

The mechanism of effective components compatibility of Astragalus and Nelumbinis folium on the prevention and treatment of dyslipidemia by regulating cholesterol reversal transport

Jian Chen

Hebei University of Chinese Medicine

Cheng Shi

Hebei University of Chinese Medicine

Yufang Zhang

Hebei University of Chinese Medicine

Ya Gao

Hebei University of Chinese Medicine

Dantong Li

Hebei University of Chinese Medicine

Ying Gu

Hebei University of Chinese Medicine

Yixin Zhang (✉ hbzyx123@163.com)

Hebei University of Chinese Medicine

Muqing Zhang

Hebei Province Hospital of Traditional Chinese Medicine

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Abstract

Background

Astragalus saponins (AS) and *Nelumbinis folium* alkaloids (NFA) are the main active components of *Astragalus membranaceus* and *Nelumbo nucifera Gaertn.* This research investigated the beneficial effects and mechanisms of AS-NFA in preventing and treating dyslipidemia.

Methods

Rats were fed a high-fat diet (HFD) for to establish a dyslipidemia model and HepG2 cells were induced by cholesterol and 25-hydroxycholesterol to establish a steatosis cell model, treating with AS-NFA. The levels of blood lipids and liver function were detected by automatic biochemical analyzer. HE staining and oil red O staining were used to observe the pathological morphological changes and steatosis. The uptake of Dil-HDL by HepG2 cells was observed and detected by fluorescence microscopy. The mRNA and protein expression levels of Cholesterol reverse transport (RCT)-related mRNA and protein. in rat liver and HepG2 cells were detected by RT-PCR, immunofluorescent staining and Western Blot.

Results

AS-NFA had remarkable effects on regulating blood lipids, improving liver function, alleviating pathological injury of liver tissues, and reducing hepatic lipid accumulation. AS-NFA dramatically reduced total cholesterol (TC), total bile acid (TBA) levels, and lipid accumulation, and increased the intake of HDL in HepG2 cells. AS-NFA can regulate RCT by upregulating scavenger receptor class B type 1 (SR-B1), recombinant cytochrome P450 7A1 (CYP7A1), and farnesoid X receptor (FXR) in the HFD rats and HepG2 steatosis cells. The effects of AS-NFA on the SR-B1/CYP7A1/FXR signaling pathway were abolished when SR-B1 was inhibited by BLT-1 (a selective SR-B1 inhibitor) in HepG2 cells.

Conclusion

AS-NFA has an efficient lipid-regulating effect mediated by activation of the SR-B1/CYP7A1/FXR signaling pathway to regulate RCT. Our findings provide new insight and evidence for the discovery of a new lipid-regulating drug for the prevention and treatment of dyslipidemia in the clinic.

Introduction

Dyslipidemia is a disorder of lipid metabolism caused by abnormal fat metabolism or transport, which is one of the high-risk pathogenic factors for cardiovascular disease (1, 2). The prevalence of dyslipidemia among adults in China is as high as 40.40%; this rate has increased over time with younger people involved(3). Chemical drugs such as statins and niacin are often used to treat dyslipidemia in clinical

practice. Despite their satisfactory lipid-lowering effects, they are often accompanied by adverse effects such as liver and kidney damage, skin allergy, and rhabdomyolysis(4, 5). Therefore, the use of reliable and efficacious natural active ingredients for the treatment of metabolic diseases continues to be actively researched in the field of pharmaceutical sciences.

Reverse cholesterol transport (RCT) is a cholesterol metabolism pathway involved in the transport of cholesterol from macrophages to the liver where it is metabolized into bile and excreted(6–8). A growing body of evidence suggests that RCT is an endogenous defense mechanism in disorders of lipid metabolism that protects cells from excessive cholesterol accumulation, thus allowing cells to excrete cholesterol and maintain homeostasis(9–12). Scavenger receptor class B type (SR-B1) is a receptor physiologically related to high-density lipoprotein (HDL) that helps transport excessive cholesterol to the liver in the form of cholesteryl esters (CE)(13, 14). Recombinant carboxylesterase 1 (CES1) has a cholesteryl ester hydrolase activity and hydrolyzes CE to free cholesterol (FC)(15).

Recombinant cytochrome P450 7A1 (CYP7A1) is the initiator and rate-limiting enzyme in the classical BA synthesis pathway that converts cholesterol to BA (16, 17). BA combines with the farnesoid X receptor (FXR) and then activates FXR, thus promoting RCT in the liver (18). Studies have shown that long-term intake of HFD led to an increase in total cholesterol (TC) synthesis, a decrease in high-density lipoprotein cholesterol (HDL-C) content, and a decrease in SR-B1, CYP7A1, and FXR expression in the liver. This in turn inhibits the RCT process and leads to disorders in cholesterol metabolism(19). Therefore, improving RCT to maintain cholesterol homeostasis appears to be an attractive therapeutic strategy to reduce dyslipidemia risk.

Astragalus membranaceus (Fisch.) Bunge belongs to Leguminosae—an attractive medicine in China. It is rich in saponins, polysaccharides, flavonoids, and other chemical components, and has great nutritional and medicinal properties. Astragalus saponins (AS) as the primary active component in *Astragalus* impacts cardiovascular protection and regulates blood lipid metabolism(20, 21). In particular, Astragaloside IV regulates the expression of PTP1B to inhibit SREBP-1c and its downstream fatty acid synthase to regulate lipid metabolism(22). *Nelumbo nucifera* Gaertn. is a perennial aquatic herb that belongs to Nymphaeaceae. *Nelumbinis folium* has gained widespread popularity as a Chinese herbal medicine and tea beverage to resolve heatstroke, lose weight, and reduce lipids(23). Alkaloids and flavonoids are the main active components of *Nelumbinis folium*(24). According to previous studies, *Nelumbinis folium* alkaloids (NFA) have been associated with hypolipidemic, anti-obesity, and anti-aging activity(23). Wang reported that nuciferine could prevent weight gain, mediate the gut microbiota composition, significantly alleviate inflammation, and improve lipid metabolism disorders and liver injury in HFD-fed obese rats(25).

Here, we investigated the prevention and treatment effect of AS-NFA on dyslipidemia and its effect on RCT-related genes and proteins in HFD-induced rats. We then induced a steatosis model of HepG2 cells using cholesterol and 25-hydroxycholesterol. Based on SR-B1/CYP7A1/FXR signaling pathway, we aimed

to uncover the potential molecular mechanism by which AS-NFA regulates RCT in the prevention and treatment of dyslipidemia.

Materials and Methods

Chemicals and reagents

We purchased the following: TC, triglycerides (TG), HDL-C, low-density lipoprotein cholesterol (LDL-C), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) kit (201001, 201102, 210201, 201102, 201001, 201201, Icubio, Shenzhen, China); total bile acid (TBA) kit (E003-1-1, Jiancheng, China); Oil Red O solution (G1015, Servicebio, Wuhan, China); hematoxylin-eosin solution (C210504, BA4024, Baso, Zhuhai, China); total RNA extraction kit (LS1040, Promeg, America); SureScript first-strand cDNA synthesis kit and BlazeTaq SYBR Green qPCR Mix 2.0 (QP057, QP042, Yijin, Guangzhou, China); SR-B1, carboxylesterase 1 (CES1), CYP7A1, and FXR (DF6479, DF2612, DF6661, DF12511, Affinity, Jiangsu, China); small heterodimer partner (SHP) antibody (bs-4311R, Bioss, Beijing, China); liver receptor homolog-1 (LRH-1) antibodies antibody (sc-393369, Santa Cruz, America); DMEM culture medium (c1995500bt, Thermo Fisher, Shanghai, China); fetal bovine serum (11012 – 8611, Tianhang, Zhejiang, China); phosphate buffer solution, penicillin-streptomycin (BL302A, BL505A, LABGIC, Beijing, China); trypsin-EDTA solution (cc0128, Leagene, Beijing, China); cholesterol, 25-hydroxycholesterol (S11040, S28281, Yuanye, Shanghai China); BLT-1 (CSN27294, CSNpharm, America); cell counting Kit-8 (GK10001, glpbio, America); and Oil Red O solution, Dil-HDL (G1262, H8910, Solarbio, Beijing, China).

Animals and experimental design

Sixty male SD rats (average weight 160–180 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (experimental animal license number: SCXK (Jing) 2016-0011). All rats were housed in a specific environment ($22 \pm 2^\circ\text{C}$, $50 \pm 5\%$ humidity, light/dark cycle 12 h/12 h) with free access to food and water. After 7 days of adaptive feeding, the rats were randomly divided into six groups (control, HFD, low, medium, high, and sim, $n = 10$ per group). Except for the control group, all rats were fed HFD for 6 weeks. The rats in the control and HFD groups intragastrically received the same volume of physiological saline while the rats in the low, medium, high, and sim groups received AS-NFA ($17 + 40$, $34 + 80$, $68 + 160 \text{ mg}\cdot\text{kg}^{-1}$) and sim ($0.126 \text{ g}\cdot\text{kg}^{-1}$) for the last 4 weeks via gavage. The dosage and ratio of AS-NFA administration were determined based on preliminary experiments(26). The rats were weighted once a week. The feces of each rat were collected at week 6 and stored for -80°C . On the last day of the sixth week, the rats were fasted overnight and then anesthetized with 3% pentobarbital sodium. Blood samples were collected into 10 mL centrifuge tubes and stored for 20 min at room temperature and then centrifuged at 4°C (4000 rpm) to obtain serum samples. The livers were then partially collected and stored at -80°C .

Hematoxylin-eosin (H&E) staining

Liver tissue was fixed with 4% paraformaldehyde solution, dehydrated, and embedded in paraffin, and 5- μ m sections were then stained with H&E. The stained slides were observed, and images were obtained with BX43 biological microscope (Olympus, Shanghai, China).

Oil Red O staining

Frozen liver tissue was cut into 5- μ m slices and stained with Oil Red O. Images were collected with a biological microscope. HepG2 cells were spread in six-well plates and fixed with 4% paraformaldehyde solution. Samples were washed with distilled water and stained with the Oil Red O kit following the steps in the instructions. Images were captured with the ICX41RFL microscope (Shunyu, Ningbo, China).

Biochemical index analysis

The serum contents or activities of TC, TG, HDL-C, LDL-C, TBA, ALT, and AST were detected with the corresponding kits using an automatic biochemical analyzer (ICUBIO, Shenzhen, China). Liver tissue and feces were ground at 4 °C to make 10% homogenate. This was centrifuged at 3000 rpm for 15 min, and the supernatant was collected. The contents of TC and TBA in liver tissue and feces were detected with a semi-automatic biochemical analyzer (HOMAG, Germany). The HepG2 cells were collected after digestion with trypsin and lysed with RIPA Lysis Buffer. TC and TBA contents of the liver homogenates and cell lysates were detected by measuring the absorbance at 510 or 405 nm using a VICTOR Nivo microplate reader (Perkin Elmer, Massachusetts, Germany).

Immunofluorescence staining

The liver tissue was embedded and sectioned. The sections were blocked for 30 min. Sections of the liver tissue were incubated overnight at 4°C with primary antibodies to SR-B1 and FXR. After rinsing with PBS, goat anti-rabbit IgG was incubated for 1 h at room temperature. The nucleus was then stained with DAPI for 15 min. Immunofluorescence was observed with a fluorescence microscope.

Cell culture and treatment

HepG2 cells (PVSQ8CTP7W, Procell, Wuhan, China) were cultured in DMEM supplemented with 10% FBS, 1% penicillin, and streptomycin at 37 °C under 5% CO₂. After reaching 75% confluence, cells were treated with AS-NFA (10 + 25 20 + 50, 40 + 100, 80 + 200, 160 + 400, 320 + 800, 640 + 1600 mg·mL⁻¹) for 12, 24, or 48 h to screen for optimal dose and time of administration. Cells were incubated with 10 mg·mL⁻¹ cholesterol and 1 mg·mL⁻¹ 25-hydroxycholesterol for 24 h to establish a hyperlipidemia cell model. In follow-up experiments, cells were always exposed to cholesterol and 25-hydroxycholesterol during drug treatment. A selective SR-B1 inhibitor (BLT-1) was used to restrain SR-B1 activation and investigate whether SR-B1 is necessary for AS-NFA to exert its function. Cells were pretreated with BLT-1 (10 μ mol·mL⁻¹) for 2 h.

Quantitative real-time RT-PCR

Total RNA was extracted from liver tissue and HepG2 cells using Eastep Super Total RNA Extraction Kit according to the instructions. Total RNA concentrations were determined by an ultra-micro

spectrophotometer (METTLER TOLEDO, Shanghai, China). cDNA was obtained using the SureScript First-Strand cDNA Synthesis Kit. The mRNA expression levels of β -actin, SR-B1, CES1, CYP7A1, FXR, SHP, and LRH-1 were detected by BlazeTaq SYBR Green qPCR Mix 2.0 using a QuantStudio™ 1 real-time PCR system (Thermo Fisher Scientific, Massachusetts, USA). Relative expression levels were detected. The primers were synthesized by Servicebio and the sequences of oligonucleotide primers are indicated in Table 1–2.

Table 1
List of primer sequences of the liver.

Gene	Sequence
SR-B1	TTCGTTTCCAGCCAGACAGG
	ACCCAAGGTCATTAGCAGC
CES1	CAATTCCAGTGGCCGTTGAG
	TCGTACATGTATGTGCGGGC
CYP7A1	GGGCAGGCTTGGAATTTTG
	AGTGAGCATTGGTCCCGAAG
FXR	CACTGACACGCCCTTTTTGC
	TGGAGGATAAAACGAGGCGG
SHP	CCAGACACTCCTGGATGGTG
	TGGATGCCAAGTCCCAAGAA
LRH-1	TATTGCACCTGCACCAGGAT
	GCACGTTTTTCCCGGTGTTT
β -actin	GCAGGAGTACGATGAGTCCG
	ACGCAGCTCAGTAACAGTCC

Table 2
List of primer sequences of HepG2 cells.

Gene	Sequence
SR-B1	GGTGATGATGGAGAATAAGCCC
	AGAGCCCAGAGTCGGAGTTG
CES1	GACACCGTGCATGGCAAAG
	TGCACATAGGAGGGTACGAGGT
CYP7A1	GCAAACACCATTCCAGCGACT
	GCCTCAGCGATTCTTGATTAT
FXR	CGACAAGTGACCTCGACAACAA
	GACCCTTTCAGCAAAGCAATCT
SHP	CAATGCTGTCTGGAGTCCTTCTG
	CAGGGTTCCAGGACTTCACAC
LRH-1	AAAGCGTTGTCCTTACTGTCGT
	GCCCAAACCTTATTCCTTCCTCC
β-actin	CACCCAGCACAATGAAGATCAAGAT
	CCAGTTTTTTAAATCCTGAGTCAAGC

Western blot

Total proteins were extracted from liver tissue and HepG2 cells and then lysed with RIPA Lysis Buffer. The protein concentrations were determined with an ultra-micro spectrophotometer, and then total proteins were denatured at 100°C for 10 min. Protein (60 µg) was fractionated with 10% SDS-PAGE and then transferred onto PVDF membranes. The membranes were sealed with 5% skim milk for 3 h at room temperature and incubated with specific primary antibodies against β-actin (1:10000), SR-B1, CES1, CYP7A1, FXR, SHP, and LRH-1 (1:2000) at 4°C overnight. The membranes were then washed three times with TBST and incubated with a secondary antibody for 55 min at room temperature. Proteins were visualized using an Intelligent Image Workstation (6000Plus, Guangzhou Biolight Biotechnology Co., Ltd, Guangzhou, China).

Statistical analysis

Data were analyzed by SPSS version 26 and showed as mean ± SEM. Statistical analysis used one-way ANOVA followed by the Tukey test. All statistical tests with $P < 0.05$ were considered significantly significant.

Results

Effects of AS-NFA on serum, liver, and feces biochemical indices in rats

After consistent consumption of HFD for six weeks, serum levels of TC, TG, LDL-C, TBA, AST, and ALT were significantly increased while the level of HDL-C was significantly decreased in HFD-fed rats versus the control group ($P < 0.01$) (Fig. 1A-G). The results indicated that a rat model of dyslipidemia was successfully replicated. Administration of AS-NFA at various doses greatly decreased TC, TG, LDL-C, TBA, AST, and ALT contents or activities, and increased HDL-C content compared with the HFD group ($P < 0.05$, $P < 0.01$). Moreover, the TC and TBA contents in the liver and feces of the control group were significantly lower than those in the HFD group ($P < 0.01$) (Fig. 1H-K). After AS-NFA intervention, the TC and TBA content in the liver decreased, while the TC and TBA in the feces increased ($P < 0.05$, $P < 0.01$).

Effects of AS-NFA on histopathology and liver lipid accumulation in rats

H&E staining of the control group showed that the central veins of the liver tissue were clear, the hepatic cords were radial, the cells were closely arranged, the nucleus was centered, and there were no obvious lipid droplet vacuoles. In the HFD group, however, the hepatocytes were swollen, and some of them were swollen like balloons; there was a lightly stained cytoplasm and many lipid droplet vacuoles. The larger ones could fill the whole cell and squeeze the nucleus to one side.

After the administration, the hepatocytes in all groups were arranged more neatly. The nucleus was normalized, and the balloon-like changes and lipid droplet vacuoles were improved to different degrees (Fig. 2A). The Oil Red O staining showed no red intracellular lipid droplets in the hepatocytes in the control group. However, the HFD group had diffuse spherical lipid droplets and fat vacuoles in the liver tissue. These lipid droplets were significantly reduced, and the lipid deposition was significantly improved in all groups after drug administration (Fig. 2B).

Effects of AS-NFA on SR-B1/CYP7A1/FXR signaling pathway in rats

The expression levels of SR-B1, CES1, CYP7A1, FXR, SHP mRNA, and protein were markedly decreased in the liver of dyslipidemia rats versus control group ($P < 0.01$) (Fig. 3–4). However, the up-regulation of SR-B1, CES1, CYP7A1, FXR, SHP mRNA and protein were reversed by AS-NFA ($P < 0.05$, $P < 0.01$). However, there was no significant difference in the mRNA and protein expression of LXR-1 in each group ($P > 0.05$).

The immunofluorescence staining was used to inspect the expression of SR-B1 and FXR protein in the liver. The results showed that the positive expression of SR-B1 and FXR was detectable in the control group (Fig. 5). However, the positive expression of SR-B1 and FXR in the HFD group was markedly

increased versus the control group. In contrast, the positive expression of SR-B1 and FXR was substantially decreased by AS-NFA treatment.

Effects of AS-NFA on lipid accumulation in HepG2 cells

To examine the cytotoxicity of AS-NFA in HepG2 cells, the cells were treated with a range of concentrations (10 + 25, 20 + 50, 40 + 100, 80 + 200, 160 + 400, 320 + 800 and 640 + 1600 mg.mL⁻¹) of AS-NFA, and cell viability was determined using the CCK-8 kit (Fig. 6A). When the concentration of AS-NFA was higher than 160 + 400 mg.mL⁻¹, the proliferation of HepG2 cells was significantly inhibited with increased drug concentration and intervention time ($P < 0.01$). Therefore, 80 + 200 mg.mL⁻¹ and 60 + 400 mg.mL⁻¹ were used as the low and high doses of the drug with treatment for 24 h in follow-up experiments. The results indicated that lipid droplets and the levels of TC and TBA were increased strikingly in the model group of HepG2 cells ($P < 0.01$) (Fig. 6B-D). The HepG2 cells were then co-incubated with cholesterol, 25-hydroxycholesterol and AS-NFA. The number of the cellular lipid droplets and the levels of TC and TBA were significantly decreased versus the model group ($P < 0.01$), which was reversed by pro-treatment with BLT-1 ($P < 0.05$, $P < 0.01$).

Effects of AS-NFA on Dil-HDL uptake in HepG2 cells

Dil-HDL was used to explore whether AS-NFA could promote HDL uptake. Fluorescence micrographs showed that the red fluorescence was obviously decreased in the model group of HepG2 cells. Enhanced fluorescence intensity was observed in AS-NFA-treated cells and was reversed by pro-treatment with BLT-1 (Fig. 6E).

Effects of AS-NFA on SR-B1/CYP7A1/FXR signaling pathway in HepG2 cells

We detected the effects of AS-NFA in cholesterol and 25-hydroxycholesterol-treated HepG2 cells. In contrast to the control group, the expression levels of SR-B1, CES1, CYP7A1, FXR, SHP mRNA and protein were markedly decreased in the model group (Fig. 7–8) ($P < 0.01$). After AS-NFA treatment, the expression levels of SR-B1, CES1, CYP7A1, FXR, SHP mRNA and protein increased ($P < 0.05$, $P < 0.01$), but these effects were reversed by BLT-1 ($P < 0.05$, $P < 0.01$). There was no significant difference in the mRNA and protein expression of LRH-1 in each group ($P > 0.05$).

Discussion

Dyslipidemia is a type of metabolic disease recognized as a significant public health concern worldwide(27). Chinese herbals can be a primary source of active compounds for anti-dyslipidemia. Recently, component-based Chinese medicine has been characterized by safety, high efficiency, controllable quality, and low toxicity and side effects. In addition, component-based Chinese medicine acts on multiple targets and participates in complex pathological processes of disease(28). Component compatibility not only maintains the core advantages of traditional Chinese medicine, but also integrates modern drug design technology to lay the foundation for the discovery of new traditional Chinese

medicine preparations. Drug homologous foods such as *Astragalus* and *Nelumbinis folium* have favorable effects on dyslipidemia and related metabolic diseases(23, 29). Among the bioactive compounds from medicinal foods, saponins and alkaloids have demonstrated promising lipid-regulating effects(30, 31). ATS and NFTA are the main components of *Astragalus* and *Nelumbinis folium*. Although previous studies have reported that ATS and NFTA can regulate lipid metabolism, it is not explicit that the potential mechanisms that regulation of RCT can improve dyslipidemia. Here, we found that AS-NFA can improve lipid metabolic disorders and then reduce hepatic pathological damage and lipid deposition in HepG2 cells. The underlying mechanisms can activate SR-B1/CYP7A1/FXR signaling pathway and promote RCT.

The liver is the main lipid production organ, thus controlling lipid aggregation, secretion, and catabolism (32, 33). When lipid metabolism or transport in the body is imbalanced, aberrantly raised TC and TG in the serum may be deposited in liver tissue, thus resulting in severe lipid lesions. In particular, abnormally high blood cholesterol levels are a major cause of lipid deposition(34). The levels of TBA, ALT, and AST in blood can evaluate the degree of liver injury in clinical practice. Here, treatment with AS-NFA not only markedly reduced serum TC, TG, LDL-C, TBA, AST, and ALT levels—and remarkably increased serum HDL-C levels—but also ameliorated hepatic and feces TC and TBA levels. Moreover, AS-NFA improved the morphological changes of the liver tissue in HFD rats. The Oil red O staining suggests that AS-NFA decreased the lipid contents. Accordingly, our experiments demonstrated that the AS-NFA effectively regulated blood lipids, improved hepatocyte steatosis, protected liver function, reduced lipid accumulation, and exerted a better effect on preventing and controlling dyslipidemia.

RCT is the process by which HDL transports excess FC from peripheral tissue or cells to the liver, where it is metabolized to produce bile acid (BA) for excretion(35). RCT is an endogenous defense mechanism to protect cells from excessive cholesterol accumulation in disorders of lipid metabolism(9, 36). HDL acts both as a receptor and a carrier for cholesterol in the RCT, thus playing an important role in the RCT process(37, 38). In particular, the uptake of lipids by HDL bound to SR-B1 in hepatocytes is the initiating factor of RCT(39, 40).

Previously, quercetin-pretreated HepG2 cells exhibited a potent increase in selective lipid uptake and Dil-HDL binding mediated by SR-B1—this trend is consistent with an increase in SR-B1 at the transcriptional and translational levels(41). Here, Dil-HDL was co-incubated with HepG2 cells. Uptake of HDL by HepG2 cells in the model group was significantly reduced after cellular uptake. However, the administration of AS-NFA increased HDL uptake and attenuated the ability of BLT-1 to inhibit HDL uptake by the HepG2 cells, thus further confirming that lipid uptake by HepG2 cells was mediated by SR-B1.

The RCT process relies on SR-B1-mediated selective HDL-C uptake(41, 42). Therefore, hepatic SR-B1 is essential for HDL-C clearance. SR-B1, located on the cell surface, is a receptor for HDL and selectively delivers CE to hepatocytes by binding to HDL particles that lack cholesterol. They then release HDL particles back into circulation(43, 44). Knockdown of SR-B1 caused excessive accumulation of CE in HDL, thus reducing hepatic uptake of cholesterol and inhibiting HDL-mediated RCT(45).

CES1 is highly expressed in the liver and exhibits cholesteryl ester hydrolase activity; it hydrolyzes SR-B1-delivered CE to free cholesterol (FC)(15). Prior work found that CES1^{-/-} mice developed hepatic steatosis, increased obesity, and hyperlipidemia(46). In contrast, specific overexpression of CES1 in liver reduced hepatic FC level and attenuated diet-induced dyslipidemia(47). The upregulated expression of CES1 is directly associated with SR-B1-delivered HDL-CE hydrolysis—this was accompanied by increased SR-B1 expression(19). Recombinant cytochrome P450 7A1 (CYP7A1) is only expressed in the liver to maintain the balance between cholesterol and BA by catalyzing the metabolism of FC generated to BA(16, 48). Previous studies demonstrated that increased CYP7A1 promotes FC-catalyzed metabolism to produce BA and activate the receptor FXR, thus enhancing RCT and improving hypercholesterolemia(49).

FXR is a nuclear receptor of BA involved in BA and lipid metabolism(50). An increase in BA concentration can effectively activate FXR(51, 52). SHP is a transcriptional repressor directly regulated by FXR(53, 54). LRH-1 is an important target of CYP7A1 activation that promotes CYP7A1 expression(55). FXR is activated and upregulates SHP, which binds to LRH-1 to form a heterodimer to inactivate LRH-1 and inhibit CYP7A1 transcription(56, 57). This study showed that the FXR-SHP signal could regulate the expression of CYP7A1 and thus promote BA metabolism(58). Moreover, the activation of FXR increased SRB1 expression via FXR-JNK-HNF4α-SRB1 pathway to reinitiate RCT(51).

We found here that the mRNA and protein expression levels of SR-B1, CES1, CYP7A1, FXR, and SHP were decreased in liver tissue of HFD rats and HepG2 steatosis cells, but these conditions were reversed by AS-NFA. AS-NFA administration alone significantly decreased the mRNA and protein expression levels of SR-B1, CES1, CYP7A1, FXR, and SHP. Moreover, if SR-B1 was blocked by BLT-1 in HepG2 cells, then the mRNA and protein expression levels of SR-B1, CES1, CYP7A1, FXR, and SHP all decreased. In fact, unaltered LRH-1 was observed in each group of rats, which might be a sign of impaired binding of SHP and LRH-1. This in turn explains the increased CYP7A1. The effect of AS-NFA was blocked by BLT-1 after the intervention with BLT-1 and AS-NFA. Therefore, our study indicates that AS-NFA could activate SR-B1/CYP7A1/FXR signaling pathway to promote RCT. This then effectively improves lipid accumulation, thus indicating that this might be one of the underlying mechanisms of AS-NFA protection against dyslipidemia.

Conclusion

In short, we confirmed that AS-NFA could efficiently relieve dyslipidemia, pathological injury, and hepatic steatosis in HFD-fed rats. It also ameliorates lipidosis and increases the uptake rate of cholesterol in cholesterol- and 25-hydroxycholesterol-induced HepG2 cells. Our results further demonstrated that AS-NFA could activate the SR-B1/CYP7A1/FXR signaling pathway, regulate the expression of related genes and proteins, promote RCT, and improve dyslipidemia (Fig. 9). This study provided vital experimental evidence to develop AS-NFA as a nutraceutical or drug for inhibiting dyslipidemia and related metabolic disorders. RCT represents a mechanism of possible anti-dyslipidemia tactics in AS-NFA.

Abbreviations

ALT alanine aminotransferase

AST aspartate aminotransferase

BA bile acid

CE cholesteryl ester

CES1 carboxylesterase 1

CYP7A1 recombinant cytochrome P450 7A1

FC free cholesterol

FXR farnesoid X receptor

HDL-C high density lipoprotein cholesterol

LDL-C low density lipoprotein cholesterol

LRH-1 liver receptor homolog-1

RCT reverse cholesterol transport

SHP small heterodimer partner

SR-B1 scavenger receptor class B type 1

TBA total bile acid

TC total cholesterol

TG triglyceride

Declarations

Funding

Not applicable.

Availability of data and materials

The data presented in this study are available within the article.

Ethics approval and consent to participate

All experimental procedures were conducted by the ethical guidelines authorized by the Ethics Committee of Hebei University of Chinese Medicine (Certificate number: DWLL2019011).

Conflicts of interest

The authors declare that they have no competing financial interests or conflicts.

Disclosure

The authors declare no conflict of interest.

Acknowledgements

Not applicable.

Authors' contributions

Chen J and Shi C equally contributed to this work. Zhang YX and Zhang MQ conceived and designed the experiments. Chen J, Zhang YF and Li DT performed the experiments. Shi C, GAO Y and Gu Y analyzed the data. Chen J and Shi C wrote the manuscript. Zhang YX and Zhang MQ revised the manuscript. All of the authors approved the submission of the manuscript.

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Figures

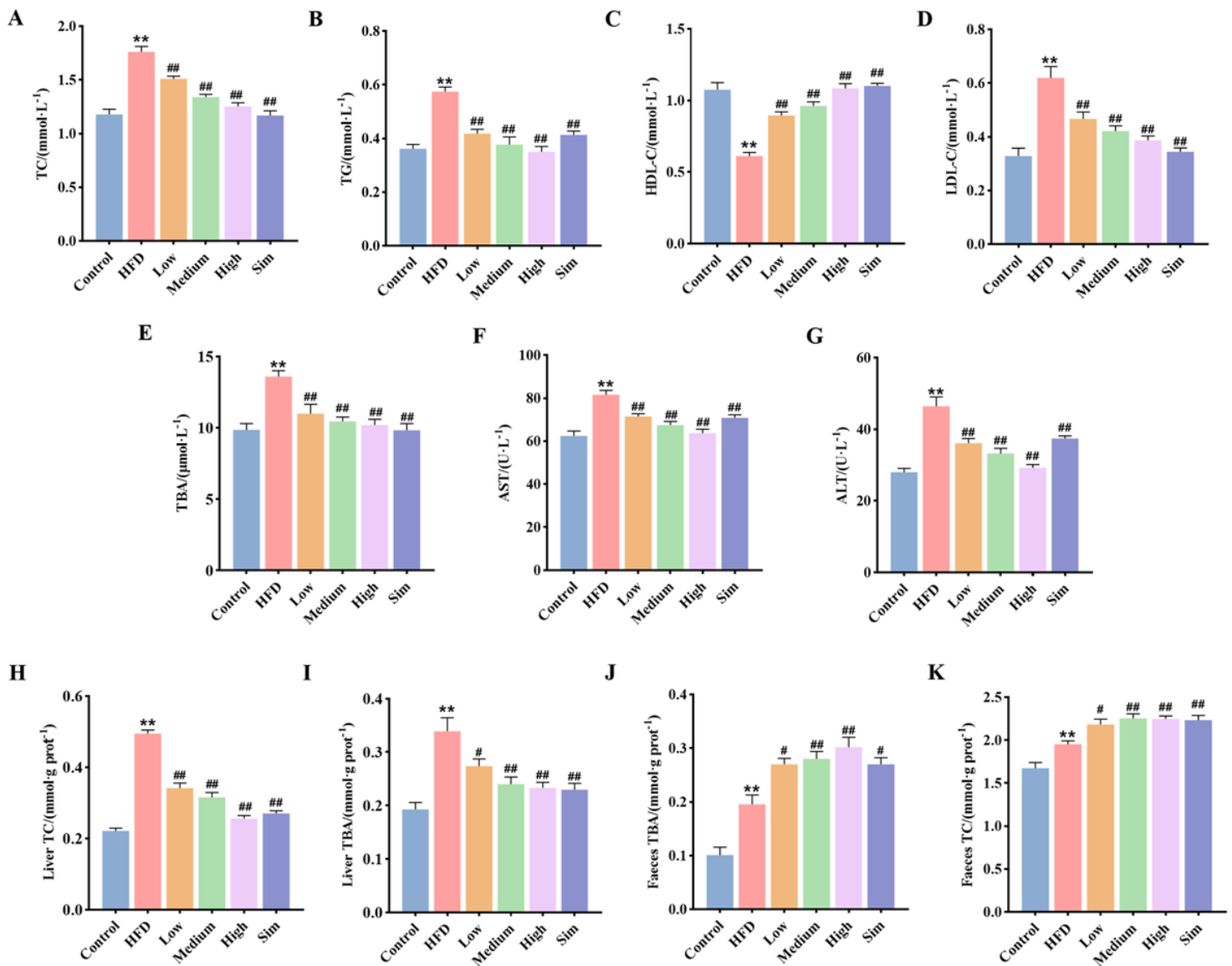
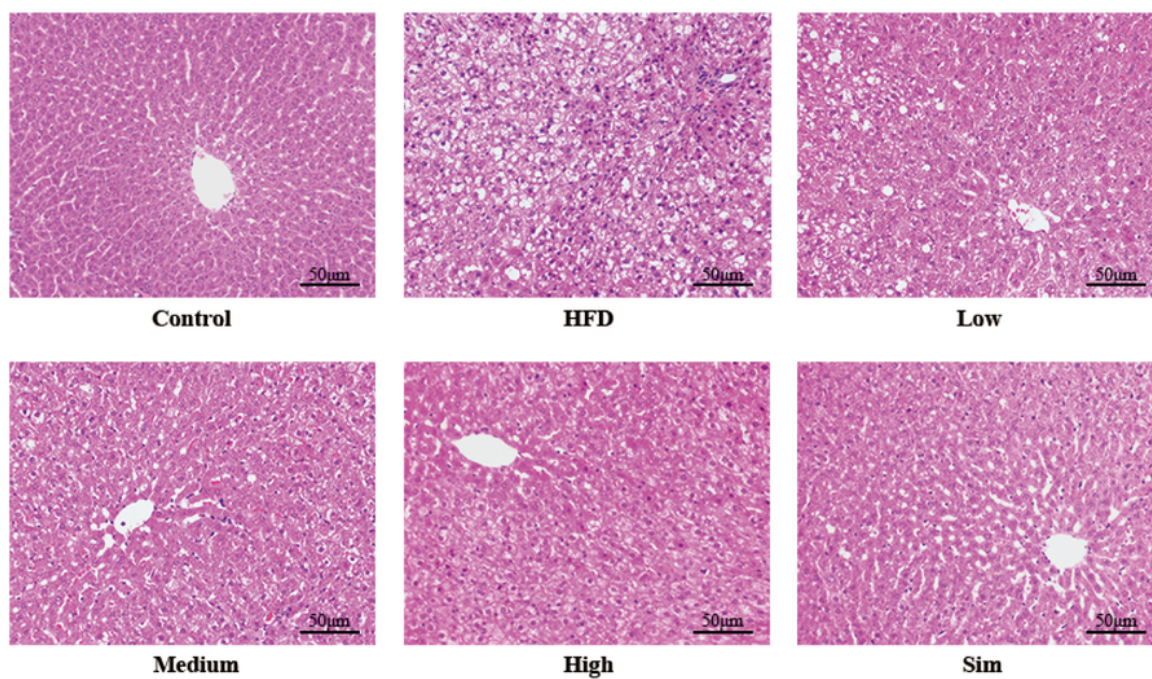


Figure 1

AS-NFA regulated the levels of TC, TG, LDL-C, TBA, AST, and ALT in dyslipidemia rats. (A-G) TC, TG, HDL-C, LDL-C, TBA, AST, and ALT levels in serum. (H-I) TC and TBA levels in liver. (J-K) TC and TBA levels in feces. All values are presented as the mean $\pm$ SEM; n=8, ** P < 0.01 vs. control, # P < 0.05, ## P < 0.01 vs. HFD.

A



B

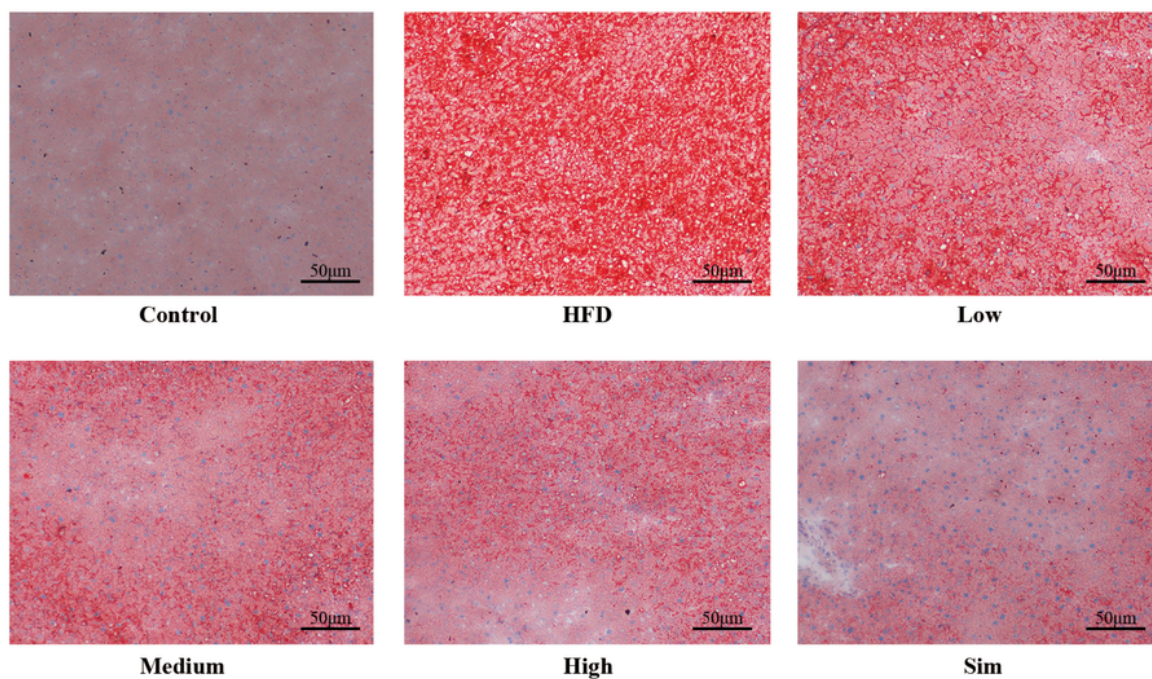


Figure 2

AS-NFA alleviated pathological injury lipid accumulation in the livers of dyslipidemic rats.(A) H&E staining of HepG2 cells (200×). (B) Oil Red O staining of HepG2 cells (200×).

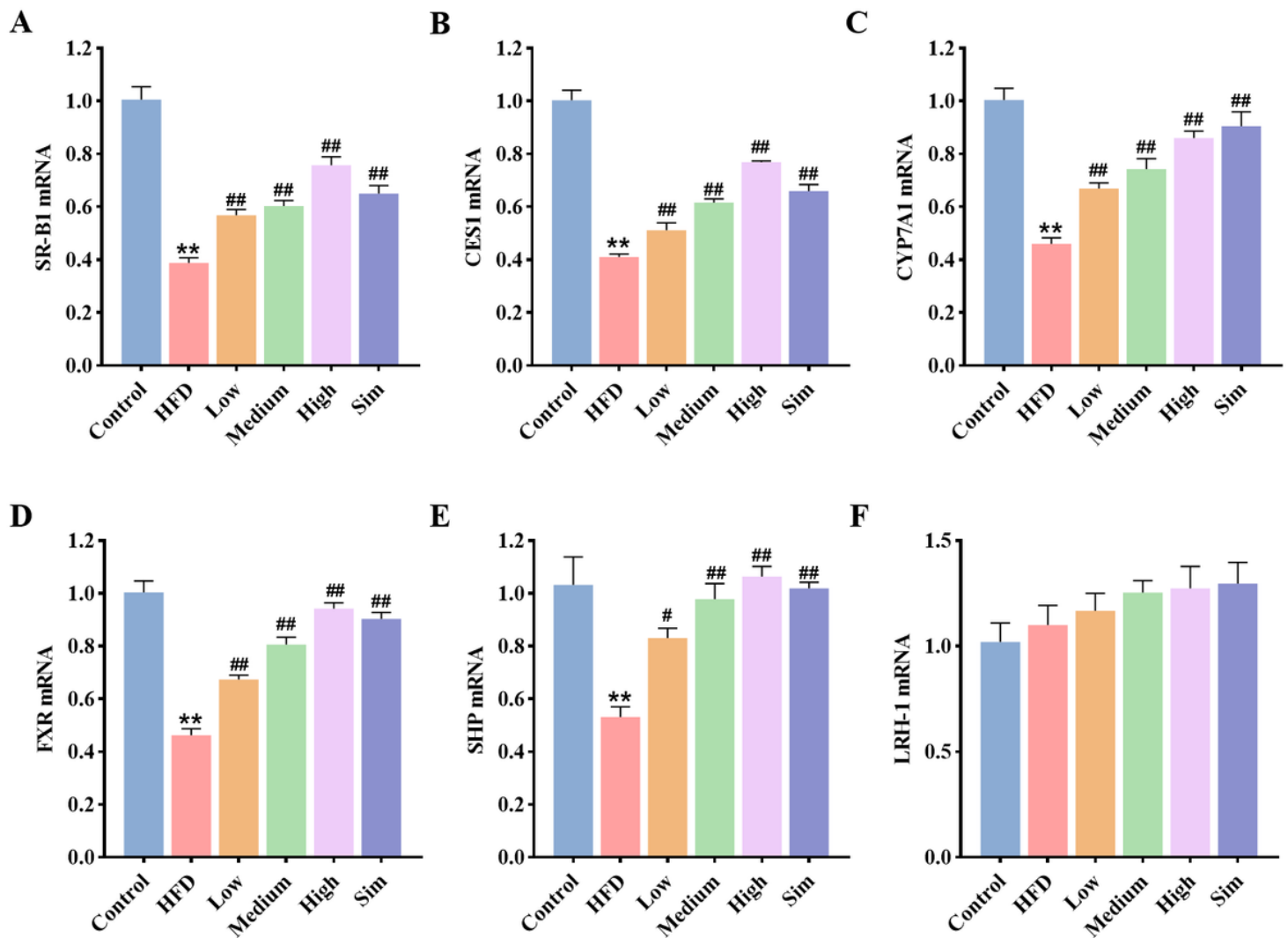


Figure 3

AS-NFA regulated the expression of genes related to the SR-B1/CYP7A1/FXR signaling pathway in the livers of dyslipidemic rats. (A-F) The relative expression levels of SR-B1, CES1, CYP7A1, FXR, SHP, and LRH-1. All values are presented as the mean $\pm$ SEM, $n=6$. ** $P < 0.01$ vs. control. # $P < 0.05$, ## $P < 0.01$ vs. HFD.

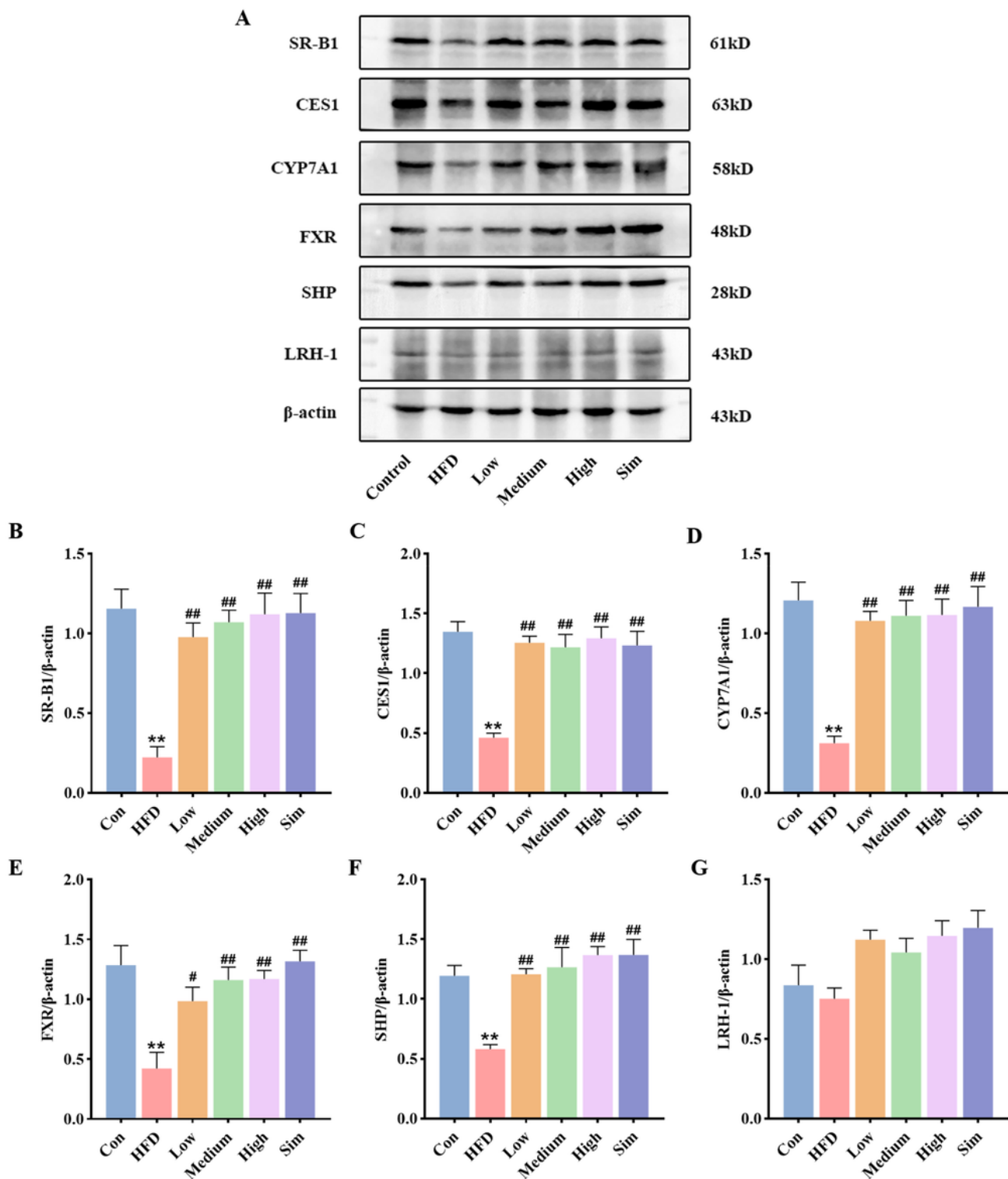


Figure 4

AS-NFA regulated the expression of protein related to the SR-B1/CYP7A1/FXR signaling pathway in the livers of dyslipidemic rats. (A-G) The relative expression levels of SR-B1, CES1, CYP7A1, FXR, SHP, and LRH-1. All values are presented as the mean $\pm$ SEM; $n=3$, $P < 0.05$, $**P < 0.01$ vs. control, $\#P < 0.05$, $##P < 0.01$ vs. HFD.

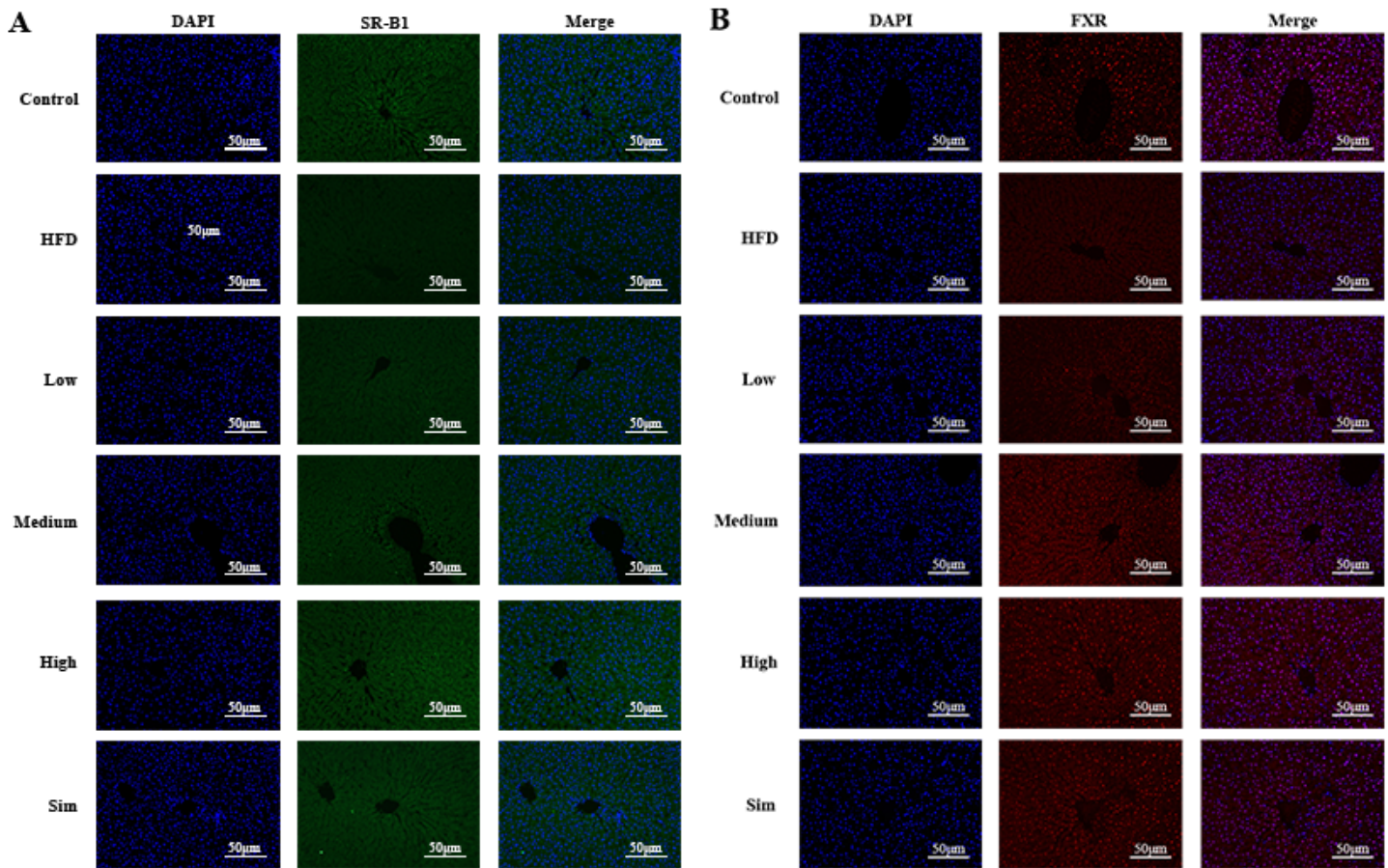


Figure 5

AS-NFA increased the expression of SR-B1 and FXR protein in the livers of dyslipidemic rats. (A) Immunofluorescence expression of SR-B1 (200×). (B) Immunofluorescence expression of FXR (200×).

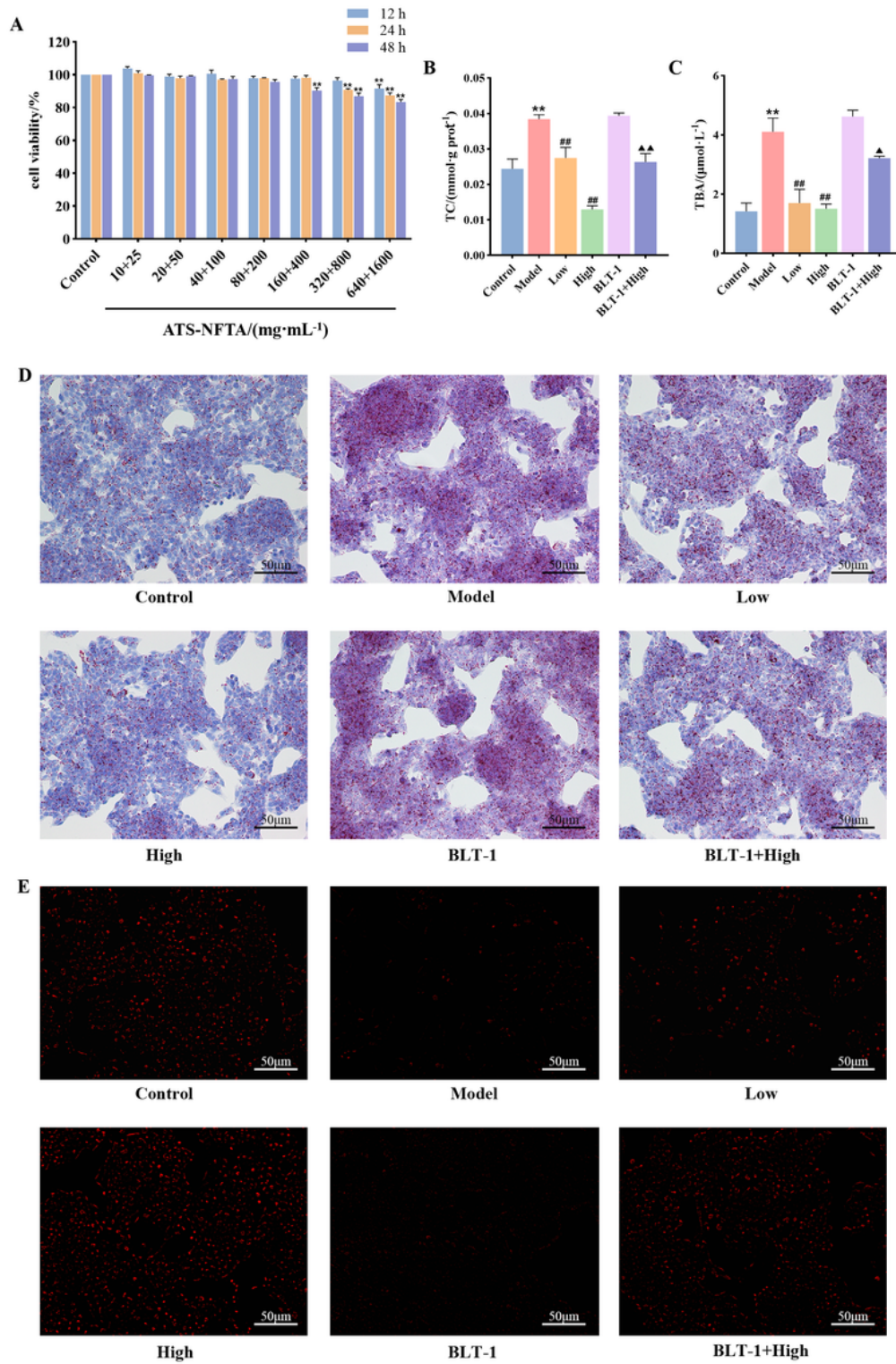


Figure 6

AS-NFA alleviated lipid accumulation and increased the intake of HDL in HepG2 cells. (A) Cell viability. (B-C) TC and TBA contents. (D) Oil Red O staining of HepG2 cells (200 $\times$). (E) the intake of HDL in HepG2 cells (200 $\times$). All values are presented as the mean $\pm$ SEM; $n=3$, ** $P < 0.01$ vs. Control, ## $P < 0.01$ vs. Model, ▲ $P < 0.05$, ▲▲ $P < 0.01$ vs. High.

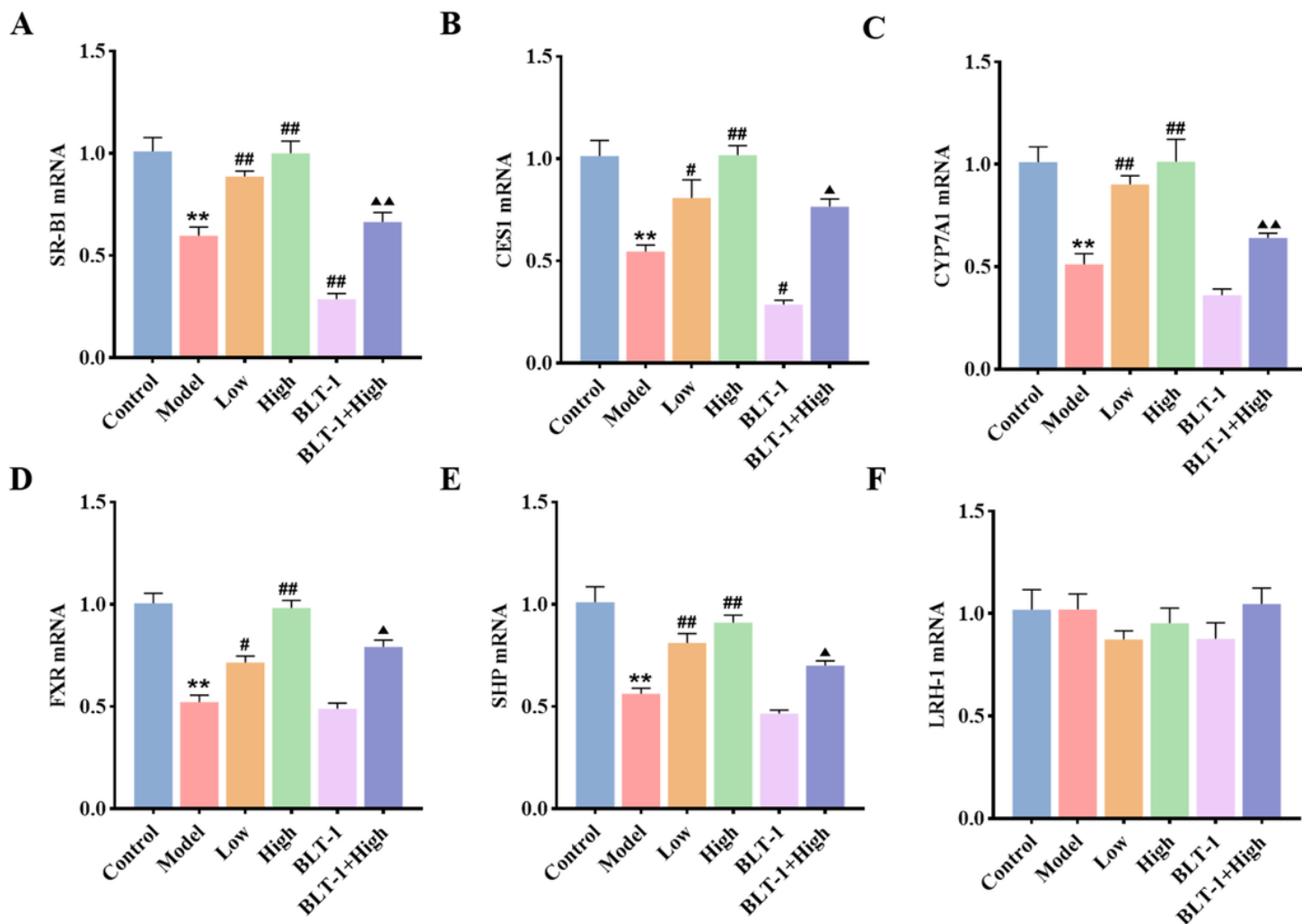


Figure 7

AS-NFA regulated the expression of genes related to the SR-B1/CYP7A1/FXR signaling pathway in HepG2 cells. (A-F) The relative expression levels of SR-B1, CES1, CYP7A1, FXR, SHP, and LRF-1. All values are presented as the mean $\pm$ SEM; $n=5$, ** $P < 0.01$ vs. control, # $P < 0.05$, ## $P < 0.01$ vs. Model, ▲ $P < 0.05$, ▲▲ $P < 0.01$ vs. High.

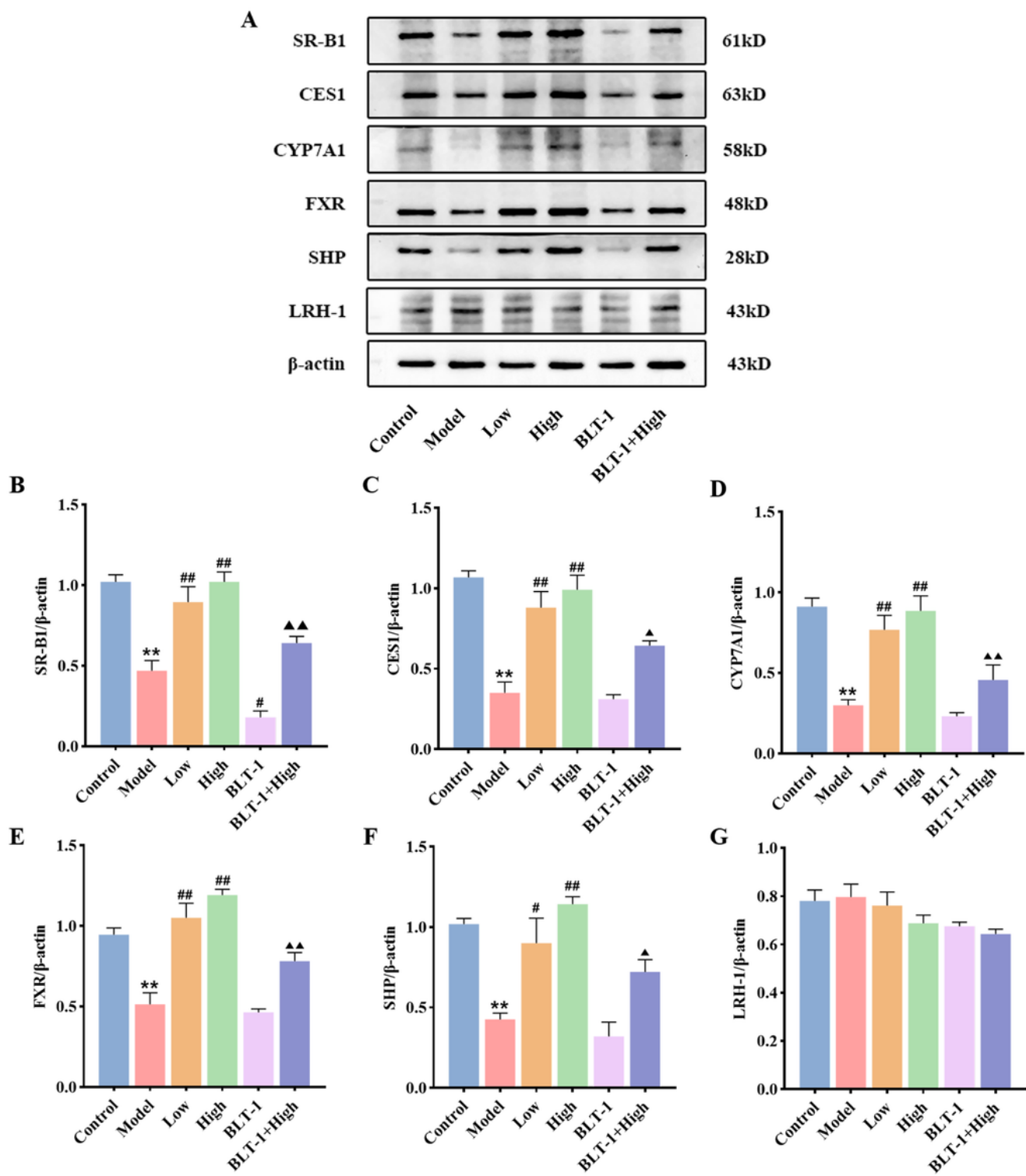


Figure 8

AS-NFA regulated the expression of protein related to the SR-B1/CYP7A1/FXR signaling pathway in HepG2 cells. (A-G) The relative expression levels of SR-B1, CES1, CYP7A1, FXR, SHP, and LRH-1. All values are presented as the mean $\pm$ SEM; $n=3$, ** $P < 0.01$ vs. control, # $P < 0.05$, ## $P < 0.01$ vs. Model, ▲ $P < 0.05$, ▲▲ $P < 0.01$ vs. High.

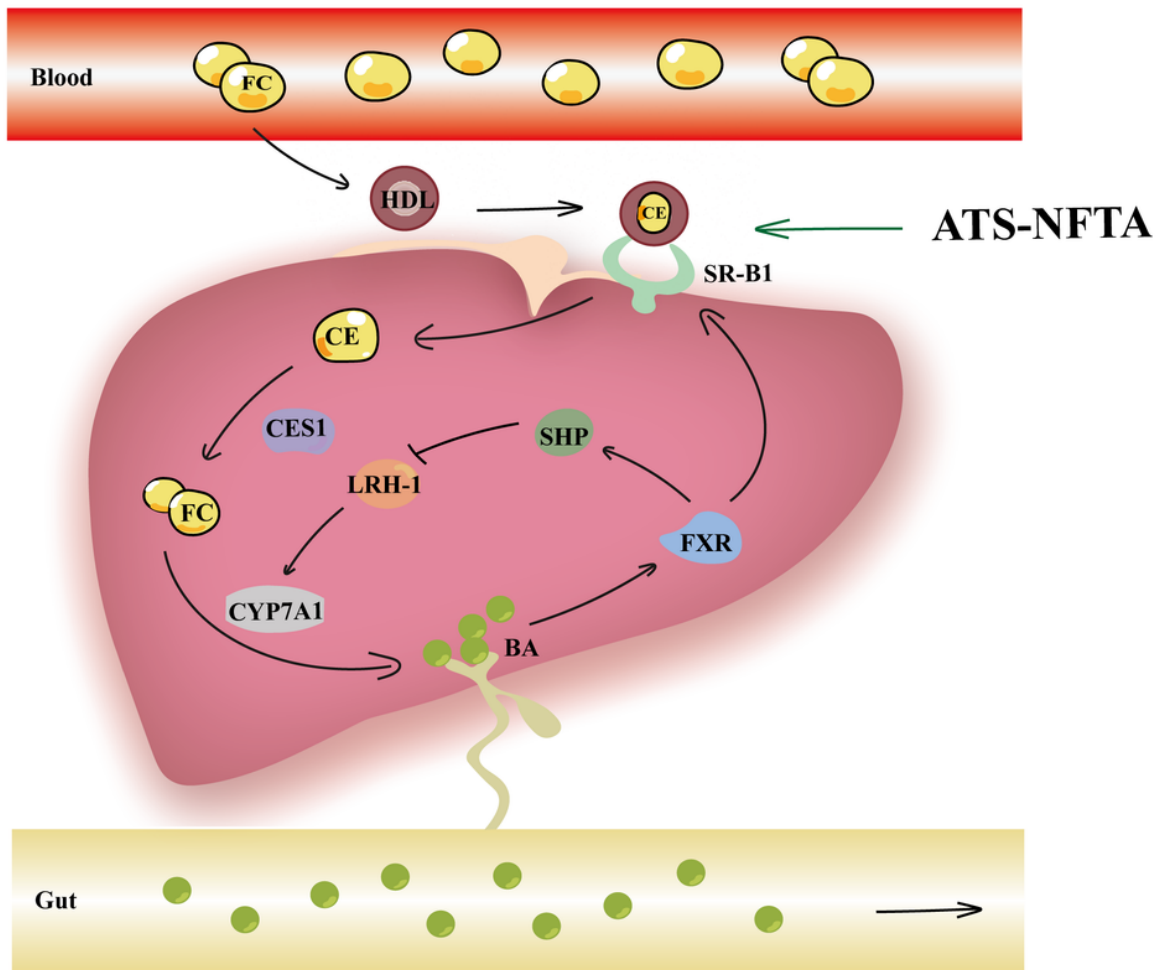


Figure 9

Schematic representation of AS-NFA regulation of RCT via SR-B1/CYP7A1/FXR signaling pathway.