

Pachymic acid modulates sirtuin 6 activity to alleviate lipid metabolism disorders

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Abstract. Pachymic acid (Pac), a major bioactive constituent of *Poria cocos*, is an antioxidant that inhibits triglyceride (TG) accumulation. To the best of our knowledge, the present study investigated for the first time whether Pac activated sirtuin 6 (SIRT6) signaling to alleviate oleic acid (OA)-palmitic acid (PA)-induced lipid metabolism disorders in mouse primary hepatocytes (MPHs). In the present study, MPHs challenged with Pac were used to test the effects of Pac on intracellular lipid metabolism. Molecular docking studies were performed to explore the potential targets of Pac in defending against lipid deposition. MPHs isolated from liver-specific SIRT6-deficient mice were subjected to OA + PA incubation and treated with Pac to determine the function and detailed mechanism. It was revealed that Pac activated SIRT6 by increasing its expression and deacetylase activity. Pa prevented OA + PA-induced lipid deposition in MPHs in a dose-dependent manner. Pac (50 μ M) administration significantly reduced TG accumulation and increased fatty acid oxidation rate in OA + PA-incubated MPHs. Meanwhile, as per the results of molecular docking and relative mRNA levels, Pac activated SIRT6 and increased SIRT6 deacetylation levels. Furthermore, SIRT6 deletions in MPHs abolished the protective effects of Pac

against OA + PA-induced hepatocyte lipid metabolism disorders. The present study demonstrated that Pac alleviates OA + PA-induced hepatocyte lipid metabolism disorders by activating SIRT6 signaling. Overall, SIRT6 signaling increases oxidative stress burden and promotes hepatocyte lipolysis.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as acquired metabolic stress-induced liver injury, characterized by increased hepatic lipid accumulation in the absence of excessive alcohol consumption (1). According to the degree of pathological changes, NAFLD can be divided into simple fatty liver, non-alcoholic steatohepatitis (NASH), and NASH-associated cirrhosis.

The homeostasis of lipid metabolism in the liver plays a major role in the development of NAFLD. The homeostasis of intrahepatic lipid levels mainly depends on the balance between the synthesis of intrahepatic triglycerides (TGs) and β -oxidation of fatty acids (2,3). Glucose and free fatty acids increase as a result of overnutrition and reduced physical activity, causing overload in the liver and other organs of the human body. In turn, this overload causes oxidative stress and inflammation, thus accelerating the process of NAFLD (4,5). Fatty acid β -oxidation is the main pathway of lipid metabolism. Peroxisome proliferator-activated receptors (PPAR- α , PPAR- β/δ , and PPAR- γ) are members of the nuclear receptor superfamily, acting as ligand-inducible transcription factors that play crucial roles in β -oxidation (6-8). In the present study, fatty acid catabolism was impaired following the deletion of PPAR- α in hepatocytes, resulting in hepatic lipid accumulation in a mouse model (9). Therefore, improving β -oxidation (by upregulating PPAR- α) may be beneficial in ameliorating the effects of NASH. In addition, oxidative stress can induce inflammation in NAFLD and regulate the expression of tumor necrosis factor-alpha and interleukin-6, thus advancing the development of hepatitis (10). Nuclear factor erythroid

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2-related factor 2 (Nrf2) has an important role in anti-oxidant stress; during oxidative stress, Nrf2 dissociates from kelch-like ECH-associated protein 1 and translocates into the nucleus to increase the expression of anti-oxidant genes and improve damage caused unto the liver by oxidative stress (11,12). Many studies have confirmed that in NAFLD models, the regulation of Nrf2 pathway activation is an important mechanism that plays a role in liver protection.

SIRT6 is an NAD⁺-dependent class III deacetylase that is characterized by its unique chromatin location (13). In the liver, SIRT6 potentially interacts with PPAR- α and Nrf2 to maintain fatty acid oxidation rate and improve oxidative stress during high-fat diet feeding (14). Furthermore, SIRT6 is a histone H3 lysine 9 (H3K9) deacetylase on the promoters of many genes and plays an essential role in glycolysis and lipid metabolism (15,16). Hepatocyte-specific SIRT6 deletion predisposes mice to NASH fatty liver; similarly, in humans with fatty liver, SIRT6 levels are lower than those with a normal liver (15). In conclusion, SIRT6 plays a critical role in fat metabolism and may serve as a therapeutic target for treating fatty liver disease (17).

Pachymic acid (Pac), an active compound isolated from *Poria cocos*, is a traditional Chinese herbal drug used to fortify the spleen and alleviate edema. Pac has remarkable effects on phlegm and fluid retention in the body, as seen in metabolic diseases including NAFLD and obesity (18,19). Previously, Pac was reported to have antitumor, anti-inflammatory, antioxidant, and hypoglycemic effects (20-22). A 2019 study reported that *Poria cocos* protected mice from hepatic steatosis by inhibiting TG accumulation (23). Although *Poria cocos* acid exhibits antioxidant and anti-lipid accumulation characteristics in many cell models, few studies on the anti-NAFLD activity of Pac have been performed. Our study identified, for the first time, that Pac can activate SIRT6 and protect mouse primary hepatocytes (MPHs) against oleic acid (OA)-palmitic acid (PA)-induced NAFLD, via SIRT6/PPAR- α and SIRT6/Nrf2 pathways.

Materials and methods

Materials. Pachymic acid (purity >97%) was purchased from Yuanye Biological Technology Co. Ltd. (Shanghai, China).

Source of animals. Male C57BL/6J mice (6-8 weeks) were purchased from the Model Animal Research Center of Guangzhou University of Chinese Medicine. Hepatocytes SIRT6 deficiency mice (6-8 weeks) were kindly provided by Professor Jinhan He (Department of Pharmacy, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, China) and Professor Yongsheng Chang (National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China) and used as previously described (24).

Cell protocols. Male C57BL/6J mice and hepatocytes SIRT6 deficiency mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium 80 mg/kg and mouse hepatocytes (MPHs) were isolated and cultured in a RPMI-1640 medium, as previously described (7). Briefly, sterilize the body surface of mice with 75% ethanol, cut the skin, open the abdominal

cavity, separate the inferior vena cava, place a catheter at the distal end of the inferior vena cava, inject 1-2 ml of heparin immediately after the blood return, and then inject 50 ml of perfusion solution I (1,000 ml Krebs Ringer with Glucose + 2 ml 50 mM EGTA), after the injection of heparin, and cut the open vein to bleed, and rapidly perfusion for about 3 min clarify the blood of the mouse was removed by this procedure. During this period, 0.015 g collagenase was dissolved in 30 ml of perfusate II (1,000 ml Krebs Ringer with Glucose + 1372 μ l 2 M CaCl₂), mixed well and maintained at 37°C. Then perfusion liquid II was infused, and the perfusion was stopped at a slow speed for about 6 min until the liver collapsed and cracked. The whole liver was removed and placed in a culture dish. After adding 20 ml of basic culture medium, it was transferred to the ultraclean table for operation. Tear the liver, filter it with a 70 μ m cell net, collect the filtrate, 800 rpm/min, centrifuge for 3 min, and discard the supernatant. Then, Freshly prepared MPHs were suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum, and plated in 6-well culture plates at 0.5x10⁶ cells/well. After attachment, MPHs were washed with PBS, and media was replaced with RPMI-1640 medium supplemented with 10% fetal bovine serum and penicillin-streptomycin. Then, MPHs were exposed to 200 μ M OA and 100 μ M PA (OA&PA), and OA&PA with different densities of Pac.

CCK-8 assay. MPHs were seeded in 96 well plates, where five wells were repeated. After culturing the MPHs in a cell incubator for 12 h, the cells were placed in a new medium, containing Pac. Blank and control wells were incubated for 24 h after administration of the Pac medium. The medium was aspirated, added, and incubated with the pre-mixed medium containing CCK-8 (100 ml 1640 medium and 10 ml CCK-8 solution), and the OD value was then measured at 450 nm, using a microplate reader.

Biochemistry analysis. MPHs were harvested after a 24-h incubation and assayed for triglycerides (TG) and total cholesterol (TC) levels using the commercially available enzymatic assay kits (Jiancheng Co.) according to the manufacturer's instructions.

Oil Red O Staining. The MPHs were stained with Oil Red O to determine their differentiation. After washing with phosphate buffered saline (PBS) and fixing with 4% paraformaldehyde, for 30 min, MPHs were then washed twice with PBS, stained with 60% saturated Oil Red O for 10-15 min, and washed with 60% isopropanol. Finally, adipocytes were imaged using a light microscope (Nikon x200).

Reactive oxygen species (ROS) analysis. For Cellular ROS determination, Cells were seeded in 12-well plates and treated as above, followed by incubation with DHE 5 μ M (KeyGEN Co.) for 30 min at 37°C in the dark. Then, cells were washed with PBS for three times and visualized under a fluorescence microscope (Nikon x200).

Hepatic lipid accumulation and lipoperoxidation. MPHs were seeded in six-well plates and treated as described above. MPHs were washed by a 1640 basic medium and stained with

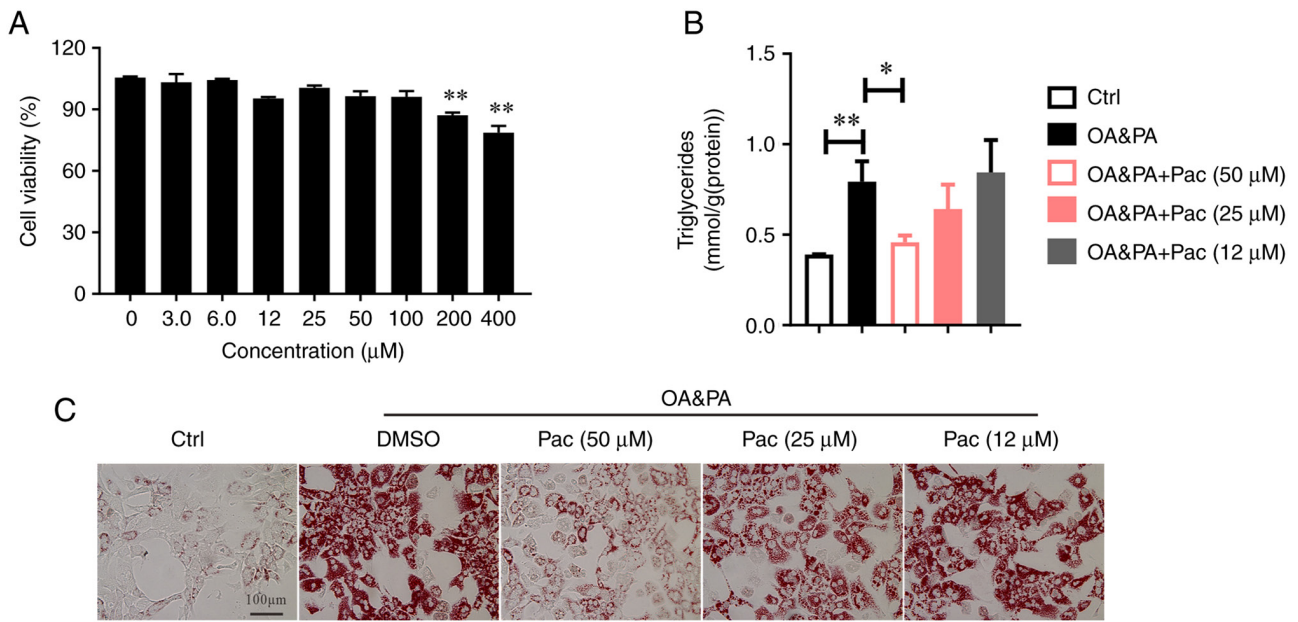


Figure 1. Pac accelerates lipid catabolism in mice primary hepatocytes. (A) Cell Counting Kit-8 analysis; (B) hepatic triglycerides levels; (C) Oil Red O-stained mouse primary hepatocytes (magnification, x200). Data are presented as means \pm SEM; n=3-4. *P<0.05, **P<0.01. OA, oleic acid; PA, palmitic acid; Pac, pachymic acid.

BODIPY 581/591 C11 and BODIPY 493/503 (Invitrogen; Thermo Fisher Scientific, Inc.) to investigate hepatic lipid accumulation and lipoperoxidation, respectively. Cell nuclei were stained with DAPI and visualized using fluorescence microscopy (Leica x200).

Quantitative Real-Time PCR Analysis. Total mRNA of liver tissues or MPHs was extracted with a TRIzol reagent. Reverse transcription was performed using a high-capacity cDNA reverse-transcription kit (Applied Biological Materials Inc.). cDNA was subjected to qPCR analysis with the PowerUp™ SYBRTM Green Master Mix (Abclonal Co.). All genes expression were standardized with β -actin and specific primer sequences were shown in Table SI.

Western blot analysis. Total protein and nuclear protein were extracted from cultured cells according to the manufacturer's instruction (Beyotime Co.). The concentrations were determined by BCA assay kit (Beyotime Co.). In total, equal amounts of the protein (20-60 μ g) were fractionated by 10% SDS-polyacrylamide gel, and separated proteins were transferred onto PVDF membranes. The membranes were incubated overnight at 4°C with various primary antibodies including anti-SIRT6 (13572-1-AP, Proteintech), anti-PPAR α (A18252, Abclonal), anti-CPT1a (A20746, Abclonal), anti-H3K9ac (A7256, Abclonal), anti-H3K56ac (A2391, Abclonal), anti-Nrf2 (16396-1-AP, Proteintech), anti-SOD2 (A19576, Abclonal), anti-HO-1(A19062, Abclonal), and anti- β -ACTIN (Ac026, Abclonal) and followed by an incubation with a secondary antibody. Finally, the blots were observed using BIO-RAD Gel Doc XR from Science and Technology Innovation Center of Guangzhou University of Chinese Medicine.

Molecular Modeling and Docking Study. The processing and optimization of molecular docking is completed by Glide

module in Schrödinger Maestro software. Protein processing uses the SIRT6 Preparation Wizard module (25,26). The Pac structure was downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) and optimized using the molecular mechanics program Minimize to get the most stable structure. The three-dimensional crystal structure of the SIRT6 protein was downloaded from the RCSB PDB database (<https://www.rcsb.org/structure/5MF6>). Preconditioning, optimization and minimization of receptors (constraint minimization using OPLS3e force field). The compound structure is prepared according to the default settings of the LigPrep module. When screening in Glide module, the prepared receptor is introduced. According to the protein structure original ligand as the docking site (x=115.08, y=26.49, z=-22.42), the docking box is set to 20x20x20Å. Finally, molecular docking and screening were carried out by standard precision (SP) method.

Statistical analysis. The data are analyzed using GraphPad Prism (Version 8.0) and presented as means \pm SEM. Statistical analysis was performed using the one-way analysis of variance followed by post hoc Tukey test for comparisons. Value of P<0.05 was considered statistical significance.

Results

Pac accelerated lipid catabolism and alleviated lipid peroxidation in MPHs. To investigate the role of Pac in abnormal lipid metabolism, OA&PA and Pac-incubated MPHs were used. According to our hypothesis, Pac displays lower cytotoxicity (Fig. 1A). In addition, Pac treatment effectively reduced intracellular TG levels in OA&PA-incubated MPHs, in a dose-dependent manner (P<0.05, Fig. 1B). Subsequent Oil Red O staining revealed decreased lipid deposition in OA&PA-incubated MPHs, following treatment with Pac (Fig. 1C).

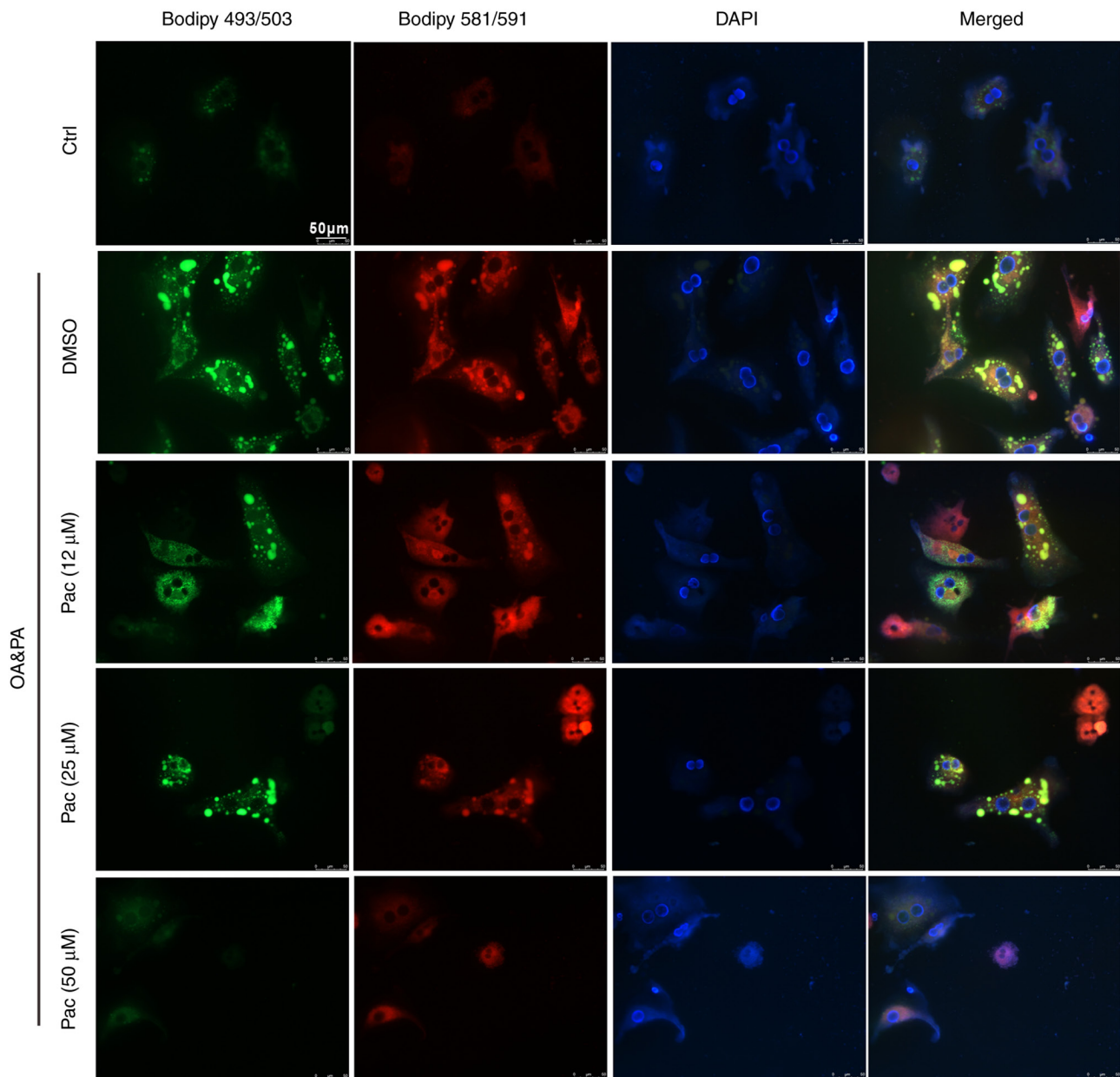


Figure 2. Pac treatment reduces hepatic lipid accumulation and lipoperoxidation in OA + PA incubated MPHs. Bodipy 493/503 and Bodipy 581/591-stained MPHs (magnification, x200). MPHs, mouse primary hepatocytes; OA, oleic acid; PA, palmitic acid; pac, pachymic acid.

Accumulating evidence indicates that oxidative stress damages the structure of cell membranes and leads to cell death-important mechanisms in the development of NAFLD (27). Thus, BODIPY 581/591 C11 and BODIPY 493/503 staining were used to test the levels of lipid accumulation and lipoperoxidation in OA&PA and Pac-incubated MPHs. Treatment with Pac decreased intracellular oxidative stress (due to lipid deposition) in a dose-dependent manner (Fig. 2). Collectively, these data suggest a potential role of Pac in promoting lipid metabolism.

Pac accelerates lipid catabolism by upregulating β -oxidation. Mechanistic studies have revealed that free fatty acids enter hepatocytes and provide energy through mitochondrial β -oxidation, or TG formation stored in hepatocytes. As NAFLD progresses, the β -oxidation of fatty acids is inhibited, leading

to excessive lipid accumulation in hepatocyte (4). Further mechanistic analyses showed that administration of Pac (50 μ M) significantly increased the fatty acid oxidation rate in OA&PA-incubated MPHs ($P < 0.01$, Fig. 3B), while lipogenesis was only slightly affected by this administration (Fig. 3A). Pac treatment significantly upregulated the genes involved in fatty acid oxidation, along with a slight suppression of lipogenic genes (Fig. 3C), which led to decreased lipid deposition in OA&PA-incubated MPHs ($P < 0.05$, $P < 0.01$, Fig. 3D). Western blot analyses also indicated that Pac treatment increased the expression of PPAR- α and Cpt1a (Fig. 3E-F). Overall, these data suggest that Pac functions as a potent positive regulator of fatty acid oxidation and hepatic steatosis.

Pac can activate SIRT6. Previous studies have shown that SIRT6 can improve the β -oxidation of fatty acids by activating

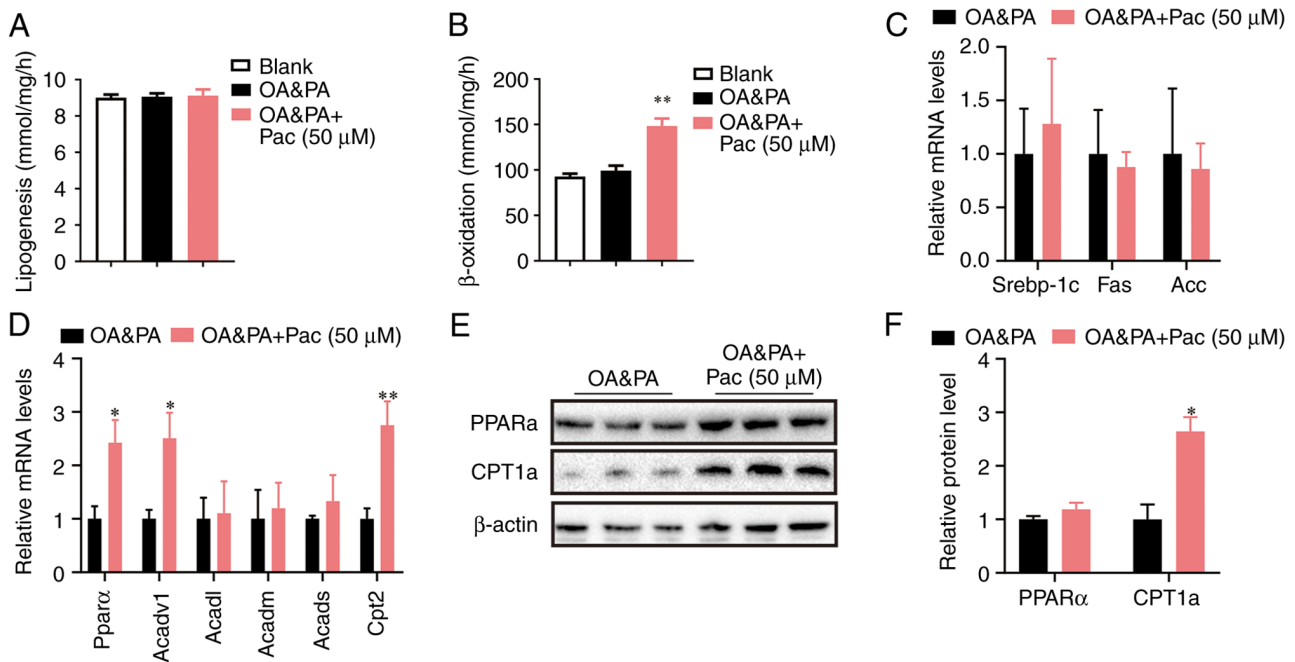


Figure 3. Pac accelerates lipid catabolism by upregulating β -oxidation. (A) Lipogenesis in MPHs; (B) fatty acid oxidation rate in MPHs; (C) relative expression of lipogenesis and of (D) lipogenesis fatty acid β -oxidation; (E) Western blotting and (F) quantification of hepatic PPAR- α and CPT1a protein in MPHs. Data are presented as means \pm SEM; n=3-4. *P<0.05, **P<0.01 vs. OA + PA. MPHs, mouse primary hepatocytes; OA, oleic acid; PA, palmitic acid; pac, pachymic acid; srebp-1c, sterol regulatory element-binding transcription factor 1; Acadvl, acyl-CoA dehydrogenase very long chain; Acadm, acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain; Acads, Acyl-CoA dehydrogenase, C-2 to C-3 short chain; Cpt2, carnitine palmitoyltransferase II; CPT1a, carnitine palmitoyltransferase 1.

PPAR- α and also inhibit liver damage caused by ROS, where these effects are observed when SIRT6-Nrf2 interactions increase (14,28). To verify whether SIRT6 plays an important role in lipid accumulation in MPHs treated with Pac, we conducted docking analyses between Pac and SIRT6. Our data indicated notable binding affinity via hydrophobic interactions (Fig. 4A). We further performed qPCR analysis to confirm that Pac increased SIRT6 expression *in vitro* (P<0.05, Fig. 4B).

SIRT6 normally functions as a transcriptional repressor by deacetylating H3K9 and H3K56 on histones that bind to gene promoters. Therefore, we tested the expression of H3K9 and H3K56 in OA&PA and Pac-cultured MPHs-with or without Pac treatment. As expected, Pac increased the deacetylase activity of SIRT6 by inhibiting H3K9 and H3K56 expression (P<0.05, P<0.01, Fig. 4C-D). Overall, these data indicate that Pac can potentially activate SIRT6 by altering its expression and enzyme activity. This interaction may increase PPAR- α to mediate fatty acid oxidation and increase Nrf2, thereby reducing lipid peroxidation.

Pac failed to alter OA&PA induced lipid deposition in Sirt6-deficient MPHs. To further confirm the effects of SIRT6 in regulating Pac-induced therapeutic effects, MPHs isolated from liver-specific SIRT6-deficient mice were cultured in an OA&PA containing medium for 24 h, followed by co-treatment with Pac (50 μ M) for another 24 h. Interestingly, Pac failed to reduce TG content in MPHs with a SIRT6-deficient state (P<0.05, Fig. 5A). Moreover, SIRT6 deficiency abrogated Pac-induced therapeutic effects on lipid accumulation, as shown by Oil Red O staining and BODIPY 581/591 C11 and BODIPY 493/503 staining in MPHs (Figs. 5B and 6). These

data suggest that the effects of Pac are SIRT6-dependent during OA&PA-induced cellular damage in MPHs.

Pac alleviated OA&PA induced oxidative stress dependent on Sirt6 in MPHs. Multiple studies have indicated that long-term, high-fat diet exposure can lead to oxidative stress and ROS overproduction (29). Therefore, we investigated the effects of Pac on OA&PA-induced oxidative stress. Pac treatment significantly increased protein levels associated with antioxidant activity (including that of SOD2, HO1, and NRF2) in OA&PA-incubated MPHs derived from wild-type mice (P<0.05, Fig. 7A and B). Meanwhile, Pac treatment also reduced ROS levels in OA&PA and Pac-incubated MPHs, however, in MPHs derived from SIRT6-deficient mice (KO) Pac treatment did not alter ROS levels (Fig. 7C). Moreover, Pac failed to reduce the expression of NRF2, SOD2 and HO1 in SIRT6-deficient MPHs (Fig. 7D). Overall, these data suggest that Pac counteracts OA&PA-induced oxidative stress in MPHs, via the activation of SIRT6.

Discussion

In the current study, we demonstrated that Pac attenuated OA&PA-induced lipid accumulation and oxidative stress by upregulating SIRT6/PPAR- α and SIRT6/Nrf2 pathways. We first found that Pac has a protective effect on liver TG accumulation in the models of MPHs cells with OA&PA treatment. Furthermore, we found that the inhibitory effect of Pac on hepatocyte TG accumulation was related to β -oxidation of fatty acids rather than inhibition of lipogenesis. Pac can also inhibit oxidative stress by increasing the expression of antioxidant

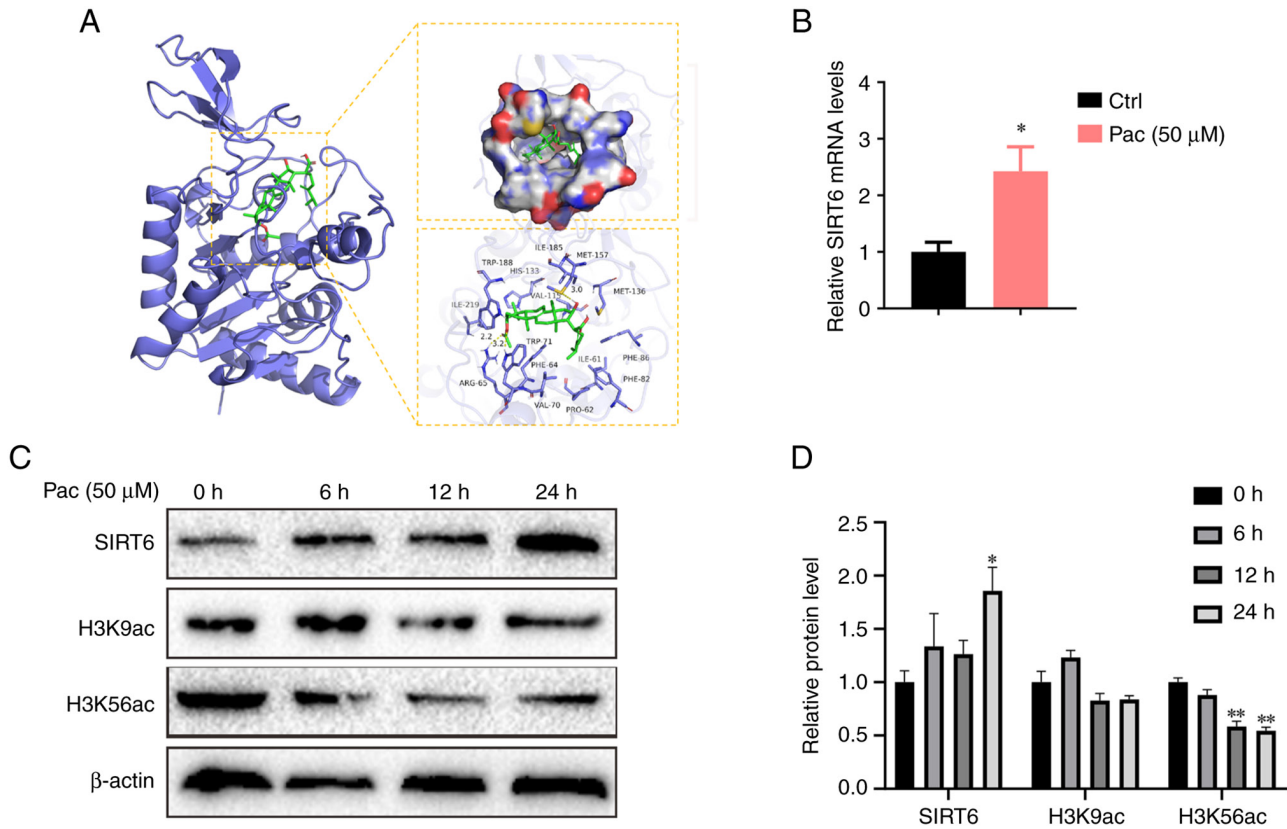


Figure 4. Pac can activate SIRT6. (A) Molecular docking model of PA and SIRT6; (B) hepatic SIRT6 expression *in vitro*; (C) western blotting and (D) quantification of SIRT6 enzymatic activity. Data are presented as means \pm SEM; n=3-4. *P<0.05 vs. ctrl or 0 h and **P<0.01. SIRT6, sirtuin 6; pac, pachymic acid; PA, palmitic acid; H3K9ac, histone H3 N ϵ -acetyl-lysines 9; H3K56ac, histone H3 N ϵ -acetyl-lysines 56.

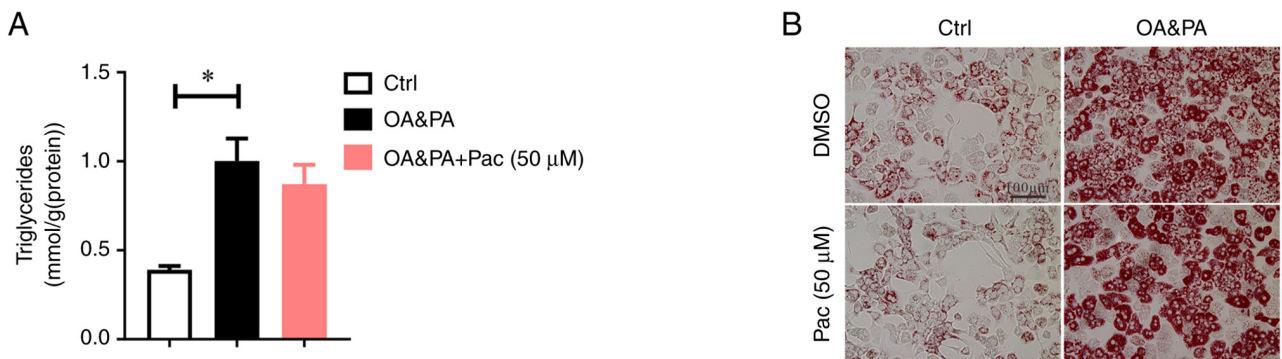


Figure 5. Pac fails to alter OA + PA-induced lipid deposition in SIRT6-deficient MPHs. (A) Hepatic TG levels; (B) Oil Red O-stained MPHs (magnification, \times 200). Data are presented means \pm SEM; n=3-4. *P<0.05. MPHs, mouse primary hepatocytes; OA, oleic acid; PA, palmitic acid; pac, pachymic acid; SIRT6, sirtuin 6.

genes, such as NRF2, HO1, and SOD2. In addition, docking analyses indicated notable binding affinity between Pac and SIRT6, via hydrophobic interactions. To further confirm the effects of SIRT6 in regulating Pac-induced therapeutic effects, MPHs isolated from liver-specific SIRT6-deficient mice were cultured in an alcohol-containing medium. Interestingly, SIRT6 deficiency can abrogate Pac-induced therapeutic effects on lipid accumulation in hepatocellular carcinoma. In addition, in our study, the relieving effect of Pac on oxidative stress (due to lipid accumulation) was abolished in SIRT6-deficient MPHs. Collectively, Pac can alleviate lipid accumulation and oxidative stress in hepatocellular cells through SIRT6/PPAR- α

and SIRT6/Nrf2 pathways, proving that Pac may be a promising agent for the treatment of OA-induced lipid metabolism disorders.

Under the influence of OA&PA, the inhibition of mitochondrial β -oxidation and generation of oxidative stress (caused by lipid accumulation) aggravates lipid metabolism disorders in MPHs, forming a loop that contributes to the main pathogenesis of NAFLD. Recent studies have proposed that the 'multiple hit model', which includes insulin resistance, hormone secretion from fat tissue, nutritional factors, altered intestinal flora along with genetic and epigenetic factors, may lead to NAFLD (30,31). The multiple hit model indicates that

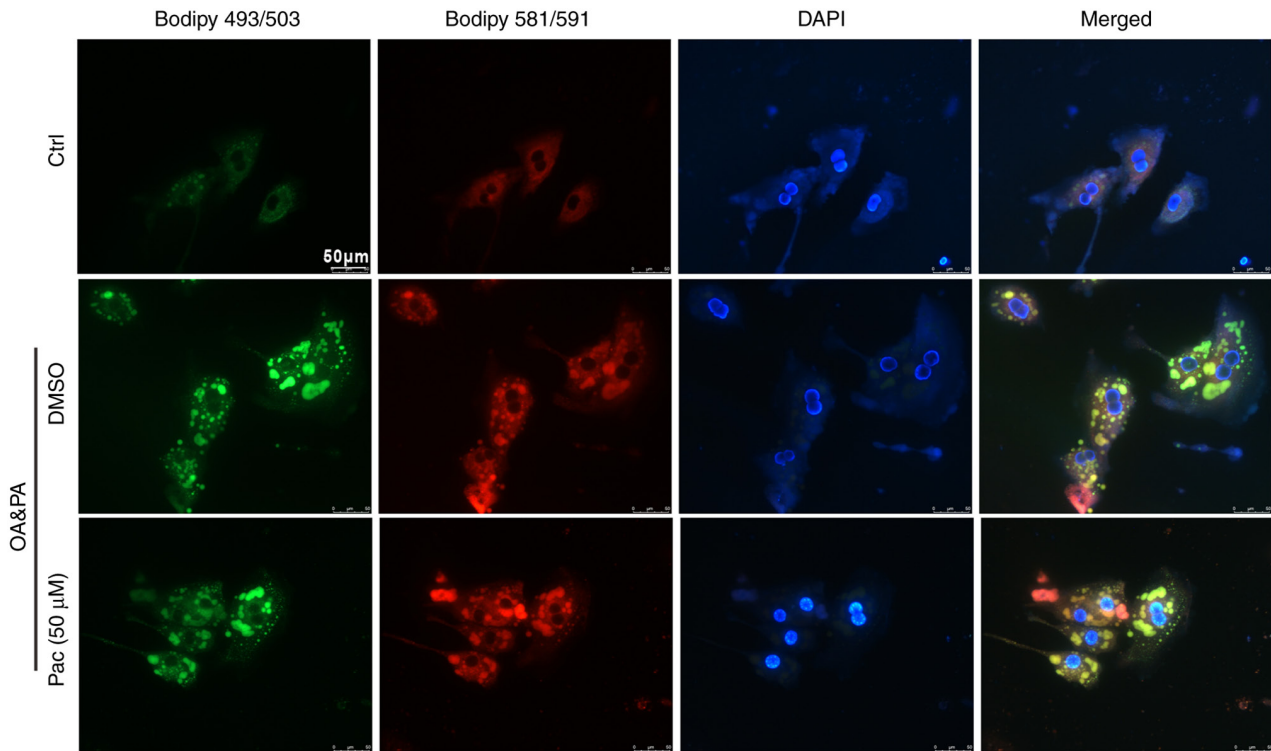


Figure 6. Pac fails to alter hepatic lipid accumulation and lipoperoxidation of SIRT6-deficient MPHs, after OA + PA treatment. Bodipy 493/503 and Bodipy 581/591-stained MPHs (magnification, x200). MPHs, mouse primary hepatocytes; OA, oleic acid; PA, palmitic acid; pac, pachymic acid; SIRT6, sirtuin 6.

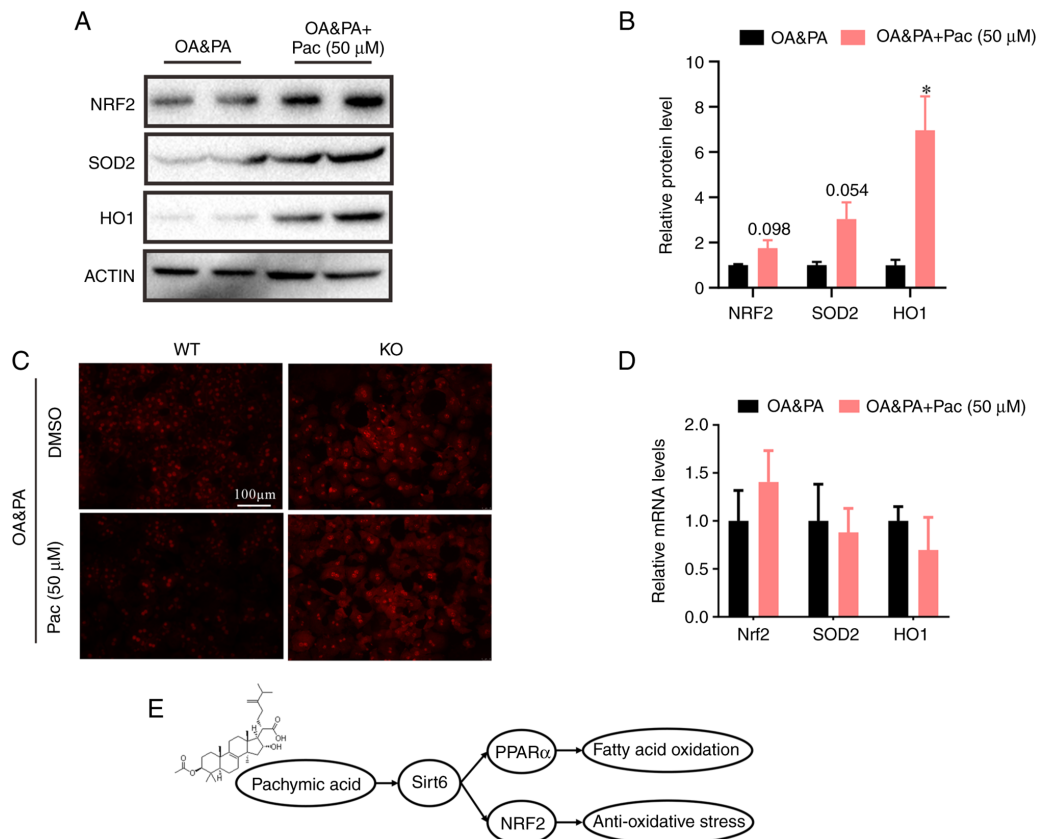


Figure 7. Pac alleviates OA + PA-induced oxidative stress that is dependent on SIRT6 in MPHs. (A) Western blotting and (B) quantification of hepatic NRF2, SOD2 and CPT1 α protein in MPHs; (C) Reactive oxygen species levels in WT MPHs and SIRT6-deficient MPHs (magnification, x200). (D) Expression of Nrf2, SOD2 and HO1 in SIRT6-deficient MPHs. (E) Schematic view of the protective effect of Pa on OA + PA-induced NAFLD that was dependent on the SIRT6-PPAR- α -Nrf2 axis. Data are presented as means \pm SEM; n=3-4. *P<0.05 vs. OA + PA. MPHs, mouse primary hepatocytes; OA, oleic acid; PA, palmitic acid; pac, pachymic acid; SIRT6, sirtuin 6; NRF2, nuclear factor erythroid 2-related factor 2; SOD2, superoxide dismutase 2; CPT1 α , carnitine palmitoyltransferase 1; WT, wild-type; KO, knockout.

fatty acid accumulation is a key factor in NAFLD development. Lipid accumulation in the liver causes an imbalance between lipid acquisition and decomposition. Lipid acquisition pertains to diet and the ingestion of circulating lipids, while lipid decomposition mainly includes the oxidation of free fatty acids, ultimately leading to oxidative stress and liver damage (32). Therefore, disorders involving the oxidation and synthesis of fatty acids play an important role in the pathogenesis of NAFLD (33). Reducing fatty acid accumulation and oxidative stress may thus be an effective way to treat NAFLD. As ample evidence suggests, Pac is a therapeutic agent since it protects against liver function damage through its relevant antioxidant and anti-lipid accumulation characteristics in many cell models (20,21). However, we must realize that our knowledge of the mechanisms Pac operates in NAFLD is still inadequate.

SIRT6 is an important nuclear deacetylase and plays an important role in lipid metabolism and oxidative stress (28,34,35). Liver-specific SIRT6-deficient mice spontaneously develop hypoglycemia and show increased TG synthesis, fatty liver formation and oxidative stress. Studies have shown that mice overexpressing SIRT6 have reduced accumulation of visceral fat; improved blood lipid levels, glucose tolerance, and insulin secretion; and increased expression of selective PPAR regulatory genes, where these traits affect the steady state of lipids (14). And a recent study demonstrated that Nrf2 and SIRT6 protein-protein interactions confer an antioxidant function in APAP-induced hepatotoxicity (28). From this observation it might be inferred that Pac's protection against NAFLD might at least in part depend on its regulation of the SIRT6. As we expected, docking analysis indicated a good binding affinity of Pac and Sirt6 via hydrophobic interaction and qPCR analyses further confirmed Pac could increase expression of SIRT6 *in vitro*.

Moreover, the activation of SIRT6/PPAR- α (in promoting fatty acid oxidation) and antioxidant effect of SIRT6/Nrf2 interactions could be a new target for the treatment of NAFLD. In this work, through the results of western blot and of qPCR analyses we also showed that the treatment with Pac increased SIRT6's anti-lipid accumulation and anti-oxidative stress action. However, this effect was abolished by SIRT6's deficiency and meanwhile the genes of β -oxidation (PPAR- α) and antioxidant stress (Nrf2) have no significant changes. Taken together, the hepatoprotective mechanisms Pac operates are tightly associated with the regulation of the SIRT6/PPAR- α and SIRT6/NRF2 signaling pathway.

A growing body of researches showed that Pac plays an important role in the treatment of many diseases, however, it has not yet been applied to clinical treatment of diseases in any way. Although we have verified that Pac could modulate SIRT6/PPAR- α and SIRT6/NRF2 pathway to alleviate hepatocyte lipid metabolism disorders firstly, our experiments lacked validation of clinical case or animal samples and just verified on cells. Thus, in the future works, we will continue to verify at the animal level and further study how sirt6 regulates NRF2 and PPAR- α at the molecular level.

In conclusion, we demonstrated that Pac prevents hepatic lipid metabolism disorders. Furthermore, we found that Pac could effectively ameliorate hepatocyte lipid metabolism disorders by targeting the activation of SIRT6/PPAR- α ,

promoting fatty acid oxidation and SIRT6/NRF2 antioxidant activity. This study suggests that Pac is a potential agent for the treatment of NAFLD and related diseases.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CHL, YYX and CPS contributed to the conception of the work. ZSP, YLC, KJT, ZZL, JLL and YHG assisted with experimental preparation and data collection. ZSP and YLC fed the animals. ZSP and KJT drafted the manuscript. All authors contributed to manuscript revision, and have read and approved the final manuscript. CHL and CPS confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All animal experiments were conducted under protocols approved by and in accordance with the guidelines of the Animal Ethics Committee of Guangzhou University of Chinese Medicine (approval no. 20220805003).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Juanola O, Martinez-Lopez S, Frances R and Gomez-Hurtado I: Non-Alcoholic fatty liver disease: Metabolic, genetic, epigenetic and environmental risk factors. *Int J Environ Res Public Health* 18: 5227, 2021.
- Byun S, Seok S, Kim YC, Zhang Y, Yau P, Iwamori N, Xu HE, Ma J, Kemper B and Kemper JK: Fasting-induced FGF21 signaling activates hepatic autophagy and lipid degradation via JMJD3 histone demethylase. *Nat Commun* 11: 807, 2020.
- Alves-Bezerra M and Cohen DE: Triglyceride metabolism in the liver. *Compr Physiol* 8: 1-8, 2017.
- Secor JD, Fligor SC, Tsikis ST, Yu LJ and Puder M: Free fatty acid receptors as mediators and therapeutic targets in liver disease. *Front Physiol* 12: 656441, 2021.
- Jiang G, Chen D, Li W, Liu C, Liu J and Guo Y: Effects of wogonoside on the inflammatory response and oxidative stress in mice with nonalcoholic fatty liver disease. *Pharm Biol* 58: 1177-1183, 2020.

6. Kersten S and Stienstra R: The role and regulation of the peroxisome proliferator activated receptor alpha in human liver. *Biochimie* 136: 75-84, 2017.
7. Sun N, Shen C, Zhang L, Wu X, Yu Y, Yang X, Yang C, Zhong C, Gao Z, Miao W, *et al*: Hepatic Kruppel-like factor 16 (KLF16) targets PPAR α to improve steatohepatitis and insulin resistance. *Gut* 70: 2183-2195, 2021.
8. Wahli W and Michalik L: PPARs at the crossroads of lipid signaling and inflammation. *Trends Endocrinol Metab* 23: 351-363, 2012.
9. Montagner A, Polizzi A, Fouche E, Ducheix S, Lippi Y, Lasserre F, Barquissau V, Régnier M, Lukowicz C, Benhamed F, *et al*: Liver PPAR α is crucial for whole-body fatty acid homeostasis and is protective against NAFLD. *Gut* 65: 1202-1214, 2016.
10. Yang DK and Jo DG: Mulberry fruit extract ameliorates nonalcoholic fatty liver disease (NAFLD) through inhibition of mitochondrial oxidative stress in rats. *Evid Based Complement Alternat Med* 2018: 8165716, 2018.
11. Wu W, Peng G, Yang F, Zhang Y, Mu Z and Han X: Sulforaphane has a therapeutic effect in an atopic dermatitis murine model and activates the Nrf2/HO1 axis. *Mol Med Rep* 20: 1761-1771, 2019.
12. Yan C, Sun W, Wang X, Long J, Liu X, Feng Z and Liu J: Punicalagin attenuates palmitate-induced lipotoxicity in HepG2 cells by activating the Keap1-Nrf2 antioxidant defense system. *Mol Nutr Food Res* 60: 1139-1149, 2016.
13. Van Gool F, Galli M, Gueydan C, Kruys V, Prevot PP, Bedalov A, Mostoslavsky R, Alt FW, De Smedt T and Leo O: Intracellular NAD levels regulate tumor necrosis factor protein synthesis in a sirtuin-dependent manner. *Nat Med* 15: 206-210, 2009.
14. Naiman S, Huynh FK, Gil R, Glick Y, Shahar Y, Touitou N, Nahum L, Avivi MY, Roichman A, Kanfi Y, *et al*: SIRT6 promotes hepatic beta-oxidation via activation of PPAR α . *Cell Rep* 29: 4127-4143 e8, 2019.
15. Kim HS, Xiao C, Wang RH, Lahusen T, Xu X, Vassilopoulos A, Vazquez-Ortiz G, Jeong WI, Park O, Ki SH, *et al*: Hepatic-specific disruption of SIRT6 in mice results in fatty liver formation due to enhanced glycolysis and triglyceride synthesis. *Cell Metab* 12: 224-236, 2010.
16. Kuang J, Chen L, Tang Q, Zhang J, Li Y and He J: The role of Sirt6 in obesity and diabetes. *Front Physiol* 9: 135, 2018.
17. Kanfi Y, Peshti V, Gil R, Naiman S, Nahum L, Levin E, Kronfeld-Schor N and Cohen HY: SIRT6 protects against pathological damage caused by diet-induced obesity. *Aging Cell* 9: 162-173, 2010.
18. Ding B, Ji X, Sun X, Zhang T and Mu S: In vitro effect of pachymic acid on the activity of Cytochrome P450 enzymes. *Xenobiotica* 50: 913-918, 2020.
19. Akihisa T, Nakamura Y, Tokuda H, Uchiyama E, Suzuki T, Kimura Y, Uchikura K and Nishino H: Triterpene acids from *Poria cocos* and their anti-tumor-promoting effects. *J Nat Prod* 70: 948-953, 2007.
20. Kim TG, Lee YH, Lee NH, Bhattarai G, Lee IK, Yun BS and Yi HK: The antioxidant property of pachymic acid improves bone disturbance against AH plus-induced inflammation in MC-3T3 E1 cells. *J Endod* 39: 461-466, 2013.
21. Lee YH, Lee NH, Bhattarai G, Kim GE, Lee IK, Yun BS, Hwang PH and Yi HK: Anti-inflammatory effect of pachymic acid promotes odontoblastic differentiation via HO-1 in dental pulp cells. *Oral Dis* 19: 193-199, 2013.
22. Lu C, Ma J and Cai D: Pachymic acid inhibits the tumorigenicity of gastric cancer cells by the mitochondrial pathway. *Anticancer Drugs* 28: 170-179, 2017.
23. Kim JH, Sim HA, Jung DY, Lim EY, Kim YT, Kim BJ and Jung MH: *Poria cocos* Wolf extract ameliorates hepatic steatosis through regulation of lipid metabolism, inhibition of ER stress, and activation of autophagy via AMPK activation. *Int J Mol Sci* 20: 4801, 2019.
24. Chen L, Liu Q, Tang Q, Kuang J, Li H, Pu S, Wu T, Yang X, Li R, Zhang J, *et al*: Hepatocyte-specific Sirt6 deficiency impairs ketogenesis. *J Biol Chem* 294: 1579-1589, 2019.
25. Friesner RA, Banks JL, Murphy RB, Halgren TA, Klicic JJ, Mainz DT, Repasky MP, Knoll EH, Shelley M, Perry JK, *et al*: Glide: A new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J Med Chem* 47: 1739-1749, 2004.
26. Fazi R, Tintori C, Brai A, Botta L, Selvaraj M, Garbelli A, Maga G and Botta M: Homology model-based virtual screening for the identification of human helicase DDX3 inhibitors. *J Chem Inf Model* 55: 2443-2454, 2015.
27. Chen Z, Tian R, She Z, Cai J and Li H: Role of oxidative stress in the pathogenesis of nonalcoholic fatty liver disease. *Free Radic Biol Med* 152: 116-141, 2020.
28. Zhou Y, Fan X, Jiao T, Li W, Chen P, Jiang Y, Sun J, Chen Y, Chen Z, Guan L, *et al*: SIRT6 as a key event linking P53 and NRF2 counteracts APAP-induced hepatotoxicity through inhibiting oxidative stress and promoting hepatocyte proliferation. *Acta Pharm Sin B* 11: 89-99, 2021.
29. Rives C, Fougerat A, Ellero-Simatos S, Loiseau N, Guillou H, Gamet-Payrastré L and Wahli W: Oxidative stress in NAFLD: Role of nutrients and food contaminants. *Biomolecules* 10: 1702, 2020.
30. Fang YL, Chen H, Wang CL and Liang L: Pathogenesis of non-alcoholic fatty liver disease in children and adolescence: From 'two hit theory' to 'multiple hit model'. *World J Gastroenterol* 24: 2974-2983, 2018.
31. Buzzetti E, Pinzani M and Tsochatzis EA: The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism* 65: 1038-1048, 2016.
32. Pawlak M, Lefebvre P and Staels B: Molecular mechanism of PPARalpha action and its impact on lipid metabolism, inflammation and fibrosis in non-alcoholic fatty liver disease. *J Hepatol* 62: 720-733, 2015.
33. Ferramosca A and Zara V: Modulation of hepatic steatosis by dietary fatty acids. *World J Gastroenterol* 20: 1746-1755, 2014.
34. Kawahara TL, Michishita E, Adler AS, Damian M, Berber E, Lin M, McCord RA, Ongaigui KC, Boxer LD, Chang HY and Chua KF: SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life Span. *Cell* 136: 62-74, 2009.
35. Li Z, Xu K, Guo Y, Ping L, Gao Y, Qiu Y, Ni J, Liu Q and Wang Z: A high-fat diet reverses metabolic disorders and premature aging by modulating insulin and IGF1 signaling in SIRT6 knockout mice. *Aging Cell* 19: e13104, 2020.



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