Biomedicine & Pharmacotherapy 100 (2018) 191–197

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Silybum marianum oil attenuates hepatic steatosis and oxidative stress in high fat diet-fed mice

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ARTICLE INFO

Keywords:
Silybum marianum oil
NAFLD
Hepatic steatosis
Oxidative stress

ABSTRACT

In the present study, the effects of *Silybum marianum* oil (SMO) on hepatic steatosis and oxidative stress were investigated during the development of nonalcoholic fatty liver disease (NAFLD) in high fat diet (HFD)-fed mice. The results showed that body weight, fat mass, and serum biochemical parameters such as triglyceride, free fatty acid, glucose and insulin were reduced by SMO treatment. Meanwhile, SMO decreased the histological injury of liver and the levels of hepatic triglyceride, cholesterol and free fatty acid in HFD-fed mice. SMO administration elevated the activities of superoxide dismutase (SOD) and catalase (CAT) and reduced the level of malondialdehyde (MDA) in the liver. Enzyme linked immunosorbent assay showed that SMO significantly decreased the levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in HFD mice. Furthermore, the mRNA levels of sterol regulatory element binding protein 1c (SREBP-1c), fatty acid synthase (FAS) and liver X receptor α (LXRα) were lower, but peroxisome proliferator-activated receptor α (PPARα) was higher in mice treated with SMO compared with the HFD group. The results indicated that SMO could play a certain protective role against HFD-induced NAFLD, and the protective effects might be associated with attenuating lipid accumulation, oxidative stress and inflammation, improving lipid metabolism.

1. Introduction

Obesity is a metabolic state associated with hepatic abnormalities and increased risk of chronic diseases such as type 2 diabetes, cardiovascular diseases, and so on. Obesity is described as a major risk factor for nonalcoholic fatty liver disease (NAFLD) [1]. NAFLD is characterized by abnormal lipid deposition in the liver without alcohol consumption and is the most common and prevalent liver disease worldwide [2]. NAFLD is a continuum of hepatic injuries, which progress from simple hepatic steatosis to nonalcoholic steatohepatitis, fibrosis, and cirrhosis and also increase the risk of metabolic disorders [3,4]. Because of the high prevalence and its subsequent risk, NAFLD has be regarded as health and social problems which should be well treated [5].

The pathogenesis of NAFLD is complex, and the underlying mechanisms remain largely unknown. Various genetic and environmental factors (e.g., lipid peroxidation, insulin resistance, oxidative stress, inflammation, mitochondrial dysfunction and cellular apoptosis) contribute to the occurrence of NAFLD [3–7]. Nowadays, there are no effective pharmacological treatments for NAFLD. The methods for treating NAFLD involve rational diet, exercise, and pharmaceutical therapy (e.g., metformin, statins and fibrates). However, these drugs have some adverse effects or contraindications, and no consensus exists on the most effective drug therapies [4,5,8]. Thus, the beneficial effects of dietary supplements on NAFLD are gaining increasing attention. Recently, the effects of natural bioactive substances, including antioxidants, lipid-lowering agents, anti-inflammatory compounds, and insulin sensitizers, have been evaluated in many studies [8].

Silybum marianum L. Gaernt in family Asteraceae is one of the important hepatoprotective plant. The main active substance in *S. marianum* is silymarin, which is composed of silybin, isosilybin, silydianin, and silychristine [9]. *S. marianum* oil (SMO), a by-product of silymarin production, is rich in essential fatty acids, phospholipids, sterols, and vitamin E [10]. Most of the beneficial effects of oils that contain essential fatty acids, vitamin E, squalene, and phenolic compounds have been shown to be due to their antioxidant activity and their capacity to prevent lipid oxidation [11]. Furthermore, it has been found that fish oil, olive oil, canola oil, rice bran oil and other natural products and

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https://doi.org/10.1016/j.biopha.2018.01.144
Received 31 October 2017; Received in revised form 8 January 2018; Accepted 28 January 2018

vegetable oil have the function of preventing or treating NAFLD [12–15]. Some studies have indicated that SMO has antioxidant effects *in vitro* and *in vivo*, and it can reduce plasma cholesterol and triglyceride levels, counteract the damaging effects of CCl_4-induced injury in hepatocytes [16–18]. We previously demonstrated that SMO had a protective effect in D-gal-induced aging mice. It could attenuate lipid peroxidation, modulate the expression of apoptosis-related factors, and alleviate mitochondrial damage [19]. The pathogenesis of NAFLD is closely related to obesity, oxidative stress, mitochondrial damage and apoptosis, so we hypothesized that SMO might improve NAFLD. However, the protective effects and underlying mechanisms of SMO on NAFLD have not been explored. The main objective of the present study was to evaluate the potential protective effects of SMO during the development of NAFLD in a mouse model of long-term HFD-induced obesity. We further investigated the potential mechanisms against NAFLD.

2. Materials and methods

2.1. Animal treatment

ICR mice (male, 2-month-old) were purchased and housed in an animal house at the Laboratory Animal Research Center of Jiangsu University, Zhenjiang, China. The license number of the mice was SCXK (SU) 2013-0011. During the entire experimental period, all the animals were allowed free access to diet and water. After acclimation for a week, the mice were randomized into 4 groups ($n = 10$/group). Control: mice were fed normal diet based on a commercial diet (Xietong Organism, Jiangsu,China) in conjunction with distilled water through intragastric administration for 8 weeks; HFD: mice were fed high fat diet (w/w, 70.5% normal diet + 10% lard + 10% yolk powder + 8% sucrose + 1.5% cholesterol) in conjunction with distilled water through intragastric administration for 8 weeks; SMO 5 mL/kg: mice were fed high fat diet for 8 weeks and intragastric administered with 5 mL/kg SMO every day; SMO 10 mL/kg: mice were fed high fat diet for 8 weeks and intragastric administered with 10 mL/kg SMO every day; SMO was extracted from *S. marianum* seed kernels by AY Mantianxue Food Manufacturing Co. LTD (Anyang, China). The chemical compositions of SMO were analyzed in our previous study [19]. All the experimental procedures were approved by the Laboratory Animal Management Committee of Jiangsu University and adhered to guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. At the end of the experiment, mice were fasted overnight, and then blood and liver samples were collected for biochemical and molecular biological determinations.

2.2. Liver morphological analysis

Small pieces of mice liver (5 × 5 mm) were fixed in Bouin solution, dehydrated in ethanol, cleared in toluene and embedded in paraffin. The liver sections were deparaffinized and processed routinely for hematoxylin-eosin (H&E) and then analyzed with a light microscopy (Olympus, Japan).

2.3. Lipid profiles in serum and liver

The serum were assayed for triglyceride (TG), total cholesterol (TC), high-density lipoproteins cholesterol (HDL-C), low-density lipoproteins cholesterol (LDL-C) levels using an Olympus AU2700 Clinical Chemistry Analyzer (Olympus Inc., Japan). The contents of free fatty acid (FFA) and TG, TC in liver were measured by commercial kits (Nanjing Jiancheng Institute of Biological Engineering, China) according the manufacturer's instructions.

2.4. Biochemical measurement

The levels of glucose in serum were assayed using an Olympus AU2700 Clinical Chemistry Analyzer (Olympus Inc., Japan). The levels of insulin (INS), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were measured using enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., China) according the manufacturer's instructions. Homeostasis model assessment of insulin resistance index (HOMA-IR) was calculated as HOMA-IR = serum glucose (mmol/L) × serum insulin (mIU/L)/22.5.

2.5. Oxidative stress evaluation

Superoxide dismutase (SOD) and catalase (CAT) activities and malondialdehyde (MDA) levels were measured using commercial assay kits (Nanjing Jiancheng Institute of Biological Engineering, China) according the manufacturer's instructions.

2.6. RNA extraction and real-time PCR

Total RNA was extracted from liver tissues using Trizol reagent (Takara, Japan) following the manufacturer's directions, and cDNA was synthesized using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan) according to the manufacturer's instructions. For RT-PCR, each reaction was prepared according to the manufacturer's instructions using Go Taq qPCR Master Mix kit (Promega, USA). The reaction conditions were as follows: initial denaturation at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 10 min. β-actin was used as internal reference during the whole procedure. The relative expression of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method [20]. The primer sequences used were shown in Table 1.

2.7. Statistical analysis

All the results were expressed as mean ± SD. Statistical analysis was performed using SPSS software package, version 17.0. The values were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. $p < 0.05$ was considered to indicate statistical significance.

Table 1
Primer sequences used for RT-PCR.

mRNA	Forward Primer (5'-3')	Reverse Primer (5'-3')
PPARα	GGCGTTTCCTGAGACCCT	ATGTTGGATGGATGTGGC
LXRα	CTGATTCTGCAACGGAGTTGT	GACGAAGCTCTGTCGGCTC
SREBP-1c	TCTGGAGACATCGCAAACAAG	TGGTAGACAACAGCCGCATC
FAS	TGCACAGAAGGGAAGGAGTACATG	ACAGCCAGGAGAATCGCAGTAGA
β-actin	GATCTGGCACCACACCTTC	ATCTTTTCACGGTTGGCCTT

Table 2
Effect of SMO on body weight, abdominal and epididymal fats in mice.

Groups	Initial body weight (g)	Final body weight (g)	Abdominal fat (g)	Epididymal fat (g)
Control	23.78 ± 0.86[a]	37.93 ± 2.27[a]	0.12 ± 0.05[a]	0.49 ± 0.12[a]
HFD	24.51 ± 0.83[a]	43.24 ± 2.13[b]	0.33 ± 0.08[b]	1.12 ± 0.28[b]
SMO 5 mL/kg	24.24 ± 1.19[a]	38.24 ± 2.19[a]	0.24 ± 0.04[c]	0.79 ± 0.15[c]
SMO 10 mL/kg	23.98 ± 0.96[a]	40.62 ± 2.52[ab]	0.26 ± 0.07[c]	0.95 ± 0.16 [c]

The data are expressed as the mean ± SD; Different letters indicate a significant difference ($P < 0.05$).

3. Results

3.1. Effects of SMO on body weight, abdominal and epididymal fats in HFD-fed mice

As shown in Table 2, the initial body weight of mice in all four groups were similar ($P > 0.05$). The final body weight of HFD mice was significantly higher than that of the control ($P < 0.05$). SMO treatment could significantly decrease the body weight at the end of the study ($P < 0.05$). In the HFD group, abdominal and epididymal fats in mice were significantly greater than that of the control ($P < 0.05$). However, SMO supplement in both of the intervention groups could markedly decrease abdominal and epididymal fats ($P < 0.05$).

3.2. Histopathological examination

Fig. 1 showed the photomicrograph of sections of the liver of all the experimental groups. There were no alterations in the control mice, it showed normal cellular architecture with distinct hepatocyte structure. However, disordered hepatic lobule structures were observed in the HFD group. It exhibited severe wide spread lipid vacuoles and ballooning degeneration inside the majority of liver cells. Treatment with SMO prevented the histopathological changes in liver tissue, and the morphology also got recovered to normal.

3.3. Effects of SMO on serum glucose, insulin and HOMA-IR levels

The mean changes in serum glucose and insulin for the different groups were shown in Fig. 2. The glucose and insulin levels of the HFD group were significantly increase compared with the control group ($P < 0.05$). After 8 weeks of treatment, the SMO group showed a significant reduction compared with the HFD group ($P < 0.05$). These findings indicated that the serum insulin level increased significantly in association with the elevated glucose in the HFD group and SMO had obvious glucose lowering effects. In addition, a high fat diet induced a significant increase of IR in the HFD group mice compared with the control group, as indicated by the high HOMA-IR. SMO administration could decrease the HOMA-IR level compared with the HFD group (Fig. 2).

3.4. Effects of SMO on lipid metabolism in HFD-fed mice

Lipid metabolism relevant parameters were detected in the experimental groups (Table 3). At the end of the 8th week, the serum TC, TG, HDL, LDL and FFA levels significantly increased in the HFD mice in comparison to the control group ($P < 0.05$). SMO could significantly decreased the levels of serum TG and FFA ($P < 0.05$). However, compared with the HFD group, TC, HDL and LDL levels in SMO groups did not show significantly difference among the three groups ($P > 0.05$). In liver, the levels of TG, TC and FFA in the HFD group

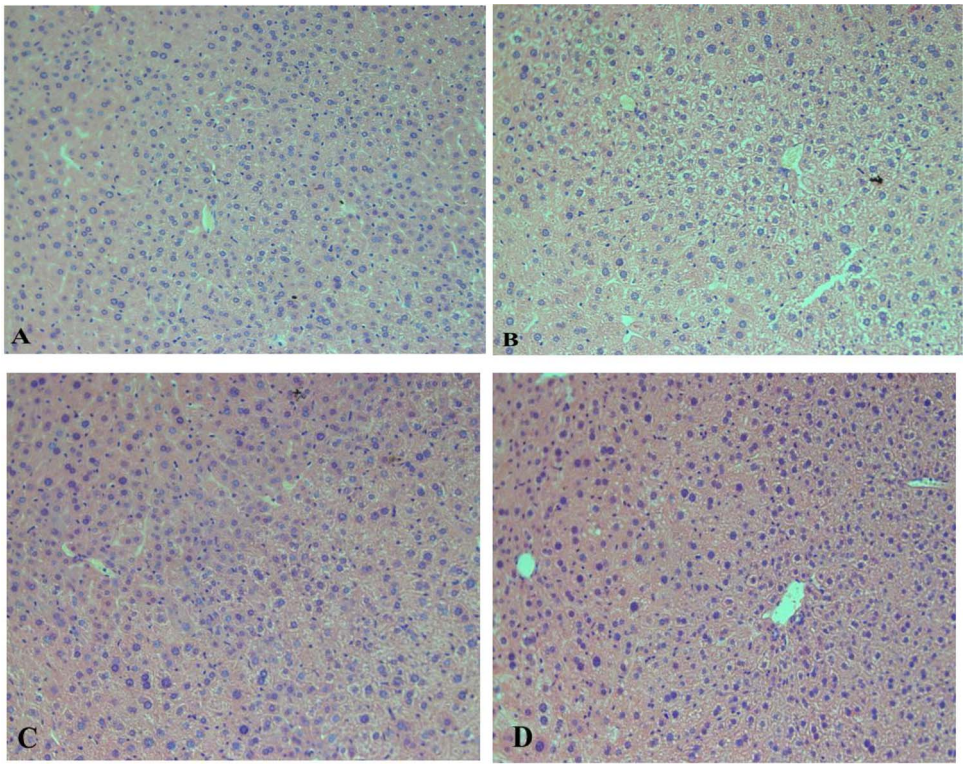

Fig. 1. Histopathological examination using H&E staining (200 magnification) A: Control; B: HFD; C: SMO 5 mL/kg; D: SMO 10 mL/kg.

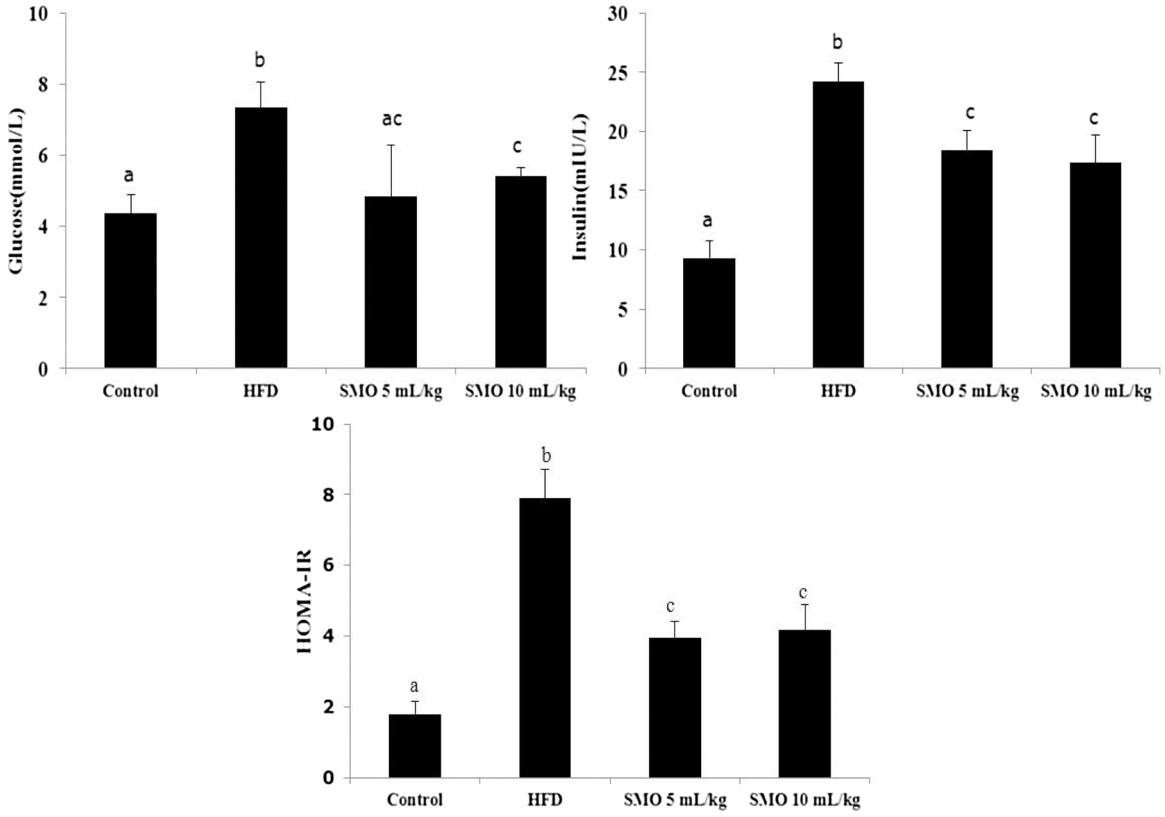

Fig. 2. Effects of SMO on serum glucose, insulin and HOMA-IR levels. The data are expressed as the mean ± SD; Different letters indicate a significant difference ($P < 0.05$).

were dramatically elevated compared with that of the control group ($P < 0.05$). SMO could markedly reduce hepatic TG, TC and FFA to a much lower level ($P < 0.05$).

3.5. Effects of SMO on TNF-α and IL-6 levels in HFD-fed mice

The results showed that HFD resulted in a significant increase in the levels of TNF-α and IL-6 in serum and liver ($P < 0.05$). The increase of TNF-α and IL-6 levels could be inhibited by SMO. The results suggested that SMO could attenuate inflammation induced by HFD feeding (Fig. 3).

3.6. Effects of SMO on oxidative stress in HFD-fed mice

Effect of SMO on oxidative stress status in HFD-fed mice was assessed (Table 4). HFD resulted in a significant decrease in SOD and CAT activities in livers, which was significantly inhibited by SMO ($P < 0.05$). Moreover, hepatic MDA level in the HFD group was significantly increased compared with that of the control group ($P < 0.05$). In contrast, SMO could markedly reduce hepatic MDA level to a normal level. The results suggested that SMO could attenuate oxidative stress induced by HFD feeding.

3.7. Effects of SMO on mRNA levels of genes related to hepatic lipid metabolism

The mRNA levels of hepatic SREBP-1c (sterol regulatory element binding protein 1c), FAS (fatty acid synthase) and LXRα (liver X receptor α) were significantly elevated compared with the control group ($P < 0.05$). While, SMO could dramatically reduce the mRNA levels of SREBP-1c, FAS and LXRα compared with those of the HFD group ($P < 0.05$). However, HFD caused a significant reduction in PPARα (peroxisome proliferator–activated receptor α) mRNA level compared with the control group ($P < 0.05$). Both of the two dose SMO presented increasing effect on the mRNA level of PPARα in liver compared to that of the HFD group. (Fig. 4)

4. Discussion

With the increasing incidence of obesity, NAFLD has become a

Table 3
Effects of SMO on lipid metabolism in mice.

		Control	HFD	SMO 5 mL/kg	SMO 10 mL/kg
Serum	TG(mmol/L)	1.56 ± 0.16^{a}	2.08 ± 0.26^{b}	1.32 ± 0.19^{ac}	1.15 ± 0.22^{c}
	TC(mmol/L)	3.31 ± 0.49^{a}	5.26 ± 0.05^{b}	5.23 ± 0.46^{b}	4.74 ± 0.46^{b}
	HDL(mmol/L)	3.20 ± 0.39^{a}	4.13 ± 0.08^{b}	4.51 ± 0.31 ^{b}	4.53 ± 0.25 ^{b}
	LDL(mmol/L)	0.25 ± 0.05^{a}	0.72 ± 0.06^{b}	0.72 ± 0.14^{b}	0.59 ± 0.16^{b}
	FFA (mmol/L)	0.47 ± 0.05^{a}	1.10 ± 0.27^{b}	0.40 ± 0.07 ^{a}	0.43 ± 0.09^{a}
Liver	TC(μmol/g prot)	40.02 ± 10.04^{a}	47.32 ± 10.47^{b}	39.06 ± 3.26^{a}	35.11 ± 1.92^{a}
	TG(μmol/g prot)	77.14 ± 19.50^{a}	95.13 ± 17.91^{b}	44.23 ± 10.01^{c}	57.15 ± 10.62^{c}
	FFA (μmol/g prot)	74.84 ± 10.49 ^{a}	176.95 ± 10.23 ^{b}	96.50 ± 11.15 ^{c}	100.37 ± 9.30 ^{c}

The data are expressed as the mean ± SD; Different letters indicate a significant difference ($P < 0.05$).

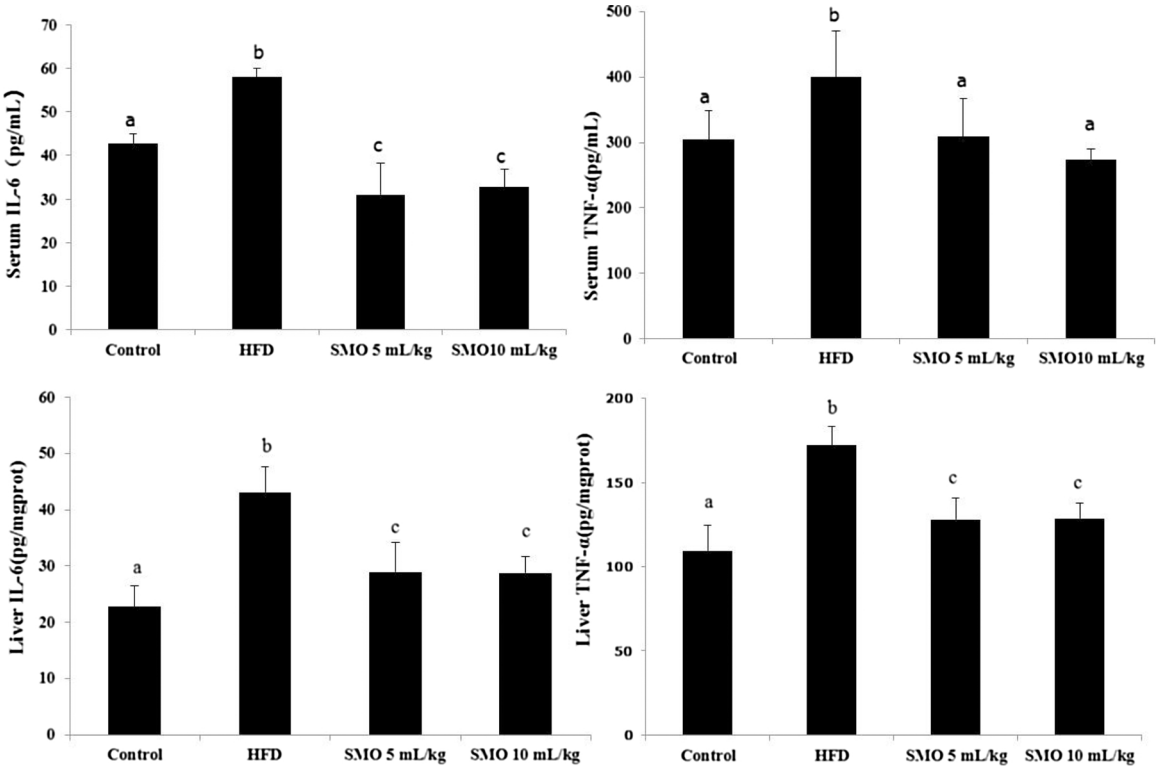

Fig. 3. Effects of SMO on TNF-α and IL-6 levels in serum and liver. The data are expressed as the mean ± SD; Different letters indicate a significant difference ($P < 0.05$).

Table 4
Effects of SMO on oxidative stress in mice.

Groups	CAT (U/mg prot)	SOD (U/mg prot)	MDA (nmol/mg prot)
Control	13.57 ± 1.45[a]	407.23 ± 58.12[a]	1.07 ± 0.21[a]
HFD	5.49 ± 1.87[b]	262.11 ± 11.68[b]	2.23 ± 0.44[b]
SMO 5 mL/kg	5.12 ± 1.61[b]	396.82 ± 23.52[ac]	1.30 ± 0.19[a]
SMO 10 mL/kg	9.16 ± 2.04[c]	348.61 ± 54.33[c]	1.32 ± 0.23[a]

The data are expressed as the mean ± SD; Different letters indicate a significant difference ($P < 0.05$).

major public health issue. In the last decades, natural substances have attracted much attention in the exploration of novel treatments for NAFLD. Mice fed a high fat diet can develop NAFLD, and this rodent animal model has been widely used [21]. The present study aimed to evaluate the protective effect of *S. marianum* oil (SMO) on long-term HFD-induced NAFLD. The dose of SMO we used was referred to the previous study [19]. We found that administration of SMO for 8 weeks was effective in regulating dyslipidemia and attenuating fat accumulation in liver, which was probably based on the suppression of oxidative stress and regulation of lipid metabolism related factors.

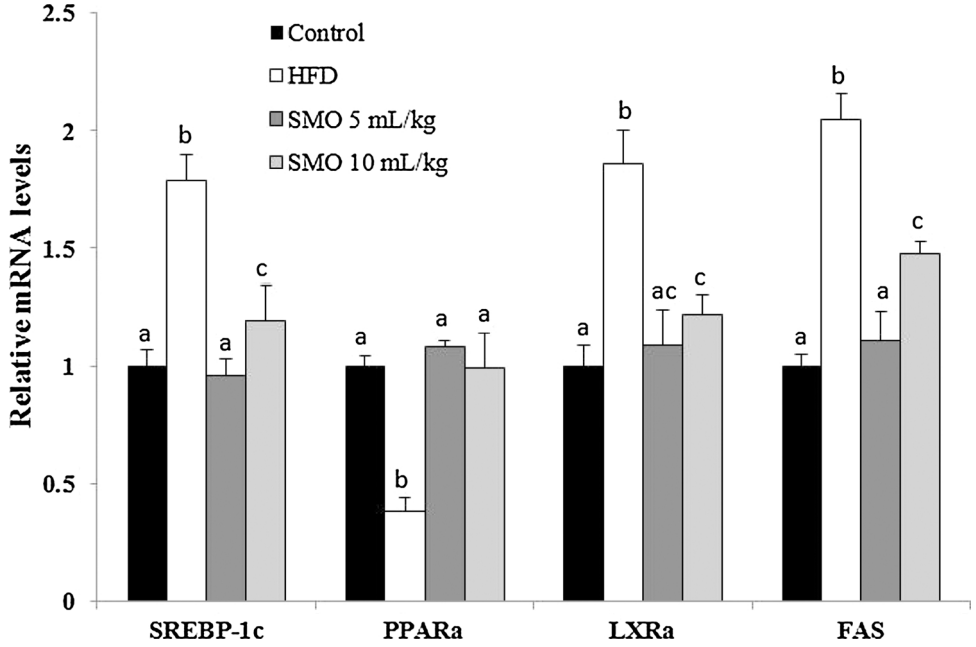

Fig. 4. Effects of SMO on mRNA levels of SREBP-1c, PPARα, LXRα and FAS. The data are expressed as the mean ± SD; Different letters indicate a significant difference ($P < 0.05$).

As known about NAFLD, insulin resistance (IR) and lipid dysregulation are the "first hit" [6]. Excessive fat contents in diet directly increase serum lipid level and hepatic lipid synthesis [22]. In the present study, HFD feeding caused the increasing of body weight and fat mass (Table 2). On the other hand, the serum TC, TG, HDL, LDL and FFA levels significantly increased in the HFD mice, whereas serum TG and FFA were significantly lower in SMO group compared with the HFD group (Table 3). Accumulation of TG is the characteristic of the initial stage of NAFLD, according to the pathological examination, some hepatic lipid deposition and enlarged hepatocytes were observed in the HFD mice. Whereas, SMO is beneficial for preventing the histopathological changes in liver tissue (Fig. 1). Meanwhile, dramatic reductions in hepatic TG, TC and FFA levels were also observed in both of the two SMO groups. Studies have shown that hepatic steatosis may induce IR [3,4]. In the present study, feeding the mice with HFD resulted in dramatic increases in the levels of serum glucose, insulin and HOMA-IR. Whereas, SMO supplement could markedly reverse these changes (Fig. 2). Based on these findings, we believed that dietary SMO could significantly ameliorate liver injury and reduce IR and lipid profiles. This is consistent with the previous reports [16,18].

Oxidative stress and the inflammatory response have emerged as an important mechanism involved in the development and progression of NAFLD [23]. Lipid oxidation in the liver is a main source of ROS. Following the accumulation of hepatic TG, excess fatty acid oxidation increases production of ROS resulting in hepatic oxidative stress [24]. SOD, GSH-Px, and CAT are the most important antioxidant enzymes that inhibit free radical formation and these enzymes also act as the primary defence system against ROS during oxidative stress. MDA is used as an indicator of oxidative damage under conditions of oxidative stress [25]. In the present study, the activities of SOD and CAT were found to be significantly lower and the levels of MDA were significantly higher in the HFD mice compared with the control group (Table 4). However, SMO could diminish oxidative stress through decreasing MDA levels and elevating antioxidant enzyme activities (SOD and CAT) in HFD-fed mice. This could be associated with its potential antioxidant properties reported previously [16,17,19]. TNF-α and IL-6 are the most well-known mediators of the early inflammatory response. They can alter adipose tissue function, influence adipogenesis, and are involved in the metabolic complications of obesity with NAFLD [26,27]. In this study, the levels of TNF-α and IL-6 in serum and liver were significantly higher in the HFD group compared with the control group. Whereas treatment with SMO significantly reduced the levels of TNF-α and IL-6 compared with the HFD group (Fig. 3). These results indicated that the administration of SMO was effective in reducing inflammatory response.

It is generally known that NAFLD is mainly caused by abnormal lipid metabolism from high-energy diets such as high fat diet. Excessive fat contents in diet directly increase hepatic lipid synthesis. Some studies showed that the LXR-SREBP-1c axis plays a central role in the up-regulation of genes involved in de novo lipogenesis in response to dietary fat. Because LXRs are important regulators of lipid metabolism, it stimulate new lipogenesis, positively inducing SREBP-1c, which is a transcription factor responsible for activating genes of the lipogenic enzymes ACC and FAS [28,29]. Whereas PPARα is abundantly expressed in liver and it can promote fatty acid oxidation, lipid transport and gluconeogenesis. Treatment with PPARα agonists has been demonstrated to protect against dietary-induced NAFLD in several different animal models [30–32]. In the present study, we observed that the mRNA levels of LXRα, SREBP-1c and FAS significantly increased and the level of PPARα significantly decreased in the HFD mice. Whereas SMO could decrease the mRNA levels of LXRα, SREBP-1c and FAS, along with increase the mRNA levels of PPARα compared with the HFD group (Fig. 3). This meant that hepatic fatty acid synthesis and fatty acid oxidation were significantly improved in SMO groups. The results indicated that SMO prevented hepatic lipid accumulation by regulating lipid metabolism in long-term HFD-fed mice. Of course, further researches *in vivo* are needed.

In conclusion, the results of this study indicate that SMO has a protective effect in the obese mice with long-term HFD-induced NAFLD. SMO can reduce serum and hepatic lipid levels, inhibit the oxidative stress and inflammation in liver. Moreover, SMO can also regulate the hepatic lipid metabolism with the decrease of the mRNA levels of LXRα, SREBP-1c and FAS and the increase of the mRNA levels of PPARα. Based on these findings, SMO could be considered as a potent dietary supplement for preventing NAFLD-related metabolic diseases. However, further research should be performed to confirm the effect of SMO on insulin sensitivity and the expression of lipid metabolism related proteins, etc.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This research was supported by grants from University natural science foundation of Jiangsu Province (16KJB550001), Postdoctoral research funding project of Jiangsu Province (1601058A), and key research and development plan of Zhenjiang city (NY2016020). We express our gratitude to the Laboratory Animal Research Center of Jiangsu University. We also thank our colleagues at the School of Food and Biological Engineering who provided assistance with this study.

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