pAMPK expression in the liver tissue of NAFLD mice [38]. Resveratrol could reduce total cholesterol and triacylglycerol in serum and reduce liver adipogenesis through increased phosphorylation of ACC to regulate fatty acid synthesis [47]. Our results also demonstrate that fisetin could significantly increase ACC phosphorylation in HFD-induced obese mice and oleic acid-induced hepatocytes *in vitro*. Activated ACC could catalyze acetyl CoA to produce malonyl-CoA for elongated fatty acid chains [24]. Recent research found that phosphorylation of AMPK could stimulate phosphorylation of its substrate, which suppresses ACC phosphorylation [10]. Interestingly, dephosphorylation of ACC is activated and phosphorylation of ACC switched off [24]. Thus, we thought that fisetin could effectively increase sirt1 and pAMPKα expression to block the synthesis of fatty acid chains.

Hepatocytes took up excessive free fatty acids and switched transcription factors on for lipogenesis, causing lipid accumulation, and these free fatty acid would reduce AMPK activity and disturb the energy balance [6, 9]. Previous studies reported that AMPK phosphorylation can suppress the expression of lipogenesis-related transcription factors, including SREBP-1c, PPAR, and C/EBP [48]. In this study, the HFD mice had increased expression of SREBP-1c, C/EBPα, and PPAR-γ, and fisetin significantly suppressed SREBP-1c, C/EBPα and PPAR-γ expression in liver tissue. Similarly, fatty liver cells treated with fisetin exhibited attenuated SREBP-1c, PPAR-γ, and C/EBPα expression *in vitro*. Previous studies confirmed that C/EBPα and C/EBPβ are able to assist adipocyte differentiation and increase lipid accumulation in adipocytes and hepatocytes [49]. However, our results demonstrated that fisetin does not significantly regulate C/EBPβ expression in cellular and animal models. The results confirm that fisetin has the ability to block lipid synthesis in hepatocytes, mainly by blocking the transcription factors SREBP-1c, C/EBPα, and PPAR-γ.

ACC could switch on the synthesis of fatty acid chains and stimulate FAS expression [24]. Therefore, activated AMPK could reduce ACC activity to block FAS production for lipid synthesis. SREBP-1c could bind to the FAS gene promoter to give assist fatty acid chain synthesis in hepatocytes [49]. Obviously, HFD-induced obese mice, liver tissue could significantly FAS expression, and fisetin could reduce FAS production compared to obese mice. We found that fisetin can reduce FAS expression in oleic acid-induced fatty liver cells. Cell and animal experiments confirmed that fisetin has the ability to block FAS expression to reduce lipid synthesis in liver tissue, improving hepatic steatosis.

Another strategy for improving hepatic steatosis is to accelerate lipid metabolism in hepatocytes [50]. Many studies have suggested that a good lifestyle and regular exercise promote liver lipid metabolism and restore normal liver function [39, 51]. In addition,

appropriate diet could regulate the liver lipid metabolism and attenuate the development of NAFLD. Previous studies found that quercetin not only inhibits the differentiation of adipocytes and lipid synthesis, but also enhances the decomposition of lipids in obese mice [44]. Quercetin has also been shown to promote lipolysis in the livers of NAFLD mice. α-Linolenic acid-rich flaxseed oil also increases lipolysis through enhanced ATGL expression, preventing alcoholic hepatic steatosis in mice [16]. Ginsenoside Rb2 also suppresses lipid accumulation in the liver by upregulating ATGL and phosphorylation of HSL in obese mice [52]. In the current study, fisetin markedly increased ATGL and pHSL production in the livers of obese mice and fatty hepatocytes *in vitro*, indicating a direct role for ATGL and HSL in lipolysis in the mouse liver. ATGL can hydrolyze TGs to from diglycerides and release a free fatty acid, and phosphorylation of HSL hydrolyzes diglycerides to from monoglycerides and releases a free fatty acid [53]. Consequently, fisetin is effective at reducing lipid accumulation, improving hepatic steatosis in obese mice.

Excess TGs, which are broken down into excess free fatty acids and released into the blood to interfere with metabolism, stimulate inflammatory responses in the liver and vasculature, deteriorating into insulin resistance, metabolic syndrome, and cardiovascular disease [54, 55]. Therefore, these free fatty acids need to be immediately converted into energy via β-oxidation or the formation of cholic acid through fecal exclusion to reduce the inflammatory response [56, 57]. In β-oxidation, CPT-1 functions to transfer the acyl group of a long-chain fatty acyl-CoA to form acyl carnitines [24]. Studies have shown that AMPK and sirt1 activation can enhance CPT-1 expression, promoting β-oxidation [58]. Resveratrol could increase AMPK activity to inhibit ACC activity, reducing fatty acid synthesis; resveratrol promotes lipolysis and increases β-oxidation, decreasing lipid accumulation in hepatocytes [59]. Celastrol also increases sirt1/AMPK expression, promoting β-oxidation for lipolysis and decreasing inflammatory and oxidative damage in the livers of NAFLD mice [13]. We used IHC to confirm CPT-1 expression in the liver and found that fisetin can significantly recover CPT-1 expression. Therefore, we thought that fisetin could accelerate the decomposition of fatty acids and reduce inflammatory damage. In addition, the decomposition of fatty acids will result in liver cell oxidative damage [25, 37]. Using BODIPY 581/591 C11 fluorescent dye, we found that fisetin ameliorates lipid peroxidation in oleic acid-induced fatty liver cells. Thus, fisetin could accelerate the decomposition of fatty acids and reduce lipid peroxidation causing cell damage.

We found that the caloric intake of both F20 and HFD mice did not differ from the normal mice. However, the F20 and HFD mice had greater food efficiency than the normal mice and the HFD contained more fat energy, which could significantly increase body weight and lead to the accumulation of excess oil droplets, interfering with carbohydrate metabolism for glycogen synthesis. Interestingly, fisetin could significantly decrease the epididymal adipose tissue and body weight. PAS stain demonstrated that fisetin could recover glycogen accumulation in hepatocytes compared to HDF-induced obese mice and has the potential to regulate lipid accumulation and glycogen synthesis to maintain liver metabolic function.

Adipose tissue secretes leptin, which binds to the leptin receptor of hypothalamic neurons to suppress appetite and reduce body weight [60]. Studies have shown that blood levels of leptin are higher in obesity, and reducing leptin levels would improve insulin resistance and metabolic syndrome [61]. Fisetin significantly reduced serum leptin levels, but appetite was not affected. Therefore, whether fisetin regulates insulin sensitivity needs to be studied further.

Our experimental results confirmed that fisetin can inhibit body weight and epididymal adipose tissue weight. Fisetin also significantly suppresses lipid accumulation in the liver tissue of obese mice through activation of the sirt1/AMPK pathway and enhances lipolysis and β-oxidation to ameliorate hepatic steatosis. We conclude that fisetin is an excellent anti-obesity flavonoid for blocking NFLD development.

Acknowledgements

This study was supported in part by grants from the Chang Gung Memorial Hospital (CMRPF1G0231, CMRPF1F0083, and CMRPF1C0201) and from the Ministry of Science and Technology in Taiwan (106-2320-B-255-008-MY3 and 106-2320-B-255-007-MY3).

Designed the experiments: WCH and CJL; Performed the experiments: CHW, YLC, CLW, and CYC; Analysis and interpretation of data: WCH, CHW, and YLC; Drafting the manuscript: WCH and CJL.

Disclosure Statement

The authors have no conflicts of interest to declare.

References

1 Reccia I, Kumar J, Akladios C, Virdis F, Pai M, Habib N, Spalding D: Non-alcoholic fatty liver disease: A sign of systemic disease. Metabolism 2017;72:94-108.
2 Kitade H, Chen G, Ni Y, Ota T: Nonalcoholic fatty liver disease and insulin resistance: new insights and potential new treatments. Nutrients 2017;9:E387.
3 Hazlehurst JM, Woods C, Marjot T, Cobbold JF, Tomlinson JW: Non-alcoholic fatty liver disease and diabetes. Metabolism 2016;65:1096-1108.
4 He XX, Wu XL, Chen RP, Chen C, Liu XG, Wu BJ, Huang ZM: Effectiveness of omega-3 polyunsaturated fatty acids in non-alcoholic fatty liver disease: a meta-analysis of randomized controlled trials. PLoS One 2016;11:e0162368.
5 Tian J, Goldstein JL, Brown MS: Insulin induction of SREBP-1c in rodent liver requires LXRalpha-C/EBPbeta complex. Proc Natl Acad Sci U S A 2016;113:8182-8187.
6 Wang Y, Viscarra J, Kim SJ, Sul HS: Transcriptional regulation of hepatic lipogenesis. Nat Rev Mol Cell Biol 2015;16:678-689.
7 Carling D: The AMP-activated protein kinase cascade--a unifying system for energy control. Trends Biochem Sci 2004;29:18-24.
8 Choi YJ, Lee KY, Jung SH, Kim HS, Shim G, Kim MG, Oh YK, Oh SH, Jun DW, Lee BH: Activation of AMPK by berberine induces hepatic lipid accumulation by upregulation of fatty acid translocase CD36 in mice. Toxicol Appl Pharmacol 2017;316:74-82.
9 Lage R, Dieguez C, Vidal-Puig A, Lopez M: AMPK: a metabolic gauge regulating whole-body energy homeostasis. Trends Mol Med 2008;14:539-549.
10 Lim CT, Kola B, Korbonits M: AMPK as a mediator of hormonal signalling. J Mol Endocrinol 2010;44:87-97.
11 Peng IC, Chen Z, Sun W, Li YS, Marin TL, Hsu PH, Su MI, Cui X, Pan S, Lytle CY, Johnson DA, Blaeser F, Chatila T, Shyy JY: Glucagon regulates ACC activity in adipocytes through the CAMKKbeta/AMPK pathway. Am J Physiol Endocrinol Metab 2012;302:E1560-1568.
12 Chen H, Liu X, Chen H, Cao J, Zhang L, Hu X, Wang J: Role of SIRT1 and AMPK in mesenchymal stem cells differentiation. Ageing Res Rev 2014;13:55-64.
13 Zhang Y, Geng C, Liu X, Li M, Gao M, Fang F, Chang Y: Celastrol ameliorates liver metabolic damage caused by a high-fat diet through Sirt1. Mol Metab 2017;6:138-147.
14 Li M, Sharma A, Yin C, Tan X, Xiao Y: Metformin ameliorates hepatic steatosis and improves the induction of autophagy in HFD induced obese mice. Mol Med Rep 2017;16:680-686.
15 Yang S, Lv Q, Luo T, Liu L, Gao R, Chen S, Ye P, Cheng Q, Li Q: Metformin inhibits expression and secretion of PEDF in adipocyte and hepatocyte via promoting AMPK phosphorylation. Mediators Inflamm 2013;2013:429207.
16 Miotto PM, Horbatuk M, Proudfoot R, Matravadia S, Bakovic M, Chabowski A, Holloway GP: alpha-Linolenic acid supplementation and exercise training reveal independent and additive responses on hepatic lipid accumulation in obese rats. Am J Physiol Endocrinol Metab 2017;312:E461-E470.

17 Rusli F, Boekschoten MV, Zubia AA, Lute C, Muller M, Steegenga WT: A weekly alternating diet between caloric restriction and medium fat protects the liver from fatty liver development in middle-aged C57BL/6J mice. Mol Nutr Food Res 2015;59:533-543.
18 Sabater AG, Ribot J, Priego T, Vazquez I, Frank S, Palou A, Buchwald-Werner S: Consumption of a mango fruit powder protects mice from high-fat induced insulin resistance and hepatic fat accumulation. Cell Physiol Biochem 2017;42:564-578.
19 Huang CZ, Tung YT, Hsia SM, Wu CH, Yen GC: The hepatoprotective effect of *Phyllanthus emblica* L. fruit on high fat diet-induced non-alcoholic fatty liver disease (NAFLD) in SD rats. Food Funct 2017;8:842-850.
20 Chen YJ, Wallig MA, Jeffery EH: Dietary broccoli lessens development of fatty liver and liver cancer in mice given diethylnitrosamine and fed a Western or control diet. J Nutr 2016;146:542-550.
21 Gupta V, Mah XJ, Garcia MC, Antonypillai C, van der Poorten D: Oily fish, coffee and walnuts: Dietary treatment for nonalcoholic fatty liver disease. World J Gastroenterol 2015;21:10621-10635.
22 Li C, Nie SP, Zhu KX, Ding Q, Xiong T, Xie MY: *Lactobacillus plantarum* NCU116 improves liver function, oxidative stress and lipid metabolism in rats with high fat diet induced non-alcoholic fatty liver disease. Food Funct 2014;5:3216-3223.
23 Valenti L, Riso P, Mazzocchi A, Porrini M, Fargion S, Agostoni C: Dietary anthocyanins as nutritional therapy for nonalcoholic fatty liver disease. Oxid Med Cell Longev 2013;2013:145421.
24 Ducharme NA, Bickel PE: Lipid droplets in lipogenesis and lipolysis. Endocrinol 2008;149:942-949.
25 Huang WC, Chang WT, Wu SJ, Xu PY, Ting NC, Liou CJ: Phloretin and phlorizin promote lipolysis and inhibit inflammation in mouse 3T3-L1 cells and in macrophage-adipocyte co-cultures. Mol Nutr Food Res 2013;57:1803-1813.
26 Sak K, Kasemaa K, Everaus H: Potentiation of luteolin cytotoxicity by flavonols fisetin and quercetin in human chronic lymphocytic leukemia cell lines. Food Funct 2016;7:3815-3824.
27 Pal HC, Pearlman RL, Afaq F: Fisetin and its role in chronic diseases. Adv Exp Med Biol 2016;928:213-244.
28 Seo SH, Jeong GS: Fisetin inhibits TNF-alpha-induced inflammatory action and hydrogen peroxide-induced oxidative damage in human keratinocyte HaCaT cells through PI3K/AKT/Nrf-2-mediated heme oxygenase-1 expression. Int Immunopharmacol 2015;29:246-253.
29 Kim SC, Kim YH, Son SW, Moon EY, Pyo S, Um SH: Fisetin induces Sirt1 expression while inhibiting early adipogenesis in 3T3-L1 cells. Biochem Biophys Res Commun 2015;467:638-644.
30 Watanabe M, Hisatake M, Fujimori K: Fisetin suppresses lipid accumulation in mouse adipocytic 3T3-L1 cells by repressing GLUT4-mediated glucose uptake through inhibition of mTOR-C/EBPalpha signaling. J Agric Food Chem 2015;63:4979-4987.
31 Kim DH, Do MS: BAFF knockout improves systemic inflammation via regulating adipose tissue distribution in high-fat diet-induced obesity. Exp Mol Med 2015;47:e129.
32 Melnyk RB, Boshes M: Efficiency of food utilization during body weight gain in dormice (Glis glis). Physiol Behav 1980;24:1017-1021.
33 Liou CJ, Huang YL, Huang WC, Yeh KW, Huang TY, Lin CF: Water extract of *Helminthostachys zeylanica* attenuates LPS-induced acute lung injury in mice by modulating NF-kappaB and MAPK pathways. J Ethnopharmacol 2017;199:30-38.
34 Liou CJ, Huang WC: Casticin inhibits interleukin-1β-induced ICAM-1 and MUC5AC expression by blocking NF-κB, PI3K-Akt, and MAPK signaling in human lung epithelial cells. Oncotarget 2017;8:101175-101188.
35 Huang WC, Fang LW, Liou CJ: Phloretin attenuates allergic airway inflammation and oxidative stress in asthmatic mice. Front Immunol 2017;8:134.
36 Liou CJ, Lai XY, Chen YL, Wang CL, Wei CH, Huang WC: Ginkgolide C Suppresses Adipogenesis in 3T3-L1 Adipocytes via the AMPK Signaling Pathway. Evid Based Complement Alternat Med 2015;2015:298635.
37 Heindel JJ, Newbold R, Schug TT: Endocrine disruptors and obesity. Nat Rev Endocrinol 2015;11:653-661.
38 Tian Y, Ma J, Wang W, Zhang L, Xu J, Wang K, Li D: Resveratrol supplement inhibited the NF-kappaB inflammation pathway through activating AMPKalpha-SIRT1 pathway in mice with fatty liver. Mol Cell Biochem 2016;422:75-84.
39 Romero-Gomez M, Zelber-Sagi S, Trenell M: Treatment of NAFLD with diet, physical activity and exercise. J Hepatol 2017; 67:829-846.
40 Feng X, Yu W, Li X, Zhou F, Zhang W, Shen Q, Li J, Zhang C, Shen P: Apigenin, a modulator of PPARgamma, attenuates HFD-induced NAFLD by regulating hepatocyte lipid metabolism and oxidative stress via Nrf2 activation. Biochem Pharmacol 2017;136:136-149.

41 Wu L, Yan M, Jiang J, He B, Hong W, Chen Z: Pure total flavonoids from citrus improve non-alcoholic fatty liver disease by regulating TLR/CCL signaling pathway: A preliminary high-throughput 'omics' study. Biomed Pharmacother 2017;93:316-326.
42 Gu M, Zhao P, Huang J, Zhao Y, Wang Y, Li Y, Fan S, Ma YM, Tong Q, Yang L, Ji G, Huang C: Silymarin ameliorates metabolic dysfunction associated with diet-induced obesity via activation of farnesyl X Receptor. Front Pharmacol 2016;7:345.
43 Yin Y, Gao L, Lin H, Wu Y, Han X, Zhu Y, Li J: Luteolin improves non-alcoholic fatty liver disease in db/db mice by inhibition of liver X receptor activation to down-regulate expression of sterol regulatory element binding protein 1c. Biochem Biophys Res Commun 2017;482:720-726.
44 Liu L, Gao C, Yao P, Gong Z: Quercetin alleviates high-fat diet-induced oxidized low-density lipoprotein accumulation in the liver: implication for autophagy regulation. Biomed Res Int 2015;2015:607531.
45 Wang Z, Pini M, Yao T, Zhou Z, Sun C, Fantuzzi G, Song Z: Homocysteine suppresses lipolysis in adipocytes by activating the AMPK pathway. Am J Physiol Endocrinol Metab 2011;301:E703-712.
46 Ross FA, Hawley SA, Auciello FR, Gowans GJ, Atrih A, Lamont DJ, Hardie DG: Mechanisms of paradoxical activation of AMPK by the kinase inhibitors SU6656 and sorafenib. Cell Chem Biol 2017; 24:813-824.
47 Ji G, Wang Y, Deng Y, Li X, Jiang Z: Resveratrol ameliorates hepatic steatosis and inflammation in methionine/choline-deficient diet-induced steatohepatitis through regulating autophagy. Lipids Health Dis 2015;14:134.
48 Gaidhu MP, Fediuc S, Anthony NM, So M, Mirpourian M, Perry RL, Ceddia RB: Prolonged AICAR-induced AMP-kinase activation promotes energy dissipation in white adipocytes: novel mechanisms integrating HSL and ATGL. J Lipid Res 2009;50:704-715.
49 Mota de Sa P, Richard AJ, Hang H, Stephens JM: Transcriptional regulation of adipogenesis. Compr Physiol 2017;7:635-674.
50 Fu A, Shi X, Zhang H, Fu B: Mitotherapy for fatty liver by intravenous administration of exogenous mitochondria in male mice. Front Pharmacol 2017;8:241.
51 Gibson PS, Lang S, Dhawan A, Fitzpatrick E, Blumfield ML, Truby H, Hart KH, Moore JB: Systematic review: Nutrition and physical activity in the management of paediatric non-alcoholic fatty liver disease. Aliment Pharmacol Ther 2014;40:1155-1170.
52 Huang Q, Wang T, Yang L, Wang HY: Ginsenoside Rb2 alleviates hepatic lipid accumulation by restoring autophagy via induction of Sirt1 and activation of AMPK. Int J Mol Sci 2017;18:E1063.
53 Frühbeck G, Méndez-Giménez L, Fernández-Formoso JA, Fernández S, Rodríguez A: Regulation of adipocyte lipolysis. Nutr Res Rev 2014;27:63-93.
54 Neuschwander-Tetri BA: Non-alcoholic fatty liver disease. BMC Med 2017;15:45.
55 Geeraerts X, Bolli E, Fendt SM, Van Ginderachter JA: Macrophage metabolism as therapeutic target for cancer, atherosclerosis, and obesity. Front Immunol 2017;8:289.
56 Ali AT, Hochfeld WE, Myburgh R, Pepper MS: Adipocyte and adipogenesis. Eur J Cell Biol 2013;92:229-236.
57 Lumeng CN, Saltiel AR: Inflammatory links between obesity and metabolic disease. J Clin Invest 2011;121:2111-2117.
58 Szkudelski T, Szkudelska K: Effects of AMPK activation on lipolysis in primary rat adipocytes: studies at different glucose concentrations. Arch Physiol Biochem 2017;123:43-49.
59 Elgebaly A, Radwan IA, AboElnas MM, Ibrahim HH, Eltoomy MF, Atta AA, Mesalam HA, Sayed AA, Othman AA: Resveratrol supplementation in patients with non-alcoholic fatty liver disease: systematic review and meta-analysis. J Gastrointestin Liver Dis 2017;26:59-67.
60 Coppari R, Bjorbaek C: Leptin revisited: its mechanism of action and potential for treating diabetes. Nat Rev Drug Discov 2012;11:692-708.
61 Cui H, Lopez M, Rahmouni K: The cellular and molecular bases of leptin and ghrelin resistance in obesity. Nat Rev Endocrinol 2017;13:338-351.