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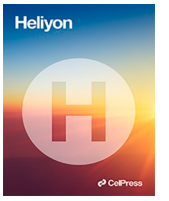

Cefminox sodium alleviates the high-fat high-sugar-fed mice's hepatic fatty accumulation via multiple pathways

Leming Xiao [a,1], Chengrui Liang [a,1], Jing Gao [a], Yin Wang [a], Yanzi Guo [b], Kan Chen [a], Xiaoyuan Jia [a,*]

[a] College of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou, China
[b] The Second Affiliated Hospital of Shaanxi Traditional University, Xianyang, China

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ABSTRACT

The increasing global prevalence of nonalcoholic fatty liver disease (NAFLD) starves for effective therapy, but no agent has been approved yet. We sought to evaluate the therapy of cefminox sodium (CMNX) on fatty accumulation in animal and cell models and explore the underlying mechanisms. The results revealed that CMNX reduced the gain of the liver and alleviated fatty accumulation both in high-fat high-sugar diet (HFHSD) mice's livers and WRL-68 cells. In HFHSD mice's livers and FFAs exposure hepatic cells, ACC1, SREBP-1c, and CYP2E1 were enhanced expression, which were reversed by CMNX treatment. In addition, PPARγ, PPARα, PCK1, and ACSL4 expressions were increased in CMNX-treated WRL-68 cells. These findings suggest that CMNX improves fatty accumulation in HFHSD mice/hepatic cells by restraining fatty acid synthesis and facilitating fatty acid oxidation.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a worldwide chronic liver disease with a 25 % prevalence. Generally, from simple fatty liver to non-alcoholic steatohepatitis (NASH), cirrhosis, and even primary liver cancer can be classified as NAFLD [1,2]. The early feature of NAFLD is hepatic steatosis and can be histologically diagnosed once fat accumulation proceeds in at least 5 % of all hepatocytes. Because NAFLD is correlated strongly with type II diabetes and cardiovascular diseases, it is also considered a multisystem disease [3,4]. Up to now, there are no pharmacological agents approved by the FDA for this disease [5], and the limited remedies are calorie restriction, exercise, and other lifestyle interventions [6].

Peroxisome proliferator-activated receptors (PPARs) are members of the supergene family of nuclear receptors, including α, β/δ, and γ isoforms. As crucial transcription factors, PPARs take part in regulating lipid and glucose homeostasis, inflammation, and fibrogenesis [7–9]. Therefore, PPARs are considered one of the promising targets to treat NAFLD, and several compounds targeting PPARs have been tested in the trials. Recently, a phase II trial manifested the benefits of one PPARα/γ agonist saroglitazar in patients with NAFLD/NASH [10]. In addition, lanifibranor, a pan-PPAR agonist, can regulate inflammatory, metabolic, and fibrogenic pathways in the occurrence of NASH. A phase IIb trial supports the potential benefits of lanifibranor in patients with NASH [11]. Cefminox sodium (CMNX), a semi-synthetic, beta-lactamase-stable cephalosporin [12], has been applied in clinical practice for many years to

* Corresponding author. College of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou, 310018, China.
E-mail address: xyjia@zstu.edu.cn (X. Jia).
[1] Leming Xiao and Chengrui Liang contributed equally to this work.

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treat infectious diseases and parasitic diseases. It is regarded as a highly safe cephalosporin antibiotic in clinical practice [13], although a rare coagulation dysfunction case was reported [14]. A recent study has shown that the antibiotic cefminox can act as a dual agonist of prostacyclin receptor and peroxisome proliferator-activated receptor-gamma (PPARγ) to inhibit the growth of pulmonary artery smooth muscle cells [15].

Based on the function of CMNX as a PPARγ agonist and the potential of targeting PPARs in NAFLD, in the current study, the benefits of CMNX on fatty accumulation were evaluated *in vivo* and *in vitro*, and the possible underlying mechanisms were elucidated.

2. Materials and methods

2.1. Animal model and treatment

Five-week-old male mice were fed on a cycle of 12 h light and 12 h dark at 18–23 °C. The mice can access food and water freely. One week later, the mice were divided into the normal diet (ND) group (n = 8) and the high-fat high-sugar diet (HFHSD) group (n = 24). The caloric content composition of the diet for ND was fat (10 %), protein (20 %), and carbohydrate (70 %), while for HFHSD the diet contained fat (60 %), protein (20 %), and carbohydrate (20 %), and water with glucose (55 %) and fructose (45 %). In week 12, two-thirds of the mice in the HFHSD group were further accepted CMNX (MedChemExpress) tail intravenous injection at a dose of 20 mg/kg (n = 8) or 100 mg/kg (n = 8), respectively. The injections were 3 times per week lasting 4 weeks. In week 16, the mice were sacrificed. The orbital vein blood samples were collected, and the livers were separated and then processed immediately according to the requirement of the following analysis.

2.2. Histological examination of mice livers

After being fixed with 4 % paraformaldehyde and embedded in paraffin, the mice livers were cut into 5-μm sections and stained with hematoxylin and eosin (H&E). To detect the lipid droplets, the mice lives were embedded in Tissue-Tek optimal cutting temperature compound (OCT) (SAKURA) and sliced to yield 6–8 μm sections. After rinsing in isopropanol, staining with Oil Red O working solution, differentiating in isopropanol, washing in distilled water, and counterstaining with hematoxylin, the slides of liver sections were mounted in glycerol gelatin and observed under an Olympus microscope. The Oil Red O staining results were quantified by ImageJ 1.53q software.

2.3. Biochemical analysis of mice blood and livers

The mice serums were centrifuged (3000 rpm, 10 min) and kept frozen until analysis. The serum alanine aminotransferase (ALT), aspartate transaminase (AST), total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and glucose (GLU) levels were detected utilizing commercial kits (Shanghai Yu Bo Biotech Co., Ltd.) according to the manufacturer's instructions.

To measure TC and TG parameters in mice livers, the liver (100 mg) was homogenized with ethanol at the ratio of 1:9 (w/v). Next, the supernatant was detected with TC and TG assay kits (Nanjing Jiancheng Bioengineering Institute) following the manufacturer's instructions.

2.4. Cell culture and cell model of lipid accumulation

Human hepatic cell line WRL-68 (FuHeng Biology, Shanghai, China) was cultured in MEM medium containing fetal bovine serum (10 %) (Hyclone), penicillin(100U/ml), and streptomycin(0.1 mg/mL).

To establish the cell model mimicking lipid accumulation, the mixture of free fatty acids (FFAs) was prepared as follows: 1 mol of palmitic acid (Sigma-Aldrich) was mixed with 2 mol of oleic acid (Sigma-Aldrich) and combined with fatty acid-free bovine serum albumin (Sigma-Aldrich) (0.5 %, w/v). Firstly, cells were planted at a density of 1.5×10^6/well into a 6-well plate overnight. Subsequently, the cells were maintained in a medium containing 0.6 mM FFAs for 24 h. Afterwards, the medium was displaced by a medium supplemented with CMNX. PBS adding in the medium was taken as a control.

2.5. Nile red staining

After fixing WRL-68 cells with 4 % paraformaldehyde, the cells were subjected to 1 μg/mL Nile red (Sigma-Aldrich) staining for 15min and Hoechst 33258 (Beyotime) staining for 5 min at room temperature to observe lipid droplets and nuclear stain. Images were observed and acquired utilizing an Olympus IX71 fluorescence microscopy. To compare the lipid content, the cells were harvested, stained with Nile red (0.75 μg/mL) for 10 min, and followed by flow cytometer analysis (BD, Accuri C6).

2.6. Enzyme-linked immunosorbent assay (ELISA)

The cytokines tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8) of the mouse serum were measured with ELISA assay kit (Zcibio Technology Co. Ltd.) according to the manufacturer's instructions. The cytokines contents were measured at 450 nm using Spectra MAX Plus384 microplate reader (Molecular Devices).

2.7. RNA extraction and quantitative real-time PCR (qRT-PCR)

The total RNA of mice livers or WRL-68 cells was extracted using TRIzol reagent. The RNA was converted into cDNA utilizing ready-to-go first-strand beads (Toyobo, Japan) and then amplified using SYBRR Green Realtime PCR Master Mix (Toyobo, Japan) according to the manufacturer's instructions. Relative gene expressions were assessed by the $2^{-\Delta\Delta CT}$ method using GAPDH as an internal control. Primers used for qRT-PCR are listed in Table S1.

2.8. Western blot analysis

The liver samples were crushed using a mortar and pestle by adding liquid nitrogen. Followed by adding lysis buffer, the livers were sonicated, and the proteins were quantified using the BCA Protein Quantification Kit (Vazyme, China). Proteins were separated by 12 % SDS-PAGE and then electro-transferred to a PVDF membrane. After blocking, the membranes were incubated with primary antibodies at 4 °C overnight followed by incubation with secondary antibodies (1:4000, Abclone) at RT for 2 h. The used primary antibodies were PCK1(1:2000, Bioworld), SREBP-1(1:200, Santa cruz) , CYP2E1 (1:4000, Abcam) and GAPDH(1:4000, CST). Proteins were visualized using the ELC enhanced plus kit (ABclonal) and the chemiluminescence image system (Clinx 6000EXP, China).

2.9. Statistical analysis

Data were presented as mean ± standard errors of the mean. Results from multiple groups were compared using one-way ANOVA with GraphPad Prism (version 8.3.0). $P < 0.05$ were considered significant.

3. Results

3.1. Cefminox sodium improvs hepatic steatosis in HFHSD mice

To know if CMNX affects mice with NAFLD, we established a NAFLD mouse model by feeding with HFHSD lasting ten weeks and

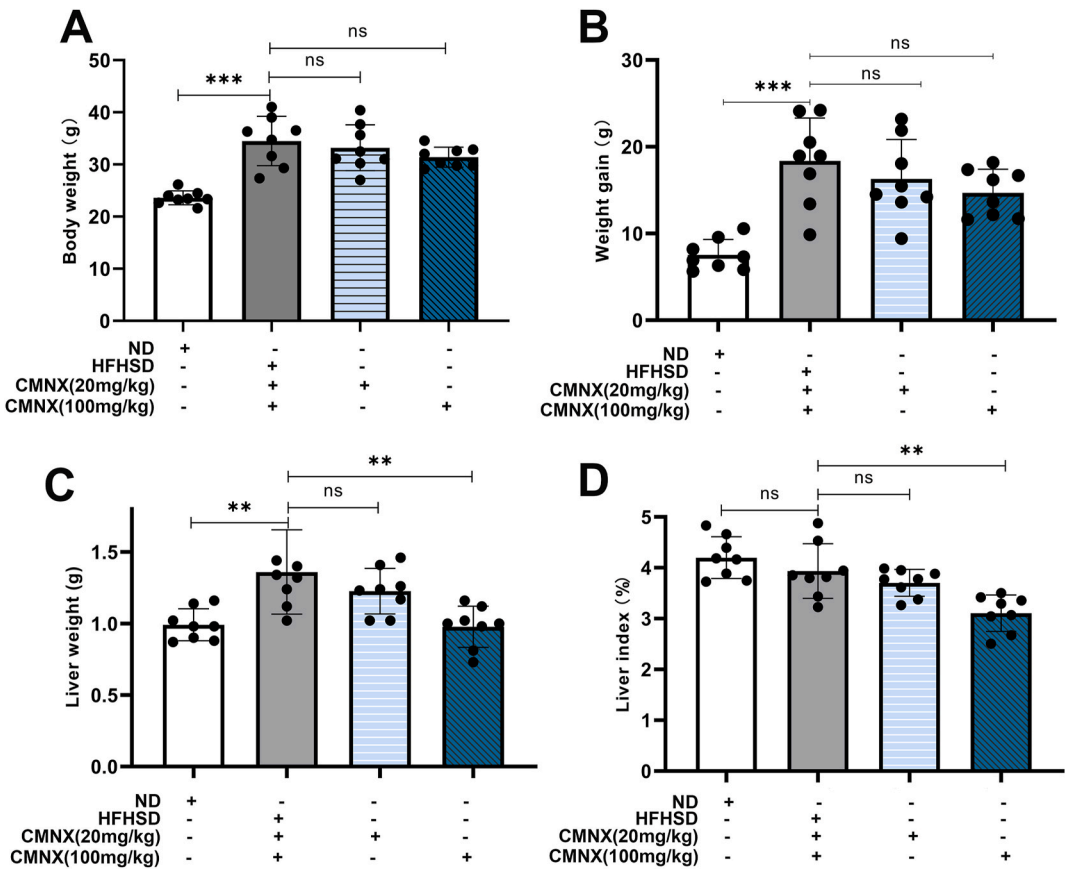

Fig. 1. CMNX reduces the liver weight and liver index in HFHSD mice. In week 16, the mice's body weight (A), weight gain (B), liver weight (C), and liver index(D). Data are mean ± SD. **P < 0.01, ***P < 0.001; ns, not significant.

intravenously injected CMNX at a dose of 20 mg/kg or 100 mg/kg for another 4 weeks. The results showed that CMNX had negligible influence on the body weight of HFHSD mice (Fig. 1A and B). Nevertheless, both the liver weight and the liver index were significantly decreased by 100 mg/kg CMNX (Fig. 1C and D).

Consistent with the foregoing, H&E staining of mice livers demonstrated that CMNX administration obviously improved the hepatic steatosis in HFHSD mice (Fig. 2A). We also assessed the lipid droplets of the mice livers performing Oil red O staining. The results (Fig. 2B and C) confirmed that CMNX significantly reduced the size of lipid droplets in mice livers. Meanwhile, the application of CMNX apparently reduced the content of TG and TC in the livers (Fig. 2D and E).

The results of biochemical markers indicated that the serum levels of ALT (Fig. 3A), TC (Fig. 3C), LDL-C (Fig. 3E), and GLU (Fig. 3G) were significantly decreased by CMNX treatment. However, AST (Fig. 3B), TG (Fig. 3D), and HDL-C (Fig. 3F) levels seemed unaltered in CMNX-treated groups. In addition, the ELISA results manifested that HFHSD feeding increased the IL-6, IL-8, and TNF-α levels, while CMNX treatment, especially at a low concentration, reversed these serum inflammatory cytokines levels, although the change of TNF-α was not obvious (Fig. 3H–J). Collectively, these results suggest that CMNX administration, to some extent, can alleviate hyperlipidemia and inflammation in mice fed on HFHSD, and a combination of CMNX and other drugs is needed to protect the liver from lipid accumulation.

3.2. *Cefminox sodium inhibits lipid accumulation in WRL-68 cells*

To identify the benefits of CMNX in human steatosis hepatocytes, we exposed WRL-68 cells to FFAs mixture to establish a hepatic steatosis model and then detected the effects of CMNX on these cells. Firstly, the cytotoxicity of CMNX and FFAs was determined by CCK8 assay (Fig. 4A and B), and the optimum concentration was selected. Subsequently, WRL-68 cells were induced by an FFAs mixture (0.5 mM) for 24 h and then treated with 0.5 mM CMNX for another 24 h. To observe the lipid droplets, WRL-68 cells were stained with Nile red and followed by fluorescent microscopy and flow cytometry (FCM) analysis. Both FCM (Fig. 4C) and the fluorescent staining (Fig. 4D) demonstrated that FFAs exposure obviously caused lipid accumulation in WRL-68 cells, which decreased in response to CMNX treatment.

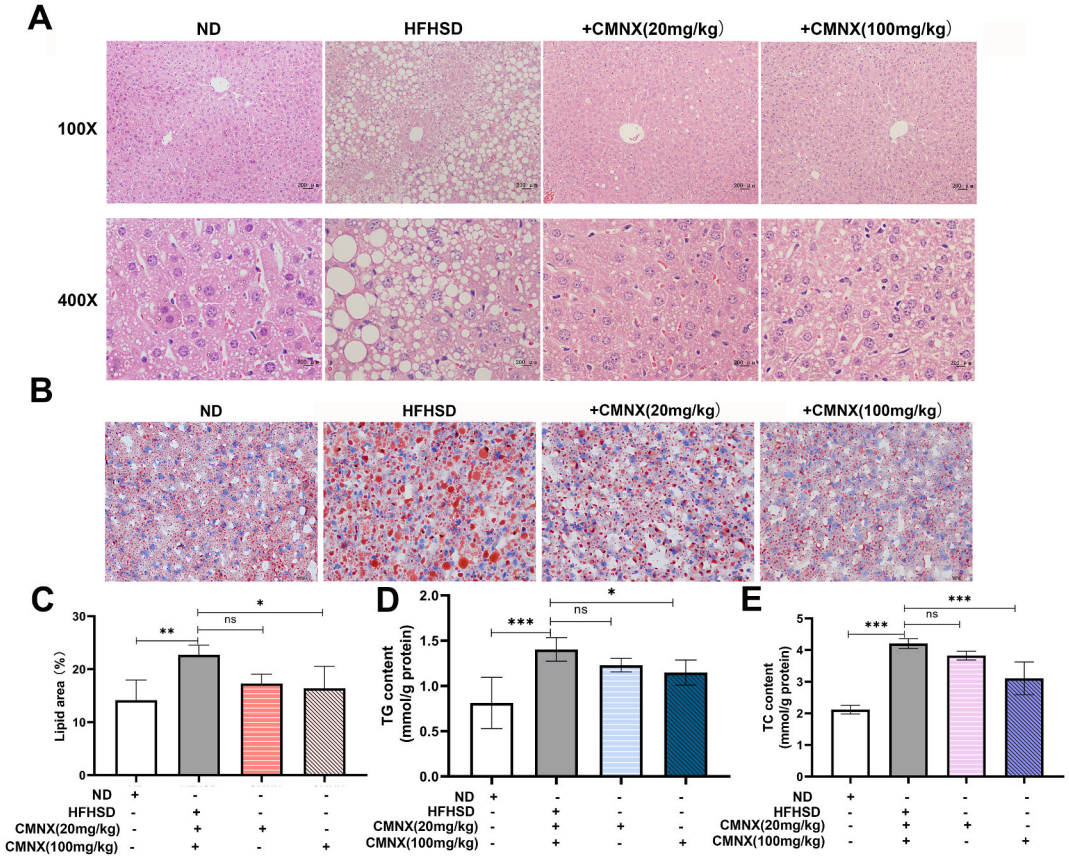

Fig. 2. CMNX attenuates hepatic steatosis in HFHSD mice. (A) Representative H&E staining images of mice liver; (B) Representative Oil Red O staining images of mice liver; (C) Quantification of Oil Red O Staining; (D) Liver triglyceride (TG) contents; (E) Liver total cholesterol (TC) contents. Data are mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant.

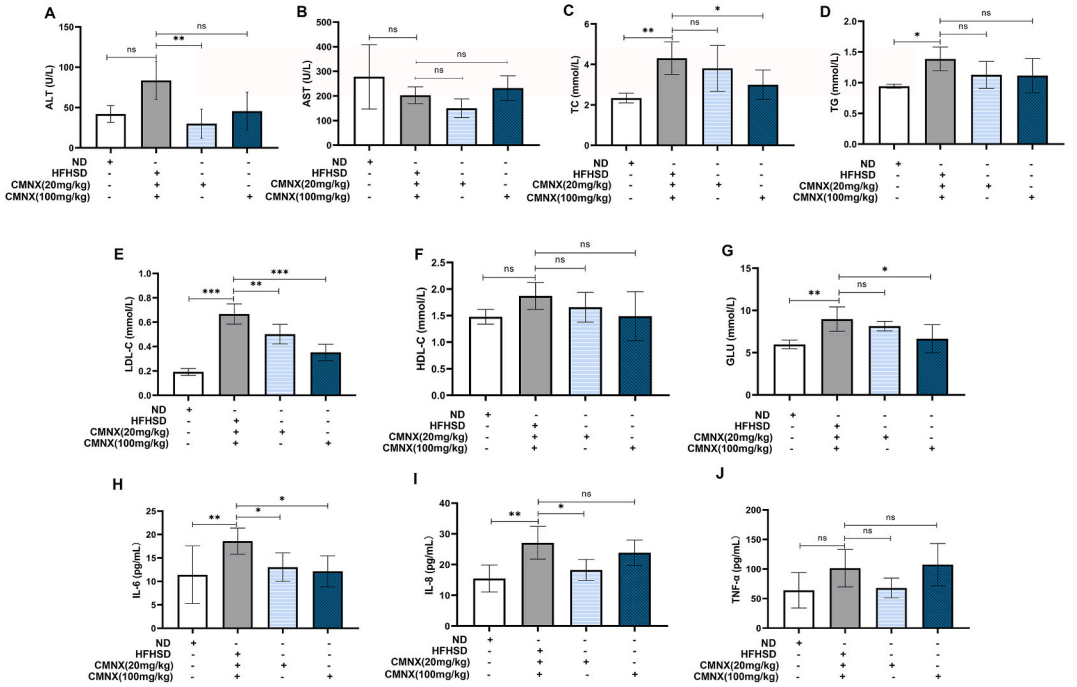

Fig. 3. The serum biochemical markers of HFHSD mice. The level of alanine aminotransferase (ALT) (A), aspartate transaminase (AST) (B), TC (C), TG (D), low-density lipoprotein-cholesterol (LDL-C) (E), high-density lipoprotein-cholesterol (HDL-C) (F), and glucose (Glu) (G) in mice serums. The serum concentration of inflammatory cytokines interleukin-6 (IL-6) (H), interleukin-8 (IL-8) (I), and tumor necrosis factor-α (TNF-α) (J). Data are mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant.

3.3. Cefminox sodium increases fatty acids metabolism both in vivo and in vitro

Since CMXN administration clearly alleviated the HFHSD mice's hepatic steatosis, we subsequently investigated the association between CMXN and hepatic fatty acids metabolism. The gene expressions related to *de novo* lipogenesis (DNL) were detected by qRT-PCR both in mouse liver and in WRL-68 cells. We found that the expressions of acetyl-CoA carboxylase 1 (ACC1) and sterol regulatory element-binding protein-1c (SREBP-1c) were significantly increased both in mice and in WRL-68 cells, while CMNX significantly decreased their expressions (Fig. 5A, B, 5G, 5H, Fig. 6A and B). Meanwhile, the expressions of ACC2 were notably enhanced by CMNX treatment (Figs. 5C and 6C). However, fatty acid synthase (FAS) did not show obvious change among the groups (data not shown). These suggest that the effects of CMNX may be associated with fatty acid synthesis mediated by the SREBP-1c/ACC1 pathway. Furthermore, the transcript levels of peroxisome proliferator activated receptor γ (PPARγ) were also compared among these groups, we found that CMNX remarkably increased the expression of PPARγ in animal models and hepatic cells (Figs. 5D and 6D), which supports previous study that CMNX is a PPARγ agonist [15].

As peroxisome proliferator activated receptor α (PPARα), cytochrome P4502E1 (CYP2E1), and cytochrome P4504A11 (CYP4A11) play roles in the process of fatty acid β-oxidation and/or microsomal ω-oxidation [16,17]. We thus detected the expression of CYP2E1 (Fig. 5E and G, Figs. 5I and 6E), CYP4A10 (murine cytochrome P450) (Fig. 5F) and PPARα (Fig. 6F). The cell and animal results illustrated that the CYP2E1 and CYP4A10 expressions were apparently elevated by HFHSD feeding or FFAs exposure, while CMNX treatment efficiently reversed it. The expression of PPARα was reduced by FFAs exposure but increased by CMNX in WRL68 cells. Together with the Western blot result of CYP2E1, these data indicate the participation of CMNX in promoting fatty acid oxidation.

Currently, it is reported that phosphoenolpyruvate carboxykinase 1(PCK1), a gluconeogenic rate-limiting enzyme, plays an important role in the activation of SREBPs [18]. Therefore, we measured the mRNA level of PCK1 in WRL-68 cells and noticed that CMNX significantly enhanced the expression of PCK1, although it was not affected by FFAs exposure apparently (Fig. 6G). The immunoblotting result of PCK1 in mice livers also supported the upregulation of PCK1 in CMNX treatment groups (Fig. 5G and J). Several studies reported that suppressing acyl-CoA synthetase long-chain member 4 (ACSL4) boosts mitochondrial respiration, and thus improves fatty acid β-oxidation and minimizes lipid accumulation [19]. Consistent with these studies, our mRNA measurement of ACSL4 in WRL-68 cells exhibited that CMNX administration distinctly inhibited the expression of ACSL4, which was enhanced by FFAs exposure (Fig. 6H).

4. Discussion

The surging of NAFLD worldwide urges researchers to develop new therapeutic drugs. As the major characteristic of NAFLD is

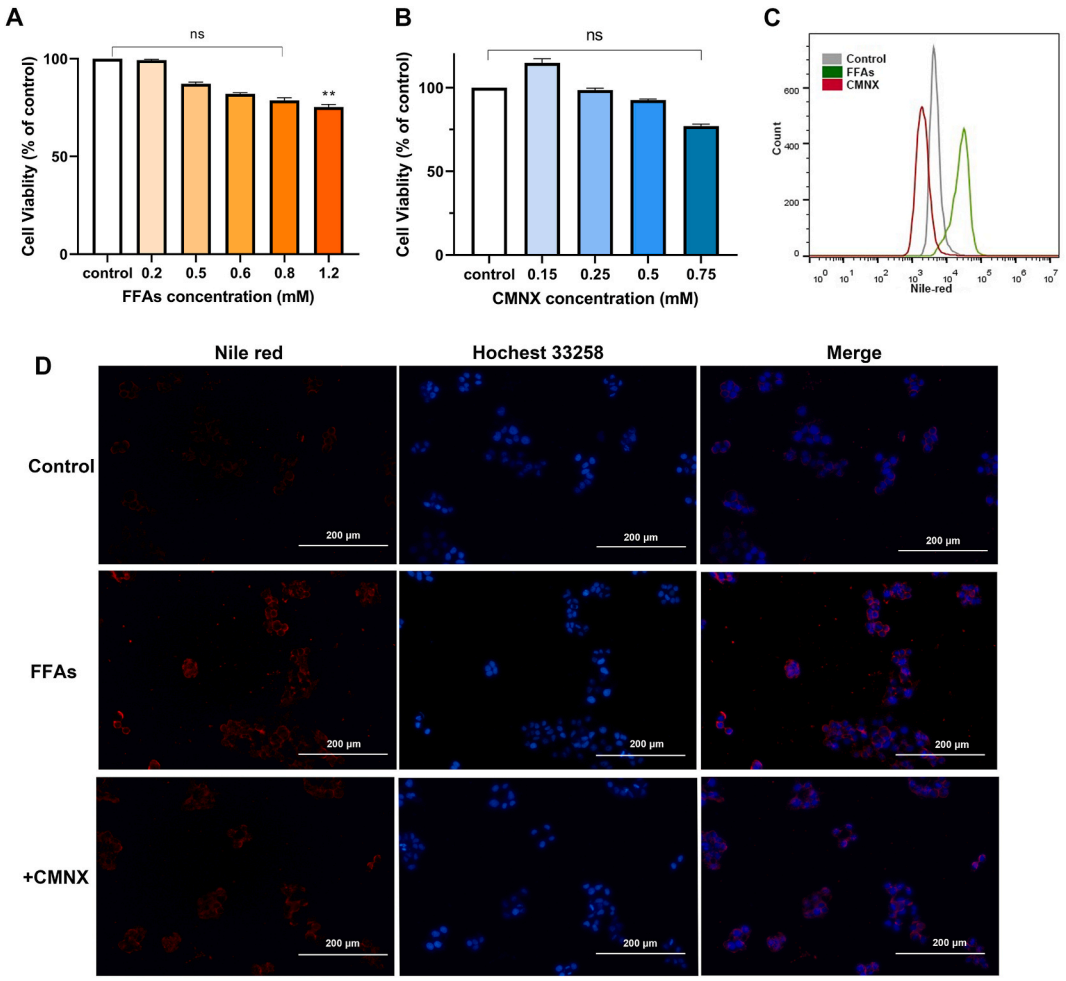

Fig. 4. CMNX attenuates fatty accumulation in human hepatic cells. CCK8 assays were performed to measure the cytotoxicity of FFAs (A) and CMNX (B) in WRL-68 cells. (C) Representative flow cytometry result of Nile red staining; (D) Representative images of Nile red and Hoechst 33258 staining. The lipid appeared red when stained with Nile red and the nuclei were blue with Hoechst 33258. Data are mean ± SD. **P < 0.01, ns, not significant.

excessive lipid deposition in hepatocytes, many studies are targeting lipid metabolism pathways. In terms of new drug discovery, starting with an old drug is a productive foundation [20]. In the present study, we found CMNX, a conventional cephalosporin antibiotic has been used safely in clinic,attenuated the lipid accumulation in HFHSD mice and FFAs-induced human hepatic cells. We further explored the possible mechanisms of actions of CMNX and found that CMNX treatment is mainly associated with the fatty acid synthesis and oxidation pathways.

Fatty acids are synthesized from acetyl-CoA. At the first committed step, acetyl-CoA carboxylase (ACC) catalyzes acetyl-CoA to malonyl-CoA. Then, fatty acid synthase(FAS) uses malonyl-CoA as a substrate to generate saturated fatty acids such as palmitate. As a key transcription factor of fatty acid biosynthesis, SREBP-1c is responsible for the pathogenesis of liver steatosis [21]. SREBP-1c positively regulates the expressions of several lipogenic enzymes such as FAS and ACC [22–24]. The elevated SREBP-1c leads to increased synthesis of fatty acids and accelerated triglyceride accumulation [23]. In our analysis, the SREBP-1c and ACC1 (not ACC2) expression in FFAs-induced cells and HFHSD mice were significantly elevated, while CMNX treatment reversed these increases. This indicates that CMNX preventing fatty accumulation is associated with the SREBP-1c/ACC1 pathway. Whether the increased ACC2 is directly related to lower lipid accumulation induced by CMNX needs to be further studied.

PPARα plays a central role in regulating gene transcription of rate-limiting enzymes involved in microsomal, peroxisomal β-oxidation, and mitochondrial ω-oxidation. In the NAFLD patients' livers, PPARα is low expressed [25]. It is considered that the weakened function of PPARα is related to hepatic lipid accumulation [26]. Using PPARα knockout mouse model, Montagner et al. found hepatocyte-restricted PPARα deletion damaged fatty acid catabolism and aggravated hepatic lipid accumulation [25]. The activation of PPARα can lower the plasma TG and LDL-cholesterol levels [16,27]. In our results, the downregulated PPARα mRNA level by FFAs exposure was increased after CMNX treatment, although the variation is slight. CYP4A11 and CYP2E1 can initiate the process of lipid oxidation as inducible microsomal cytochrome P-450s and catalyze lipid peroxides as key microsomal fatty acid hydroxylases

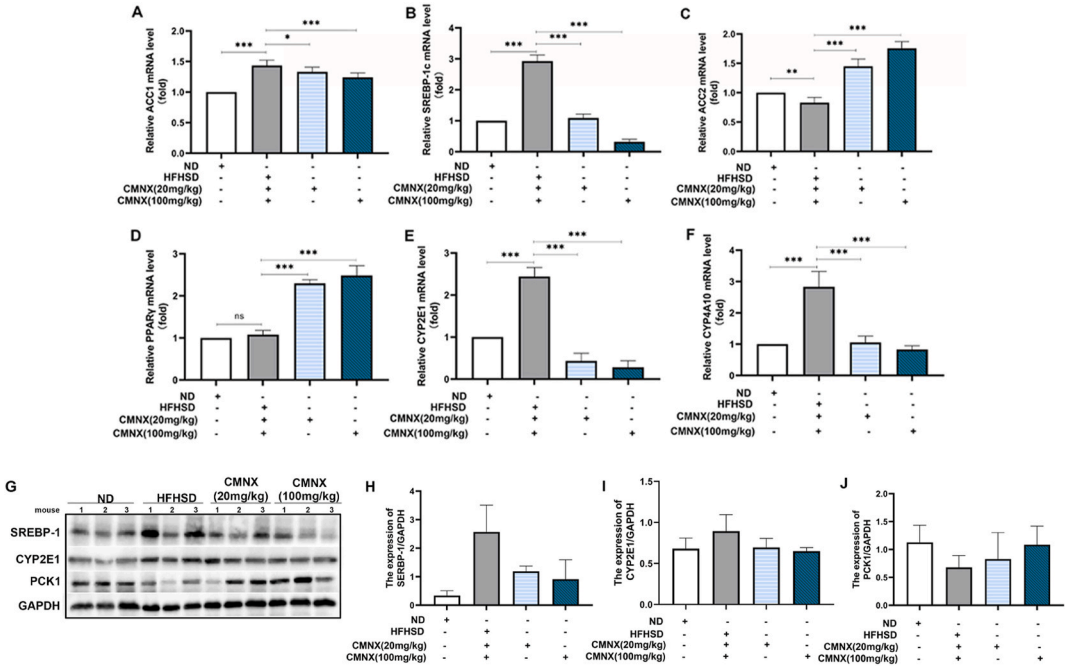

Fig. 5. QRT-PCR and Western blot assays with mouse liver. The transcript levels of acetyl-CoA carboxylase 1 (ACC1) (A), sterol regulatory element-binding protein-1c (SREBP-1c) (B), acetyl-CoA carboxylase 2 (ACC2) (C), peroxisome proliferator-activated receptor γ (PPARγ) (D), cytochrome P450 2E1(CYP2E1) (E) and cytochrome P450 4A10 (CYP4A10) (F). Western blot showing the levels of SREBP-1, CYP2E1, and phosphoenolpyruvate carboxykinase 1 (PCK1) of the mice livers(G), and the results were normalized (H, I, J). Data are mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant.

in murine nonalcoholic steatohepatitis [17,28]. Increased hepatic CYP2E1 and CYP4A11 levels were reported in NAFLD patients [17, 28]. CYP4A11 plays important roles in the metabolism of fatty acids, promotes the production of reactive oxygen species (ROS), and facilitates the progression of NAFLD [29]. In high-fat diet rats, deletion of the *Cyp2e1* prominently reduces visceral fat and restrains liver lipid deposition and insulin resistance [30]. As shown in our data, the expression of CYP4A10 and CYP2E1 were enhanced in HFHSD mice, while CMNX treatment efficiently reduced their expressions, together with the expression of PPARα in WRL-68 cells, we conclude that CMNX treatment promotes lipid oxidation.

As the members of nuclear receptor family, PPARs perform pivotal roles in the transcriptional regulation of lipid and glucose metabolism, as well as inflammation [16,31]. PPARγ, another one of the three PPARs isotypes, regulates adipogenesis and lipid metabolism indispensably [16,31]. Although the hepatic PPARγ expressions are elevated in NAFLD patients [16], the treatment of some PPARγ agonists in NAFLD patients shows improvement of insulin sensitivity and steatosis without effect on liver fibrosis [16,32]. Here, we provided that CMNX treatment remarkably promoted PPARγ expressions in both mice and cells, supporting a role for CMNX as a PPARγ agonist.

It's reported that PCK1 plays critical roles not only in the activation of SREBPs and lipogenesis but also in the HCC progression [18]. The expression of PCK1 is decreased in hepatocellular carcinoma (HCC), while up-regulating PCK1 can antagonize HCC via activating gluconeogenesis, inhibiting glycolysis, enhancing energy, and oxidative stress [33,34]. This study demonstrated that CMNX treatment increased the PCK1 levels in hepatic cells and mice livers, which suggests the potential anti-HCC effect of CMNX. Considering the close relationship between HCC and NAFLD [35], further research of CMNX on HCC patients that developed from NAFLD should be investigated. In addition, currently, ACSL4 is reported as an alternative potential therapeutic target for NAFLD therapy [19]. The main function of ACSL4 is to catalyze the addition of coenzyme A (CoA) groups to fatty acids to form fatty acyl CoA [23]. Our results suggest the lipid-lowering effect of CMNX may be also associated with the downregulation of ACSL4.

In conclusion, this study illustrated the benefits of CMNX in treating the early stage of NAFLD, which provides a novel strategy for NAFLD therapy. We cannot deny our study is insufficient, and more experimental evidence such as intervention experiments and specifying the primary regulatory mechanisms of CMNX will contribute to using CMNX as a potential therapeutic drug.

Ethics statement

The animal experiments were approved by the Ethics Committee of the Zhejiang Sci-Tech University (ZSTU) and followed protocol code IACUC-20210614-15.

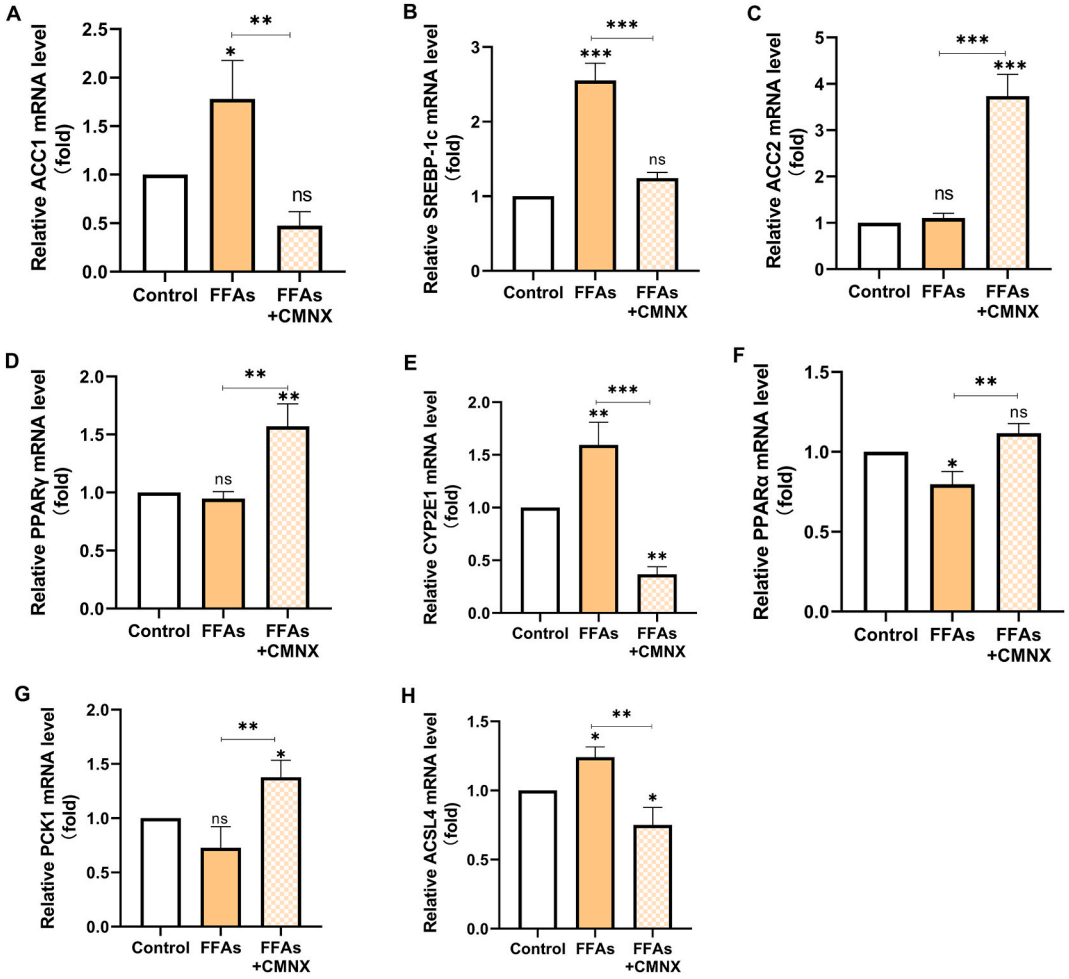

Fig. 6. QRT-PCR assays with WRL-68 cells. The relative mRNA levels of ACC1 (A), SREBP-1c (B), ACC2 (C), PPARγ (D), CYP2E1 (E), PPARα (F), PCK1 (G), and acyl-CoA synthetase long-chain member 4 (ACSL4) (H). Data are mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant.

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Data availability statement

Data included in article/supplementary material/referenced in article.

CRediT authorship contribution statement

Leming Xiao: Investigation. **Chengrui Liang:** Investigation. **Jing Gao:** Investigation, Funding acquisition. **Yin Wang:** Investigation. **Yanzi Guo:** Methodology. **Kan Chen:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Xiaoyuan Jia:** Writing – original draft, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21973.

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