Available online at www.sciencedirect.com
ScienceDirect
Chinese Journal of Natural Medicines 2021, **19**(6): 401-411
doi: 10.1016/S1875-5364(21)60039-0
Chinese Journal of Natural Medicines

•Research article•

Silybin alleviates hepatic lipid accumulation in methionine-choline deficient diet-induced nonalcoholic fatty liver disease in mice *via* peroxisome proliferator-activated receptor *α*

CUI Shuang[1Δ], PAN Xiao-Jie[1Δ], GE Chao-Liang[1, 2], GUO Yi-Tong[1], ZHANG Peng-Fei[1], YAN Ting-Ting[1, 3], ZHOU Ji-Yu[1], HE Qing-Xian[1], CHENG Long-Hao[1, 2], WANG Guang-Ji[1], HAO Hai-Ping[1*], WANG Hong[1*]

[1] *State Key Laboratory of Natural Medicines, Key Laboratory of Drug Metabolism & Pharmacokinetics, China Pharmaceutical University, Nanjing 210009, China;*
[2] *Department of Pharmacy, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, China;*
[3] *Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA*

Available online 20 Jun., 2021

[ABSTRACT] Nonalcoholic fatty liver disease (NAFLD) is regarded as the most common liver disease with no approved therapeutic drug currently. Silymarin, an extract from the seeds of *Silybum marianum*, has been used for centuries for the treatment of various liver diseases. Although the hepatoprotective effect of silybin against NAFLD is widely accepted, the underlying mechanism and therapeutic target remain unclear. In this study, NAFLD mice caused by methionine-choline deficient (MCD) diet were orally administrated with silybin to explore the possible mechanism and target. To clarify the contribution of peroxisome proliferator-activated receptor *α* (PPAR*α*), PPAR*α* antagonist GW6471 was co-administrated with silybin to NAFLD mice. Since silybin was proven as a PPAR*α* partial agonist, the combined effect of silybin with PPAR*α* agonist, fenofibrate, was then evaluated in NAFLD mice. Serum and liver samples were collected to analyze the pharmacological efficacy and expression of PPAR*α* and its targets. As expected, silybin significantly protected mice from MCD-induced NAFLD. Furthermore, silybin reduced lipid accumulation *via* activating PPAR*α*, inducing the expression of liver cytosolic fatty acid-binding protein, carnitine palmitoyltransferase (Cpt)-1a, Cpt-2, medium chain acyl-CoA dehydrogenase and stearoyl-CoA desaturase-1, and suppressing fatty acid synthase and acetyl-CoA carboxylase *α*. GW6471 abolished the effect of silybin on PPAR*α* signal and hepatoprotective effect against NAFLD. Moreover, as a partial agonist for PPAR*α*, silybin impaired the powerful lipid-lowering effect of fenofibrate when used together. Taken together, silybin protected mice against NAFLD *via* activating PPAR*α* to diminish lipid accumulation and it is not suggested to simultaneously take silybin and classical PPAR*α* agonists for NAFLD therapy.

[KEY WORDS] Silybin; NAFLD; PPAR*α*; Lipid metabolism; Fenofibrate
[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2021)06-0401-11

Introduction

Nonalcoholic fatty liver disease (NAFLD) is currently regarded as the most common liver disease worldwide, and about 25% of the world population suffers from NAFLD [1-3]. This incidence is much higher in obese and diabetic population. NAFLD is projected to become the most common indication for liver transplantation in the next decade [4]. Despite its high prevalence around the world, there are currently no approved pharmaceutical therapeutics, highlighting the urgent need to discover and develop effective medicines. The spectrum of this entity spans from simple hepatic steatosis to

[Received on] 28-Jan.-2021
[Research funding] This work was supported by the National Natural Science Foundation of China (Nos. 81720108032, 81930109, 82073926, and 82073928), the Project for Major New Drug Innovation and Development (Nos. 2018ZX09711001-002-003 and 2018ZX09711002-001-004), Overseas Expertise Introduction Project for Discipline Innovation (No. G20582017001).
[*Corresponding author] Tel: 86-25-83271179, Fax: 86-25-83271060, E-mail: haipinghao@cpu.edu.cn (HAO Hai-Ping); E-mail: wanghong@cpu.edu.cn (WANG Hong)
[Δ]These authors contributed equally to this work.
These authors have no conflict of interest to declare.

nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and even hepatocellular carcinoma. NAFLD is initially triggered by lipid accumulation in the liver, stemmed from metabolic disorder, such as increased *de novo* lipogenesis and reduced lipid catabolism. Reducing lipid accumulation is regarded as a fundamental way to rescue NAFLD.

Traditional herbal medicines have garnered increased attention for providing therapeutic candidates for NAFLD [5-7]. *Silybum marianum* (L.) Gaernt, one of the oldest medicinal plants, has already been used in clinical practice for liver dysfunctions and gallbladder disorders. Silymarin, an extract of its seeds, consists of a range of flavonoids, including silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, and taxifolin [8, 9]. Silybin is one of the major and pharmacologically active compounds in silymarin [10]. As a safe and well-tolerated phytomedicine, silymarin/silybin has been marketed as a hepatoprotective drug in China and as a complementary protection in Europe [11]. Accumulating studies have reported the hepatoprotective activity of silybin [12], and the possible mechanisms lying in lipid lowering, antioxidant [13], anti-inflammatory, and antiviral activities [14]. The metabolic regulatory effect of silybin on lipid homeostasis may largely contribute to its hepatoprotective effect on NAFLD [14]. However, the therapeutic target and exact mechanism remain unclear.

Peroxisome proliferator-activated receptor alpha (PPARα) is a ligand-activated transcription factor. Binding of agonists within the ligand-binding site of PPARα promotes its binding to the specific DNA element in the regulatory region of complex networks of target genes [15, 16]. It has been shown that, animals with the absence of PPARα are more susceptible to hepatic steatosis and have perturbations in circulating free fatty acids [17, 18]. While PPARα agonists treatment reversed nutritional steatohepatitis in mice [17]. Above evidence indicated the crucial role of PPARα in the development and management of NAFLD.

We have previously demonstrated that silybin can bind to and activate PPARα [19]. Thus, in the present study, we intended to investigate the contribution of PPARα agonism by silybin on its hepatoprotective effect against NAFLD. Besides, since silybin was demonstrated to compromise PPARα agonism by fenofibrate, we further evaluated the combinatory effect of silybin and fenofibrate on NAFLD.

Materials and Methods

Chemicals and reagents

Silybin, fenofibrate, and palmitic acid (PA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). GW6471 was purchased MedChem Express (Monmouth Junction, NJ, USA).

Animal experiments

6- to 8-week-old male C57BL/6J mice, weighing 20−22 g, were obtained from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). They were kept in an air-conditioned animal quarter at a temperature of 25 ± 2 °C and a relative humidity of 50% ± 10% with 12-hour light/dark cycles. Animals were acclimatized to the facilities for 1 week with water and food allowed *ad libitum*. All the animal studies were approved by the Animal Ethics Committee of China Pharmaceutical University.

For all the studies, NAFLD model in mice was established by MCD diet feeding for 6 weeks as previously described [20]. Methionine-choline deficient diet (MCD, Cat No. TP 3005G) and methionine-choline supplemental diet (MCS, Cat No. TP 3005GS) were purchased from Trophic Animal Feed High-tech Co. Ltd. (Jiangsu, China). MCD and MCS formulas comprise 17% protein (as defined amino acids), 66% carbohydrate (sucrose and starch), and 10% fat (as corn oil) by weight. MCS formulas contain 3 $g \cdot kg^{-1}$ L-methionine and 2 $g \cdot kg^{-1}$ choline chloride, while MCD formulas contain no methionine or choline.

To confirm the efficacy of silybin, NAFLD mice caused by MCD diet were treated with silybin. Previously study showed that silybin administration at the dose range from 50 to 300 $mg \cdot kg^{-1} \cdot d^{-1}$ exerted excellent hepatoprotective effect against NAFLD [10, 12, 21]. Besides, silybin gavage at the dose of 50 and 150 $mg \cdot kg^{-1} \cdot d^{-1}$ was demonstrated to significantly regulate PPARα signal [19]. Taken together, the dose of silybin was selected as 50 and 150 $mg \cdot kg^{-1} \cdot d^{-1}$. Mice were randomly divided into four groups (8 mice per group) as follows: (1) the first group served as the control group in which mice were fed an MCS diet for 6 weeks; (2) mice in the second group were fed an MCD diet for 6 weeks and intragastrically treated with vehicle from the 3rd week for 4 weeks; (3) mice in the third group were fed an MCD diet for 6 weeks and intragastrically treated with silybin at 50 $mg \cdot kg^{-1} \cdot d^{-1}$ from the 3rd week for 4 weeks; (4) mice in the fourth group were fed an MCD diet for 6 weeks and intragastrically treated with silybin at 150 $mg \cdot kg^{-1} \cdot d^{-1}$ from the 3rd week for 4 weeks.

To validate the efficacy of fenofibrate on MCD diet-induced NAFLD, mice were randomly divided into three groups (6 mice per group) as follows: (1) the first group served as the control group in which mice were fed an MCS diet for 6 weeks; (2) in the second group, mice were fed an MCD diet for 6 weeks and intragastrically treated with vehicle from the 3rd week for 4 weeks; (3) in the third group, mice were fed an MCD diet for 6 weeks and intragastrically treated with fenofibrate at 50 $mg \cdot kg^{-1} \cdot d^{-1}$ from the 3rd week for 4 weeks.

To evaluate the influence of GW6471 on the hepatoprotective effect of silybin, mice were randomly divided into five groups (6 mice per group) as follows: (1) the first group serve as the control group in which mice were fed an MCS diet for 6 weeks; (2) in the second group, mice were fed an MCD diet for 6 weeks and intragastrically treated with vehicle; (3) in the third group, mice fed an MCD diet were intragastrically treated with silybin at 150 $mg \cdot kg^{-1} \cdot d^{-1}$ from the 3rd week for 4 weeks; (4) in the fourth group, mice fed an MCD diet were intragastrically treated with GW6471 at 10 $mg \cdot kg^{-1} \cdot d^{-1}$ from the 3rd week for 4 weeks; (5) in the fifth group, mice fed an

MCD diet were intragastrically treated with GW6471 (10 $mg \cdot kg^{-1} \cdot d^{-1}$) and silybin (150 $mg \cdot kg^{-1} \cdot d^{-1}$) from the 3rd week for 4 weeks.

To evaluate the combinatory effect of silybin and fenofibrate, mice were randomly divided into five groups (6 mice per group) as follows: (1) the first group serve as the control group in which mice were fed an MCS diet for 6 weeks; (2) in the second group, mice were fed an MCD diet for 6 weeks and intragastrically treated with vehicle; (3) in the third group, mice fed an MCD diet were intragastrically treated with silybin at 150 $mg \cdot kg^{-1} \cdot d^{-1}$ from the 3rd week for 4 weeks; (4) in the fourth group, mice fed an MCD diet were intragastrically treated with fenofibrate at 50 $mg \cdot kg^{-1} \cdot d^{-1}$ from the 3rd week for 4 weeks; (5) in the fifth group, mice fed with MCD diet were intragastrically treated with fenofibrate (50 $mg \cdot kg^{-1} \cdot d^{-1}$) and silybin (150 $mg \cdot kg^{-1} \cdot d^{-1}$) from the 3rd week for 4 weeks.

For all above animal studies, 24 h after the last administrations, blood samples were collected, the animals were euthanized and hepatic samples were immediately harvested and stored.

Serum biochemical analysis

Serum alanine aminotransferase (ALT) and aspartate transaminase (AST) levels were measured using an automatic blood biochemical analyzer (Beckman Counter LX20, USA). Total triglyceride (TG) and total cholesterol (TC) in liver tissues were measured as follow: lipids were extracted from liver with chloroform−methanol (2 : 1), then measured with commercial kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) following the manufacturer's instructions.

Histology evaluation

Liver tissue was fixed in 10% formaldehyde phosphate buffer saline (pH 7.4) overnight, then embedded in paraffin, sectioned. General histology was assessed in hematoxylin-eosin (H&E) stained liver sections, and blindly scored by an experienced pathologist. The NAFLD activity score (NAS) was calculated by addition of grades of steatosis (0−3), inflammation (0–3) and ballooning (0–2) as previously described [22], which is based in a semi-quantitative analysis of the three diagnostic categories of NASH. Total score, indicating a prognostic status in the liver, ranges from 0 to 8. NAS from 0 to 2 was considered non-steatohepatitis, score ≥ 5 was diagnosed as NASH, score from 3 to 5 was identified as non-defining NASH.

Lipid accumulation in hepatic tissue was determined by Oil Red O staining. Freshly harvested liver tissues were embedded in optimum cutting temperature on dry ice. After sliced at −20 °C, the sections were stained with Oil Red O solution, followed by differentiation with propylene glycol solution and hematoxylin counterstaining.

RT-PCR analysis

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) was performed as previously described [23]. Briefly speaking, total RNA was isolated from mouse livers or HepG2 cells with the RNAiso Plus reagent (TakaRa Biotechnology, Dalian, China), according to the manufacturer's protocol. Purified total RNA was reverse-transcribed using the PrimeScript RT Reagent Kit (TakaRa Biotechnology, Dalian, China). Real-time PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Bedford, MA, USA) and SYBR Green Reagent Kit (Applied Biosystems, Bedford, MA, USA) to determine the mRNA expressions. Primer sequences are list in Supplemental Table S1.

Cell culture and treatment

HepG2 cells were obtain from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Hyclone, Logan, Utah, USA) and antibiotics (100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin) at 37 °C in a humidified 5% CO_2 atmosphere [24]. To investigated the effect of PA on inflammatory response, HepG2 cells were seeded in 12-well plate. 24 h after incubation, the cells were treated with PA (0.5 $mmol \cdot L^{-1}$) for 24 h and harvested for PCR analysis.

Statistical analysis

All data are presented as the mean ± SEM and were analyzed using a two-tailed Student's *t*-test was applied, for comparison of multiple groups a one-way ANOVA with Bonferroni post hoc analysis where appreciate. All tests were performed with GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA, USA) and *P* values below 0.05 were considered statistically significant.

Results

Silybin significantly attenuated MCD diet-induced steatohepatitis

In order to confirm the effect of silybin on NAFLD, C57BL/6J mice were fed an MCS or MCD diet for 6 weeks, and treated with vehicle or silybin (50 and 150 $mg \cdot kg^{-1} \cdot d^{-1}$) from the 3rd week for 4 weeks. Feeding an MCD diet for 6 weeks caused a large increase in serum ALT and AST levels, and silybin gavage significantly lowered the serum levels of ALT and AST (Fig. 1A). This hepatoprotective effect of silybin was further tested by histologic evaluations. H&E staining data showed that the MCD diet caused pronounced fat accumulation and neutrophils infiltration, and Oil Red O staining data further confirmed lipid accumulation in MCD diet-fed mice. These pathologic changes were significantly attenuated by silybin treatment dose-dependently (Fig. 1B, Table 1). Consistent with the histopathological analysis, silybin treatment caused significant decreases of hepatic fat contents (TG and TC, Fig. 1C) and inflammation (*Il-1b*, *Il-6*, and *Tnfa*, Fig. 1D). Besides, we also evaluated the effects of silybin on mice fed with MCS diet. Silybin gavage had marginal effect on serum ALT and AST levels in MCS diet-fed mice (Fig. S1). To our surprise, silybin treatment reduced hepatic TG and TC levels as well as the inflammatory genes in MCS-fed mice (Fig. S1). Taken together, these data demonstrated that

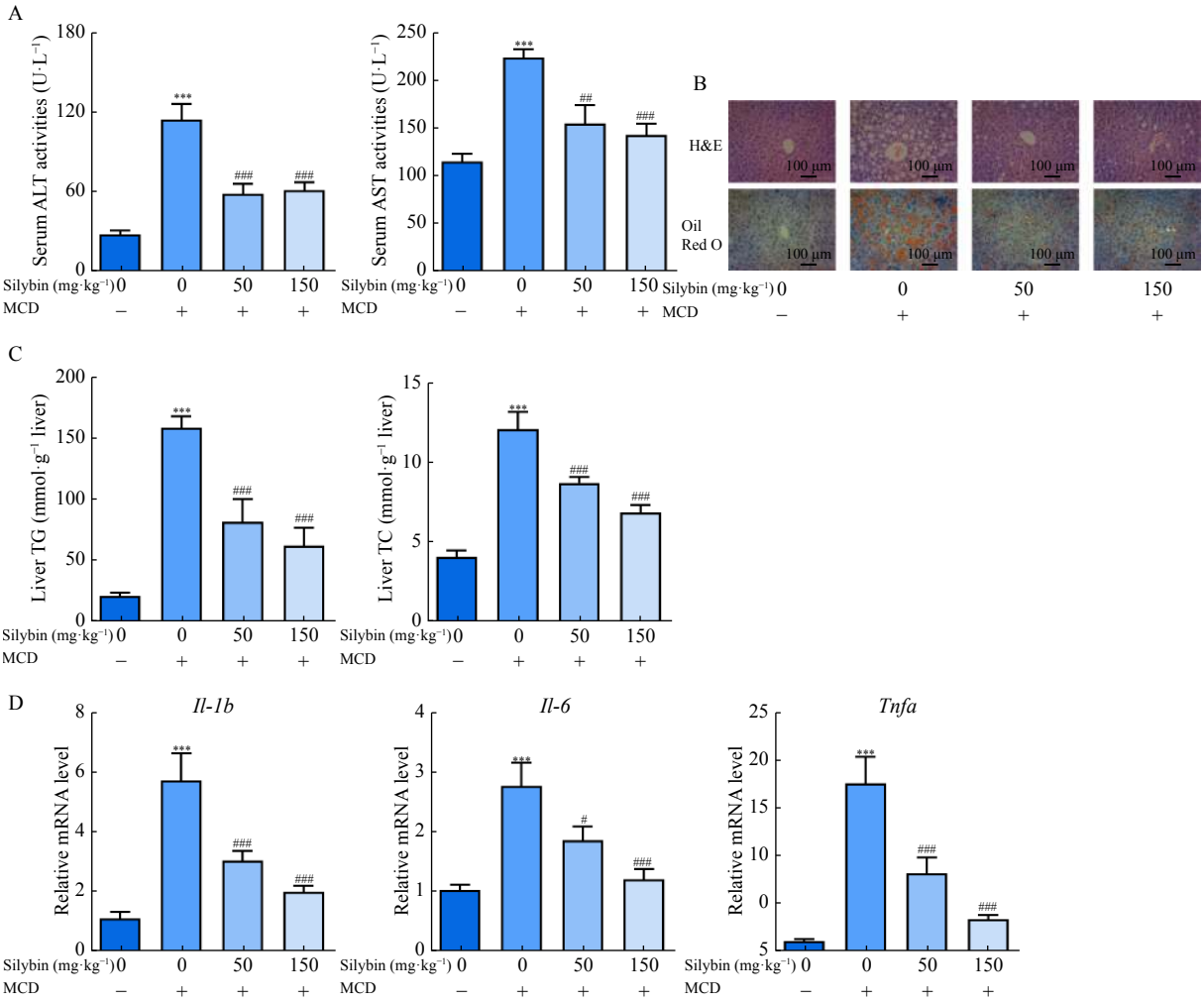

Fig. 1 Silybin significantly attenuated MCD diet-induced steatohepatitis (n = 8). (A) Serum ALT and AST levels. (B) H&E staining and Oil Red O staining of liver sections. (C) Hepatic TG and TC levels. (D) RT-PCR analysis of mRNA levels of hepatic inflammation genes. Results are mean ± SEM, $^{***}P < 0.001$ *vs* MCS, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ *vs* MCD control, as assessed with ANOVA. Scale bars 100 μm

Table 1 Silybin treatment significantly decreased NAS in MCD-fed mice (mean ± SEM, n = 8)

	MCS	MCD		
		Control	Silybin (50 mg·kg^{-1})	Silybin (150 mg·kg^{-1})
Steatosis	0	2.88 ± 0.13***	1.38 ± 0.18$^{\#\#\#}$	1.13 ± 0.23$^{\#\#\#}$
Inflammation	0	2.75 ± 0.16***	1.38 ± 0.18$^{\#\#\#}$	0.75 ± 0.16$^{\#\#\#}$
Ballooning	0	2.25 ± 0.16***	1.25 ± 0.16$^{\#\#\#}$	1.00 ± 0.19$^{\#\#\#}$
NAS	0	7.88 ± 0.13***	4.00 ± 0.38$^{\#\#\#}$	2.88 ± 0.48$^{\#\#\#}$

$^{***}P < 0.001$ *vs* MCS, $^{\#\#\#}P < 0.001$ *vs* MCD control, as assessed with ANOVA

silybin gavage significantly attenuated MCD-induced steatohepatitis.

Silybin regulated PPARα target genes involved in lipid homeostasis in NAFLD

Above results showed that silybin attenuated lipid accumulation and inflammation. According to the two-hit hypothesis of NASH development, lipid accumulation accounts for the stimulation of inflammatory response. Virtually, the mRNA expression of inflammatory cytokines, including *IL-1B*, *IL-6* and *TNFA*, were significantly enhanced HepG2 cells loaded with PA (Fig. S2), suggesting that lipid accumulation is the driver of inflammation in the development of NAFLD. We then intended to explore the possible mechanism about the effect of silybin in reducing lipid accumulation. We have

previously demonstrated that silybin acted as a partial agonist for PPAR*α* [19], a well-known therapeutic target for NAFLD treatment. PPAR*α* inhibits fatty acid synthesis by suppressing the expression of acetyl-CoA carboxylase *α* (ACC*α*) and fatty acid synthase (FAS), regulates fatty acid transport by modulating liver cytosolic fatty acid-binding protein (L-FABP), carnitine palmitoyltransferase 1a (CPT-1a), CPT-2, and promotes fatty acid metabolism by upregulating medium chain acyl-CoA dehydrogenase (MCAD) and stearoyl-CoA desaturase-1 (SCD-1) [25]. Results showed that the mRNA expression of *Ppara* was decreased in MCD-fed mice. Besides, this downregulation was observed for much of its target genes, including *L-fabp*, *Cpt-2*, *Mcad* and *Scd-1*. Additionally, the suppressive target genes of *Ppara*, including *Acca* and *Fas*, were upregulated. Silybin treatment reversed the mRNA expression of above genes (Fig. 2). What's more, in mice fed with MCS diet, silybin alone treatment slightly activated PPARα signal as the mRNA expression of *L-fabp*, *Cpt-1a*, *Mcad*, and *Scd-1* were induced (Fig. S3). These data indicated that silybin treatment restored the disturbed PPARα signal, especially its targets involved in lipid homeostasis in NAFLD.

PPARα agonist, fenofibrate, rapidly attenuated MCD diet-induced steatohepatitis

As stated above, PPAR*α* is an important therapeutic target for NAFLD treatment, we then validated the therapeutic effect of fenofibrate, a classical PPAR*α* agonist, on MCD-induced NAFLD. As expected, fenofibrate treatment significantly reduced serum ALT and AST levels in MCD diet-induced NAFLD mice (Fig. 3A). Besides, H&E staining showed that fenofibrate administration obviously alleviated steatosis and inflammation in MCD-fed NAFLD mice. Meanwhile, Oil Red O staining showed that fenofibrate reduced lipid deposition in the liver tissue (Fig. 3B). The excellent effect of fenofibrate on lipid disposition has also demonstrated by quantitative analysis of hepatic TG and TC levels (Fig. 3C). Fenofibrate treatment robustly upregulated the mRNA expression of *L-fabp*, *Cpt-1a*, *Cpt-2*, *Mcad* and *Scd-1*, while downregulated the mRNA expression of *Acca* and *Fas* in NAFLD mice (Fig. 3D). Above results collectively demonstrated that PPAR*α* agonism by fenofibrate restored lipid homeostasis *via* a set of target genes in MCD diet-induced NAFLD mice.

Silybin protected against MCD diet-induced NAFLD in a PPARα dependent manner

Considering the regulatory effect of silybin on PPAR*α* and the beneficial effect of PPAR*α* agonism on NAFLD management, we then wondered whether silybin exerts its hepatoprotective effect *via* PPAR*α*. GW6471, a typical PPAR*α* antagonist, was enrolled in the following study, and silybin was administrated to mice in the presence or absence of GW6471. GW6471 alone treatment had marginal effect on MCD diet induced NAFLD, as indicated by serum aminotransferase, hepatic fat contents, and histological analysis of liver sections (Fig. 4). Silybin treatment significantly reduced serum transaminases, while these effects were reversed in the presence GW6471 (Fig. 4A), suggesting that PPAR*α* agonism may mediate the hepatoprotective effect of silybin against NAFLD. Subsequently, we examined the histopathological features of liver tissues by silybin with or without GW6471. Results from H&E staining and Oil Red O staining suggested that silybin alone treatment strongly reduced hepatic lipid accumulation, while co-treatment of silybin and GW6471 slightly reduced lipid accumulation (Fig. 4B). Consistently,

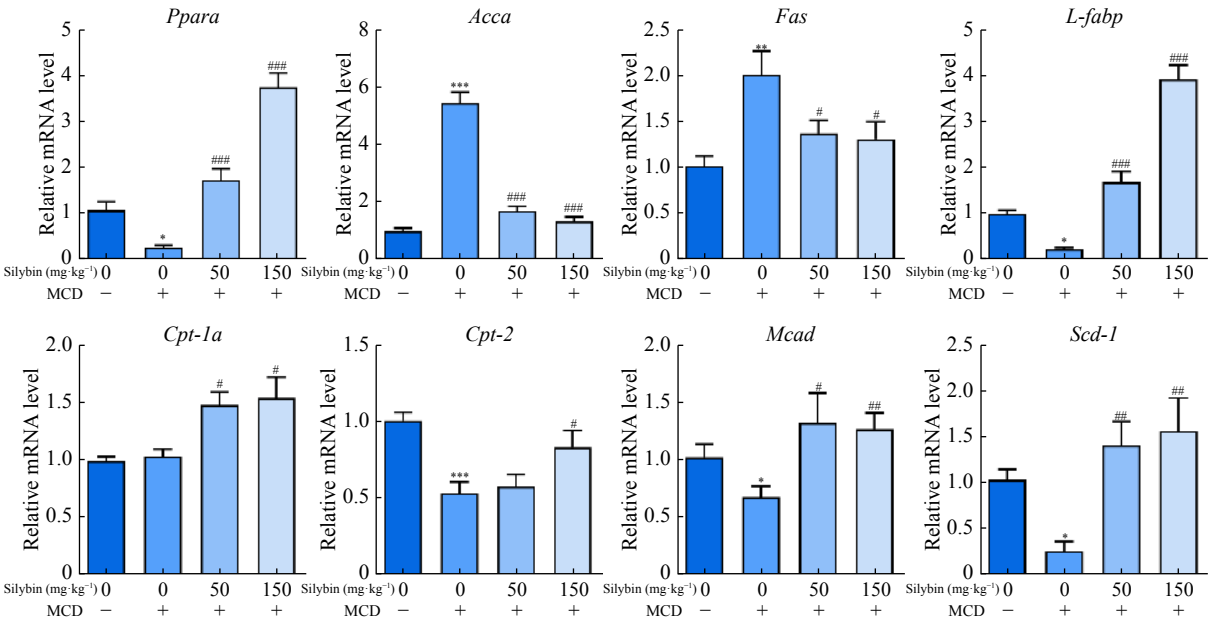

Fig. 2 Silybin regulated lipid homeostasis *via* activating PPAR*α* signal (n = 8). RT-PCR analysis of mRNA levels of genes of *Ppara* and its target genes involved in lipid homeostasis. Results are mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* MCS, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ *vs* MCD control, as assessed with ANOVA

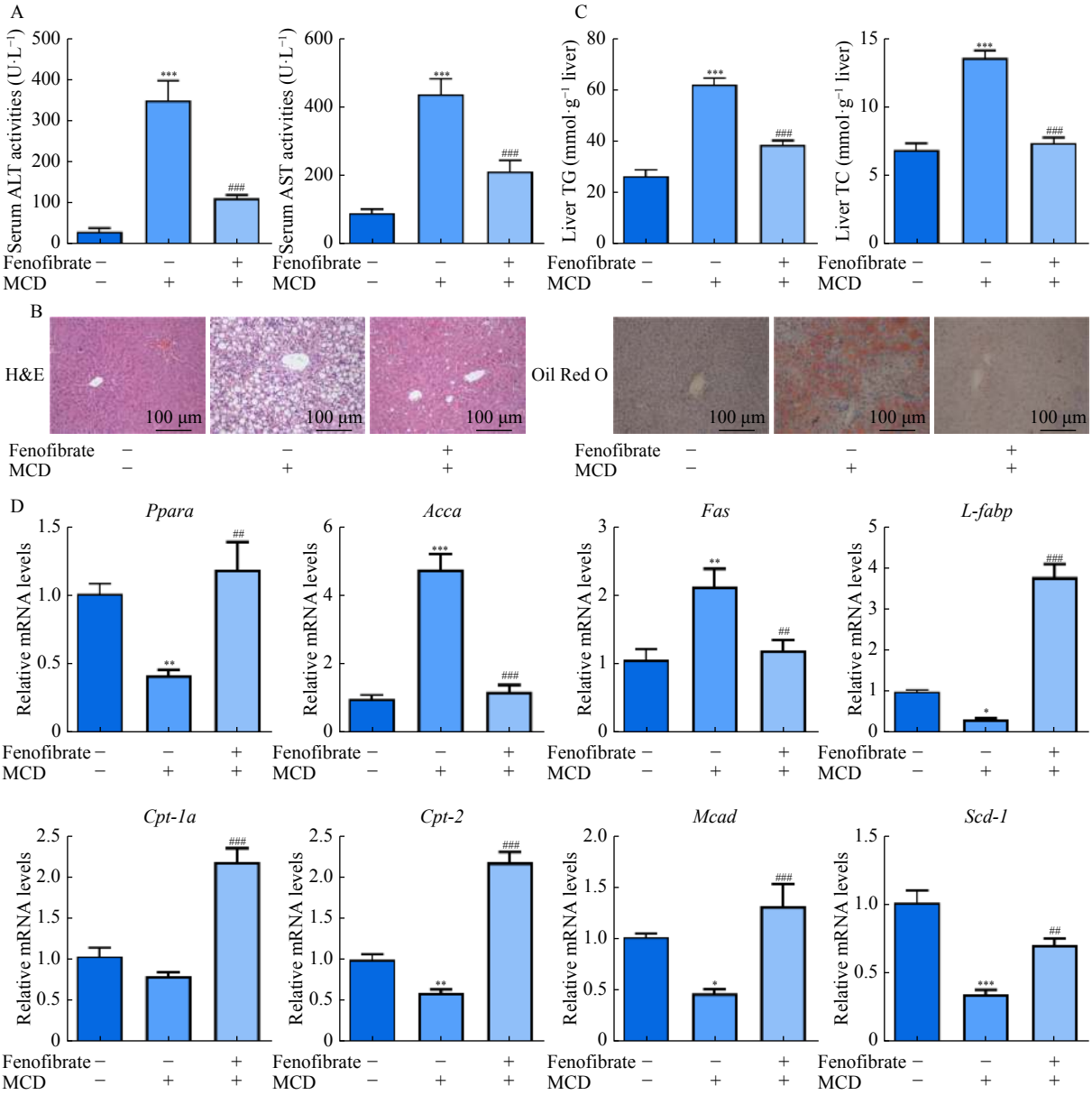

Fig. 3 PPAR*α* agonist, fenofibrate, strongly attenuated MCD diet-induced steatosis (*n* = 6). (A) Serum ALT and AST levels. (B) H&E staining and Oil Red O staining of liver sections. (C) Hepatic TG and TC levels. (D) RT-PCR analysis of mRNA levels of *Ppara* and its target genes involved in lipid homeostasis. Results are mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* MCS, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ *vs* MCD control, as assessed with ANOVA. Scale bars 100 μm

results from quantitative analysis of hepatic lipid contents indicated that GW6471 treatment impaired the lipid-lowering effect of silybin (Fig. 4C). Notably, in the presence of GW6471, silybin failed to regulate most of the genes, including *Acca, Fas, L-fabp*, *Cpt-1a, Cpt-2*, *Mcad* and *Scd-1* (Fig. 4D). Taken together, these data suggested that silybin improved steatohepatitis, at least partially, *via* activating of PPAR*α*.

Co-administration of silybin and fenofibrate resulted in an impaired therapeutic efficacy on NAFLD

Silybin has been previously demonstrated as a partial agonist for PPAR*α*. Furthermore, above results indicated that silybin exerted hepatoprotective effect against NAFLD in a PPAR*α*-dependent manner. We then suspected that silybin may impair the efficacy of classical PPAR*α* agonists. In the next study, NAFLD mice were treated with either silybin or fenofibrate alone, or both. Elevated serum ALT and AST levels caused by MCD diet were significantly reduced by single treatment of either silybin or fenofibrate. However, co-treatment of silybin and fenofibrate failed to further reduce serum levels of these serum transaminases (Fig. 5A). Histological analysis showed that silybin significantly, and fenofibrate robustly improved the severe steatosis caused by MCD diet. Silybin treatment diminished the excellent effect

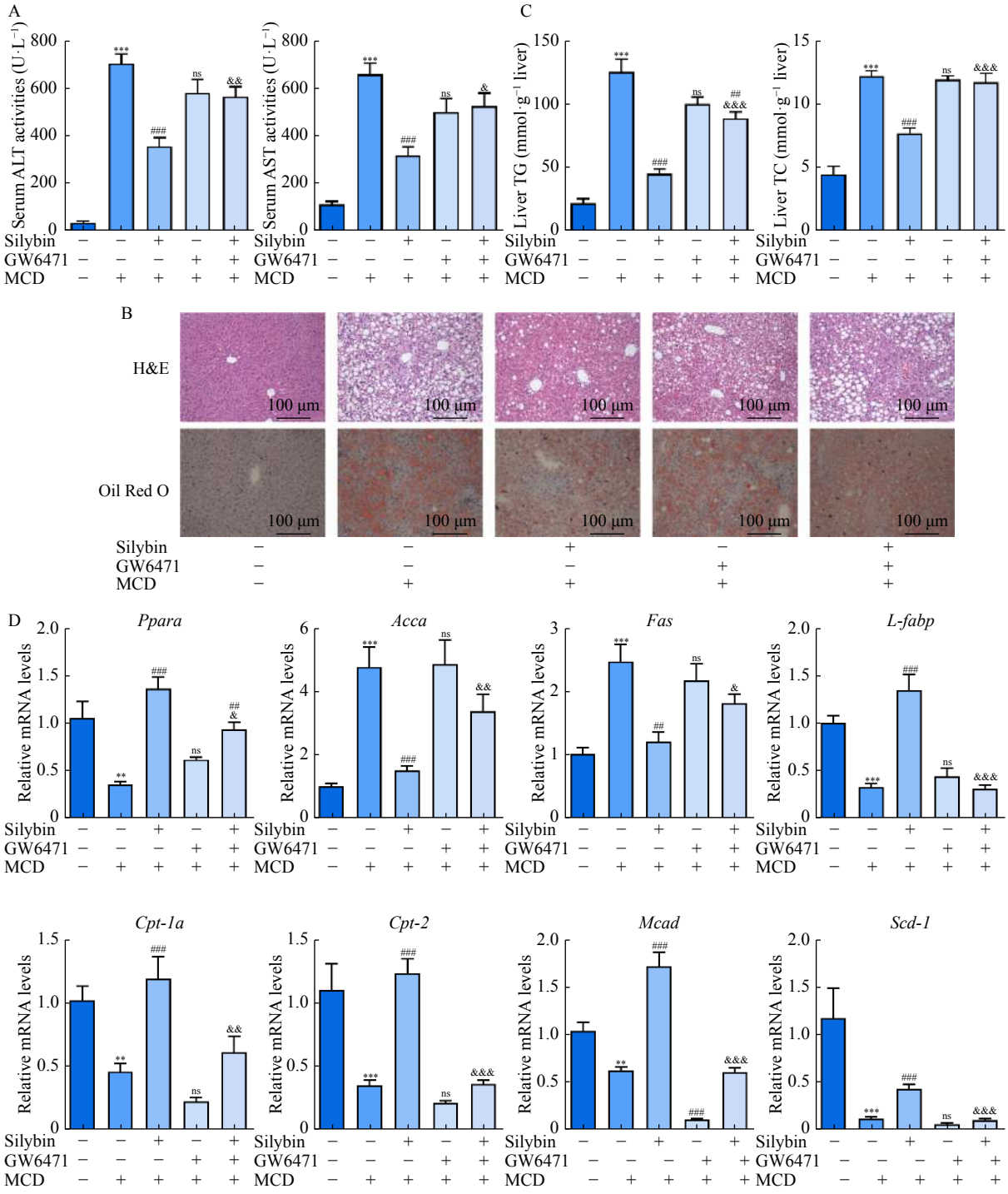

Fig. 4 Silybin protected against MCD diet-induced NAFLD in a PPAR*α* dependent manner (*n* = 6). (A) Serum ALT and AST levels. (B) H&E staining and Oil Red O staining of liver sections. (C) Hepatic TG and TC levels. (D) RT-PCR analysis of mRNA levels of *Ppara* and its target genes involved in lipid homeostasis. Results are mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* MCS, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ *vs* MCD control, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ *vs* MCD with silybin, ns *vs* MCD control, as assessed with ANOVA. Scale bars 100 μm

of fenofibrate, although co-treatment of silybin and fenofibrate showed remarkable effect compared with MCD diet-fed group (Fig. 5B), which is supported by the quantitative results of hepatic TG contents (Fig. 5C). Besides, single treatment of silybin or fenofibrate rescued the expression of *Acca*, *Fas*, *L-fabp*, *Cpt-1a*, *Cpt-2*, *Mcad*, and *Scd-1*. However, co-treatment of silybin and fenofibrate slightly impaired the excellent regulatory effects of fenofibrate on these gene expression (Fig. 5D). These observations were consistent with the histological analysis. Taken together, these results indicated

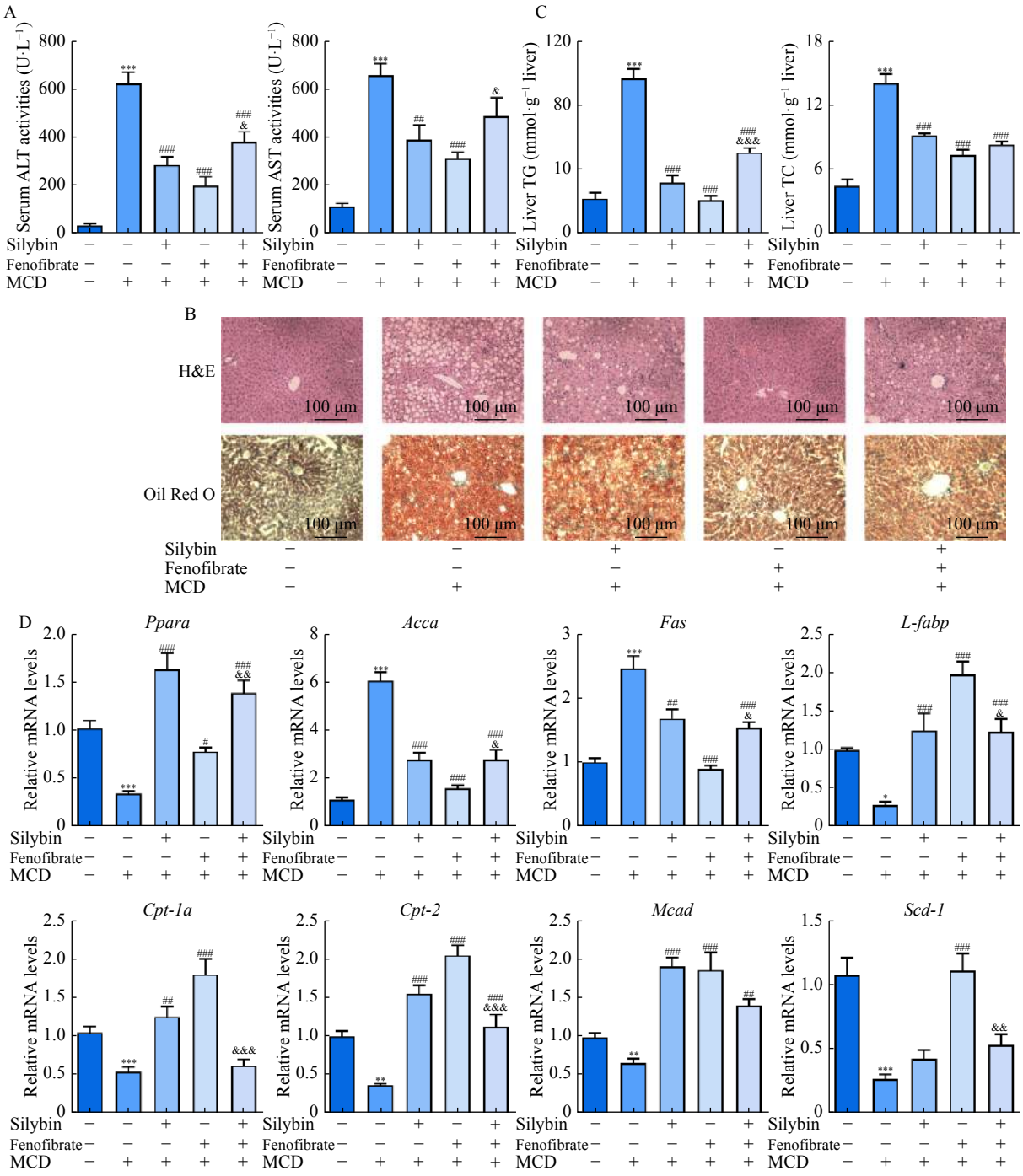

Fig. 5 Co-administration of silybin and fenofibrate resulted in an impaired therapeutic efficacy on NAFLD (n = 6). (A) Serum ALT and AST levels. (B) H&E staining and Oil Red O staining of liver sections. (C) Hepatic TC and TG levels. (D) RT-PCR analysis of mRNA levels of *Ppara* and its target genes involved in lipid homeostasis. Results are mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* MCS, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ *vs* MCD control, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ *vs* MCD with fenofibrate, as assessed with ANOVA. Scale bars 100 μm

that co-administration of silybin and fenofibrate resulted an impaired therapeutic efficacy on NAFLD.

Discussion

Over the past 2 decades, NAFLD has grown from a relative unknown disease to the most common chronic liver disease and resulted in a significant clinical and economic burden. However, there is no medicine approved by FDA for NAFLD/NASH treatment up to now. Silybin, a traditional herbal medicine, has been widely used for the treatment of NAFLD/NASH due to its excellent lipid lowering, anti-oxidant and anti-inflammatory effects. However, the molecular

mechanism and therapeutic target of silybin remain unclear, which affects the rational usage in clinic. In this study, we demonstrated that silybin reduced lipid accumulation *via* activating PPAR*α*. However, silybin attenuated the excellent effect of fenofibrate, a typical PPAR*α* agonist, when administrated together.

PPAR*α*, a member of PPAR family, functions mainly as a ligand-activated transcription factor. This nuclear factor is highly expressed in metabolically active tissues and regulates the expression of large number of genes involved in energy homeostasis, lipid metabolism [26], glucose metabolism, insulin sensitivity and inflammatory response. In detail, PPAR*α* promotes lipid catabolism chiefly through activating targets involved in fatty acid *β*-oxidation, including L-FABP, CPT-1a, CPT-2, MCAD and SCD-1, suppressing the expression of ACC*α* and FAS [25]. Due to its excellent activity in maintaining lipid homeostasis, PPAR*α* is widely accepted as a promising target for the treatment of various metabolic diseases, including NAFLD/NASH. The exact role of PPAR*α* in NAFLD comes from the evidence that deficiency of PPAR*α* in MCD-fed mice results in a worsening of steatosis and hepatitis [17, 18]. From then on, synthetic agonists of PPAR*α* have been used for NAFLD therapy. Fenofibrate, a well-known PPAR*α* agonist that is indicated for the treatment of hypertriglyceridemia, was demonstrated to improve hepatic steatosis in animal models [27]. Besides, activation of PPAR*α* by Wy-14643 was demonstrated to reverse fibrosis indirectly by reducing stimuli, including lipid accumulation and peroxides [28]. Consistently, in a small pilot trial with biopsy-confirmed NAFLD patients, the percentage of patients with abnormal serum ALT and AST levels (> 45 $IU \cdot L^{-1}$) decreased significantly after fenofibrate treatment. Besides, a control biopsy after fenofibrate treatment revealed a decrease in the grade of hepatocellular ballooning degeneration, but the grade of steatosis, lobular inflammation, fibrosis or NAS score did not change significantly [29]. Our study also provided evidence for the usage of PPAR*α* agonist in NAFLD/NASH treatment.

Silybum marianum, commonly known as “milk thistle”, is one of the oldest and thoroughly researched plants in the treatment of liver diseases, gallbladder diseases, diabetes and cancer. The extract of milk thistle is being used as a general medicinal herb from as early as 4th century, and is accepted as a favored medicine for hepatobiliary diseases since 16th century. Virtually, silymarin is one of the top 10 most popular natural products consumed by western society and the most commonly consumed botanical medicine reported in patients with chronic liver injuries [30]. A placebo-controlled, double-blind, phase III, randomized clinical trial also supported the benefit of silybin on NAFLD patients. Patients receiving silybin showed significant improvements in liver enzyme levels and liver histology [31]. In another clinical study, administration of silybin for 6 months, the proportion of NAFLD patients showed a statistically significant improvements in metabolic markers, oxidative stress, and endothelial dysfunction, compared with placebo consumption [32]. Although the benefits of silybin have been widely realized, the molecular mechanism and therapeutic target have not been delineated. The possible mechanism of its hepatoprotective effect often explained by its antioxidant, free radical screening, and anti-inflammation properties. As well known, accumulated lipid in hepatocyte is the leading cause of inflammation and oxidative stress. Indeed, in cultured steatotic hepatocytes, silybin incubation was previously demonstrated to counteract lipid excess [33, 34]. Thus, in the current study, we intended to explore the possible mechanism for the lipid lowering effect of silybin. We systemic analyzed the genes involved in de novo synthesis (FAS and ACC*α*), lipid uptake (L-FABP), and mitochondrial *β*-oxidation (CPT-1a, CPT-2, MCAD and SCD-1). Interestingly, silybin treatment significantly upregulated the hepatic expression of *L-fabp*, *Cpt-1a*, *Cpt-2*, *Mcad*, and *Scd-1*, and downregulated the hepatic expression of *Acca* and *Fas* in MCD diet-induced NAFLD mice. This is consistent with previous findings that silybin promoted lipid catabolism by increasing the expression of CPT-1a [33, 34]. Additional study also showed that silybin treatment enhanced the expression and activity of SCD-1 and the expression of L-FABP [35]. Interestingly, above genes are tightly controlled by PPAR*α*. More importantly, we have previously demonstrated that silybin could bind to and activate PPAR*α* [19]. In line with our conclusion, another recent study also indicated that silybin upregulated the expression and the activity of PPAR*α* [36]. Thus, we suspected that silybin attenuated lipid accumulation by activating this nuclear receptor. Supporting this, we provided evidence in the present study that co-administration of PPAR*α* antagonist, GW6471, abolished the beneficial effect of silybin on NAFLD, especially reduced lipid accumulation. Taken together, our results, as well as evidence from other studies, demonstrated that silybin attenuated lipid accumulation *via* activating PPAR*α*.

However, it should be kept in mind that silybin is a partial agonist for PPAR*α*. When used alone, silybin significantly activated PPAR*α* signal, which could be abolished by PPAR*α* antagonist GW6471. When co-administrated with typical PPAR*α* agonists, silybin may attenuate their regulatory function on PPAR*α* signal. In the present study, co-administration of silybin impaired the hepatoprotective effect of fenofibrate, a well-known PPAR*α* agonist. Nowadays, it is generally accepted that combination strategy is necessary to achieve a satisfying effect in NAFLD therapy [24, 37]. Both silybin and PPAR*α* agonist serve as promising candidates of choice for the treatment of NAFLD. These data suggest that it should be avoid to take silybin and fenofibrate, as well as other classical PPAR*α* agonists, at the same time.

Conclusion

This study revealed the essential role of PPAR*α* in the lipid lowering effect of silybin in MCD diet-induced NAFLD mice. Silybin was demonstrated as a partial agonist for PPAR*α*. When used alone, silybin attenuated lipid accumula-

tion in NAFLD mice *via* PPAR*α*, and this effect was abolished by PPAR*α* antagonist GW6471. However, when co-administrated with strong PPAR*α* agonist fenofibrate, silybin impaired the powerful hepatoprotective effect of fenofibrate. Thus, it should be avoided to simultaneously take silybin and classical PPAR*α* agonists, two promising candidates for NAFLD therapy.

Supplementary Materials

Table S1 Primer sequences in PCR analysis.

Fig. S1 Effects of silybin on liver functions in mice with MCS diet.

Fig. S2 PA significantly triggered inflammatory response.

Fig. S3 Effects of silybin on PPARα signal in mice with MCS diet.

References

[1] Younossi Z, Tacke F, Arrese M, *et al*. Global perspectives on nonalcoholic fatty liver disease and nonalcoholic steatohepatitis [J]. *Hepatology*, 2019, **69**(6): 2672-2682.

[2] Younossi Z, Anstee QM, Marietti M, *et al*. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention [J]. *Nat Rev Gastroenterol Hepatol*, 2018, **15**(1): 11-20.

[3] Leng YR, Zhang MH, Luo JG, *et al*. Pathogenesis of NASH and promising natural products [J]. *Chin J Nat Med*, 2021, **19**(1): 12-27.

[4] Wong RJ, Aguilar M, Cheung R, *et al*. Nonalcoholic steatohepatitis is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States [J]. *Gastroenterology*, 2015, **148**(3): 547-555.

[5] Yan T, Yan N, Wang P, *et al*. Herbal drug discovery for the treatment of nonalcoholic fatty liver disease [J]. *Acta Pharm Sin B*, 2020, **10**(1): 3-18.

[6] Chen T, Zhong FJ, Hong YM, *et al*. Effect of Trifolium pratense extract on methionine-choline-deficient diet-induced steatohepatitis in C57BL/6 mice [J]. *Chin J Nat Med*, 2014, **12**(3): 194-198.

[7] Ma LL, Yuan YY, Zhao M, *et al*. Mori Cortex extract ameliorates nonalcoholic fatty liver disease (NAFLD) and insulin resistance in high-fat-diet/streptozotocin-induced type 2 diabetes in rats [J]. *Chin J Nat Med*, 2018, **16**(6): 411-417.

[8] Graf T, Cech N, Polyak S, *et al*. A validated UHPLC-tandem mass spectrometry method for quantitative analysis of flavonolignans in milk thistle (*Silybum marianum*) extracts [J]. *J Pharm Biomed Anal*, 2016, **126**: 26-33.

[9] Drouet S, Tungmunnithum D, Lainé É, *et al*. Silybum marianum gene expression analysis and metabolite profiling of silymarin biosynthesis during *Milk thistle* ((L.) Gaertn.) fruit ripening [J]. *Int J Mol Sci*, 2020, **21**(13): 4730-4387.

[10] Xie Y, Hao H, Wang H, *et al*. Reversing effects of silybin on TAA-induced hepatic CYP3A dysfunction through PXR regulation [J]. *Chin J Nat Med*, 2013, **11**(6): 645-652.

[11] Marmouzi I, Bouyahya A, Ezzat SM, *et al*. The food plant *Silybum marianum* (L.) Gaertn: phytochemistry, ethnopharmacology and clinical evidence [J]. *J Ethnopharmacol*, 2021, **265**: 113303-113325.

[12] Sun R, Xu D, Wei Q, *et al*. Silybin ameliorates hepatic lipid accumulation and modulates global metabolism in an NAFLD mouse model [J]. *Biomed Pharmacother*, 2020, **123**: 109721-109730.

[13] Liu Y, Xu W, Zhai T, *et al*. Silibinin ameliorates hepatic lipid accumulation and oxidative stress in mice with non-alcoholic steatohepatitis by regulating CFLAR-JNK pathway [J]. *Acta Pharm Sin B*, 2019, **9**(4): 745-757.

[14] Cui CX, Deng JN, Yan L, *et al*. Silibinin Capsules improves high fat diet-induced nonalcoholic fatty liver disease in hamsters through modifying hepatic de novo lipogenesis and fatty acid oxidation [J]. *J Ethnopharmacol*, 2017, **208**: 24-35.

[15] van Diepen JA, Jansen PA, Ballak DB, *et al*. PPAR-alpha dependent regulation of vanin-1 mediates hepatic lipid metabolism [J]. *J Hepatol*, 2014, **61**(2): 366-372.

[16] Feng S, Wang H, Wang Y, *et al*. Apatinib induces 3-hydroxybutyric acid production in the liver of mice by peroxisome proliferator-activated receptor *α* activation to aid its antitumor effect [J]. *Cancer Sci*, 2019, **110**(10): 3328-3339.

[17] Ip E, Farrell GC, Robertson G, *et al*. Central role of PPARalpha-dependent hepatic lipid turnover in dietary steatohepatitis in mice [J]. *Hepatology*, 2003, **38**(1): 123-132.

[18] Montagner A, Polizzi A, Fouché E, *et al*. Liver PPAR*α* is crucial for whole-body fatty acid homeostasis and is protective against NAFLD [J]. *Gut*, 2016, **65**(7): 1202-1214.

[19] Wang H, Yan T, Xie Y, *et al*. Mechanism-based inhibitory and peroxisome proliferator-activated receptor *α*-dependent modulating effects of silybin on principal hepatic drug-metabolizing enzymes [J]. *Drug Metab Dispos*, 2015, **43**(4): 444-454.

[20] Zhou J, Cui S, He Q, *et al*. SUMOylation inhibitors synergize with FXR agonists in combating liver fibrosis [J]. *Nat Commun*, 2020, **11**(1): 240-255.

[21] Li X, Wang Y, Xing Y, *et al*. Changes of gut microbiota during silybin-mediated treatment of high-fat diet-induced non-alcoholic fatty liver disease in mice [J]. *Hepatol Res*, 2020, **50**(1): 5-14.

[22] Kleiner D, Brunt E, Van Natta M, *et al*. Design and validation of a histological scoring system for nonalcoholic fatty liver disease [J]. *Hepatology*, 2005, **41**(6): 1313-1321.

[23] Xie Y, Wang H, Cheng X, *et al*. Farnesoid X receptor activation promotes cell proliferation *via* PDK4-controlled metabolic reprogramming [J]. *Sci Rep*, 2016, **6**: 18751-18761.

[24] Zhou J, Huang N, Guo Y, *et al*. Combined obeticholic acid and apoptosis inhibitor treatment alleviates liver fibrosis [J]. *Acta Pharm Sin B*, 2019, **9**(3): 526-536.

[25] Yoon M. The role of PPARalpha in lipid metabolism and obesity: focusing on the effects of estrogen on PPARalpha actions [J]. *Pharmacol Res*, 2009, **60**(3): 151-159.

[26] Yi HW, Zhu XX, Huang XL, *et al*. Selenium-enriched Bifidobacterium longum protected alcohol and high fat diet induced hepatic injury in mice [J]. *Chin J Nat Med*, 2020, **18**(3): 169-177.

[27] Lefere S, Puengel T, Hundertmark J, *et al*. Differential effects of selective- and pan-PPAR agonists on experimental steatohepatitis and hepatic macrophages [J]. *J Hepatol*, 2020, **73**(4): 757-770.

[28] Ip E, Farrell G, Hall P, *et al*. Administration of the potent PPARalpha agonist, Wy-14, 643, reverses nutritional fibrosis and steatohepatitis in mice [J]. *Hepatology*, 2004, **39**(5): 1286-1296.

[29] Fernández-Miranda C, Pérez-Carreras M, Colina F, *et al*. A pilot trial of fenofibrate for the treatment of non-alcoholic fatty liver disease [J]. *Dig Liver Dis*, 2008, **40**(3): 200-205.

[30] Abenavoli L, Capasso R, Milic N, *et al*. Milk thistle in liver diseases: past, present, future [J]. *Phytother Res*, 2010, **24**(10): 1423-1432.

[31] Loguercio C, Andreone P, Brisc C, *et al*. Silybin combined with phosphatidylcholine and vitamin E in patients with nonalcoholic fatty liver disease: a randomized controlled trial [J]. *Free Radic Biol Med*, 2012, **52**(9): 1658-1665.

[32] Federico A, Dallio M, Masarone M, *et al*. Evaluation of the effect derived from silybin with Vitamin D and Vitamin E administration on clinical, metabolic, endothelial dysfunction, oxidative stress parameters, and serological worsening markers in nonalcoholic fatty liver disease patients [J]. *Oxid Med Cell Longevity*, 2019, **2019**: 8742075-8742086.
[33] Vecchione G, Grasselli E, Voci A, *et al*. Silybin counteracts lipid excess and oxidative stress in cultured steatotic hepatic cells [J]. *World J Gastroenterol*, 2016, **22**(26): 6016-6026.
[34] Grasselli E, Baldini F, Vecchione G, *et al*. Excess fructose and fatty acids trigger a model of non-alcoholic fatty liver disease progression *in vitro*: protective effect of the flavonoid silybin [J]. *Int J Mol Med*, 2019, **44**(2): 705-712.
[35] Salamone F, Galvano F, Cappello F, *et al*. Silibinin modulates lipid homeostasis and inhibits nuclear factor kappa B activation in experimental nonalcoholic steatohepatitis [J]. *Transl Res*, 2012, **159**(6): 477-486.
[36] Liu X, Xu Q, Long X, *et al*. Silibinin-induced autophagy mediated by PPAR*α*-sirt1-AMPK pathway participated in the regulation of type I collagen-enhanced migration in murine 3T3-L1 preadipocytes [J]. *Mol Cell Biochem*, 2019, **450**(1-2): 1-23.
[37] Dufour JF, Caussy C, Loomba R. Combination therapy for non-alcoholic steatohepatitis: rationale, opportunities and challenges [J]. *Gut*, 2020, **69**(10): 1877-1884.

Cite this article as: CUI Shuang, PAN Xiao-Jie, GE Chao-Liang, GUO Yi-Tong, ZHANG Peng-Fei, YAN Ting-Ting, ZHOU Ji-Yu, HE Qing-Xian, CHENG Long-Hao, WANG Guang-Ji, HAO Hai-Ping, WANG Hong. Silybin alleviates hepatic lipid accumulation in methionine-choline deficient diet-induced nonalcoholic fatty liver disease in mice *via* peroxisome proliferator-activated receptor *α* [J]. *Chin J Nat Med*, 2021, **19**(6): 401-411.

Dr. WANG Hong is the Associate professor and Tutor of doctoral degree candidates in Drug Metabolism and Pharmacokinetics of China Pharmaceutical University. He obtained his Ph.D. degree in China Pharmaceutical University in 2015. He then performed postdoctoral research at Postdoctoral Research Station of Traditional Chinese Medicine. Dr. WANG focuses on drug development and therapeutic strategy investigation for metabolic diseases based on metabolic regulation of nuclear receptors. For example, he demonstrated that enhanced FXR SUMOylation in activated hepatic stellate cells reduced the transcriptional activity of FXR, which gave explanation for the limited pharmacological activity of FXR agonist for fibrotic patients. Furthermore, he proposed the combined strategy of FXR agonist and SUMOylation inhibitor for the treatment of NASH and fibrosis, which significantly improves the pharmacological activity of FXR agonist. Dr. WANG also focuses on drug target identification & validation and pharmacological mechanism exploration for natural medicines. For example, he demonstrated that silybin served as a partial agonist of PPARα to regulate lipid metabolism and prevent NASH development. Dr. WANG has been in charge of several scientific research projects, including the National Natural Science Foundation of China and China Postdoctoral Science Foundation. He also participates in several significant scientific research projects, such as the Project for Major New Drug Innovation.