Ophiopogonin D ameliorates non-alcoholic fatty liver disease in high-fat diet-induced obese mice by improving lipid metabolism, oxidative stress and inflammatory response

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Abstract. Lipid metabolic disorders, oxidative stress and inflammation in the liver are key steps in the progression of non-alcoholic fatty liver disease (NAFLD). Ophiopogonin D (OP-D), the main active ingredient of *Ophiopogon japonicus*, exhibits several pharmacological activities such as antioxidant and anti-inflammatory activities. Therefore, the current study aimed to explore the role of OP-D in NAFLD in a high-fat diet (HFD)-induced obesity mouse model. To investigate the effect of OP-D on NAFLD *in vivo*, a NAFLD mouse model was established following feeding mice with HFD, then the mice were randomly treated with HFD or HFD + OP-D for 4 weeks. Subsequently, primary mouse hepatocytes were isolated, and enzyme-linked immunosorbent assay, reverse transcription-quantitative PCR western blotting and immunofluorescence analysis were used for assessment to explore the direct effect of OP-D *in vitro*. The results of the present study indicated that OP-D could ameliorate NAFLD in HFD-induced obese mice by regulating lipid metabolism and antioxidant and anti-inflammatory responses. Additionally, OP-D treatment decreased lipogenesis and inflammation levels *in vitro*, suggesting that the NF-κB signaling pathway may be involved in the beneficial effects of OP-D on NAFLD.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) represents a spectrum of liver injury diseases, including simple steatosis, non-alcoholic steatohepatitis as well as fibrosis and liver cirrhosis (1). Notably, the progressive form of NAFLD can lead to hepatocellular carcinoma and liver-related mortality (2,3). With the continuous increase in the prevalence of metabolic syndromes, such as type 2 diabetes mellitus (T2DM), obesity and hyperlipidemia, the morbidity of NAFLD has increased in parallel, affecting ~25% of the population worldwide, thus significantly contributing to the disease burden (2,4). At present, NAFLD is considered to be one of the most common chronic liver diseases worldwide (5). However, to date, there have been no approved pharmacological approaches for this condition. Therefore, the development of novel therapeutic strategies to improve the progression of NAFLD is important.

The pathogenesis and treatment of NAFLD have been widely investigated; however, they are still not entirely understood. The most accepted mechanism of NAFLD is the 'multiple hits' theory. According to this theory, NAFLD can be caused by several factors, such as insulin resistance (IR), dyslipidemia, oxidative stress (OS), endoplasmic reticulum stress and inflammation (6,7). Notably, dyslipidemia is a primary feature of NAFLD (8), while OS (free radical damage) and inflammation are considered as the most significant mechanisms leading to hepatic cell death and tissue injury (9,10). Therefore, regulating lipid metabolism, OS and the inflammatory response may be a particular approach for ameliorating the progression of NAFLD.

Ophiopogonin D (OP-D) is a significant pharmacological ingredient of the traditional Chinese medicine *Ophiopogon japonicus*. It has been reported that OP-D exhibits several pharmacological effects, including antioxidant and anti-inflammatory activities, while it also inhibits venous thrombosis (11-13). A recent study showed that OP-D can improve renal function by inhibiting OS and inflammation in streptozotocin-induced diabetic nephropathy rats (14). Therefore, the current study aimed to explore the role of OP-D

on NAFLD in a mouse model of high-fat diet (HFD)-induced obesity.

Materials and methods

Drugs and reagents. OP-D was obtained from Shanghai Yuanye Biotechnology Co., Ltd. Palmitic acid (PA) was purchased from Sigma-Aldrich (Merck KGaA).

Animal experiments. A total of 15 male C57BL/6J mice (age, 3-4 weeks; weight, 17-20 g) were purchased from Shanghai Model Organisms Centre, Inc. Mice with similar body weights were allowed to adjust to the environment for one week prior to the experiments. Subsequently, mice (age, 4-5 weeks) were randomly divided into the following three groups: Normal chow diet (ND); HFD; and HFD + OP-D group. Mice were given free access to food and distilled water. Mice fed with ND (cat. no. D12450B; 10% fat, 70% carbohydrates and 20% protein; Research Diets, Inc.) or HFD (cat. no. D12492; 60% fat, 20% carbohydrate and 20% protein; Research Diets, Inc.). Following feeding for 12 weeks, HFD-induced obese mice were randomly allocated to the test groups for pharmacological studies. Mice in the HFD + OP-D group continued to be fed with HFD and were intragastrically administered with 5 mg/kg/day OP-D (dissolved in 0.5% of carboxymethyl cellulose) for the following 4 weeks, according to preliminary experiments and previous studies (14,15). Mice in the other groups were treated with the same volume of 0.5% sodium carboxyl methyl cellulose solution (in order to protect the gastric mucosa of mice) according to a previous study (16,17). At the end of the experiment (16 weeks), all mice were sacrificed by cervical dislocation. All mice were housed in specific pathogen-free facility under a 12-h light/dark cycle, a relative humidity of 50% and a controlled temperature of 22±1°C. All animal procedures were approved by the Medical Ethics Committee of Wuhan University of Science and Technology (Wuhan, China; approval number, 2022139).

Serum biochemical measurements. At the end of the experiment, mice were fasted overnight and blood samples from the tail were collected to detect blood glucose levels using a blood glucose meter (Bayer AG). The mice were then sacrificed, and blood samples were collected by aortic puncture with centrifugation (1,300 x g for 10 min at 4°C) to obtain serum, and stored at -80°C for the blood chemistry measurements. The levels of insulin, IL-6, IL-1β and TNFα in serum were detected using the corresponding commercially available ELISA kits (insulin: cat. no. KCD-E1025; IL-6: cat. no. KCD-E1002; IL-1β: cat. no. KCD-E10010; TNFα: cat. no. KCD-E1018; Shanghai Kechuangda Biomedical Technology Co., Ltd.), according to the manufacturer's instructions. The serum levels of triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using an automatic biochemical analyzer (Hitachi, Ltd.). In addition, the serum levels of free fatty acids (FFA) were detected by a commercially available colorimetric assay kit (cat. No. A042-1-1, Nanjing Jiancheng Bioengineering Institute). The homeostatic model assessment for insulin resistance (HOMA-IR) index was calculated as follows: HOMA-IR=[fasting serum glucose (mmol/l) x fasting serum insulin (μU/ml)]/22.5.

Liver histology. Liver tissues from mice in each group were collected and fixed in 10% formaldehyde neutral buffer solution for 24 h at 4°C, followed by washing with tap water, dehydration in alcohol and embedding in paraffin. Subsequently, the 5-μm thick transversal sections were obtained, deparaffinized, dehydrated in ethanol (50-100%) and cleared with xylene. The sections were stained using hematoxylin and eosin (H&E) solution for 5 min at room temperature (Wuhan Servicebio Technology Co., Ltd.), according to the manufacturer's protocols. For Oil-red O staining, liver tissues were frozen in optimal cutting temperature compound at -20°C and 10-μm thick sections were obtained, followed by staining with Oil-red O solution (Wuhan Servicebio Technology Co., Ltd.) for 10 min at room temperature, according to the manufacturer's protocols. Images were observed using a NIKON imaging workstation (NIKON digital sight DS-FI2; Nikon Corporation).

Hepatic biochemical analysis. The commercially available Triglyceride Assay Kit (cat. no. ab65336; Abcam) was used to assess the levels of liver TG by measuring the optical density at a wavelength of 570 nm using spectrophotometry. The index of OS, including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) levels, was determined using the corresponding commercially available kits (MDA: cat. no. A003-1-1; SOD: cat. no. A001-1-1; CAT: cat. no. A007-1-1; GSH-Px: cat. no. A005-1-1. Nanjing Jiancheng Bioengineering Institute).

Cell cultures and treatments. Primary hepatocytes (PMHs) were isolated from mice. Briefly, 6-8-week-old male C57BL/6J mice (n=6; weight, 18-22 g) were purchased from Shanghai Model Organisms Centre, Inc. Mice were given free access to food and distilled water, and were housed in a specific pathogen-free facility under a 12-h light/dark cycle, a relative humidity of 50% and a controlled temperature of 22±1°C. Mice were anesthetized using intraperitoneal injections of sodium pentobarbital (50 mg/kg). After the liver tissue was separated, the mice were sacrificed by cervical dislocation. The liver tissue was then collected, minced and filtered after portal vein perfusion. PMHs were purified and cultured in DMEM (cat. no. G4510; Wuhan Servicebio Technology Co., Ltd.) supplemented with 1% penicillin/streptomycin solution and 10% FBS (cat. no. G0004; Wuhan Servicebio Technology Co., Ltd.). To assess cell viability, PMHs (5x10³) were seeded into 96-well plates for 24 h at 37°C. The following day, when plates reached ~80% confluency, cells were treated with various concentrations (0-20 μmol/l) of OP-D for an additional 24 h at 37°C. Cell viability was assessed using a Cell Counting Kit-8 assay (Wuhan Servicebio Technology Co., Ltd.). Briefly, 10 μl CCK-8 solution added into each well, which were then incubated at 37°C with 5% CO_2 for 2 h. The optical density was measured at a wavelength of 450 nm. To establish an *in vitro* model of lipid accumulation, hepatocytes were treated with PA (400 μmol/l) for 24 h at 37°C. Then the cells were treated with or without 10 mol/l OP-D for another
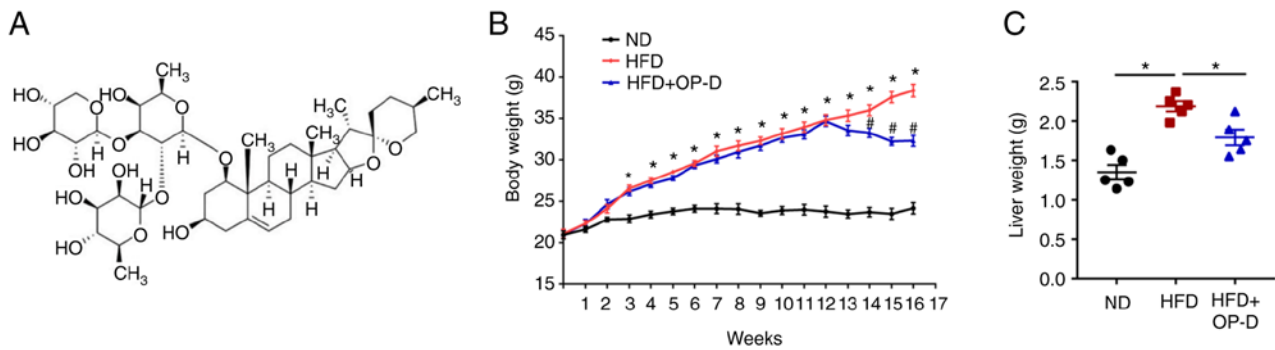
Figure 1. OP-D treatment suppresses HFD-induced body weight gain in HFD-fed mice. Mice were fed with ND or HFD for 16 weeks. Mice in the OP-D intervention group were administrated 5 mg/kg/day OP-D for the last 4 weeks (n=5). (A) Chemical structure of OP-D. (B) Body weight. (C) Liver weight. [*]P<0.05 vs. the ND group or as indicated; [#]P<0.05 vs. the HFD group. OP-D, Ophiopogonin D; HFD, high fat diet; ND, normal chow diet.

24 h. Cultured cells were collected, isopropyl alcohol was added into 2×10^6 cells according to the proportion of 200 μl homogenizing medium, and centrifugation was performed at 4°C and 10,000 x g for 10 min to obtain the lysed cells. The content of TG in lysed cells was measured using the TG Colorimetric Assay kit (cat. no. E-BC-K261-M; Elabscience Biotechnology, Inc.) following manufacturer's protocols. The optical density was measured at a wavelength of 510 nm.

Measurements of cytokine levels in culture supernatants. The levels of TNF-α, IL-1β and IL-6 in PMH supernatants were determined using ELISA, according to the manufacturer's instructions (Shanghai Kechuangda Biomedical Technology Co., Ltd.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from liver tissues or cells and isolated using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the protocol provided by the manufacturer. The isolated RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. qPCR was performed using SYBR Green PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used are listed in Table SI. All reactions were conducted in triplicate and the thermal cycling conditions were as follows: 30 sec at 95°C, followed by 50 cycles of 95°C for 5 sec and 60°C for 30 sec. The mRNA expression levels of target genes were calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to those of GAPDH (18).

Western blot analysis. Liver tissues or cells were lysed with RIPA buffer containing protease inhibitors (Roche Applied Science). A BCA kit (Beyotime Institute of Biotechnology) was used to detect protein concentrations. The extracted proteins (20 μg of each protein) were loaded onto 10% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes (MilliporeSigma) after electrophoresis. Blots were blocked with 5% bovine serum albumin (cat. no. GC305006; Wuhan Servicebio Technology Co., Ltd.) at 4°C for 2 h and then incubated with primary antibodies against GAPDH (1:2,000; cat. no. 5174), P65 (1:1,000; cat. no. 8242), phosphorylated (p-)P65 (Ser468) (1:1,000;

cat. no. 3039), IκBα (1:1,000; cat. no. 9242), p-IκBα (1:1,000; cat. no. 2859), TNFα (1:1,000; cat. no. 3707) (all CST Biological Reagents Co., Ltd.) at 4°C overnight, The membranes were then incubated with HRP-conjugated anti-IgG secondary antibodies (cat. no. A0192; 1:2,000; Beyotime Institute of Biotechnology) at room temperature for 45 min. GAPDH was used as an internal reference. The intensity of protein bands was assessed using the ImageJ software (version 1.8.0; National Institutes of Health).

Immunofluorescence (IF) analysis. IF staining was carried out according to standard procedures (19). Briefly, cells were first fixed with 95% ethanol for 15 min at -20°C. The cells were then incubated and blocked with blocking solution (5% BSA/PBS) for 30 min at room temperature, followed by being incubated overnight with a primary antibody against p65 (1:200; cat. no. 8242; Cell Signaling Technology, Inc.) at 4°C. Subsequently, incubation with Alexa Fluor® 647-conjugated goat anti-rabbit IgG (1:400; cat. no. ab150079; Abcam) at 4°C for 1 h was performed. The coverslips were mounted on microscope slides with fluorescence reagent containing 10 μg/ml DAPI (Thermo Fisher Scientific, Inc.). IF images were captured using the Fluoview FV1000 confocal microscope (Olympus Corporation).

Statistical data analysis. Data are presented as the mean ± SEM. All analyses were performed using GraphPad Prism 8.0 (GraphPad Software; Dotmatics) or SPSS 22.0 (IBM Corp.) software. Data among groups were compared using one-way ANOVA followed by Dunnett's test. P<0.05 was considered to indicate a statistically significant difference.

Results

OP-D attenuates body weight gain in HFD-treated mice. OP-D is a pharmacological compound of *Ophiopogon japonicus*, a traditional Chinese medicine herb (14). The molecular structure of OP-D is illustrated in Fig. 1A. The current study aimed to explore whether OP-D administration could inhibit body weight gain in mice fed with HFD. The results showed that the body weight of mice in the HFD + OP-D group was notably reduced compared with that in the HFD group at weeks 14-16 (Fig. 1B). Additionally, OP-D administration significantly
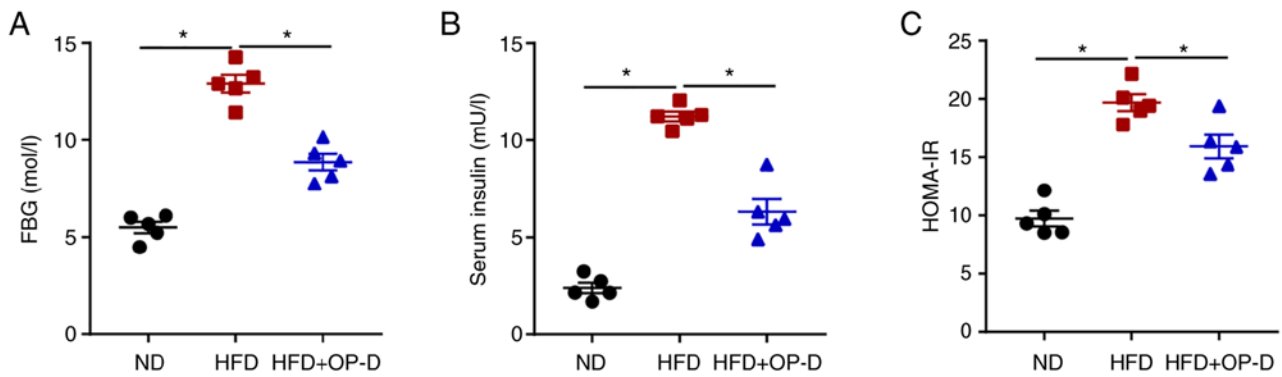
Figure 2. OP-D improves glucose homeostasis and insulin resistance in HFD fed mice. (A) Levels of FBG. (B) Levels of serum insulin. (C) Index of HOMA-IR (n=5). *P<0.05. OP-D, Ophiopogonin D; HFD, high fat diet; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment of insulin resistance; ND, normal chow diet.

reduced liver weight in HFD-treated mice compared with those in the HFD group (Fig. 1C).

OP-D improves glucose homeostasis and IR in mice fed with HFD. Subsequently, the present study investigated whether OP-D could regulate glucose and insulin homeostasis in HFD-fed mice. Consistent with previous studies (20,21), HFD-treated mice exhibited elevated glucose and insulin levels compared with ND control mice. The effect of the HFD on the levels of fasting blood glucose (FBG) and insulin was significantly reduced by OP-D treatment (Fig. 2A and B). Furthermore, the HOMA-IR index suggested that mice in the HFD + OP-D group displayed significantly reduced IR compared with those in the HFD group (Fig. 2C). These findings indicated that OP-D could exert a significant role in improving glucose homeostasis and IR in HFD-induced obese mice.

OP-D improves lipogenesis and hepatic steatosis in NAFLD mice. To further explore whether OP-D could prevent hepatic steatosis, H&E staining was first carried out. H&E staining showed widespread vacuolation in the liver tissue of HFD mice. However, OP-D treatment alleviated hepatic steatosis (Fig. 3A). In addition, mice in the HFD + OP-D group displayed a smaller area of hepatic lipid droplets compared with mice in the HFD group (Fig. 3A). Treatment with OP-D significantly reduced TG levels in the liver compared with the HFD group, thus suggesting that OP-D could attenuate lipid accumulation (Fig. 3B). Furthermore, TC, TG, FFA and LDL-C levels were significantly reduced in the serum of HFD mice treated with OP-D compared with mice treated with vehicle (Fig. 3C-F). Subsequently, the mRNA expression levels of lipid metabolism-related genes were detected. The data showed that the expression levels of lipid droplet formation-related genes [3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), fat storage-inducing transmembrane protein (Fitm)1 and Fitm2], lipid uptake-related genes [fatty acid-binding protein 1 (Fabp1), fatty acid transporter 1 (Fatp1) and lipoprotein lipase (Lpl)] and lipogenesis-related genes [fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1) and peroxisome proliferator-activated receptor (PPAR)-γ] were significantly decreased by treatment of

HFD-fed mice with OP-D (Fig. 3G-I). By contrast, the expression of fatty acid β-oxidation-related genes [PPAR-α, peroxisomal acyl-coenzyme A oxidase 1 (Acox1) and carnitine O-palmitoyl transferase 1α (Cpt1α)] were significantly upregulated after OP-D treatment compared with the HFD group (Fig. 3J). Furthermore, the HFD-induced increased levels of ALT and AST in HFD mice were significantly reduced by OP-D (Fig. 3K and L). Taken together, these results suggested that OP-D could exert a protective role in the development of NAFLD.

OP-D attenuates OS and inflammation in NAFLD mice. Lipid peroxidation, OS and inflammation serve a critical role in the progression of NAFLD (22). Therefore, the levels of OS in the liver tissue were determined in the present study. The results revealed that the hepatic levels of MDA, an oxidative marker, were significantly reduced following treatment of HFD-induced NAFLD mice with OP-D (Fig. 4A). Inversely, the levels of the antioxidative markers SOD, CAT and GSH-Px were significantly increased in the livers of mice in the HFD + OP-D group compared with the HFD group that did not receive OP-D treatment (Fig. 4B-D). Furthermore, the mRNA expression levels of nuclear respiratory factor 1 and transcription factor A mitochondria, two mitochondrial regulators, were significantly increased in the HFD + OP-D group compared with the HFD group (Fig. 4E). Subsequently, the inflammatory response in mice treated with or without OP-D was investigated. As shown in Fig. 4F-H, the serum levels of the cytokines TNFα, IL-6 and IL-1β were significantly increased in HFD mice compared with ND mice. However, the secretion levels of these inflammatory mediators were restored following OP-D administration. Additionally, OP-D administration significantly reversed the increased mRNA expression levels of TNFα, IL-6 and Il-1β after induction by HFD (Fig. 4I-K). Furthermore, the significantly increased gene expression levels of the inflammatory mediators, including matrix metalloproteinase 9 (MMP9), monocyte chemoattractant protein 1 (MCP1) and vascular cell adhesion protein 1 (VCAM1), were also significantly reduced in the HFD + OP-D group compared with the HFD group (Fig. 4L). These findings suggested that OP-D could reduce OS and inflammation in HFD-induced NAFLD mice.
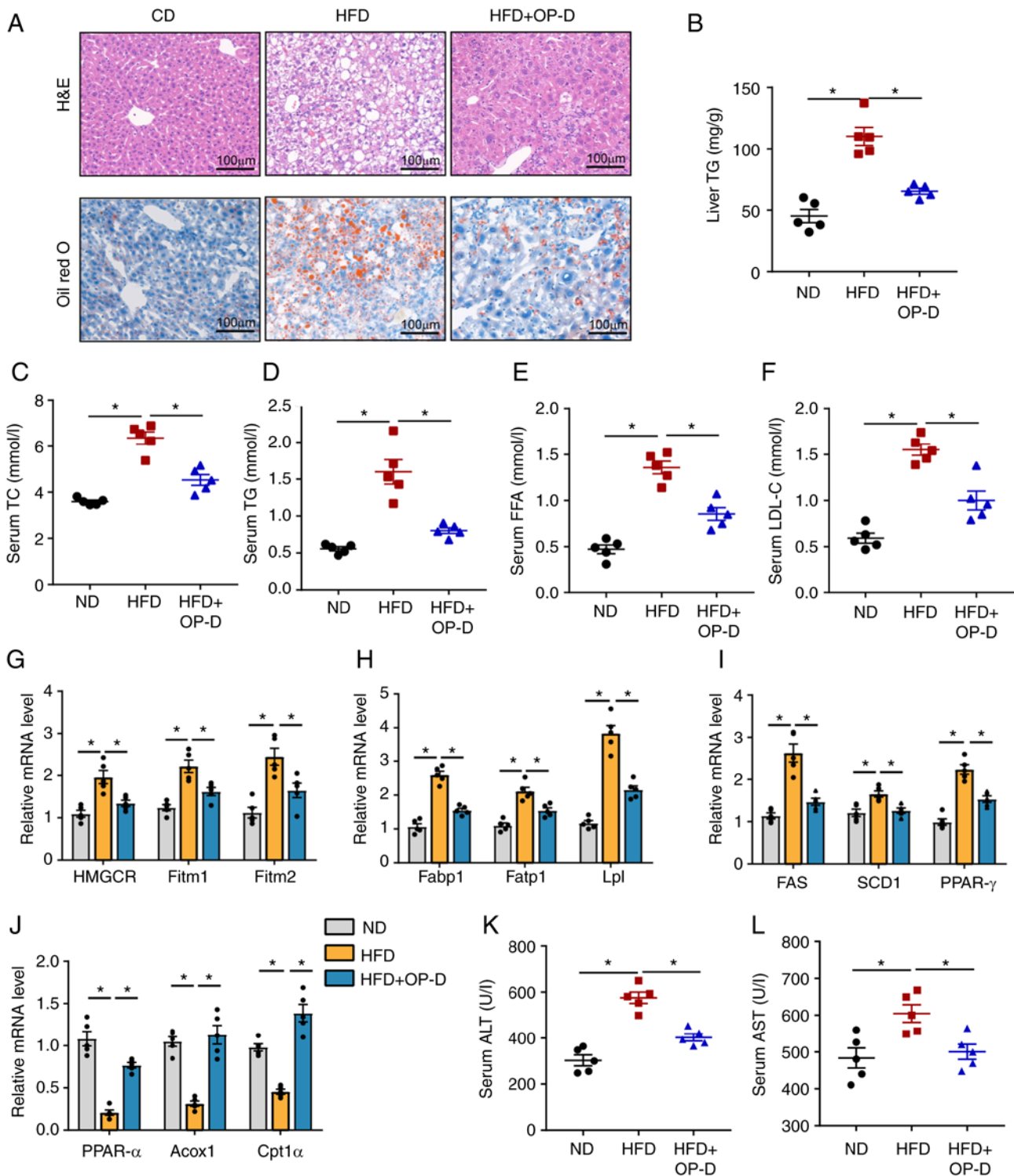
Figure 3. OP-D improves lipogenesis and hepatic steatosis in HFD-induced NAFLD mice. (A) Representative images of H&E (upper panel) and Oil-Red O (lower panel) stained liver tissue. Scale bar, 100 μm. (B) Content of TG in the liver. Levels of (C) TC, (D) TG, (E) FFA and (F) LDL-C in the serum. Relative mRNA expression of (G) cholesterol synthesis and efflux-related genes (HMGCR, Fitm1 and Fitm2), (H) uptake-related genes (Fabp1, Fatp1 and Lpl), (I) fatty acid synthesis genes (FAS, SCD1 and PPAR-γ) and (J) fatty acid oxidation genes (PPAR-α, Acox1 and Cpt1α). Levels of (K) ALT and (L) AST in the serum (n=5). *P<0.05. OP-D, Ophiopogonin D; HFD, high fat diet; ND, normal chow diet; NAFLD, non-alcoholic fatty liver disease; H&E, hematoxylin and eosin; TC, total cholesterol; TG, triglycerides; FFA, free fatty acids; LDL-C, low-density lipoprotein cholesterol; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; Fitm, fat storage-inducing transmembrane protein; Fabp1, fatty acid-binding protein 1; Fatp1, fatty acid transport protein 1; Lpl, lipoprotein lipase; FAS, fatty acid synthase; SCD1, stearoyl-CoA desaturase-1; PPAR-γ, peroxisome proliferator-activated receptor γ; PPAR-α, peroxisome proliferators-activated receptor α; Acox1, acyl-CoA oxidase 1; Cpt1α, carnitine palmitoyl transferase 1α; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

OP-D alleviates lipogenesis and inflammatory responses in vitro. The current study aimed to explore whether OP-D could exert a direct effect on PMHs. Therefore, the changes in fat deposition were evaluated in PMHs treated with different concentrations of PA for different time points (data not shown). Consistently, treatment with 400 μmol/l PA for 24 h was the
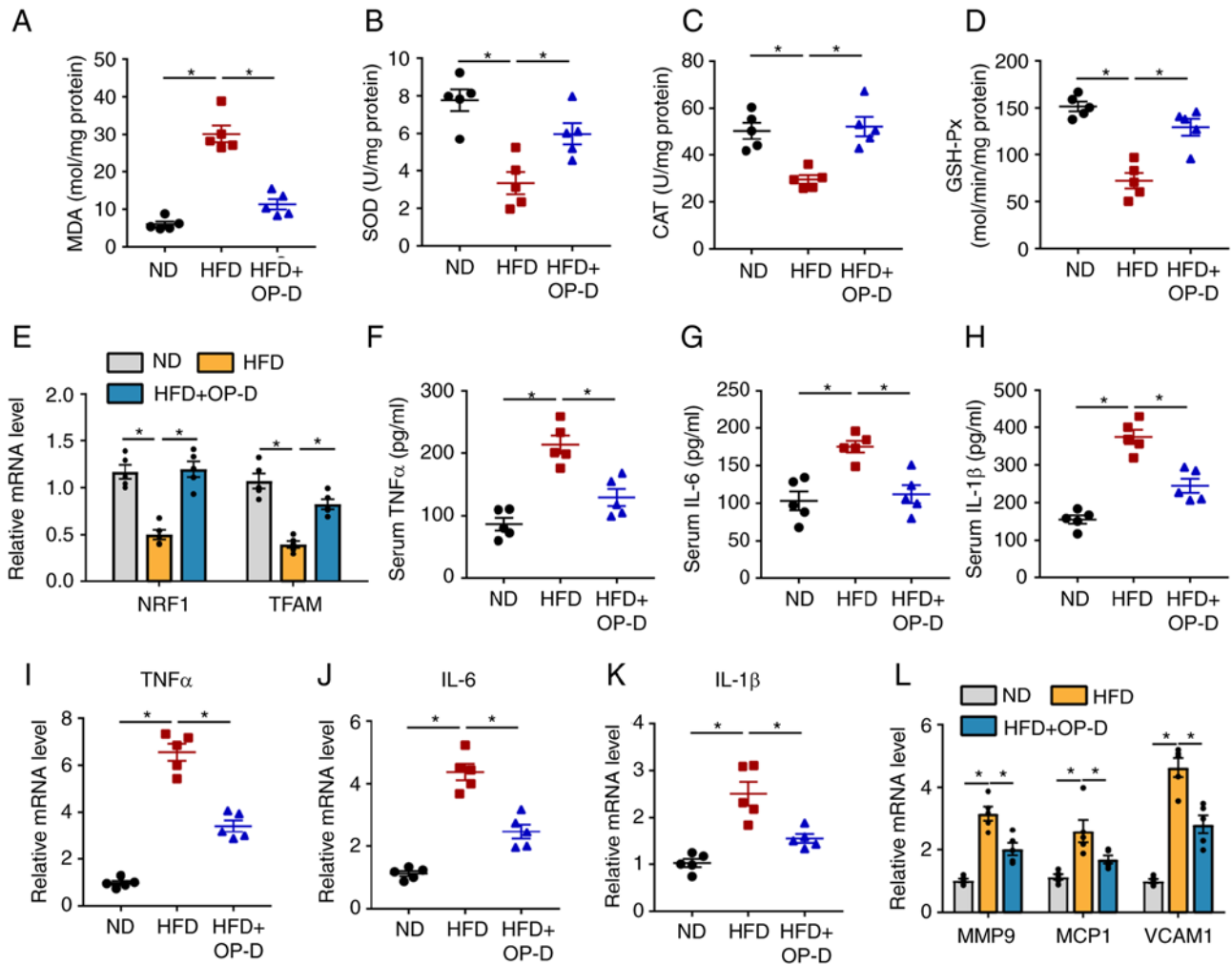
Figure 4. OP-D attenuates the levels of oxidative stress and inflammation in NAFLD mice. (A) MDA content, activities of (B) SOD, (C) CAT and (D) GSH-Px from the livers of mice with different interventions. (E) Relative mRNA expression levels of NRF1 and TFAM. Levels of (F) TNFα, (G) IL-6 and (H) IL-1β in the serum. Relative mRNA expression levels of (I) TNF-α, (J) IL-6 and (K) IL-1β. (L) Relative mRNA expression of MMP9, MCP1 and VCAM1 (n=5). *P<0.05. OP-D, Ophiopogonin D; NAFLD, non-alcoholic fatty liver disease; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; HFD, high fat diet; ND, normal chow diet; NRF-1, nuclear respiratory factor 1; TFAM, mitochondrial transcription factor A; MMP9, matrix metalloproteinase 9; MCP1, monocyte chemoattractant protein-1; VCAM1, vascular cell adhesion protein 1.

optimum condition, as previously described (23). Since the CCK8 result showed that 0-20 µmol/l OP-D did not affect the cell viability of PMHs, the concentration of 10 µmol/l OP-D for 24 h was selected as the optimal condition (Fig. 5A). Treatment with OP-D significantly reduced TG levels in lysed PMHs compared with the PA-only treated group (Fig. 5B). The mRNA expression levels of lipid synthesis-related genes, such as acetyl-CoA carboxylase 1 (ACC1), FAS and SCD1, were significantly reduced in cells treated with OP-D compared with the PA group (Fig. 5C). Consistent with previous studies (11,24), PA significantly increased the levels of TNFα, IL-1β and IL-6 from PMHs, while OP-D reversed this effect (Fig. 5D-F). Additionally, OP-D could significantly reduce the mRNA expression levels of TNFα, IL-6 and IL-1β compared with the PA-only treated group (Fig. 5G). Collectively, these results indicated that OP-D could alleviate lipogenesis and inflammation *in vitro*.

NF-κB signaling is involved in the regulation of OP-D in PMHs. The results suggested that OP-D could alleviate inflammatory responses, which have a significant effect on the progression of HFD-induced NAFLD in mice. Therefore, the present study further explored the mechanisms underlying the effect of OP-D on NAFLD in PMHs. It has been reported previously that the inflammatory NF-κB signaling pathway plays a crucial role in regulating inflammatory responses in NAFLD (25). In the current study, western blot analysis demonstrated that cell treatment with OP-D significantly reduced the PA-induced protein expression levels of p-P65, p-IκBα and TNFα in hepatocytes (Fig. 6A-D). OP-D also inhibited the PA-induced nuclear translocation of P65 (Fig. 6E). These results suggested that the NF-κB signaling pathway could mediate the beneficial effects of OP-D on NAFLD.

Discussion

NAFLD is a common pathological disease of the liver, affecting ~25% of the global population, which increases the risk of hepatic and extrahepatic complications (2,4). Although several phytochemicals are currently used worldwide for the
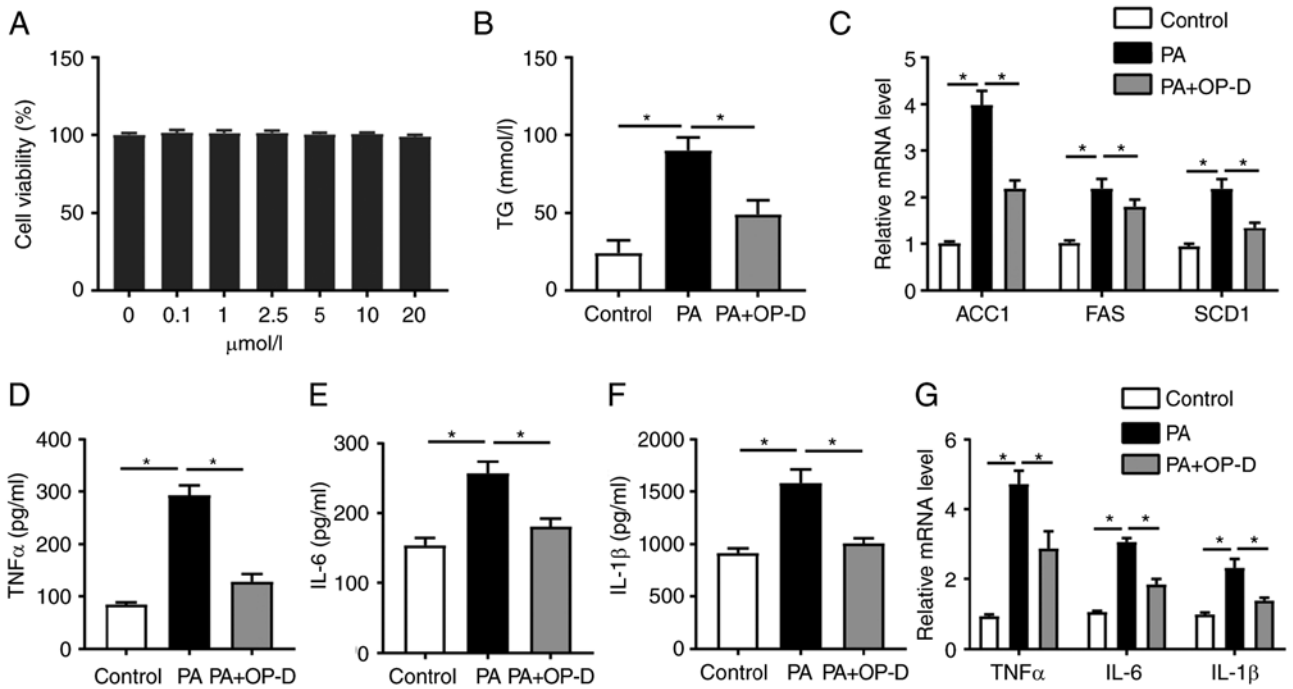
Figure 5. OP-D alleviates lipogenesis and inflammatory responses *in vitro*. Primary mouse hepatocytes were isolated and treated with 400 mmol/l PA with or without 10 μmol/l OP-D. (A) Effect of OP-D on cell viability. (B) Serum levels of TG. (C) Relative mRNA expression levels of ACC1, FAS and SCD1. Levels of (D) TNFα, (E) IL-6 and (F) IL-1β in the serum. (G) Relative mRNA expression level of TNFα, IL-6 and IL-1β (n=3). *P<0.05. OP-D, Ophiopogonin D; PA, palmitic acid; TG, triglycerides; ACC1, acetyl-CoA carboxylase 1, SCD1, stearoyl-CoA desaturase-1; FAS, fatty acid synthase.
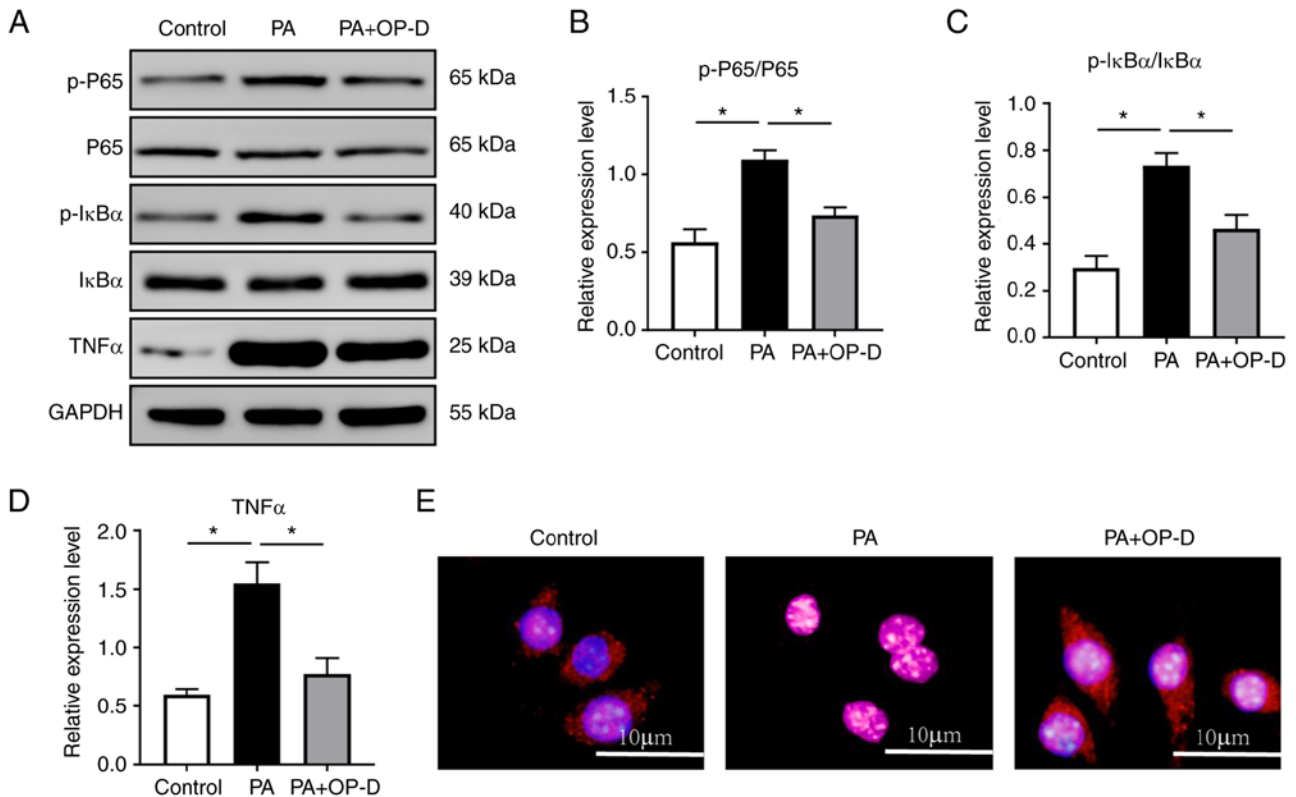

Figure 6. NF-κB signaling pathway is involved in the regulation of OP-D *in vitro*. (A) Representative western blot images and protein levels of (B) p-P65/P65, (C) p-IκBα/IκBα and (D) TNFα. (E) Representative images of P65 nuclear translocation (n=3). Scale bar, 10 μm. *P<0.05. OP-D, Ophiopogonin D; PA, palmitic acid; p-, phosphorylated.

clinical treatment of NAFLD (26,27), the efficacy and safety of these drugs still remains unknown. Therefore, studies on the mechanisms underlying the development of NAFLD and potential protective therapeutic interventions are important.

Ophiopogon japonicus is a common herb in traditional Chinese medicine, that has been widely used for thousands of years due to its high nutritional and medicinal value (11-13). However, the mechanisms underlying the effects of *Ophiopogon japonicus* still require investigation. Emerging evidence has suggested that OP-D, a steroidal glycoside of *Ophiopogon japonicus*, exhibits several biological activities. Previous studies have demonstrated that OP-D can exert an anti-inflammatory effect on PM2.5-injured alveolar epithelial cells (28), protect human endothelial cells from OS injury (29) and alleviate diabetic myocardial injuries by reducing lipid accumulation and mitochondrial injury (15).

The current study aimed to investigate the possible therapeutic effects of OP-D on HFD-induced NAFLD. The main findings of the present study were as follows: i) OP-D alleviated obesity in a mouse model; ii) OP-D potentially played a beneficial role in NAFLD via the regulation of lipogenesis, OS injury and inflammation; and iii) the NF-κB signaling pathway was potentially involved in the regulation of OP-D in NAFLD.

NAFLD, currently known as 'metabolic dysfunction-associated fatty liver disease', is considered a hepatic manifestation of metabolic syndrome (30). The disease is closely associated with metabolic disorders, such as obesity, dyslipidemia, IR and T2DM (31). Obesity and being overweight are the most common causes of metabolic diseases and NAFLD (32). The present study showed that OP-D suppressed HFD-induced body weight gain in mice, which prompts further research on the effects of OP-D on NAFLD. It has been widely reported that IR and dyslipidemia are key events in the progression of NAFLD (32-34). In the current study, the results revealed that OP-D improved lipid metabolic disorders and IR in HFD-induced obese mice. These findings were consistent with those of a previous study that demonstrated that OP-D can improve hyperglycemia in diabetic rats (14).

OS plays an essential role in the occurrence and progression of NAFLD (9,35). OS refers to the imbalance of oxidants and antioxidants, which can lead to severe failure of cell function and eventually to cell death (36). Previous studies have demonstrated that the levels of the hepatic oxidation markers MDA and ROS are increased, while those of the antioxidative stress markers CAT, GSH-Px and SOD are decreased in NAFLD (36-38). The results of the current study showed that OP-D reduced OS, as evidenced by the reduced activity of MDA, and increased antioxidant activities, as evidenced by the increased SOD, CAT and GSH-Px activities.

The regulation of inflammatory responses has been recognized as a pivotal pathway for maintaining homeostasis and preventing the progression of NAFLD (9,39,40). It has been reported that NF-κB signaling plays a critical role in the regulation of inflammation (25). Once the NF-κB pathway is activated, pro-inflammatory cytokines, such as TNFα, IL-1β and IL-6, are widely expressed (41). The present study demonstrated that OP-D alleviated inflammatory responses in HFD-induced obese mice and verified that its anti-inflammatory effect was associated with the NF-κB pathway *in vitro*.

The current study indicated the effect of OP-D on alleviating hepatic steatosis via antioxidant and anti-inflammatory responses through the NF-κB signaling pathway; however, there are still certain limitations. Firstly, the particular upstream pathways involved in OP-D promoting P65 activation were not elucidated, and it could not be excluded that other mechanisms, such as mitochondrial OS, autophagy and ferroptosis could be involved in the aforementioned process. Secondly, the effects of OP-D on other cell types, such as Kupffer cells and sinusoidal endothelial cells in the liver, were not investigated. Finally, OP-D significantly reduced body weight, thus supporting the notion of a reduction in obesity; however, it cannot be excluded that muscle may also be reduced by OP-D administration. Therefore, further studies on the roles of OP-D on muscle volume or muscle strength are needed. Overall, further evidence is needed to clarify the mechanism underlying the effect of OP-D on improving NAFLD.

It has been reported that OP-D is safe for clinical use and the drug has little toxicity in animal subacute toxicity experiments (42,43). However, a recent study reported that OP-D can cause hemolysis in Kunming mice, but there is no further data to confirm this (44). In the present study, no specific side effects of OP-D on the HFD mice were observed. The side effects of OP-D still need to be investigated further.

In summary, the present study demonstrated that OP-D could alleviate NAFLD in HFD-induced obese mice by improving lipogenesis, inflammation and oxidative stress injury. In addition, NF-κB signaling could play an essential role in the beneficial effects of OP-D on NAFLD. Therefore, the above findings indicated that OP-D could be a potential therapeutic agent for NAFLD.

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

XH and QJ conducted the animal experiments. CS and YC performed the *in vitro* experiments. XH and YC wrote the manuscript. JZ and QW designed the study and conducted data analysis. QW is the guarantor of this work. XH and QW confirm the authenticity of all the raw data All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the Medical Ethics Committee of Wuhan University of Science and Technology (Wuhan, China; approval no. 2022139).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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