Co-administration of obeticholic acid and simvastatin protects against high-fat diet-induced non-alcoholic steatohepatitis in mice

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Abstract. Non-alcoholic steatohepatitis (NASH) has no approved therapy. The farnesoid X nuclear receptor (FXR) agonist obeticholic acid (OCA) has shown promise as a drug for NASH, but can adversely affect plasma lipid profiles. Therefore, the present study aimed to investigate the effects and underlying mechanisms of OCA in combination with simvastatin (SIM) in a high-fat diet (HFD)-induced model of NASH. C57BL/6J mice were fed with a HFD for 16 weeks to establish the NASH model. The mice were randomly divided into the following five groups: HFD, HFD + OCA, HFD + SIM, HFD + OCA + SIM and control. After 16 weeks, the mice were sacrificed under anesthesia. The ratios of liver weight to body weight (Lw/Bw) and of abdominal adipose tissue weight to body weight were calculated. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol, triglycerides and low-density lipoprotein were measured. Liver sections were stained with hematoxylin and eosin. The protein levels of FXR, small heterodimeric partner (SHP) and cytochrome P450 family 7 subfamily A member 1 (CYP7A1) in the liver were detected by western blotting, while the mRNA levels of FXR, SHP, CYP7A1, bile salt export pump, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), sterol regulatory element binding protein-1 (SREBP1) and fatty acid synthase (FASN) were examined by reverse transcription-quantitative polymerase chain reaction. The administration of OCA with or without SIM reduced the liver inflammation score compared with those of the HFD and HFD + SIM groups, with no significant difference between the HFD + OCA and HFD + OCA + SIM groups. The steatosis score followed similar trends to the inflammation score. In HFD-fed mice, OCA combined with SIM prevented body weight gain compared with that in HFD and HFD + OCA groups, and reduced the Lw/Bw ratio compared with that in the HFD and HFD + SIM groups. In addition to preventing HFD-induced increases of ALT and AST, the combination of OCA and SIM reduced the mRNA levels of IL-6, TNF-α, SREBP1 and FASN. On the basis of these results, it may be concluded that the strategy of combining OCA with SIM represents an effective pharmacotherapy for NASH.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent liver disorder in advanced countries, and primarily involves the dysregulation of liver metabolism and inflammation. The term NAFLD covers a broad spectrum of potentially serious conditions, including hepatic steatosis and non-alcoholic steatohepatitis (NASH), and may result in hepatic cirrhosis and hepatocellular carcinoma (HCC) (1,2). As the progressive form of NAFLD, 15-25% of cases of NASH progress to cirrhosis within 10-15 years. NASH-associated cirrhosis carries a high risk for HCC, and it has been reported that 30-50% of HCC cases occur in patients with non-cirrhotic NASH (3). In 2011, NASH was predicted to become the predominant indication for liver transplantation within the next decade (4). Furthermore, other NASH-associated complications include hypertension, diabetes mellitus and cardiovascular disease (CVD) that also have associated increased risks of morbidity and mortality (3,5). Therefore, pharmaceutical experts are eager to develop novel therapeutic approaches for NASH. However, even though more than 35 drugs have been investigated in preclinical and clinical studies, there are as yet no approved therapies for NASH (6). An understanding of the pathogenesis of NAFLD is fundamental to the development of new therapeutic approaches, as it facilitates the identification of potential therapeutic targets.

NASH is reported to be associated with inappropriate functioning of the farnesoid X nuclear receptor (FXR), which

is activated by bile acid (BA) in direct proportion to its lipophilicity, and serves a critical role in BA, lipid and carbohydrate metabolism (7). When lipophilic BAs bind to and activate the FXR, insulin sensitivity is increased and hepatic gluconeogenesis and circulating triglycerides are decreased. The FXR is principally expressed in the liver, intestine and kidney (7). Obeticholic acid (OCA) is a potent, selective FXR agonist, which is a promising new drug for NASH, based on preclinical studies of acute hepatic inflammation and fibrosis (8,9) as well as clinical studies (10,11). OCA has shown the ability to ameliorate the histological features of NASH in the Farnesoid X nuclear ligand OCA for non-cirrhotic, non-alcoholic steatohepatitis (FLINT) study (11) and to mitigate hepatic fibrosis in the REGENERATE study (12). However, as noted in the FLINT study (11) and a number of studies using animals (13,14), OCA can adversely affect plasma lipoprotein profiles via the elevation of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels and reduction of high-density lipoprotein cholesterol (HDL-C) levels. This significant drawback has impeded the introduction of OCA into clinical practice. To address and potentially overcome this obstacle, we hypothesized that the use of statins as lipid-lowering agents would prevent the changes in cholesterol levels associated with OCA.

Statins are effective cholesterol-lowering drugs widely used in the treatment and prevention of CVD. They can reduce LDL-C ('bad' cholesterol) by 20-60%, lower triglyceride (TG) levels and slightly increase HDL ('good' cholesterol) (15,16). In addition, guidelines published in 2018 (3) recommended the use of statins in the management of dyslipidemia, a common comorbidity of NASH. Nevertheless, the use of statins in certain types of liver disease remains controversial, because statins can induce liver injury, characterized by elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in some patients. Therefore, statins should not be administered to patients with liver failure or liver decompensation. However, liver toxicity should be of little concern in patients with non-cirrhotic NASH, in whom statins are expected to be efficacious in treating hyperlipemia. Therefore, the present study examined a therapeutic strategy using a combination of OCA and simvastatin (SIM) for combating high-fat diet (HFD)-induced NASH in mice. The aim of the study was to explore the mechanisms and effects of a combination of OCA and SIM in the HFD-induced NASH model.

Materials and methods

Experimental animals and reagents. A total of 30 male C57BL/6J mice (age, 8 weeks; weight, 23.68±1.16 g; purchased from Beijing HFK Bioscience Co. Ltd.) were bred and housed as previously described (17). NASH was induced by a HFD comprising 0.2% cholesterol and 42% calories from fat (TD.88137 adjusted calories diet; Envigo Tekland). OCA (cat. no. OCA-161101) was a gift from North China Pharmaceutical Group Corp. SIM tablets (20 mg/tablet; national medicine permission number, H20083840) were purchased from Shandong Lukang Pharmaceutical Group Saite Co., Ltd.

Experimental design. The C57BL/6J mice were randomly divided into five groups (n=6/group): i) control group, mice fed normal chow diet; ii) HFD group, mice fed the HFD; iii) HFD + OCA group, mice fed the HFD with OCA (10 mg/kg/day) provided as a dietary admixture; iv) HFD + SIM group, mice fed the HFD diet supplemented with SIM (20 mg/kg/day); v) HFD + OCA + SIM group, mice fed the HFD supplemented with OCA (10 mg/kg/day) and SIM (20 mg/kg/day). The mice were provided with ad libitum access to water and food. At the end of the 16-week experiment, the mice were sacrificed by cervical dislocation performed under diethyl ether anesthesia by a trained individual. The successful induction of anesthesia by diethyl ether prior to cervical dislocation was confirmed by observation of the following parameters: Respiration decreased in frequency and increased in depth, eyelid and cornea reflexes disappeared, muscle tension and the reflex response reduced and no response to pain or other stimulation was exhibited. Death was confirmed by checking for a lack of heartbeat, pupillary response to light and respiration. Following sacrifice, blood samples and liver tissues were collected for analysis. The livers and abdominal adipose tissue were isolated and weighed in order to calculate the ratio of liver weight to body weight (Lw/Bw) and the ratio of abdominal adipose tissue weight to body weight (Aw/Bw). Livers were fixed in 10% formalin at room temperature for 48 h for histological analysis or snap-frozen in liquid nitrogen followed by storage at -80˚C in a freezer until required. The blood samples were centrifuged at 1,000 x g for 10 min at 4˚C. The serum supernatants were collected, divided into aliquots and stored at -80˚C for subsequent biochemical analyses on the serum biochemical indices. All animal care and experimental protocols were in accordance with the Animal Management Rules of the Ministry of Health of the People's Republic of China. The animal protocol was approved by the Ethics Committee of the Third Hospital of Hebei Medical University.

Measurement of serum biochemical indices. Serum ALT, AST, TC, TG and LDL levels were quantitated by an enzymatic kinetic method using an automatic biochemical analyzer (Olympus AU2700; Olympus Corporation) according to the manufacturer's instructions.

Histological examination. The paraffin-embedded liver sections (5 μm thick) were treated with hematoxylin solution for 5 mins and eosin solution for 1 min for Hematoxylin and eosin (H&E) staining at room temperature. The liver sections were scored for hepatic steatosis and inflammation as described previously using Brunt's criteria and the histological scoring system for NAFLD issued by the Pathology Committee of the Nonalcoholic Steatohepatitis Clinical Research Network (18). Briefly, steatosis was expressed as the percentage of the total liver section affected by microvesicular or macrovesicular steatosis. Hepatic inflammation was analyzed by counting the number of inflammatory foci per field at x200 magnification in five overlapping fields per specimen using a light microscope. Liver samples were scored independently for NASH severity by two pathologists who were blinded to the source of the samples.

Western blot analysis. Total proteins were extracted from liver tissue using radio-immunoprecipitation buffer (Wanleibio Co., Ltd.) and the protein concentration was evaluated

Table I. Primers for quantitative polymerase chain reaction.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
FXR (NR1H4)	GCTAATGAGGACGACAGCGAAGG	GTCTGTTGGTCTGCCGTGAGTTC
SHP (NR0B2)	GTCCGACTATTCTGTATGCACT	CTACTGTCTTGGCTAGGACATC
CYP7A1	GTGATGTTTGAAGCCGGATATC	TTTATGTGCGGTCTTGAACAAG
BSEP (ABCB11)	ATGAAGCCATTGCCGACCAGATG	GACTGACAGCGAGAATCACCAAGG
IL-6	ACTTCCATCCAGTTGCCTTCTTGG	TTAAGCCTCCGACTTGTGAAGTGG
TNF-α	GCGACGTGGAACTGGCAGAAG	GCCACAAGCAGGAATGAGAAGAGG
SREBP1	TAGAGCATATCCCCCAGGTG	GGTACGGGCCACAAGAAGTA
FASN	AAGGACCTGTCTAGGTTTGATGC	TGGCTTCATAGGTGACTTCCA
GAPDH	GGCATGGACTGTGGTCATGAG	TGCACCACCAACTGCTTAGC

FXR, farnesoid X receptor; SHP, small heterodimeric partner; CYP7A1, cytochrome P450 family 7 subfamily A member 1; BSEP, bile salt export pump; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; SREBP1, sterol regulatory element binding protein-1; FASN, fatty acid synthase.

using a bicinchoninic acid kit (Wanleibio Co., Ltd.). Then, ~40 μg sample proteins/lane were separated using 10 or 12% SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes (EMD Millipore) by electroblotting. The membranes were blocked for 60 min at room temperature in a buffer containing 0.1% Tween-20 and 5% milk, and then incubated overnight at 4˚C with primary antibodies against FXR (ab235094; Abcam), small heterodimeric partner (SHP; ab186874; Abcam), cytochrome P450 family 7 subfamily A member 1 (CYP7A1; ab65596; Abcam) and β-actin (WL01845; Wanleibio Co., Ltd.). After washing, the membranes were incubated with HRP-conjugated secondary antibodies (WLA023; Wanleibio Co., Ltd.) for 1 h at room temperature, and then the protein bands were visualized using the enhanced chemiluminescence reagent (Wanleibio Co., Ltd.). β-actin served as a loading control. The intensity of each protein band was quantified using ImageJ 1.46r software (National Institutes of Health).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated and extracted from frozen liver tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized from the RNA using a PrimeScript™ RT reagent kit (RR037A; Takara Bio, Inc.) according to the instructions suggested by the manufacturer. qPCR was performed on an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using TB Green™ Premix Ex Taq™ II (RR820A; Takara Bio, Inc.). The thermocycling conditions used were as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec, and finally, annealing and extension at 60˚C for 34 sec. The expression levels of the target mRNAs were normalized against the endogenous reference gene glyceraldehyde-phosphate dehydrogenase. The relative amount of each gene was measured using the $2^{-\Delta\Delta Cq}$ method (19). All RT-qPCR reactions were conducted in triplicate. The primers used for RT-qPCR are shown in Table I.

Statistical analysis. Each experiment was repeated 3 times. All data are presented as the mean ± standard deviation. Statistical analysis was carried out by one-way analysis of variance followed by Tukey's post hoc tests for evaluating differences between groups using IBM SPSS Statistics 26 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

OCA combined with SIM ameliorates NASH histological features in a HFD-induced mouse model. C57BL/6J mice were fed a HFD for 16 weeks to establish a NASH mouse model. The characteristic histological features of NASH were confirmed by H&E staining, which included micro- and macrovesicular steatosis, ballooning degeneration and the massive infiltration of cells (Fig. 1A).

The HFD group exhibited a significantly increased inflammation score compared with the control group ($P<0.05$; Fig. 1B). All three intervention groups exhibited significantly lower liver inflammation scores compared with the HFD group ($P<0.05$). Moreover, in the HFD + OCA and HFD + OCA + SIM groups, the liver inflammation scores were markedly reduced compared with that of the HFD + SIM group ($P<0.05$), and no significant difference was observed between the HFD + OCA and HFD + OCA + SIM groups. The steatosis scores mirrored the inflammation scores. Notably, the combined administration of OCA and SIM to the HFD-fed mice significantly alleviated the steatosis score compared with those of the two monotherapy groups ($P<0.05$), whereas no statistically significant difference in steatosis score was observed between the HFD + SIM and HFD groups.

Co-administration of OCA and SIM prevents body weight gain in HFD-fed mice. As weight loss is a factor that can improve the clinical and metabolic features of NASH (20), the changes in the body weights of the mice were carefully recorded. The body weight increase was significantly higher in the HFD-fed mice compared with the control group ($P<0.05$; Table II and Fig. 2A). The mice in the HFD + OCA group gained a similar amount of body weight to those in the

Table II. Effect of HFD and experimental treatments on body weight and the Lw/Bw and Aw/Bw ratios of C57BL/6J mice.

Variable	Control	HFD	HFD + OCA	HFD + SIM	HFD + OCA + SIM	F-value	P-value
Body weight gain (g)	5.60±1.34	26.83±1.60[a]	23.67±4.37	16.00±2.00[b]	15.00±6.52[b,c]	25.327	<0.001
Lw/Bw (%)	3.46±0.44	8.05±0.96[a]	4.74±0.87[b]	7.31±1.39[c]	2.83±1.59[b,d]	23.055	<0.001
Aw/Bw (%)	2.41±1.07	5.86±0.49[a]	5.79±0.39	5.53±0.31	4.18±2.35	7.725	0.001

HFD, high fat diet; OCA, obeticholic acid; SIM, simvastatin; Lw/Bw, liver weight/body weight ratio; Aw/Bw, abdominal adipose tissue weight/body weight ratio. [a]P<0.05 vs. control group; [b]P<0.05 vs. HFD group; [c]P<0.05 vs. HFD + OCA group; [d]P<0.05 vs. HFD + SIM group.

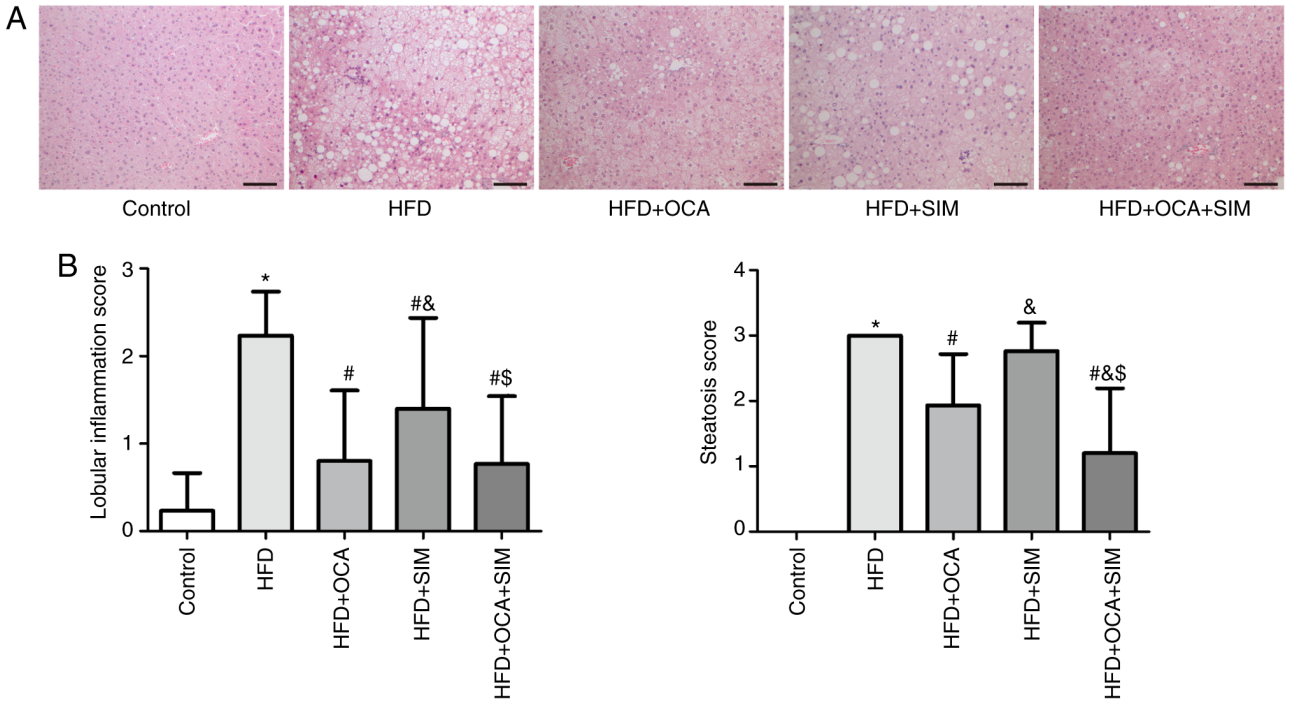

Figure 1. Histological analysis of the NASH model with OCA and/or SIM treatment. (A) Liver tissue from different groups was stained with hematoxylin and eosin (magnification, x200). (B) Lobular inflammation and steatosis scores were calculated by two separate pathologists to evaluate the NASH severity. Data are presented as the mean ± SD. NASH, non-alcoholic steatohepatitis; OCA, obeticholic acid; SIM, simvastatin; HFD, high-fat diet. *P<0.05 vs. control group; #P<0.05 vs. HFD group; &P<0.05 vs. HFD + OCA group; $P<0.05 vs. HFD + SIM group.

HFD group, whereas SIM alone significantly reduced body weight gain compared with that of the HFD group (P<0.05). Furthermore, the co-administration of OCA and SIM significantly prevented body weight gain compared with that of the HFD and HFD + OCA groups (P<0.05).

The Lw/Bw and Aw/Bw ratios were calculated in order to determine the weight changes of the liver and abdominal adipose tissue. The Lw/Bw and Aw/Bw ratios in the HFD group were significantly increased compared with those in the control group (P<0.05), indicating that the weight of the liver and the abdominal adipose tissue increased in NASH. When compared with the HFD group, the HFD + OCA group exhibited a decreased Lw/Bw ratio (P<0.05) but no difference in the Aw/Bw ratio, while the HFD + SIM group exhibited no significant difference in Lw/Bw or Aw/Bw ratios. Notably, the HFD + OCA + SIM group presented a prominent and significant reduction in Lw/Bw ratio compared with the HFD and HFD + SIM groups (P<0.05), but no significant difference compared with the HFD + OCA group.

Co-administration of OCA and SIM alleviates the pro-inflammatory response and liver damage in NASH. The inflammatory response of the liver was assessed via the measurement of serum ALT and AST levels. The HFD group showed significantly increased levels of ALT and AST compared with the control group (P<0.05; Table III and Fig. 2B). In the HFD + OCA and HFD + OCA + SIM groups, the increases in ALT and AST induced by the HFD were significantly reversed. Furthermore, the combination of OCA and SIM appeared to exhibit a stronger effect than OCA alone, but the difference between the HFD + OCA and HFD + OCA + SIM groups did not reach statistical significance. Although the HFD + SIM group exhibited a slight but non-significant reduction in ALT and AST levels compared with the HFD group, the results suggest that SIM is safe to administer to subjects with NASH in this model.

To further investigate the anti-inflammatory mechanism of OCA and/or SIM therapies, the expression levels of the pro-inflammatory cytokines interleukin-6 (IL-6) and tumor

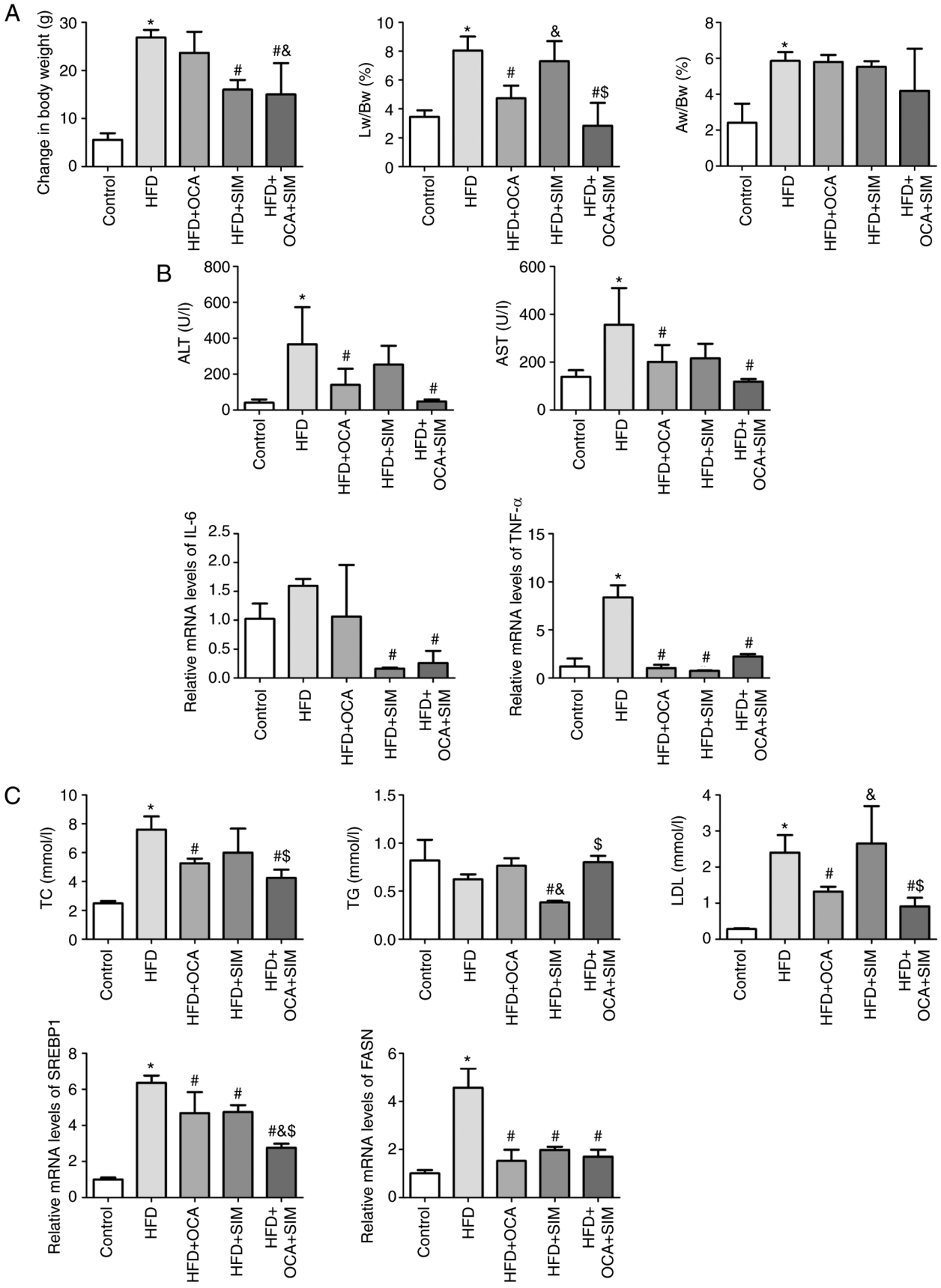

Figure 2. Co-administration of OCA and SIM prevents body weight gain, alleviates the pro-inflammatory response and liver damage and downregulates lipid metabolism in HFD-fed mice. (A) Changes in weights, Lw/Bw ratios and Aw/Bw ratios in the five groups. (B) Markers of liver damage, the inflammatory response and (C) lipid metabolism were evaluated. Data are presented as the mean ± SD (n=6/group). HFD, high-fat diet; OCA, obeticholic acid; SIM, simvastatin; Lw/Bw, ratio of liver weight to body weight; Aw/Bw, ratio of abdominal adipose tissue weight to body weight; ALT, alanine transaminase; AST, aspartate aminotransferase; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; TC, total cholesterol; TG, triglyceride; LDL, low-density lipoprotein; SREBP1, sterol regulatory element binding protein-1; FASN, fatty acid synthase. *P<0.05 vs. control group; #P<0.05 vs. HFD group; &P<0.05 vs. HFD + OCA group; $P<0.05 vs. HFD + SIM group.

Table III. Effect of HFD and experimental treatments on serum transaminases and circulating lipids.

Variable	Control	HFD	HFD + OCA	HFD + SIM	HFD + OCA + SIM	F-value	P-value
ALT (U/l)	40.00±18.68	365.83±206.62[a]	139.67±90.28[b]	253.33±104.04	47.80±11.21[b]	7.481	0.001
AST (U/l)	138.80±27.67	355.67±153.72[a]	201.17±70.80[b]	216.00±60.92	118.60±11.24[b]	6.322	0.002
TC (mmol/l)	2.49±0.16	7.60±0.91[a]	5.26±0.32[b]	6.00±1.67	4.25±0.57[b,c]	33.172	<0.001
TG (mmol/l)	0.82±0.21	0.62±0.05	0.76±0.08	0.38±0.02[b,d]	0.80±0.07[c]	9.794	<0.001
LDL (mmol/l)	0.29±0.02	2.40±0.49[a]	1.32±0.13[b]	2.65±1.04[d]	0.91±0.24[b,c]	15.610	<0.001

HFD, high fat diet; OCA, obeticholic acid; SIM, simvastatin; ALT, alanine transaminase; AST, aspartate aminotransferase; TC, total cholesterol; TG, triglyceride; LDL, low-density lipoprotein. [a]P<0.05 vs. control group; [b]P<0.05 vs. HFD group; [c]P<0.05 vs. HFD + SIM group; [d]P<0.05 vs. HFD + OCA group.

necrosis factor-α (TNF-α) were assessed by RT-qPCR. Compared with the control group, the HFD-fed mice exhibited increased mRNA expression of IL-6, which was attenuated somewhat by the administration of OCA; however, neither of these changes were statistically significant. The HFD + SIM and HFD + OCA + SIM groups presented significant reductions in the mRNA expression levels of IL-6 when compared with the HFD group (P<0.05). With regard to TNF-α, the mRNA expression level in the HFD group was increased compared with that in the control group (P<0.05). By sharp contrast, the three interventions significantly reduced the mRNA expression levels of TNF-α relative to those in the HFD group (P<0.05).

Effect of OCA and/or SIM on lipid metabolism. Lipid metabolism is a key determinant of NASH, and OCA has been documented to have an adverse effect on plasma lipoprotein profiles in patients, as characterized by elevated TC and LDL-C levels and reduced HDL-C levels. Therefore, serum lipoproteins level were examined and the mRNA expression levels of sterol regulatory element binding protein-1 (SREBP1) and fatty acid synthase (FASN) were measured by RT-qPCR. The HFD-fed mice were found to have significantly elevated levels of TC and LDL (P<0.05), and a non-significant reduction in the levels of TGs compared with those in the control group (Table III and Fig. 2C). These changes in serum lipoproteins were attenuated by the administration of OCA and/or SIM. The levels of TGs in the HFD + SIM group were significantly decreased compared with those in the HFD, HFD + OCA and HFD + OCA + SIM groups (P<0.05). However, a modest increase in TC and LDL levels was observed in the HFD + SIM group compared with the HFD + OCA and HFD + OCA + SIM groups.

The mice in the HFD group presented a significant increase in the mRNA expression levels of SREBP1 and FASN compared with those in the control group, and these increases were attenuated by the administration of OCA or SIM alone or in combination (P<0.05). Notably, no difference was observed in the FASN mRNA expression levels of the three intervention groups, but the HFD + OCA + SIM group exhibited a significant reduction in the mRNA expression level of SREBP1 compared with the HFD + OCA and HFD + SIM groups (P<0.05).

Effect of OCA and/or SIM on the FXR signaling pathway. OCA is a potent, selective FXR agonist. To determine whether SIM enhances the effect of OCA on the FXR signaling pathway, the hepatic expression levels of FXR and its downstream genes SHP, CYP7A1 and bile salt export pump (BSEP) were assessed.

No significant difference in the protein expression levels of FXR was observed between the HFD and control groups (Fig. 3A), while the protein expression levels of FXR in the HFD + OCA and HFD + SIM groups were significantly increased compared with those in the HFD group (P<0.05). Notably, the FXR protein level in the HFD + SIM group was significantly higher than that in the HFD + OCA group (P<0.05), while it was markedly and significantly reduced in the HFD + OCA + SIM group compared with the HFD, HFD + OCA and HFD + SIM groups (P<0.05). The mRNA expression level of FXR was detected by RT-qPCR. The HFD group presented a significant decrease in FXR mRNA expression (P<0.05), and the FXR mRNA levels in the HFD + OCA and HFD + OCA + SIM groups were significantly higher than those in the HFD and HFD + SIM groups (P<0.05).

SHP is a downstream target gene of FXR (21). The SHP protein expression level of the HFD-fed mice was slightly lower than that of the control group, but the difference was not significant. The protein expression levels of SHP in the three intervention groups were significantly increased compared with those in the HFD group (P<0.05). Importantly, the HFD + SIM group exhibited a higher protein level of SHP compared with the HFD + OCA and HFD + OCA + SIM groups (P<0.05). The trends in SHP mRNA expression levels were similar to those of SHP protein, with the exception that the SHP mRNA level in the HFD + SIM group appeared to be lower than those of the HFD + OCA and HFD + OCA + SIM groups (Fig. 3B).

CYP7A1 is the rate-limiting enzyme in cholesterol synthesis, which can be regulated by FXR and its target gene SHP in the liver (22). In the present study, the protein expression level of CYP7A1 in the HFD group was increased compared with that in the control group (P<0.05); the mRNA expression level of CYP7A1 was also increased, albeit not significantly. In comparison with the HFD group, the HFD + OCA group showed a significant reduction in the expression of CYP7A1 at the protein level (P<0.05), but no difference at the mRNA level. The HFD + SIM and HFD + OCA + SIM groups were found to have significantly increased protein expression levels of CYP7A1 compared with the HFD and HFD + OCA groups

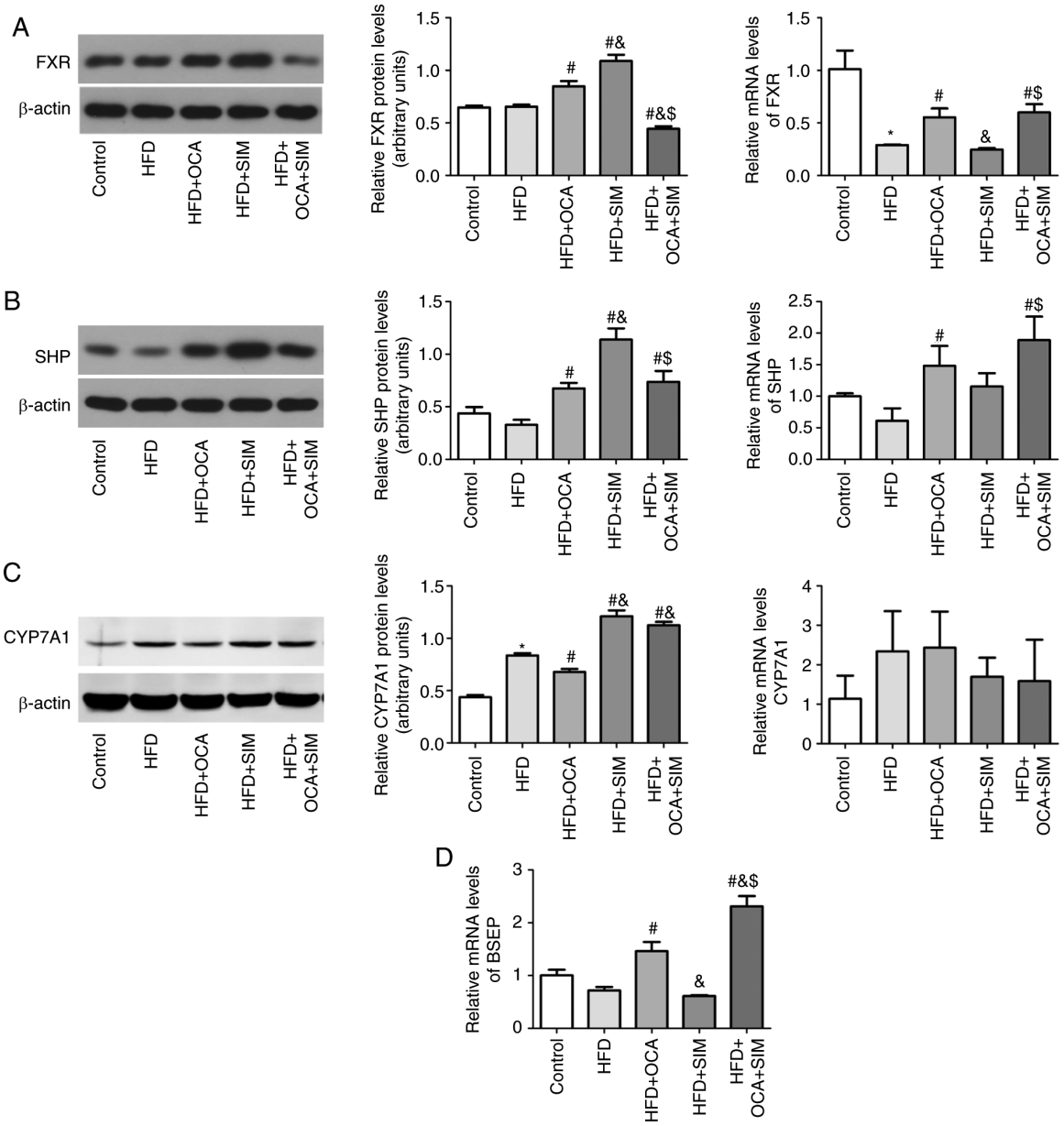

Figure 3. Effect of OCA and/or SIM on the FXR signaling pathway. Western blot and RT-qPCR analysis of (A) FXR, (B) SHP and (C) CYP7A1. (D) RT-qPCR analysis of BSEP. Data are presented as the mean ± SD (n=6/group). OCA, obeticholic acid; SIM, simvastatin; HFD, high-fat diet; FXR, farnesoid X receptor; SHP, small heterodimeric partner; CYP7A1, cytochrome P450 family 7 subfamily A member 1; BSEP, bile salt export pump; RT-qPCR, reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. control group; #P<0.05 vs. HFD group; &P<0.05 vs. HFD + OCA group; $P<0.05 vs. HFD + SIM group.

(P<0.05), but no significant difference in the mRNA expression levels of CYP7A1 was observed (Fig. 3C).

The mRNA expression levels of BSEP were detected by RT-qPCR. As shown in Fig. 3D, the mRNA expression level of BSEP was slightly reduced in the HFD-fed mice. However, the mice in the HFD + OCA and HFD + OCA + SIM groups had significantly increased BSEP mRNA expression levels compared with those in the HFD and HFD + SIM groups (P<0.05). Also, the BSEP level in the HFD + OCA + SIM group was higher than that in the HFD + OCA group (P<0.05).

Discussion

OCA is an FXR agonist that has been approved by the US Food and Drug Administration for use in patients with primary biliary cirrhosis, either in combination with ursodeoxycholic acid (UDCA) for patients with an inadequate response to UDCA alone, or as a monotherapy in patients who are intolerant to UDCA. The FXR regulates the synthesis of BA by cholesterol catabolism. Although OCA can stimulate FXR expression, increase the hepatic expression of SHP and consequently suppress the expression of CYP7A1 to reduce the rate of BA synthesis in the liver, it has yet to be approved for the treatment of NASH. Although numerous clinical studies, including randomized clinical trials, have confirmed the effectiveness of OCA in ameliorating NASH (10,11), its side effect of elevating cholesterol levels has prevented its progression to the clinic. Statins are competitive inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)-reductase. Via this mechanism, SIM directly

inhibits the formation of mevalonate from HMG-CoA and thus also inhibits the biosynthesis of cholesterol (23). SIM is mainly used in the clinical treatment of hyperlipidemia and effectively prevents the occurrence of cardiovascular and cerebrovascular adverse events such as coronary heart disease. We hypothesized that SIM may be able to counteract the side effects of OCA and perhaps even promote the effectiveness of OCA in the treatment of NASH.

Our previous study (24) demonstrated that a HFD induces NASH in mice. Continuous feeding with a HFD triggers severe endoplasmic reticulum stress in the liver, and hepatocytes fail to adapt to this stress; they are unable to maintain normal homeostatic levels of unfolded or misfolded proteins, leading to hepatocyte apoptosis and inflammation. In the present study, treatment with OCA and SIM was demonstrated to prevention HFD-induced liver injury in the mouse model of NASH. The results support the combination of OCA and SIM as a novel therapy for NASH, which is in line with findings of the randomized Combination of OCA and statins for monitoring of lipids (CONTROL) trial (25). In the CONTROL trial, a phase II placebo-controlled patient study, the OCA-induced increases in LDL in patients with NASH were mitigated with atorvastatin, and the combination of OCA and atorvastatin was generally safe and well-tolerated. In the present study, the co-administration of OCA and SIM resulted in lower steatosis scores than were obtained using OCA or SIM monotherapies, while OCA with or without SIM markedly reduced the hepatic inflammation score compared with those of the HFD and HFD + SIM groups. Serum ALT and AST levels were examined as they are indicators of the inflammatory response associated with liver damage. The use of OCA with or without SIM significantly prevented the HFD-induced increase of ALT and AST levels, and the co-administration of OCA and SIM exhibited the strongest effect. Although the HFD + SIM group exhibited minimal reductions in ALT and AST levels compared with the HFD group, the results indicate that the administration of SIM in subjects with NASH is safe in this model. IL-6 and TNF-α were examined as representative inflammatory factors. Hu *et al* (26) investigated INT-767, a recently identified dual FXR/TGR5 agonist, in a rat model of NASH and demonstrated that treatment with INT-767 significantly alleviated HFD-induced NASH and attenuated the pro-inflammatory response via the suppression of TNF-α and the NF-κB signaling pathway. The results of the present study were consistent with this, and showed that OCA alone or in combination with SIM was able to attenuate liver inflammation in a mice NASH model and downregulate the expression levels of IL-6 and TNF-α.

The optimization of metabolic risk factors and participation in diet management and exercise are key to the management of NASH, and weight loss alone can improve the histology of NASH. Based on the FXR ligand OA in NASH treatment (FLINT) trial, a post hoc subgroup analysis was performed by Hameed *et al* (27), which showed that OCA and weight loss had additive beneficial effects on the liver enzymes ALT and AST, and on histological features of disease activity in NASH. Based on these findings from the FLINT trial, the body weights of the mice were carefully documented in the present study, and it was observed that OCA administration exhibited no significant effect on body weight gain. This is in concordance with a previous study on NASH models using male wild-type C57BL/6J mice and Lep ob/ob (ob/ob-NASH) mice (28). By contrast, the administration of SIM alone and the co-administration of OCA and SIM significantly prevented body weight gain. The Lw/Bw and Aw/Bw ratios in the HFD group were significantly higher than those in the control group, indicating that liver and abdominal adipose tissue weights increased in NASH. Interestingly, the Lw/Bw ratio was only prominently reduced in the HFD + OCA + SIM group compared with the HFD and HFD + SIM groups. Relative to the HFD group, the HFD + OCA group exhibited a lower Lw/Bw ratio, but no difference in the Aw/Bw ratio, and the HFD + SIM group exhibited only slightly decreased Lw/Bw and Aw/Bw ratios compared with those of the HFD group. A suggested interpretation of these data is that OCA principally reduces liver weight, whereas SIM has the ability to reduce liver and abdominal adipose tissue weight as well as overall body weight. Therefore, SIM may enhance the efficacy of OCA in the treatment of NASH by reducing overall body, liver and abdominal adipose tissue weights.

The original intention of the present study was to investigate whether SIM is able to prevent or reverse the adverse effect of OCA on plasma lipoproteins. In the C57BL/6J mice fed with a HFD for 16 weeks, significantly elevated levels of TC and LDL were observed, but the levels of TG were reduced, which is not consistent with lipid metabolism in humans. These changes in plasma lipoproteins were inhibited in mice whose HFD was supplemented with OCA alone or in combination with SIM. Although serum lipid metabolism in mice is not completely consistent with clinical studies, the examination of liver histology in the present study demonstrated that OCA significantly reduced the steatosis score in the mouse model of NASH. Moreover, the mRNA expression levels of SREBP1 and FASN were detected by RT-qPCR, and the results showed that treatment with OCA alone or with SIM reduced the mRNA expression levels of SREBP1 and FASN, which are the essential gene transcription factors in lipogenesis.

It has been demonstrated that BAs are endogenous ligands for FXR, and by activating FXR, they induce SHP, which in turn suppresses CYP7A1 gene expression and thereby reduces the rate of BA synthesis in the liver (29). OCA is able to stimulate the expression of FXR, induce SHP and suppress the expression of CYP7A1. In the present study, HFD-fed mice showed no significant difference in the protein expression levels of FXR and SHP, but a marked increase in CYP7A1 protein expression compared with the control group. Regarding mRNA expression, the HFD reduced the levels of FXR and SHP, and induced CYP7A1 levels, which is entirely consistent with previously published studies (26,30). FXR is a member of the nuclear receptor superfamily, and is a ligand-activated transcriptional regulator, harboring DNA- and ligand-binding domains (7). Therefore, it seems possible that FXR may exert an effect at the transcriptional level.

The administration of OCA significantly elevated the expression of FXR and SHP at the mRNA and protein levels and reduced the CYP7A1 protein level. These results are consistent with a mechanism involving the FXR signaling pathway. The administration of SIM did not increase the FXR mRNA level compared with that in the HFD group, but elevated the SHP mRNA level and reduced the CYP7A1 mRNA level.

The HFD + SIM group showed significantly elevated protein levels of FXR, SHP and CYP7A1, but decreased expression of the corresponding mRNAs compared with those in the HFD + OCA group. There has been considerable research into statins and the FXR pathway. For example, in a study using cultured Hep3B cells, pravastatin enhanced FXR and CYP7A1 mRNA expression, and prevented cholesterol gallstone formation in human hepatocytes by increasing FXR, LXRα and CYP7A1 (31). In addition, in another study atorvastatin was shown to induce CYP7A1 in mice by suppressing FXR signaling in the liver and intestine (32). The mechanisms underlying the effect of statins on FXR and CYP7A1 remain to be clarified, but are outside the scope of the present study. Furthermore, it must be noted that the present study is limited by the lack of control groups comprising OCA and/or SIM-treated mice fed a normal diet.

In conclusion, the combination of OCA and SIM ameliorated the histological features of NASH and prevented body weight gain in an HFD-induced mouse model. The co-administration of OCA and SIM suppressed the levels of IL-6, TNF-α, SREBP1 and FASN, and alleviated the pro-inflammatory response and liver steatosis in NASH. Although the exact mechanisms of the combined therapy require further verification, the results of the study suggest that the combination of OCA and SIM may be an effective pharmacotherapy for NASH.

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Availability of data and materials

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

Authors' contributions

YMN designed the research, WCL, YGZ, LBK and QSZ performed the experiments, SXZ, RQW and WGR analyzed the data, and WCL and SXZ wrote the manuscript. WCL and YMN confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal care and experimental protocols were in accordance with the Animal Management Rules of the Ministry of Health of the People's Republic of China. The animal protocol was approved by the Ethics Committee of the Third Hospital of Hebei Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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