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# Tetrahydrocurcumin protects against nonalcoholic fatty liver disease by improving lipid metabolism and redox homeostasis

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is the most common liver metabolic disease in the world. In this study we investigated the effect of tetrahydrocurcumin (THC), a potent antioxidant, on NAFLD. We found that THC significantly reduced the body weight, serum and liver lipids, serum malondialdehyde (MDA) and fasting blood glucose (FBG) of male NAFLD mice induced by feeding a high-fat diet (HFD) for 8 weeks. A RNA-seq analysis revealed that THC also affected the expression of many genes involved in lipid metabolism in the liver of NAFLD mice, including increasing the mRNA expression of *CYP51* and *FOXQ1*. Moreover, THC significantly reduced sodium oleate-induced lipid accumulation, activated the NRF2 pathway, and up-regulated the mRNA expression of *FGF21* in HepG2 cells. Overall, our study showed that THC can improve the antioxidant capacity of liver, which suggests that dietary THC may be used to treat NAFLD.

## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD), the excessive accumulation of fat in the liver without alcohol or drug consumption, is a major chronic liver disease. NAFLD is associated with a variety of metabolic disorders, such as obesity, hyperlipidemia, hypertension and hyperglycemia (Filipovic et al., 2021; Maurice & Manousou, 2018). Pathologically, NAFLD begins with liver steatosis but may progress to nonalcoholic steatohepatitis (NASH) that may be accompanied by fibrosis, and that may even develop into liver cirrhosis or hepatocellular carcinoma (Chalasani et al., 2018; Diehl and Day, 2017; Yabiku, 2021). A study showed that NAFLD may affect as high as 25% of the global population (Younossi et al., 2016). Clearly, NAFLD is a global health challenge.

A variety of factors are believed to contribute to NAFLD, such as oxidative stress, insulin resistance, and inflammation (Buzzetti et al., 2016; Fang et al., 2018). A number of studies have shown that oxidative stress is an important factor in the pathogenesis of NAFLD; therefore, improving the antioxidant capacity could be an effective way to improve NAFLD (Arroyave-Ospina et al., 2021; Hong et al., 2021; Masarone et al., 2018; Zakaria et al., 2021). Nuclear factor erythroid-2-related factor 2 (Nrf2), a member of the Cap-n-Collar family, plays an important role in regulating oxidative stress. Inactive Nrf2 binds to Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. When oxidative stress occurs, Nrf2 dissociates from Keap1 and translocates to the nucleus to bind to the antioxidant response element, thereby promoting the transcription of target genes such as *NQO1, HO-1, GSTA1, GCLC* and *GCLM* to improve the antioxidant capacity of cells (Buendia et al., 2016; Suzuki & Yamamoto, 2015; Zhang et al., 2015). Fibroblast growth factor 21 (FGF21), a secreted protein and mainly expressed in liver, fat and muscle tissue, plays an important role in the improvement of glycolipid metabolism (Guo et al., 2020). Studies have shown that the expression of FGF21 in the liver is regulated by Nrf2 and that FGF21 participates in the process of redox homeostasis (Chen et al., 2017; Furusawa et al., 2014; Gómez-Sámano et al., 2017; Zhang, Xu, Gu, et al., 2018). However, both the circulating and liver FGF21 levels increase in obesity, a situation

*Abbreviations:* CYPs, Cytochromes p450; DEGs, Differentially expressed genes; FBG, Fasting blood glucose; FGF21, Fibroblast growth factor 21; FOXQ1, Forkhead box O1; GCLC, Glutamate cysteine ligase catalytic; GCLM, Glutamate cysteine ligase modifier; GSTA1, Glutathione S-transferase α1; HDL-C, High-density Lipoprotein Cholesterol; HFD, High-fat diet; HO-1, Heme oxygenase-1; Keap1, Kelch-like ECH-associated protein 1; LDL-C, Low-density Lipoprotein Cholesterol; MDA, Malondialdehyde; NAFLD, Non-alcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis; NQO1, NADPH quinone dehydrogenase 1; NRF2, Nuclear factor erythroid-2-related factor 2; OA, Sodium Oleate; PPAR, Peroxisome proliferator-activated receptors; TC, Total Cholesterol; TG, Triglycerides; THC, Tetrahydrocurcumin.

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called FGF21 resistance (Fisher et al., 2010; Shen et al., 2013).

Currently, there is no effective treatment for NAFLD (Jump et al., 2018). Recent studies have shown that some natural dietary components can improve NAFLD due to their antioxidant effects, such as polydatin (Zhao et al., 2018), ginsenoside (Kim et al., 2020), anthocyanin (Fan et al., 2019) and curcumin (Mokgalaboni et al., 2021). However, clinical trials have shown that curcumin is not superior to placebo in improving NAFLD, which may be related to the single curcumin dose or the body weight differences of the two groups of patients (Saadati, Hatami, Yari, et al., 2019; Saadati, Sadeghi, Mansour, et al., 2019). The polyphenol tetrahydrocurcumin (THC), was first discovered as a metabolite of curcumin (Holder et al., 1978). Later studies showed that THC also exists in the roots of some plants, such as *Zingiber mioga*, *Z. officinale*, and *Curcuma zedoaria* (Han et al., 2015; Lai et al., 2020; Yang et al., 2009). As an excellent antioxidant, THC has better bioavailability than curcumin (Liu et al., 2017; Okada et al., 2001). Previous studies have shown that THC improves liver oxidative damage induced by cadmium (Ramakrishnan et al., 2017) and arsenic (Muthumani & Miltonprabu, 2015), and that THC reduces blood sugar and blood lipid levels in diabetic rats and inhibits diet-induced liver steatosis (Pan et al., 2018; Pari & Murugan, 2007). However, it is unknown if THC has antioxidant and protective effects against NAFLD. Thus, in the current study, we tested the protective effects of THC against high-fat diet-induced NAFLD in mice. We also determined if the protective effects of THC against NAFLD involve the NRF2 signaling pathway.

## 2. Materials and methods

### 2.1. Materials

THC (98% purity) was provided by Shanghai Macklin Biochemical Co. Ltd. (Shanghai, China). Simvastatin was purchased from Merck & Co., Inc. Triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and malondialdehyde (MDA) assay kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Sodium oleate (OA) and fat-free bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. louis, MO, USA). Enhanced BCA Protein Assay Kit was obtained from Beyotime Biotechnology (Shanghai, China). Primary antibodies against HO-1 (No. DF639) and NQO1 (No. FD6437) were purchased from Affinity Biosciences (Changzhou, China). Primary antibodies against NRF2 (No. SC-365949) was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Primary antibodies against actin (No. KC-5A08) was purchased from Aksomics (Shanghai, China). HRP-conjugated anti-rabbit (No. D110056) or anti-mouse (No. D110087) secondary antibody was purchased from Sangon Biotech (Shanghai, China). All other chemicals used in this study were at least of analytical grade.

### 2.2. Animal experiments

All animal procedures and protocols in this study were approved by the Animal Care Committee of the Changshu Institute of Technology (Permit number: EAWEC1912). Six to eight-week-old male C57BL/6J mice were obtained from Laboratory Animal Center of Nantong University (Nantong, China). Mice were housed in an environmentally controlled room (23 ± 1 °C, 55% ± 5% humidity, 12-h light–dark cycle) for 1 week for acclimation and, then, randomly divided into six groups ($n$ = 12 each). Mice were fed a normal diet (CON, 10 kcal% fat, D12450J, Research Diet, Inc., New Brunswick, NJ, USA) or a high-fat diet (MOD, 60 kcal% fat, D12492i, Research Diet, Inc., New Brunswick, NJ, USA), with or without THC (25, 50 or 100 mg/kg body weight, daily oral administration), or Simvastatin (20 mg/kg body weight, daily oral administration). During the 8-week experimental period, all mice had free access to food and water. Body weight was measured weekly and food intake was recorded every day. At the end of the experiment, mice were fasted overnight, and anesthetized before blood samples were collected through *retro*-orbital bleeding. When collected, blood samples were left standing at room temperature for 2 h before being centrifuged at 3500 rpm for 15 min at 4 °C to obtain serum. Serum samples were stored at −80 °C for subsequent blood biochemical determination. The liver samples were washed with pre-chilled saline and soaked in 10 times the volume of 10% formalin. Liver samples were also stored at −80 °C for other analyses.

### 2.3. Intraperitoneal glucose tolerance test (IPGTT)

Three days before the end of the experiment, the mice were fasted overnight, and then were intraperitoneally injected with 10% D-glucose (2.0 g/kg body weight). After 30, 60, 90, 120 min of intraperitoneal injection, blood glucose levels were measured from the tail using the blood glucose meter (OneTouch Ultra, USA),

### 2.4. Biochemical analyses

To extract lipids from liver, 100 mg of liver was homogenized in 0.9 mL ethanol followed by centrifugation at 2500 rpm for 10 min to obtain the supernatant. Liver levels of TG, TC and protein and serum levels of TG, TC, LDL-C, HDL-C and MDA were determined by a microplate reader (Thermo Scientific, China) according to the instructions of the respective assay kits.

### 2.5. Hematoxylin and eosin (H & E) staining and Oil red O staining

Fresh liver tissue was fixed with 10% formalin for more than 24 h, and then embedded in paraffin after gradient dehydration. Tissue sections (3 μm thick) were stained with H & E and observed with a light microscope (Olympus, Japan).

Frozen sections of liver were fixed with 10% formalin for 15 min, and stained with Oil red O and Hematoxylin. The sections were blocked with glycerin jelly and then observed with a light microscope (Olympus, Japan).

### 2.6. RNA-Seq analysis

Total RNA preparation and subsequent RNA-seq library construction were performed using a service from Shanghai Personal Biotechnology Co., Ltd. RNA-seq libraries were paired-end (PE) sequenced on the Illumina sequencing platform. Raw sequencing data were first filtered. The filtered clean data were aligned to the mouse reference genome GRCm38.dna.primary_assembly.fa.gz (ftp://ftp.ensembl.org/pub/release-100/fasta/mus_musculus/dna/) using Tophat2 (http://ccb.jhu.edu/software/tophat/index.shtml). The expression level of each gene was calculated using HTSeq (http://htseq.readthedocs.io/). Gene expression level was expressed as FPKM (Fragments Per Kilo bases per Million fragments). Differential expression was determined using DESeq (http://www.bioconductor.org/packages/release/bioc/html/DESeq.html).

The selection condition of DEGs (Differentially expressed genes) between groups was |log2FoldChange|greater than 1 and P-value < 0.05. For GO and KEGG enrichment analysis, the hypergeometric distribution method was used to calculate the P-value. The GO enrichment analysis was classified according to molecular functions (MF), biological processes (BP), and cellular components (CC). The top 10 terms in each GO category (P-value < 0.05) and the top 20 KEGG pathways (P-value < 0.05) were selected.

### 2.7. Cell culture and treatment

HepG2 cells were maintained in Minimum Essential Medium (Gibco, USA) containing 1% penicillin/streptomycin, 1% sodium pyruvate and 10% fetal bovine serum in an atmosphere of 5% $CO_2$ at 37 °C. OA was

dissolved in methanol to obtain stock solution. HepG2 cells were incubated in medium containing 0.2 mM OA for 12 or 24 h and then treated with THC for indicated time.

### 2.8. Cell viability assay

HepG2 cells were seeded in 96-well plates at $1 \times 10^4$ cells/well. After overnight incubation, the cells were treated with THC (25, 50, 100, 125 μM) or DMSO for 24 h. Untreated cells were used as a control group. Wells without cells were used as zeroing group. Cell viability was measured by the MTT assay, Briefly, 10 μL of MTT solution (5 mg/mL) were added to each well, and then the cells were incubated for 4 h. The formazan crystals were dissolved in 100 μL of DMSO and the absorbance (OD) was measured at 490 nm.

Cell viability was calculated using this formula: Cell viability = (treatment group OD - zeroing group OD)/(control group OD- zeroing group OD), where treatment was THC or DMSO.

### 2.9. Oil red O staining of cells

HepG2 cells were seeded in 12-well plates at $3 \times 10^5$ cells/well. After overnight incubation, cells were incubated in medium containing 0.2 mM OA for 24 h and then treated with THC (25, 50, 100 μM) or pioglitazone hydrochloride (Pi, 10 μM, Sigma, USA) for 48 h. The cells were fixed with 4% formaldehyde for 30 min, and then stained with Oil red O for 10 min. The excess Oil red O was washed away and the wells were air-dried for 10 min. Isopropanol was added to dissolve Oil red O and the absorbance was measured at 450 nm.

### 2.10. Reverse transcription-quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from liver or cells using RNAiso Plus (TaKaRa, China). The concentrations of RNA were determined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The cDNA was synthesized using the PrimeScript RT reagent kit (TaKaRa, China). qPCR was performed using the TB Green Premix Ex Taq reagent kit (TaKaRa, China) and in QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, USA). Sequences of the primers are shown in Table 1. The qPCR data were analyzed using the $2^{-\Delta\Delta CT}$ method.

### 2.11. Western blot analysis

HepG2 cells were treated as described above. Total cellular protein was extracted using a lysis buffer supplemented with 1% Phenylmethanesulfonyl fluoride (PMSF, Beyotime Biotechnology, China). The protein concentrations were measured using the BCA Protein Assay Kit (Beyotime Biotechnology, China). The protein samples were separated by SDS-PAGE gels and transferred to PVDF membranes (Sigma, USA) in a transfer solution composed of 25 mM Tris-HCl (pH 8.3), 190 mM glycine, and 20% methanol. The membranes were blocked with 5% skim milk for 90 min at room temperature, and then incubated with primary antibodies overnight at 4 °C. The membranes were washed three times with TBST. Then the membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibody for 1 h at room temperature. The membranes were then washed three times with TBST and visualized using an enhanced chemiluminescence detection kit (Beyotime Biotechnology, China).

### 2.12. Statistical analysis

All data were expressed as mean ± standard deviation (SD). Data comparisons between groups were performed with one-way ANOVA in SPSS (version 24.0, IBM Inc., Chicago, IL, USA). A $P$ value $< 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Tetrahydrocurcumin inhibited weight gain in HFD-fed mice

We determined the effects of oral administration of THC (25, 50 or 100 mg/kg body weight) on the body weight and food intake of mice (Table 2). Compared with the group fed a normal diet (CON), HFD-fed mice (MOD) gained more in body weight despite eating less (Table 2). Oral administration of 50 mg/kg THC significantly decreased the body weight of the MOD mice without affecting their food intake (Table 2). The other two doses (25 and 100 mg/kg) of THC did not significantly affect body weight in MOD mice (Table 2). Interestingly, 100 mg/kg THC significantly increased the food intake of MOD mice without increasing their body weight (Table 2).

### 3.2. Tetrahydrocurcumin improved fasting blood glucose (FBG) and glucose uptake HFD-fed mice

As shown in Fig. 1A, the fasting blood glucose (FBG) level was significantly increased in the MOD group, and this increase was significantly suppressed by oral administration of 50 mg/kg THC. Similarly, compared with the CON group, the MOD group showed glucose intolerance and the area under the curve (AUC) was significantly increased and oral administration of 50 mg/kg THC significantly reduced AUC (Fig. 1B, C). These results indicated that THC can reduce FBG and improve glucose uptake in high-fat diet-fed mice.

### 3.3. Tetrahydrocurcumin reduced serum lipid and malondialdehyde (MDA) levels in HFD-fed mice

We determined the effect of THC on the levels of blood lipids in mice. Compared with the CON group, serum levels of TG, TC and LDL-C in the MOD group were significantly higher (Fig. 2A, B, C). However, THC treatment (25 or 50 mg/kg) significantly reduced serum levels of TG and LDL-C, without affecting serum HDL-C levels (Fig. 2A, C, D). We also measured serum MDA levels to determine the effect of THC on lipid

**Table 1**
Primers sequences of Reverse transcription quantitative real-time PCR.

| Gene | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|---|---|---|
| mus-Foxq1 | CGAGATCAACGAGTACCTCATGG | GCATCCAGTAGTTGTCCTTGCC |
| mus-Cyp51 | ACGCTGCCTGGCTATTGC | TTGATCTCTCGATGGGCTCTATC |
| mus-Fgf21 | CAGGGAGGATGGAACAGTGGTA | GCTGTTGGCAAAGAAACCTAGAG |
| mus-Gapdh | AGGTCGGTGTGAACGGATTTG | TGTAGACCATGTAGTTGAGGTCA |
| has-FGF21 | GCCTTGAAGCCGGGAGTTATT | GTGGAGCGATCCATACAGGG |
| has-NRF2 | AGTGGATCTGCCAACTACTC | CATCTACAAACGGGAATGTCTG |
| has-NQO1 | CCGTGGATCCCTTGCAGAGA | AGGACCCTTCCGGAGTAAGA |
| has-HO-1 | CCAGGCAGAGAATGCTGAGTTC | AAGACTGGGCTCTCCTTGTTGC |
| has-GCLC | GGCACAAGGACGTTCTCAAGT | CAGACAGGACCAACCGGAC |
| has-GCLM | CATTTACAGCCTTACTGGGAGG | ATGCAGTCAAATCTGGTGGCA |
| has-GSTA1 | GAAGCCTCCCATGGATGAGA | AGCTTCACAACAGGCACAAT |
| has-β-actin | TGGATCAGCAAGCAGGAGTA | TCGGCCACATTGTGAACTTT |

**Table 2**
Effects of tetrahydrocurcumin (THC) on body weight and food intake in mice fed a high-fat diet.

| Parameters | CON | MOD | Si | THC25 | THC50 | THC100 |
|---|---|---|---|---|---|---|
| Initial weight (g) | 20.8208 ± 1.0086[a] | 20.8583 ± 1.0988[a] | 20.8833 ± 1.0521[a] | 20.8817 ± 1.1270[a] | 20.8725 ± 1.0500[a] | 20.7950 ± 1.2338[a] |
| Final weight (g) | 24.3758 ± 1.2794[c] | 30.6508 ± 3.5727[a] | 29.1242 ± 2.3906[ab] | 29.5733 ± 3.7667[ab] | 27.7883 ± 2.7709[b] | 29.2992 ± 3.7655[ab] |
| Food intake (g) | 2.5827 ± 0.5025[a] | 2.3407 ± 0.2973[c] | 2.3183 ± 0.2490[c] | 2.4366 ± 0.2763[bc] | 2.3820 ± 0.2620[c] | 2.5461 ± 0.2694[ab] |

CON: normal diet; MOD: high-fat diet; Si: high-fat diet + simvastatin; THC25, THC50, THC100: high-fat diet + 25, 50, 100 mg/kg THC, respectively. Different letters (a-c) indicate significant difference ($P < 0.05$) between groups (n = 12).

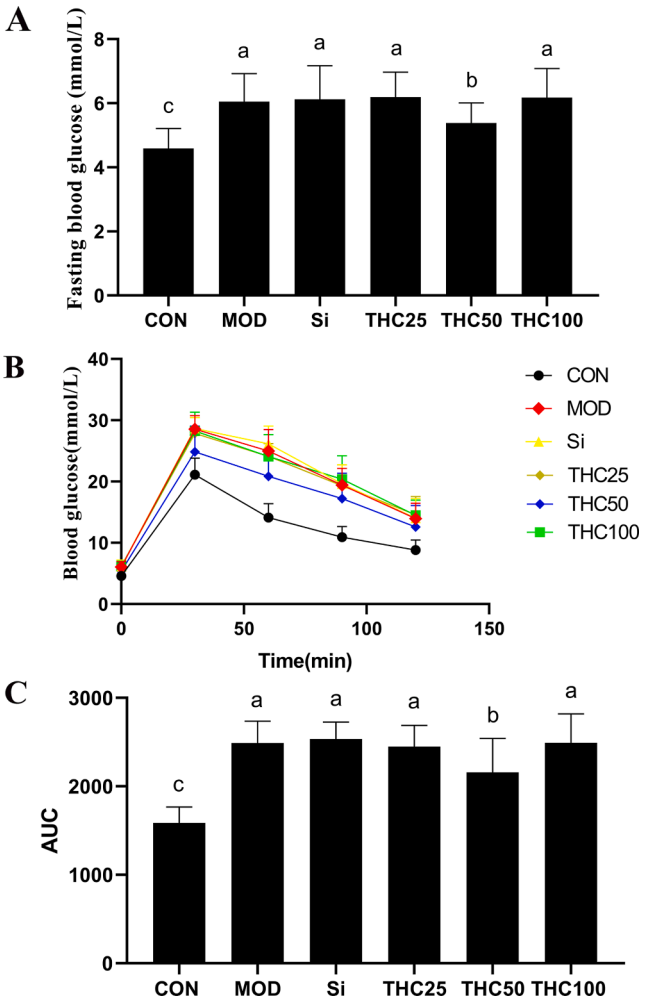


**Fig. 1. Effects of tetrahydrocurcumin (THC) on intraperitoneal glucose tolerance test (IPGTT) and fasting blood glucose (FBG) in high-fat diet-fed mice.** CON: normal diet; MOD: high-fat diet; Si: high-fat diet + simvastatin; THC25/50/100: high-fat diet + THC25/50/100 mg/kg. Before an IPGTT was performed, the fasting blood glucose (A) were measured in each group. IPGTT was performed three days before the end of the experiment to evaluate glucose tolerability as described in Materials and Methods. (B) The curve of blood glucose levels with time after intraperitoneal injection of 10% D-glucose. (C) The area under the blood glucose curve with time. Different letters (a-c) between groups (n = 12) indicate significant differences (P < 0.05).

peroxidation. High-fat diet feeding significantly increased serum MDA levels, while THC treatment significantly reduced serum MDA levels in high-fat diet-fed mice (Fig. 2E). These results indicated that THC can improve blood biochemical disorders caused by HFD.

### 3.4. Tetrahydrocurcumin reduced hepatic lipid levels in HFD-fed mice

We determiend the effect of THC on liver fat content in HFD-fed mice. Compared with the normal diet, HFD significantly increased the liver TC levels in the MOD group, but had no significant effect on the TG levels (Fig. 3A, B). THC significantly reduced liver TG and TC levels in a dose-dependent manner in HFD-fed mice, and these effects of 100 mg/kg THC were greater than those of 20 mg/kg Si (Fig. 3A, B).

Consistent with the quantitive analyses, H&E staining and Oil red O staining of liver tissue sections showed that the MOD group had more fatty vacuoles and fatty deposits compared with the CON group, and that THC and Si reversed these differences (Fig. 3C, D),

### 3.5. Effects of tetrahydrocurcumin on liver gene expression profile in HFD-fed mice

According to the above experimental results, we selected the CON, MOD and THC50 groups of mice to further explore the effect of THC on liver transcriptome through RNA-Seq. Compared with the CON group, the MOD group had 103 DEGs; compared with the MOD group, the THC50 group had 51 DEGs (Fig. 4A, B). The GO enrichment analyses indicated that the DEGs between the CON and MOD groups were mainly enriched in extracellular region part, oxidoreductase activity and response to oxygen-containing compound (Fig. 4C, D). The DEGs between the THC group and the MOD groupwere mainly enriched in USH2 complex, oxidoreductase activity and sterol biosynthetic process (Fig. 4C, D). Both sets of DEGs were enriched in the oxidoreductase activity of MF category. We also perfromed KEGG enrichment analyses on these DEGs (Fig. 4E, F). The DEGs between the CON and MOD groups were enriched in lipid metabolism-related pathways, including fatty acid biosynthesis, PPAR (peroxisome proliferator-activated receptors) signaling pathway and steroid hormone biosynthesis. The DEGs between the MOD group and THC group were enriched in steroid biosynthesis and MAPK (mitogen-activated protein kinases) signaling pathways. These results indicated that HFD affected the expression of liver oxidoreductase and lipid metabolism-related genes and that THC reversed these changes.

We also determined the effects of THC on liver mRNA expression of *Cyp51, Foxq1* and *Fgf21* by RT-qPCR. These genes were selected because they are involved in the regulation of glycolipid metabolism and oxidative stress. Based on this RT-qPCR, *Cyp51* and *Foxq1* mRNA levels were significantly increased by THC in HFD-fed mice (Fig. 4G, H). In addition, *Fgf21* mRNA expression was significantly higher in the MOD group than in the CON group, and THC treatment significantly reduced *Fgf21* expression in HFD-fed mice (Fig. 4I).

### 3.6. Tetrahydrocurcumin increased the mRNA expression of NRF2 pathway and FGF21 in HepG2 cells

To explore the mechanism of THC intervention in redox reaction, we determined the effect of THC on mRNA expression of the *NRF2* pathway in HepG2 cells. Firstly, the cytotoxicity of THC to HepG2 cells was detected by MTT and DMSO as solvent control (Fig. 5A). Cell viability was not significantly affected by 25–100 μM THC and DMSO control. However, 125 μM THC significantly decreased cell viability, which was 70% of the CON group. Therefore, 25 μM, 50 μM, and 100 μM THC were used in subsequent experiments.

As shown in Fig. 5B, HepG2 cells were treated with 25 μM, 50 μM, and 100 μM THC for 24 h, the expressions of *NRF2* and target genes *NQO1, GSTA1, GCLM* and *GCLC* in HepG2 cells were increased in a dose-

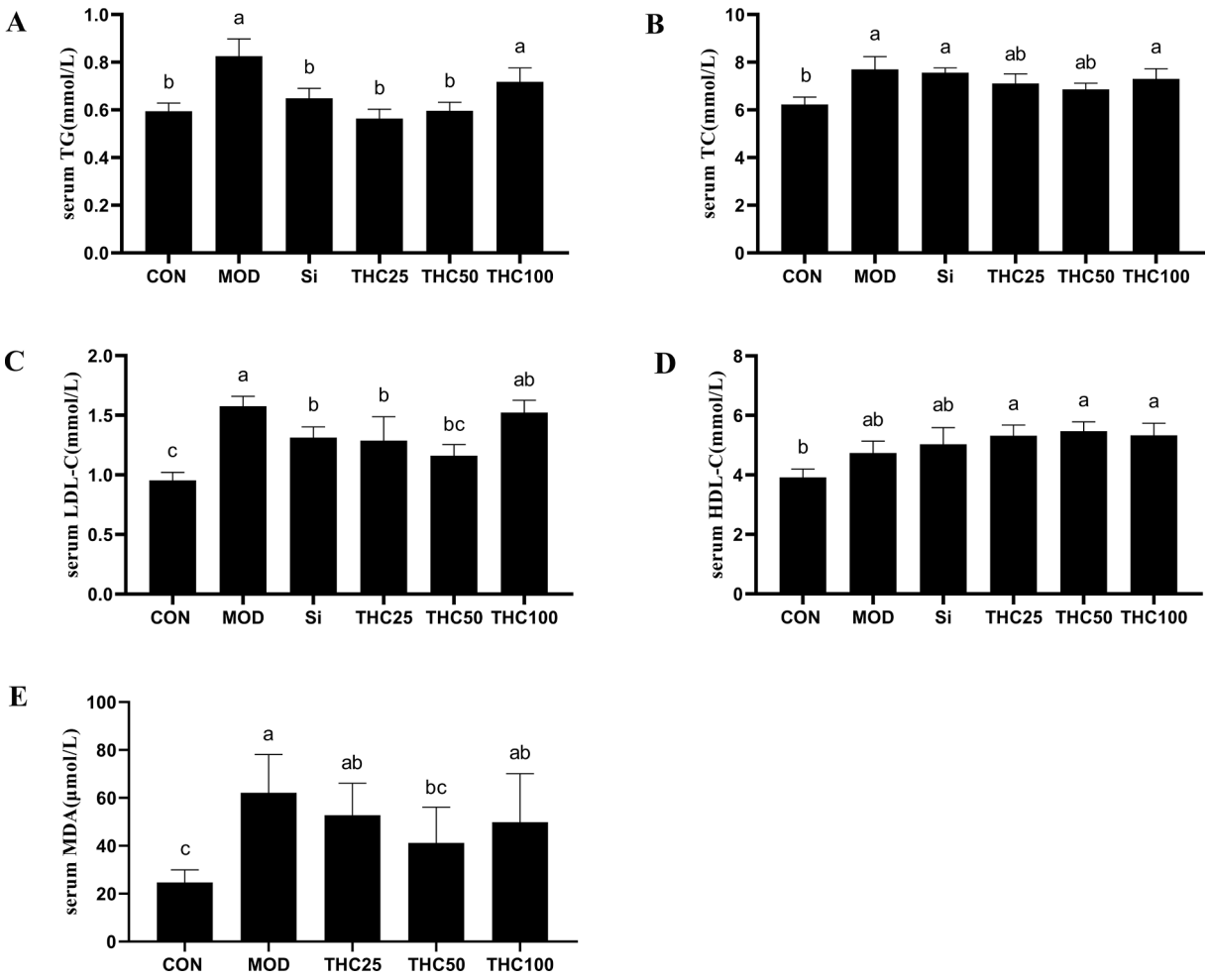


**Fig. 2. Effects of tetrahydrocurcumin (THC) on serum lipid and malondialdehyde (MDA) levels in high-fat diet-fed mice fed.** CON: normal diet; MOD: high-fat diet; Si: high-fat diet + simvastatin; THC25/50/100: high-fat diet + THC25/50/100 mg/kg. (A) Triglycerides (TG, n = 12), (B) Total Cholesterol (TC, n = 12), (C) Low-density Lipoprotein Cholesterol (LDL-C, n = 6–12), (D) High-density Lipoprotein Cholesterol (HDL-C, n = 10–12) and (E) Malondialdehyde (MDA, n = 6). Different letters (a-c) between groups indicate significant difference ($P < 0.05$).

dependent manner.

The animal experiments described above indicated that THC improved HFD-induced FGF21 resistance. Therefore, we also determined the effect of THC on the mRNA expression of *FGF21*. 25 μM and 50 μM THC significantly increased the mRNA expression of *FGF21* in HepG2 cells (Fig. 5C).

### *3.7. Tetrahydrocurcumin inhibited lipid accumulation and increased mRNA and protein expression of the NRF2 pathway in sodium oleate (OA) induced HepG2 cells*

To clarify the mechanism of THC inhibiting lipid deposition, we determined the effect of THC on the NRF2 pathway in a lipid deposition cell model. As shown in Fig. 6A, 0.2 mM OA significantly increased the intracellular lipid accumulation in HepG2 cells, whereas the THC treatment decreased the intracellular lipid level in a dose-dependent manner (Fig. 6A).

Compared with control, 0.2 mM OA significantly reduced the mRNA expression of *HO-1*, but had no effect on the expression of *NRF2, NQO1, GSTA1, GCLC* and *GCLM* (Fig. 6B, C). However, THC treatment significantly increased the mRNA expression of *NRF2, NQO1, HO-1* and *GCLM* in HepG2 cells in a dose-dependent manner (Fig. 6B,C). Similarly, a Western blot analysis showed that THC treatment significantly increased the protein expression of NRF2 and HO-1, but had no effect on NQO1 in OA-treated HepG2 cells (Fig. 6D,E,F). These results indicated that THC activated the NRF2 pathway, thereby increasing the antioxidant capacity of cells.

## 4. Discussion

The NAFLD is a globally prevalently chronic liver disease. The typical pathological feature of NAFLD is the occurrence of hepatic steatosis, accompanied by systemic metabolic dysfunction, such as weight gain, elevated blood sugar and lipids, and lipid peroxidation (Maurice & Manousou, 2018). In this study, we demonstrated that THC increases the antioxidant capacity and reduces liver fat accumulation in mice and HepG2 cells.

Consistent with previous reports (Song et al., 2019; Yamaguchi et al., 2015), our study showed that HFD induced weight gain, elevated blood lipids and blood sugar, glucose intolerance, and liver steatosis in mice. Our study also showed that THC treatment inhibited weight gain, lowered blood lipids and FBG, improved glucose tolerance, and reduced liver steatosis in mice. Our study showed that the reason for THC to reduce weight gain is not to reduce food intake. MDA is the product of lipid peroxidation, and the level of MDA reflects the degree of oxidative stress (Chu et al., 2019). As expected, HFD increased serum MDA levels and caused lipid peroxidation in this study. Interestingly, THC reduced the serum MDA content and improved the redox imbalance caused by HFD. THC did not show a dose-dependent effect on body weight and blood biochemistry, but inhibited liver fat deposition in a dose-

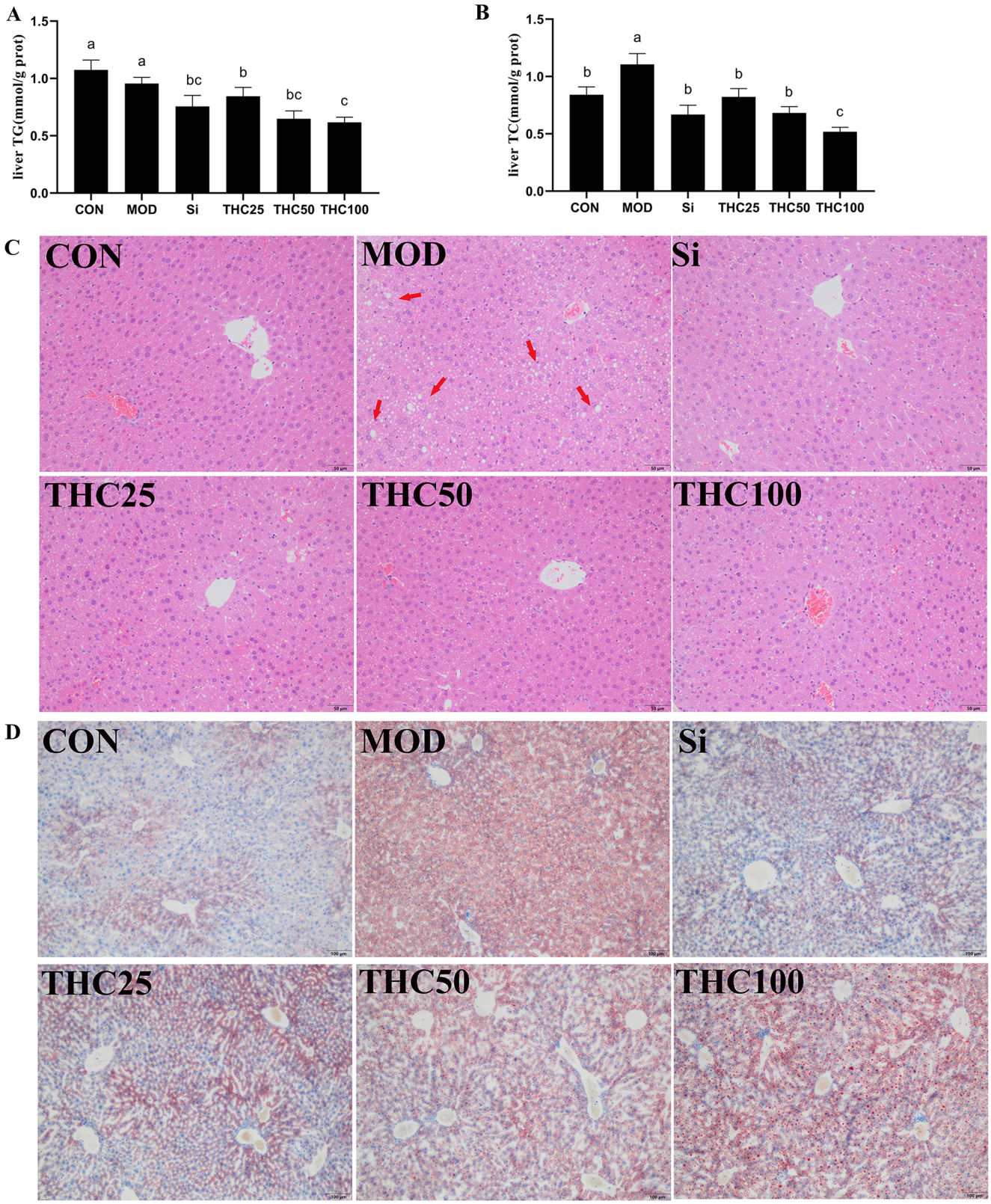


**Fig. 3. Effects of tetrahydrocurcumin (THC) on liver fat content in high-fat diet-fed mice.** CON: normal diet; MOD: high-fat diet; Si: high-fat diet + simvastatin; THC25/50/100: high-fat diet + THC25/50/100 mg/kg. (A) Triglycerides (TG) and (B) Total Cholesterol (TC) in liver. Different letters (a-c) between groups (n = 12) indicate significant differences ($P < 0.05$). (C) H&E staining of liver tissue (200×, bar = 50 μm). The red arrow points to fatty vacuoles; (D) Oil red O staining of liver tissue (100×, bar = 100 μm).

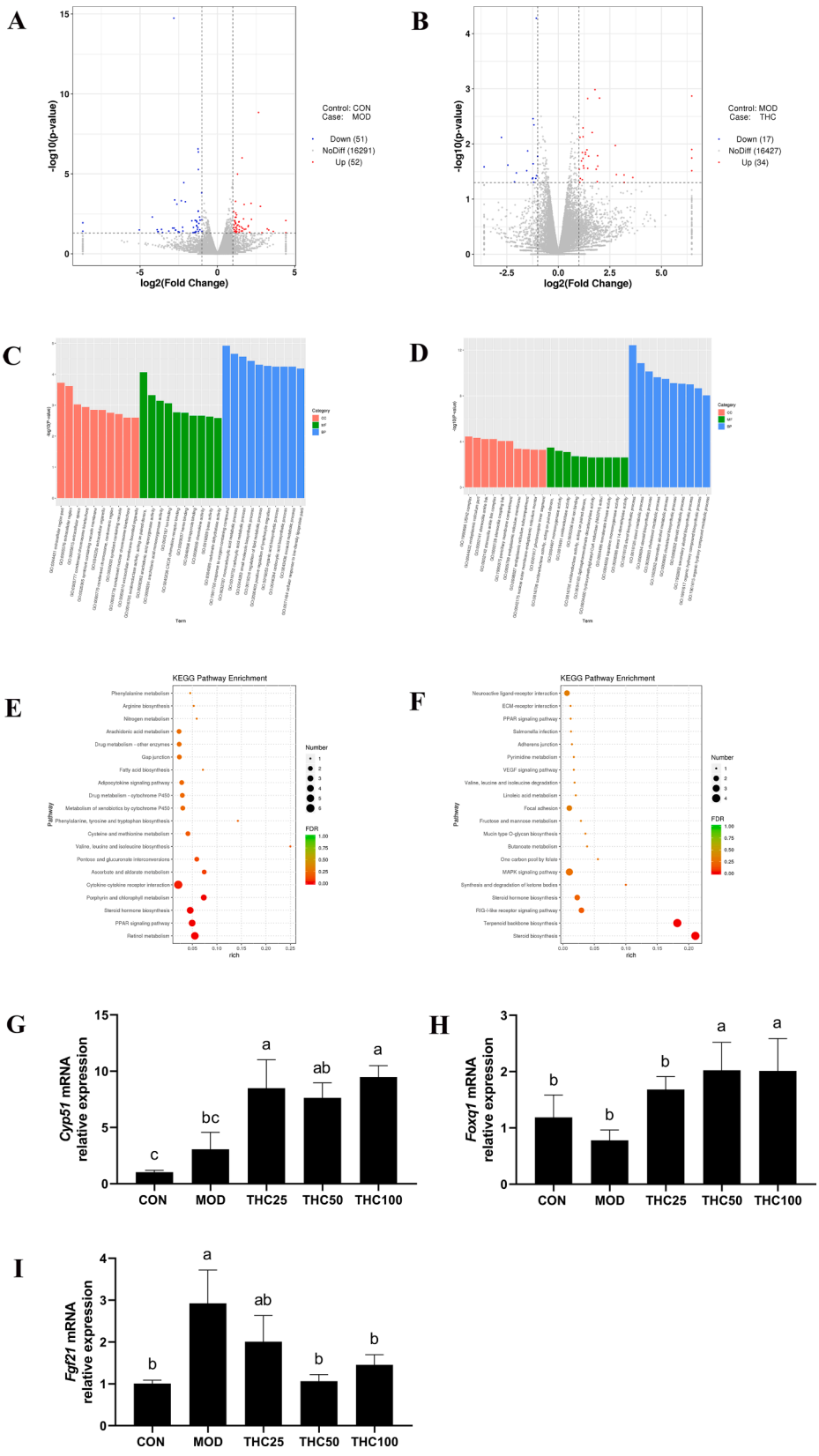


**Fig. 4. Effects of tetrahydrocurcumin (THC) on liver gene expression profile in high-fat diet-fed mice.** CON: normal diet; MOD: high-fat diet; Si: high-fat diet + simvastatin; THC25/50/100: high-fat diet + THC25/50/100 mg/kg. Differentially expressed genes (DEGs) of (A) CON vs MOD and (B) MOD vs THC were screened as described in Materials and Methods. The blue dots indicate down-regulated genes in Case group relative to Control group, and the red dots indicate the up-regulated genes in Case group relative to Control group. The GO enrichment analysis of DEGs of (C) CON vs MOD and (D) MOD vs THC were classified according to molecular function (MF), biological process (BP) and cell composition (CC). The KEGG enrichment analysis of DEGs of (E) CON vs MOD and (F) MOD vs THC were shown. 'Rich' refers to the ratio of the number of DEGs enriched in the pathway to the number of annotated genes. The greater the Rich value, the greater the degree of enrichment. The FDR value range is 0–1, and the closer to zero, the more significant the enrichment. Th relative mRNA expression levels of liver (G) *Cyp51,* (H) *Foxq1* and (I) *Fgf21* were determined by RT-qPCR. *Gapdh* was used as a reference for normalization. Different letters (a-c) between groups indicate significant difference ($P < 0.05$, $n = 4$).

dependent manner. This discrepancy may be related to the initial body weight of the mice or the short treatment time (8w). Interestingly, 100 mg/kg of THC increased food intake. The stimulatory effect of 100 mg/kg THC on food intake could cancel out some of the beneficial effects of THC at this dose on lipid metabolism. Previous studies have shown that serum THC concentrations are low in mice receiving oral administration of THC, indicating that THC has poor oral bioavailability (Novaes et al., 2017; Okada et al., 2001; Pan et al., 2018), which may explain why THC does not always have a dose-dependent effect on serum parameters in our study.

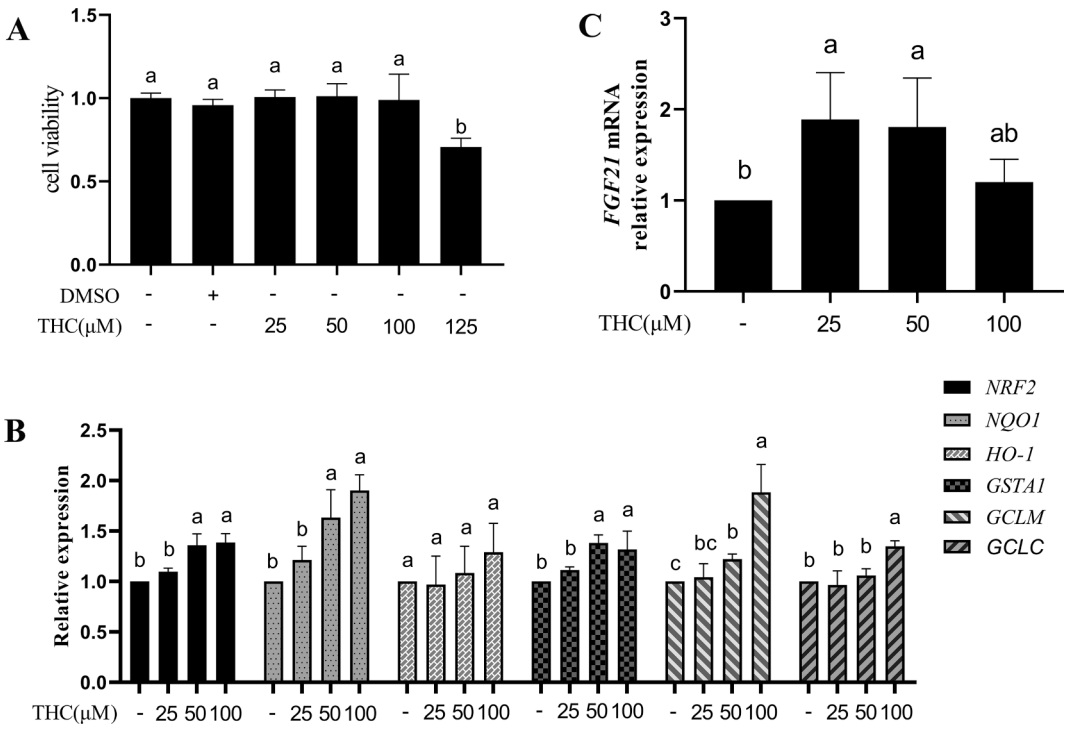


**Fig. 5. Effect of tetrahydrocurcumin (THC) on cell viability and expression of antioxidant and *FGF21* genes in HepG2 cells.** (A) Cell viability was determined after 24 h of treatment of the cells with different concentration of THC. Untreated cells as a control group. DMSO was used as a solvent control. Those without cells were set as zeroing group. The relative mRNA expression levels of (B) *NRF2* pathway and (C) *FGF21* were measured by RT-qPCR. Different letters (a-c) between groups indicate significant differences ($P < 0.05$, $n = 3$).

Previous studies reported that THC affects the expression of genes in the liver of HFD mice, such as the Cytochromes p450 (CYPs) (Jearapong et al., 2015) and peroxisome proliferator-activated receptors (PPAR) signaling pathway (Pan et al., 2018). In our study, we determined the effect of THC on the entire transcriptome of NAFLD mouse liver by RNA-seq. The RNA-seq analysis identified 103 DEGs between the CON group and MOD group, and 51 DEGs between the MOD group and THC group. The GO enrichment analysis of these DEGs indicated that they were enriched in lipid synthesis and metabolism related processes, as well as oxidoreductase activity. These results indicated that HFD may induce oxidative stress and promote steatosis and that THC activates oxidoreductase activity, thereby improving the redox imbalance and steatosis in the liver caused by HFD. The KEGG enrichment analysis indicated that these DEGs were enriched in the pathways related to fat synthesis and metabolism, such as fatty acid synthesis, PPAR signaling pathway, steroid biosynthesis and MAPK signaling pathway. These enrichments indicated that HFD changed the synthesis and metabolism of fat in mice and that THC treatment affected the related pathways of fat metabolism and regulated the dynamic balance of lipids.

CYP51 is an important member of the CYP family and a key enzyme for cholesterol synthesis. Increased cholesterol content reduces CYP51 expression (Pikuleva, 2006). A diet supplemented with chardonnay grape seed flour up-regulated the expression of liver CYP51, thereby reducing plasma cholesterol content (Kim et al., 2014). CYP51 plays an important role in liver function. Liver-specific knockout of CYP51 led to progressive liver damage and fibrosis (Urlep et al., 2017). This study showed that THC increased the expression of *Cyp51* in the liver, thereby exerting a hepatoprotective effect and reducing the cholesterol content in the liver and serum. Forkhead box Q1 (FOXQ1), also known as HFH1, is a member of the FOX protein family. FOXQ1 was initially discovered to have the effect of regulating the development of hair follicles (Hong et al., 2001). Later studies found that FOXQ1 promotes osteogenic differentiation (Xiang et al., 2020), and is associated with the metastasis and poor prognosis of a variety of human cancers (Kaneda et al., 2010; Qiao et al., 2011). A recent study showed that decreased expression of FOXQ1 in the liver may promote the development of T2DM (Cui et al., 2016). Overexpression of liver FOXQ1 reduces the symptoms of hyperglycemia in db/db mice and diet-induced obese mice (Cui et al., 2016). In this study, THC significantly increased the expression of *Foxq1* in the liver, thereby contributing to lowering blood sugar and improving glucose tolerance. Surprisingly, this study showed that THC reduced liver *Fgf21* expression. FGF21 is a polypeptide hormone synthesized primarily in the liver, that has an energy-regulating effect, including reducing body weight and regulating metabolic disorders (Fisher & Maratos-flier, 2016; Markan et al., 2014). However, studies have shown that in HFD-induced obese mice, the level of endogenous FGF21 increased significantly without a beneficial effect, contributing to FGF21 resistance (Fisher et al., 2010). Our study suggests that THC might improve FGF21 resistance in HFD-fed mice by reducing FGF21 expression in the liver.

FGF21 expression is positively regulated by Nrf2 in the liver and participates in the regulation of oxidative stress (Chen et al., 2017; Furusawa et al., 2014; Gómez-Sámano et al., 2017; Zhang, Xu, Gu, et al., 2018). In *in vivo* study, we proved that THC improves HFD-induced liver FGF21 resistance. Furthermore, the effect of THC on the expression of FGF21 under normal physiological conditions was determined. The results showed that THC significantly increased the expression of *FGF21* gene in HepG2 cells, indicating that THC may promote the secretion of FGF21. Similar to the results of our study, dietary curcumin up-regulates liver FGF21 expression and improves FGF21 resistance caused by HFD (Zeng et al., 2017), which may be because curcumin and THC have similar chemical structures. However, the effect of THC on the FGF21 receptor has not been explored in this study, and further research is needed in subsequent experiments.

As an important regulator of oxidative stress, Nrf2 activities include transcription, mRNA processing, translation, subcellular localization, self-protein stability and binding protein activity such as that of Keap1 (Tonelli et al., 2018). Our study showed that OA significantly increased

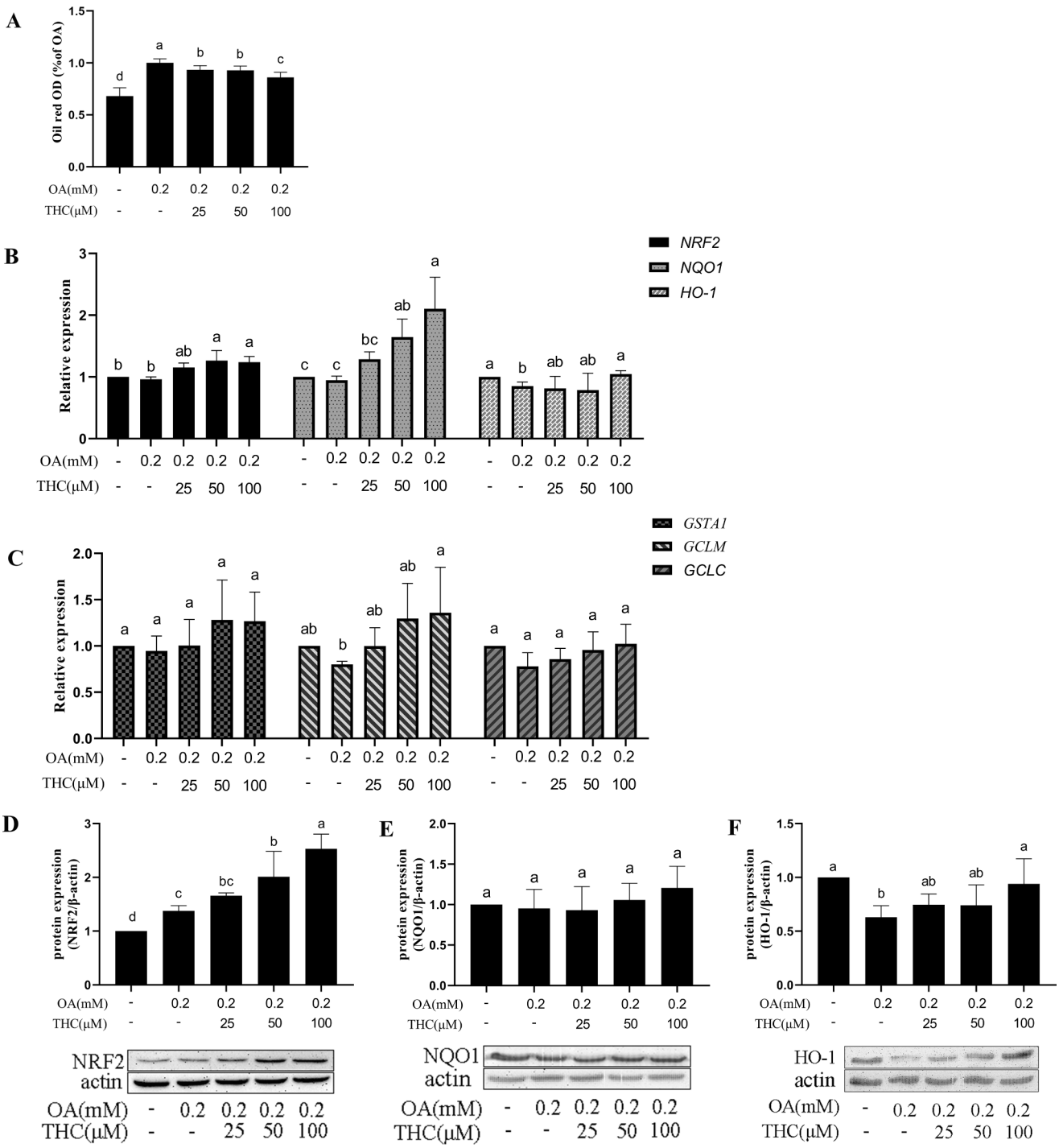


**Fig. 6. Effect of tetrahydrocurcumin (THC) on lipid content and expression of the NRF2 pathway in HepG2 cells.** HepG2 cells were treated with 0.2 mM sodium oleate (OA) for 24 h, and then with THC for 48 h. Oil red O was used to determine the (A) Intracellular lipid content. RT-qPCR and Western blot were used to analyze the expression of NRF2 signaling pathway. (B) Relative expression of *NRF2, NQO1 and HO-1* mRNA; (C) Relative expression of *GSTA1, GCLC and GCLM* mRNA; (D) Relative expression of NRF2 protein; (E) Relative expression of NQO1 protein; (F) Relative expression of HO-1 protein. Different letters (a-d) between groups indicate significant differences ($P < 0.05$, $n = 3$).

the protein level of NRF2 in HepG2 cells, which is consistent with the report by Zhang, Ji, Sun, et al. (2018), and significantly decreased the mRNA and protein levels of HO-1, suggesting that OA induces redox imbalance in cells. However, THC treatment increased the expression of NRF2 and downstream targets in either normal HepG2 cells or NAFLD cells. It shows that THC improves the antioxidant capacity of cells. These results are consistent with previous studies showing that THC regulates the Keap1-Nrf2 pathway and activates downstream target proteins, thereby reducing acetaminophen-induced liver damage (Luo et al., 2019).

## 5. Conclusion

Our study indicated that THC reduced fat accumulation and lipid peroxidation in HFD-induced NAFLD in mice, and affected liver transcriptome, including the expression of *CYP51, FOXQ1* and *FGF21*. Our

study also showed that THC up-regulated the expression of *FGF21*, activated the NRF2 signaling pathway, and reduced lipid accumulation. These findings suggest that dietary THC has a beneficial effect on the antioxidant capacity in NAFLD.

## Ethical statement

All animal procedures and protocols in this experiment were approved by the Animal Care Committee of the Changshu Institute of Technology (Permit number: EAWEC1912).

## 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.

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