Thesis for the Degree of Master of Science

Effects of Schizandrin A supplementation on plasma lipid profiles, hepatic lipid accumulation and antioxidant capacity in mice fed a high-fat and high-cholesterol diet

By

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by

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정미지

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요약

Schizandrin 물질은 *Schisandra chinensis* 의 lignan 성분으로써 Schizandrin (SCH) A, B 그리고 C 로 나뉜다. 이 중 SCH B 의 간 보호 효능이 이미 여러 연구들을 통해 알려져 있고 SCH C 는 항신경염증 및 알츠하이머 치료에 효과가 있음이 밝혀져 있다. 최근, SCH A 가 면역 작용을 향상시킨다는 결과가 보고되었으나 SCH A가 비알콜성 지방간에 미치는 영향에 대한 연구는 부족한 실정이다.

따라서 본 연구에서는 고지방·고콜레스테롤 식이를 급여한 마우스에서 SCH A 보충이 혈장과 간 조직 지질 축적 및 항산화 활성에 미치는 영향에 대해 밝히고자 하였다. 이를 위해 C57BL/6J 마우스를 1주간 pellet 형태의 lab. chow 식이를 제공하여 사육환경에 적응시킨 후, 난괴법에 의해 고지방·고콜레스테롤 식이를 급여한 대조군과 고지방·고콜레스테롤 식이에 SCH A 실험물질을 보충한 SCH A 군으로 나누어 실험물질을 15주 동안 급여하였다. 고지방·고콜레스테롤 식이 급여 마우스에서 SCH A 보충이 체중, 식이 섭취량, 식이 효율 및 장기(신장, 간) 와 다양한 부위별 백색지방조직(부고환 백색지방조직, 신주위 백색지방조직, 후복강 백색지방조직, 장간막 백색지방조직 및 피하의 백색지방조직) 무게에 미치는 유의적인 영향은 없었다.

혈장의 총 콜레스테롤과 HDL-콜레스테롤 수치 역시 군간 유의적인 차이가 없었다. 하지만 SCH A를 보충한 식이군에서 혈장 유리 지방산 농도가 유의적으로 감소하였으며 혈장 중성지질 농도도 대조군에 비해 감소하는 경향을 보였다. 또한 SCH A 보충이 간조직의 유리지방산, 중성지질 및 콜레스테롤 함량을 유의적으로 감소시킴을 확인할 수 있었다. 흥미롭게도 간조직에서 지질대사를 조절하는 효소들 중, 지방산과 중성지질 합성에 관여하는 fatty acid synthase와 phosphatidate phosphatase 활성도가 대조군에 비해 SCH A 보충군에서 유의하게 감소하는 반면, 지방산 산화에 관여하는 유전자 PPARα 와 CPT1a 의 발현이 현저히 증가했으며 분변의 유리 지방산 및 중성지질 배설이 유의적으로 증가하였다. 또한 CYP7A1 와 ABCA1 의 발현이 SCH A 보충에 의해 유의적으로 증가되었으며 이로 인해 간의 콜레스테롤로부터 답즙산 생합성이 촉진되고 간 조직의 콜레스테롤이 외부로 배출됨으로써 콜레스테롤의 농도가 유의적으로 저하되는 것으로 추정되었다. 아울러 SCH A 보충은 간조직의 항산화 효소인 superoxide dismutase, catalase 그리고 glutathione peroxidase 의 활성도를 증가시킴과 동시에 간조직의 지질과산화물 함량을 유의적으로 감소시키는 것으로 확인되었으며 SCH A 보충이 항산화 효소 활성 증가를 통해 간조직의 산화적 스트레스를 감소시키는 것으로 추정되었다.

이러한 결과를 종합해 보면, SCH A 보충이 간조직의 지질 대사 효소 활성도와 유전자 발현 조절 및 분변 지질 배설 증가를 통해 간조직의 지질 축적을 억제하고 항산화 효소 활성화를 통해 산화적 스트레스를 완화시킴으로써 고지방·고콜레스테롤 식이에 의해 유도된 비알콜성지방간을 완화시킬 수 있을 것으로 판단된다.

CONTENTS

Ⅰ. INTRODUCTION

1-1. Purpose of study

Bad eating habits, such as unbalanced energy intake relative to its expenditure, are closely related to disease outbreaks - including dyslipidemia, hyperlipidemia, non-alcoholic fatty liver disease (NAFLD), atherosclerosis and cardiovascular disease (CVD) [1, 2, 3]. Especially, high-fat and high-cholesterol diet have a detrimental effect on hepatic lipid accumulation [4, 5]. Because dietary fat and cholesterol are important factors that can influence hepatic lipid metabolism, they may exert large effects on the pathogenesis of liver disease such as hepatic steatosis [6, 7]. Hepatic steatosis is considered as the first step in the progression of NAFLD [7]. Approximately 20% to 30% of hepatic steatosis patients have histologic signs of fibrosis and necroinflammation. These symptoms indicate that higher risk of developing nonalcoholic steatohepatitis (NASH) and progressing to cirrhosis and terminal liver failure. [8-12]. Accordingly, NAFLD comprises a wide spectrum of disorders ranging from simple steatosis to a more advanced severe liver damage such as steatohepatitis, advanced fibrosis, and cirrhosis. So, alleviating hepatic steatosis is a promising approach to prevent the progression of NAFLD.

NAFLD is the most common liver disorder in western industrialized countries, affecting 20-40% of general population [13]. Also, 15-30% of general adult population suffers from NAFLD in Asia [14]. A large population-based survey in Korea, Japan and China indicates that the prevalence of NAFLD is now 12% to 24% in population subgroups [13]. Additionally, the longitudinal studies from Japan and China indicate that the prevalence of NAFLD has been increasing [13]. Over a 12-year period, the prevalence of NAFLD in Japan and China have more than doubled from ~13% in 1988 to ~30% in 2004 [13]. There is a growing concern about the rising development of NAFLD in clinical hepatology. So, more research is necessary to develop novel treatment strategies for NAFLD.

Nowadays, as interest in health is heightened, interest in therapeutic agents using natural substances is also increasing. Examples include Coenzyme Q10, phytochemicals and herbal medicine [15-17]. *Schisandra chinensis* is a traditional Chinese herb and it has long been used as a medicinal material in Asia [18]. So far, evidence supports its efficacy in protection against stress and in reduction of blood pressure, inflammation, oxidative stress and cancer. [4, 18-20]. Moreover, several studies have reported beneficial influences of each of components extracted from *Schisandra chinensis* [4]. Schizandrin (SCH) is the major lignan isolated from *Schisandra chinensis* [21, 22]. SCH is classified into SCH A, B and C (Figure 1). Among three substances, SCH B is known to have anti-cancer activity and hepatoprotective effect through removing reactive oxygen species [23, 24]. SCH C has been used as a natural anti-neuroinflammatory agent. Also, it ameliorates learning and memory deficits by $A\beta_{1-42}$-induced oxidative stress [25-27]. Recent studies have reported protective effects of SCH A on immune function and D-galactosamin (D-GalN)-induced acute liver failure [20]. However, the effect of SCH A on high-fat and high-cholesterol diet-induced NAFLD has not been investigated.

In a previous study, we investigated the effects of SCH A on high-fat diet (20%, w/w)-induced obesity in C57BL/6J mice. It ameliorated adiposity and hepatic steatosis. The aim of this study was to evaluate the effects of SCH A supplementation (0.05%, w/w) on plasma and hepatic lipid dysregulation in mice fed a high-fat and high-cholesterol (16% fat, 1.25% cholesterol, w/w) diet and to provide mechanistic insights into its anti-metabolic effects.

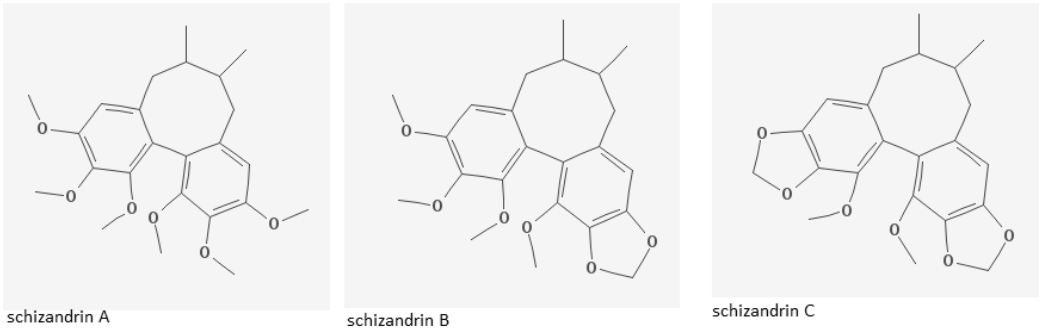

Figure 1. Chemical structure of Schizandrin A, Schizandrin B and Schizandrin C.

1-2. Background

1-2-1. Pathogenesis of nonalcoholic fatty liver disease

Excessive consumption of dietary cholesterol and fat can stimulate *de novo* fatty acid synthesis, which can cause NAFLD [28]. Especially, consumption of saturated fat-rich diet is reported to be an important risk factor of hepatic lipid accumulation [29].

On histology, excessive hepatic fat accumulation exceeding 5% of the liver weight is defined as NAFLD [30]. NAFLD describes a range of conditions caused by fat deposition within liver cell. Liver fat content reflects the equilibrium between several metabolic pathways involved in triglyceride synthesis and disposal, such as lipolysis in adipose tissue and *de novo* lipogenesis, triglyceride esterification, fatty acid oxidation in hepatic tissue. In particular, it has been demonstrated that hepatic *de novo* lipogenesis plays a significant role in NAFLD pathogenesis [31]. Because the hallmark in the pathogenesis of NAFLD is the accumulation of the triglycerides derived from the esterification of FFA and glycerol in hepatocytes, the excessive accumulation of triglyceride in hepatocytes results from imbalance of supply, consumption and disposal of triglyceride [32, 33].

The “two-hits” hypothesis in relation to the pathogenesis of NAFLD is widely accepted; the first hit includes triglyceride accumulation in the hepatocytes (steatosis), and the second hit causes inflammation and fibrosis though effects of cytokines or oxidative stress [34]. This second hit may include genetic susceptibility, dietary factors, or environmental stressors. However, the precise molecular signals that trigger this change have not yet been identified [6].

1-2-2. Lipid-regulating enzyme activity and gene expression

The regulation of triglyceride synthesis and storage is crucial in human health, because both excess and defective fat storage are related to lipid-associated disorders such as obesity, insulin resistance, diabetes, hepatic steatosis, cardiovascular disease, and cancer [35]. Especially, triglyceride may provide a source of fatty acids and diacylglycerol for membrane biosynthesis [36]. Phosphatidate phosphohydrolase (PAP) plays a crucial role in lipid homeostasis by controlling the relative proportions of its substrate phosphatidate and its product diaylglycerol [35]. The enzyme PAP catalyzes the dephosphorylation of phosphatidic acid to form diacylglycerol and P_i, which is the penultimate step in triglyceride synthesis [36, 37].

De novo fatty acid synthesis implies a complex series of reactions starting in the mitochondrial matrix and continuing in the cytosol of hepatocytes [31]. The fatty acid synthase (FAS) catalyzes the terminal steps in *de novo* synthesis of long-chain saturated fatty acids in the presence of NADPH [38]. In other words, acetyl-CoA and malonyl-CoA are catalyzed by FAS, which eventually leads to palmitic acid (16:0), the main product of *de novo* fatty acid synthesis [31].

Proliferator-activated receptor (*PPAR)a* is highly expressed in liver and functions as a lipid sensor. *PPARa* recognizes and responds to the influx of fatty acids by stimulating the transcription of specific genes [39, 40]. In addition, some of the key enzymes of fatty acid oxidation systems in liver, including carnitine palmitatoyltranferase (CPT)1, are regulated by *PPARa* [40]. Numerous studies have demonstrated that *PPARa* is crucial role in stimulation of hepatic fatty acid oxidation and up-regulates the expression of *CPT1a* [41]. CPT1 catalyzes the rate-limiting step of β-oxidation by translocating fatty acids across the mitochondrial membranes [42]. That is, mitochondrial β-oxidation is regulated by CPT1 and this process provides energy to cellular processes [40]. *De novo* lipogenesis-derived FFA and *PPARa* are important stimulators of CPT1 responsible for the entry of FFA into mitochondria [33]. And, CPT1 enzymes are encoded by the gene *CPT1a* in mammal [43].

Bile acids are synthesized from cholesterol in the liver. Entero-hepatic circulation of bile acids plays an important role in lipid absorption and secretion, and controlling of cholesterol homeostasis [44]. Most of all, cholesterol degradation to bile acids represents 50% of total elimination of cholesterol from the body each day in animal model [45]. The conversion of cholesterol to bile acids in the liver is initiated by cholesterol 7α -hydroxylase, the rate-limiting enzyme in bile acid biosynthesis pathway. Transcription from the *CYP7A1*, which encodes cholesterol 7α-hydroxylase, is regulated by hormones and dietary factors [46].

ATP-binding cassette transporter A1 (ABCA1) is essential for production of apolipoprotein AI (apo AI), which is the major protein component of HDL particles in plasma [47]. ABCA1 mediates the secretion of cellular free cholesterol to an extracelluar acceptor, apo AI. So, ABCA1 is a rate-limiting factor for HDL biogenesis, and this enzyme is encoded by the *ABCA1* gene [48, 49].

1-2-3. Activities of antioxidant enzymes

Reactive oxygen species, such as superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$), consist of radical and non-radical oxygen species formed by the partial reduction of oxygen [50]. Reactive oxygen species-mediated oxidative stress provokes oxidative damage, resulting in cell injury and death. Moreover, oxidative stress has been implicated in carcinogenesis, neurodegeneration, atherosclerosis, diabetes and aging [50].

About 2 - 4% of intracellular oxygen is converted into reactive oxygen species which could be detected in both the mitochondrial matrix and cytosol [51]. The surplus of FFA within the liver is playing an important role in reactive oxygen species generation as a result of electron leakage during mitochondrial β-oxidation in energy production (ATP) [51]. In the cytosol, reactive oxygen species enhances lipid peroxidation products such as malondialdehyde (MDA) [33]. Lipid peroxidation occurs because of the high content of unsaturated fatty acid [52]. MDA is known to form from various lipids upon oxidation as one of secondary oxidation products. As an indicator of lipid peroxidation, MDA is made up by reactive oxygen species during tissue damage and used in the measurement of oxidative stress [53]. MDA is measured using the thiobarbituric acid reactive substances (TBARS) assay [54, 55].

The defense mechanism against reactive oxygen species, mediators of liver damage, includes antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) [56]. These antioxidant enzymes are considered to be a primary defense that prevents biological macromolecule from oxidative damage. Since these enzymes are known to scavenge reactive oxygen species, any change in enzyme activities should provide indirect information about reactive oxygen species production [57]. SOD is the first line of defense in scavenging the superoxide radical ($O_2^{\cdot -}$) and catalyses the disproportionation of superoxide radical ($O_2^{\cdot -}$) into hydrogen peroxide (H_2O_2) and oxygen (O_2) [58]. However, hydrogen peroxide (H_2O_2) is a major contributor to reactive oxygen species-mediated oxidative stress [12]. Because transition metal ions such as iron and copper ions

catalyze the reaction between superoxide radical (O_2·−) and hydrogen peroxide (H_2O_2), generating hydroxyl radical (HO·) (Haber-Weiss reaction), the enzyme CAT and GPX detoxify H_2O_2 to oxygen (O_2) and water (H_2O) [57]. Therefore, these antioxidant enzymes play a crucial role in protecting macromolecule from oxidative stress (Figure 2).

Figure 2. Overview of activities of antioxidant enzymes
SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase.

Ⅱ. METERIAL AND METHOD

2-1. Experimental mouse model and diets

C57BL/6J mice (4 weeks, male, n=19) were purchased from Japan SLC Inc. through the Central Lab. Animal Inc. (Seoul. Republic of Korea). All mice were housed in cage, kept at temperature 24±2 ℃ and humidity 50±5% under 12h light-dark cycle, and fed a standard chow diet (Zeigler Rodent NIH-31, Central Lab. Animal Inc.) for 1 week after delivery. All mice were randomly divided into two groups according to their weight and fed a high-fat and high-cholesterol diet (D12336, Research Diets Inc., USA) with or without powdered schizandrin A (0.05%, w/w, Sigma Chemical company, 61281-38-7) for 15 weeks. The number of mice per group was 10 mice for CON group (high-fat and high-cholesterol diet) and 9 mice for SCH A group (high-fat and high-cholesterol diet with Schizandrin A supplementation). Body weight was measured once a week and feed was given 4g daily (Figure 3). All procedures were approved by the animal ethics committee of Pukyong National University (Approval No. 2016-12)

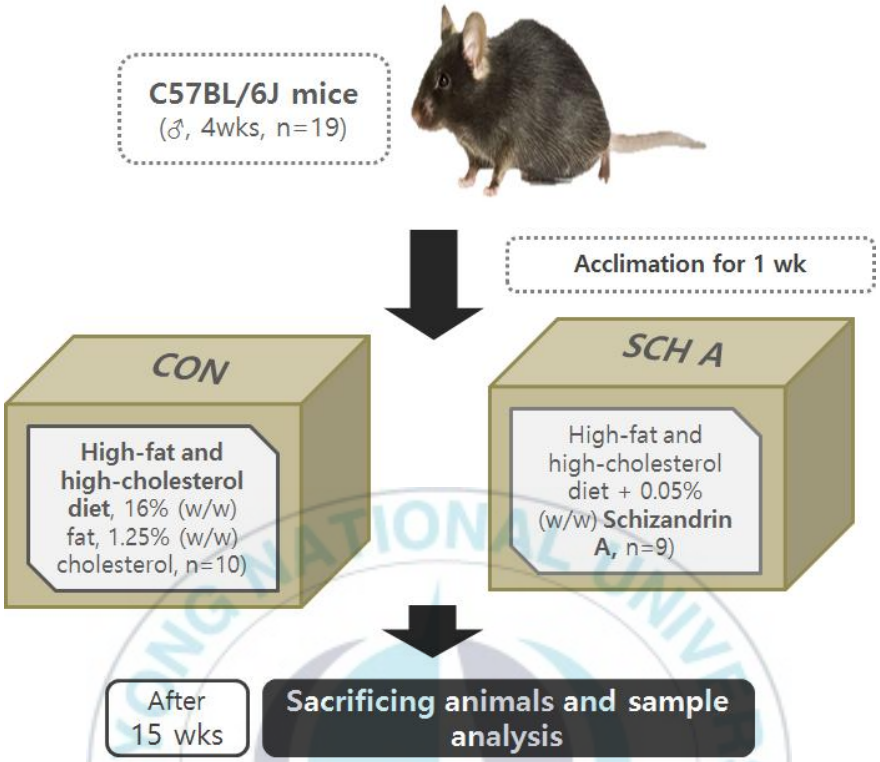

Figure 3. Experimental mouse model and diets design

Four weeks old male C57BL/6J mice were purchased from Japan SLC Inc. through the Central Lab. Animal Inc. (Seoul. Republic of Korea). After 1 week for acclimation, mice were randomly assigned to 2 groups. C57BL/6J mice were fed a high-fat and high-cholesterol diet (D12336, Research Diets Inc., USA) with or without powdered schizandrin A (0.05%, w/w, Sigma Chemical company) for 15 weeks. CON, high-fat and high-cholesterol diet; SCH A, high-fat and high-cholesterol diet with schizandrin A supplementation.

2-2. Blood analysis

Plasma total-cholesterol, triglyceride and HDL-cholesterol were measured using a commercial kit (Asan Pharm, Seoul, Korea). Also, the concentration of plasma fatty acid was measured using a commercial kit from Wako (Tokyo, Japan). HTR and AI were used the following equations : HTR, (HDL-cholesterol / total-cholesterol)*100 (%), AI, (HDL cholesterol - total cholesterol) / HDL-cholesterol.

2-3. Analysis of hepatic and fecal lipids

Hepatic lipids and feces were extracted a method of Folch et al [27], with a slight modification.

The liver tissue is homogenized by using homogenizer (Daihan, Seoul, Korea) and then filter it. The extracted lipids are dried with nitrogen gas (60 ℃). They were then dissolved in FM solution (methanol and chloroform, 2:1, v/v) and again dried with nitrogen gas 60 ℃. Then, the sample was dissolved in ethanol, and hepatic total-cholesterol and triglyceride contents were measured using commercial kit (Asan Pharm, Seoul, Korea). Also, the concentration of hepatic fatty acid was measured using a commercial kit from Wako (Tokyo, Japan).

The fecal lipid was dried for 1 week and then ground with a mortar. After then, FM solution put in the powdered fecal lipid, and left at 22 ℃ for 24 hours. And the sample was centrifuged at 3000rpm, 4 ℃ for 10 min. After the supernatant is volatilized by using nitrogen gas (50 ℃), FM solution was added, and mixing with vortex was performed. Finally, samples were subdivided into 200 ul aliquots, and volatilized by using nitrogen gas (50℃) and dissolved in ethanol.

2-4. Hepatic enzyme activity analysis

The hepatic mitochodrial and cytosol samples for the measurement were prepared according to the method of Hulcher and Oleson [59] with slight modification. Liver (0.5g) was homogenized in the pH 7.0 buffer (0.1 M Triehanolamine + 0.02 M EDTA + 2mM dithiothreitol). And then, it was centrifuged at 3,000 rpm for 15 min and separated only supernatant from sample. Supernatant was centrifuged at 13,000 rpm 15 min and added buffer

(1ml). After it also was centrifuged at 13,000 for rpm 20 min, removed only supernatant. And then, sample of removed supernatant was added buffer (1ml) and dissolved it.

Phosphatidate phosphohydrolase (PAP) activity was measured according to the method of Walton & Possmayer [60]. And carnitine palmitoyl-CoA transferase1 (CPT1) activity was measured according to the method of Markwell *et al* [60]. Their unit are expressed as nmol/min/mg protein. Fatty acid synthase (FAS) activity was measured using spectrophotometric assay according to the method of Nepokroeff et al. [62] through monitoring the malonyl-CoA-dependent oxidation of NADPH at 340 nm, in which the activity represents the oxidized NADPH nmol/min per mg of protein. Fatty acid β-oxidation was measured the reduction of NAD to NADH in the presence of palmitoyl-CoA by using spectrophotometric assay [63].

2-5. Hepatic antioxidant enzyme activity and hydrogen peroxide content

The superoxide dismutase (SOD) activity was estimated according to the method of Marklund, which is based on the color change due to the auto-oxidation of pyrogallol [64]. The catalase (CAT) activity was measured using Aebi's method, in which the decrease of hydrogen peroxide was monitored spectrophotometrically at 240 nm for 5 min using a spectrophotometer [65]. The glutathion peroxidase (GPX) activity was measured using a spectrophotometric assay, as described previously by Paglia and Valentine with a slight modification [66]. The hepatic thiobarbituric acid-reactive substances (TBARS) concentration, as a marker of lipid peroxide production, was measured spectrophotometrically by the method of Ohkawa et al [67].

2-6. Real time reverse transcription polymerase chain reaction (RT-PCR) analysis

The RNA was isolated from the liver according to the manufacturer's method using TRIzol reagent (Invitrogen Life Technoloies, Grand Island, NY) with homogenizer. DNase digestion was used to remove any DNA contamination, and RNA was re-precipitated in ethanol to ensure no phenol

contamination. For the same adjustment, RNA purity and integrity were evaluated using the Agilent 2100 Bioanalyzer (Aglient Technologies, Palo Alto, CA, USA). The same amount of RNA from each group was pooled to normalize individual differences. For cDNA synthesis, total RNA (1 μg) was reverse transcribed with the QuantiTech SYBR Green PCR kit (Qiagen, Germany). And mRNA expression was quantified by real time PCR, using the QuantiTects SYBR green PCR kit (Qiagen, Germany) on the CFX96TM real time PCR system (Bio-Rad, UK).

The cycle threshold were determined based on SYBR green emission intensity during the exponential phase. The fold change in the gene expression was determined by the $2^{-\triangle\triangle Ct}$ method [68]. Transcripts of GAPDH were amplified from the samples in order to assure normalized real time PCR detection. The genes and their forward and reverse primers are listed in Table 1.

2-7. Morphological analysis of liver fat

The liver was fixed in 10% (v/v) formaldehyde for staining with hematoxylin and eosin. Stained sample were viewed using an Research Fluorescence Microscope (Nikon, Japan) with magnifying power of ×100.

2-8. Statistical analysis

All data were expressed as mean±S.E. Student's t-test of the SPSS 23 program (SPSS, Inc., Chicago, IL, USA) was used to compare the means of the two samples of related data.

Table 1. Primer sequence for genes used for real-time PCR

Gene	Sequence
GAPDH	5′-CAAGTTCAACGGCACAGTCAAGG-3′ (F) 5′-CCACTACGACCACGACTCATACA-3′ (R)
PPARa	5′-ATGCCAAAAATATCCCTGGTTTC-3′ (F) 5′-AGATGTGCTACGACCGGAGG-3′ (R)
CPT1a	5′-ATCTGGATGGCTATGGTCAAGGTC-3′ (F) 5′-CTGAAGGTTGCGTACTGTCGTG-3′ (R)
CYP7A1	5′-TCCACCTTTGATGACATGGA-3′ (F) 5′-GTAATGTCTCACGACCGGTT-3′ (R)
ABCA1	5′-GGACATGCACAAGGTCCTGA-3′ (F) 5′-AAACTTCGAGGTCCTAAAAGAC-3′ (R)

Ⅲ. RESULT

3-1. Effect of Schizandrin A on body weight, body weight gain, food intake, food efficiency ratio and adipose tissue weight in C57BL/6J mice fed a high-fat and high-cholesterol diet

3-1-1. Effect on body weight, body weight gain, food intake and food efficiency ratio

We were monitored body weight and food intake weekly for 15 weeks. There were no significant differences in body weight, body weight gain (BWG), food intake and food efficiency ratio (FER) between CON and SCH A groups (Figure 4, Table 2). Also, BWG and food efficiency ratio were not significantly different between the two groups.

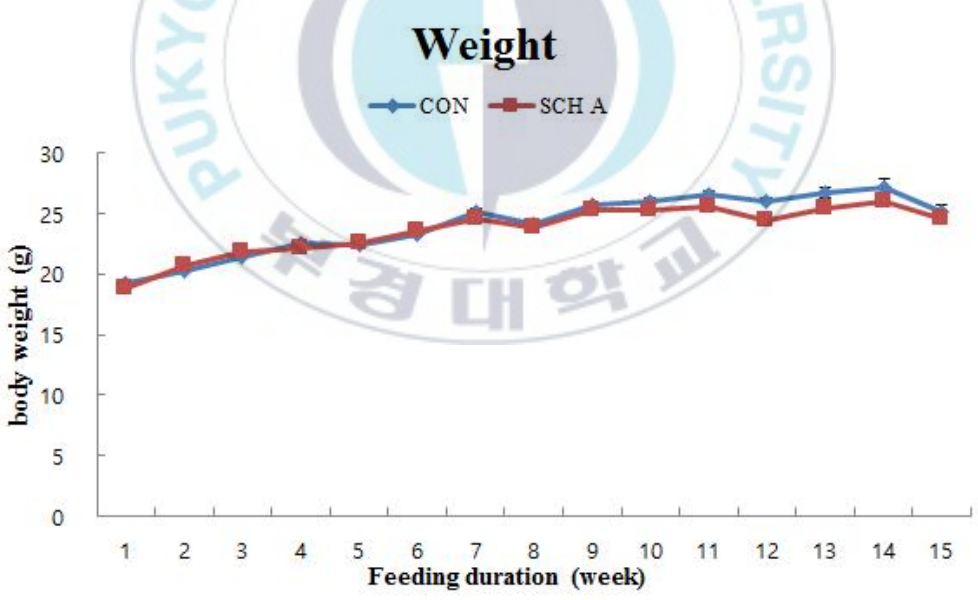

Figure 4. Effect of Schizandrin A supplementation for 15 weeks on body weight in C57BL/6J mice fed a high-fat and high-cholesterol diet. Data are mean±S.E. (n=9-10). There were no significant differences in body weight between the two groups. CON, high-fat and high-cholesterol diet; SCH A, high-fat and high-cholesterol diet+schizandrin A (0.05%, w/w).

Table 2. Effect of Schizandrin A supplementation for 15 weeks on body weight gain, food intake and food efficiency ratio in C57BL/6J mice fed a high-fat and high-cholesterol diet

	CON	SCH A
BWG (g/week)	0.40±0.02	0.38±0.03
Food intake (g/day)	3.65±0.07	3.52 ±0.05
FER	0.0155 ± 0.001	0.0154 ± 0.001

Data are mean±S.E. (n=9-10). There were no significant differences in body weight gain, food intake and FER between the two groups. CON, high-fat and high-cholesterol diet; SCH A, high-fat and high-cholesterol diet+schizandrin A (0.05%, w/w); BWG, body weight gain; FER, food efficiency ratio.

3-1-2. Effect on diverse white adipose tissue weight

To examine the anti-adiposity effect of SCH A, we measured weights of white adipose tissues (WAT) (epididymal WAT, perirenal WAT, subcutaneous WAT, retroperitoneum WAT, mesenteric WAT, visceral WAT, subcutaneous WAT and total WAT) (Figure 5). The supplementation of SCH A did not significantly alter weights of diverse adipose tissue regions in mice fed a high-fat and high-cholesterol diet.

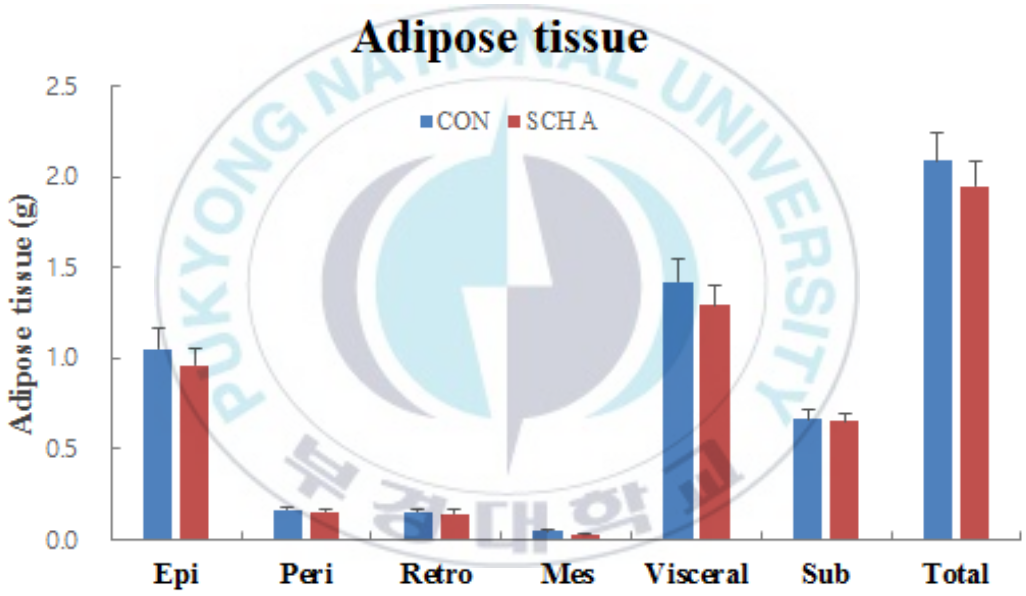

Figure 5. Effect of Schizandrin A supplementation for 15 weeks on adipose tissue weight in C57BL/6J mice fed a high-fat and high-cholesterol diet.

Data are mean±S.E. (n=9-10). There were no significant differences in adipose tissue weight between the two groups. CON, high-fat and high-cholesterol diet; SCH A, high-fat and high-cholesterol diet+schizandrin A (0.05%, w/w). Epi, epididymal WAT; Peri, perirenal WAT; Retro, retroperitoneum WAT; Mes, mesenteric WAT; Visceral, visceral WAT; Sub, subcutaneous WAT; Total, total WAT; WAT, white adipose tissue.

3-2. Effect of Schizandrin A on plasma, hepatic and fecal lipid profile in C57BL/6J mice fed a high-fat and high-cholesterol diet

3-2-1. Effect on plasma lipid profiles

Levels of plasma lipids are shown in Figure 6. Levels of plasma total-cholesterol, triglyceride and HDL-cholesterol were not significantly different between groups. Additionally, there were no significant differences in HTR and AI between groups. However, plasma free fatty acid level was significantly lowered in SCH A group compared with CON group.

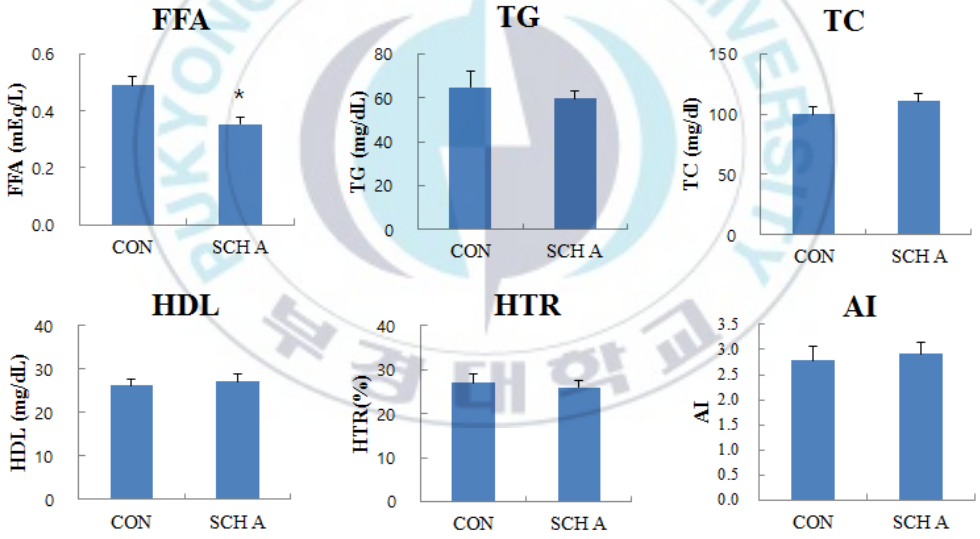

Figure 6. Effect of Schizandrin A supplementation for 15 weeks on plasma lipids levels in C57BL/6J mice fed a high-fat and high-cholesterol diet. Data are mean±S.E. (n=9-10). Statistical differences between CON and SCH A were determined using Student's t-test. : *p<0.05. CON, high-fat and high-cholesterol diet ; SCH A, high-fat and high-cholesterol diet+schizandrin A (0.05%, w/w), FFA, free fatty acid; TG, triglyceride; TC, total cholesterol; HDL, HDL-cholesterol; HTR, ratio of HDL-cholesterol to total cholesterol; AI, atherogenic index.

3-2-2. Effect on hepatic lipid profiles and liver morphology

SCH A group significantly decreased the hepatic free fatty acid, triglyceride and cholesterol contents compared to the CON group. Consistent with the hepatic lipids contents, SCH A supplementation markedly decreased lipid droplets accumulation in the liver (Figure 7).

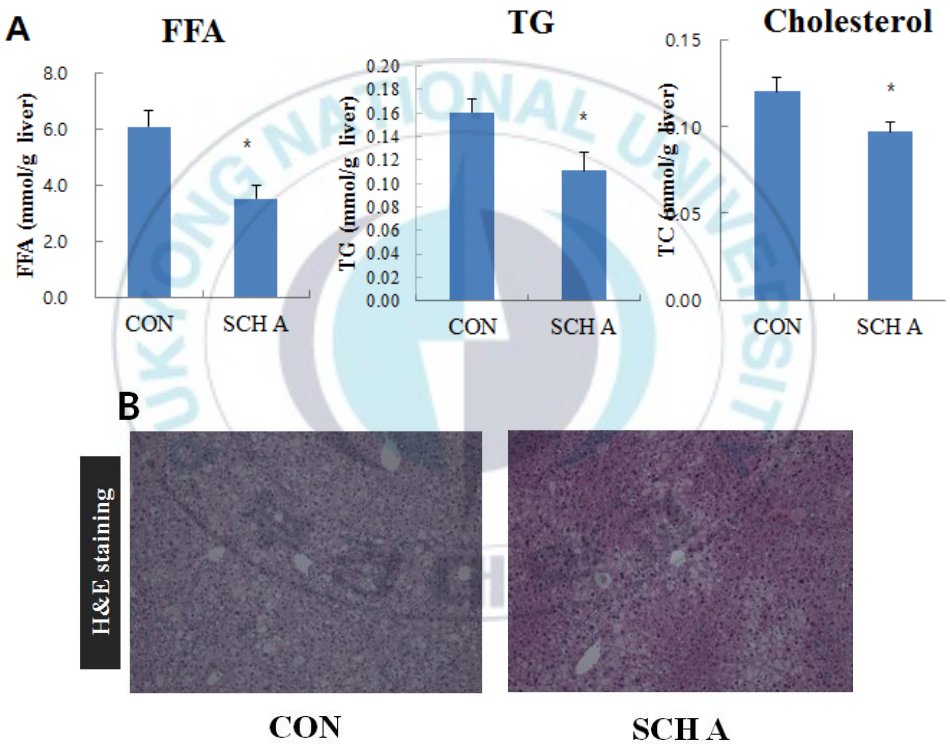

Figure 7. Effect of Schizandrin A supplementation for 15 weeks on hepatic lipids levels (A) and hepatic morphology (B) in C57BL/6J mice fed a high-fat and high-cholesterol diet. A: Representative photomicrographs of liver are shown ×100 magnification. B: Data are mean±S.E. (n=9-10). Statistical differences between CON and SCH A were determined using Student's t-test. : *p<0.05. CON, high-fat and high-cholesterol diet; SCH A, high-fat and high-cholesterol diet+schizandrin A (0.05%, w/w). H&E, hematoxylin and eosin; FFA, free fatty acid; TG, triglyceride.

3-3. Effect on fecal lipid profiles in C57BL/6J mice fed a high-fat and high-cholesterol diet

We also analyzed fecal excretion of free fatty acid, total cholesterol and triglyceride (Figure 8). SCH A group displayed more increased excretion of fecal free fatty acid and triglyceride compared to CON group. Fecal cholesterol level also tended to be higher in SCH A group than in CON group, but the difference was not statistically significant.

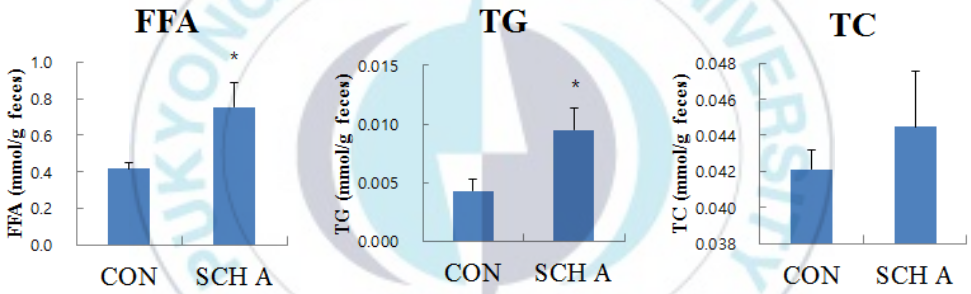

Figure 8. Effect of Schizandrin A supplementation for 15 weeks on fecal lipids excretion in C57BL/6J mice fed a high-fat and high-cholesterol diet. Data are mean±S.E. (n=9-10). Statistical differences between CON and SCH A were determined using Student's t-test. : *$p<0.05$. CON, high-fat and high-cholesterol diet; SCH A, high fat and high cholesterol diet+schizandrin A (0.05%, w/w). FFA, free fatty acid; TG, triglyceride; TC, total cholesterol.

3-4. Effect of Schizandrin A activity of hepatic enzymes related to lipid metabolism in C57BL/6J mice fed a high-fat and high-cholesterol diet

We examined the effect of SCH A on activity of hepatic lipogenic enzymes, fatty acid synthase (FAS) and phosphatidate phosphohydrolase (PAP). These lipogenic enzymes activity were significantly lower in SCH A group than in the CON group. Moreover, supplementation of SCH A tended to increase activity of CPT as well as fatty acid oxidation compared to the CON group, although the difference was not statistically significant (Figure 9).

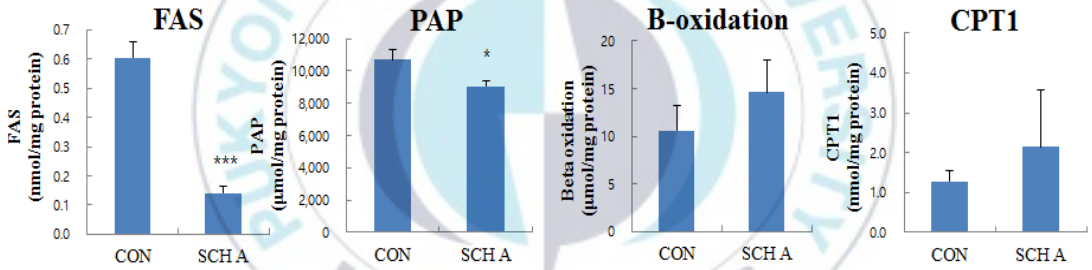

Figure 9. Effects of Schizandrin A supplementation on hepatic lipid-regulating enzymes activities in C57BL/6J mice fed a high-fat and high-cholesterol diet. Data are mean±S.E. (n=9-10). Statistical differences between CON and SCH A were determined using Student's t-test. : *p<0.05, ***p<0.001. CON, high-fat and high-cholesterol diet; SCH A, high-fat and high-cholesterol diet+schizandrin A (0.05%, w/w). FAS, fatty acid synthase; PAP, phosphatidate phosphohydrolase CPT1, carnitine palmitoyl-CoA transferase1.

3-5. Effect of Schizandrin A on mRNA expression of hepatic genes involved in lipid metabolism in C57BL/6J mice fed a high-fat and high-cholesterol diet

mRNA expression of fatty acid oxidation-related genes such as PPAR-α and CPT1a were markedly increased in liver of SCH A-supplemented mice. In addition, mRNA expression of cholesterol metabolism-related genes such as CYP7A1 and ABCA1 were also higher in the SCH A group than in the CON group. These differences were statistically significant (Figure 10).

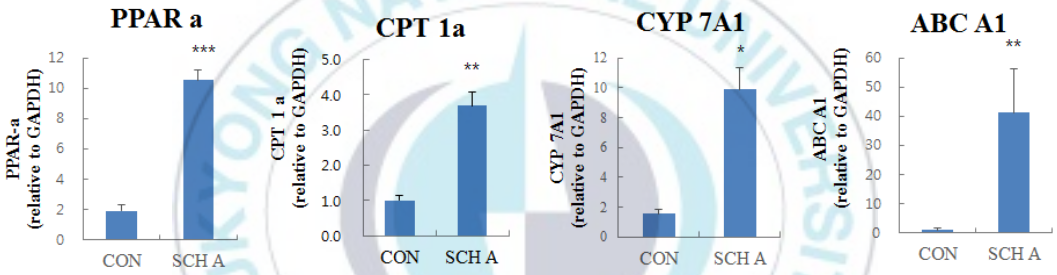

Figure 10. Effects of Schizandrin A supplementation on hepatic lipid-regulating genes expression in C57BL/6J mice fed a high-fat and high-cholesterol diet. Data are mean±S.E. (n=9-10). Statistical differences between CON and SCH A were determined using Student's t-test. : *p<0.05, **P<0.01, ***p<0.001. CON, high-fat and high-cholesterol diet; SCH A, high fat and high cholesterol diet+schizandrin A (0.05%, w/w).

3-6. Effect of Schizandrin A on hepatic oxidative stress in C57BL/6J mice fed a high-fat and high-cholesterol diet

Finally, we examined the effect of SCH A on oxidative stress in the liver (Figure 11). Supplementation of SCH A significantly decreased TBARS in the liver of mice fed a high-fat and high-cholesterol diet. The activity of antioxidant enzymes, including SOD, GPX and CAT, in the liver were significantly increased by SCH A supplementation.

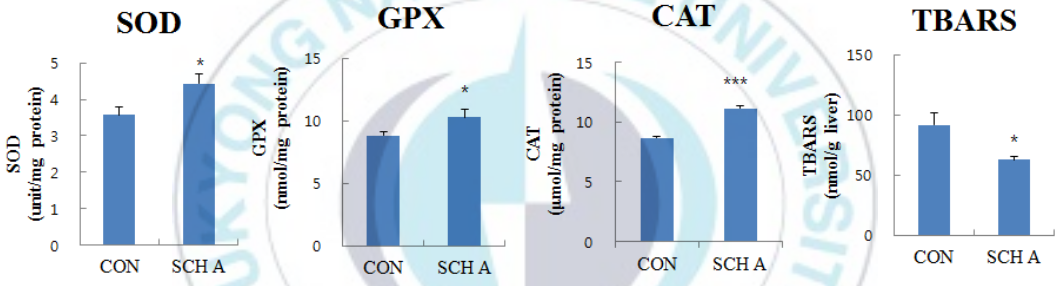

Figure 11. Effects of Schizandrin A supplementation on hepatic oxidative stress in C57BL/6J mice fed a high-fat and high-cholesterol diet. Data are mean±S.E. (n=9-10). Statistical differences between CON and SCH A were determined using Student's t-test. : *p<0.05, ***p<0.001. CON, high-fat and high-cholesterol diet; SCH A, high fat and high cholesterol diet+schizandrin A (0.05%, w/w). SOD, superoxide dismutase; GPX, glutathion peroxidase; CAT, catalase.

Ⅳ. DISCUSSION

4-1. Effect of Schizandrin A on lipid metabolism

Hepatic lipid accumulation is a hallmark of NAFLD, and hepatic steatosis is initial step of NAFLD [32]. The hepatic steatosis occurs as a result of the availability of excess free fatty acids from a high-fat and high-cholesterol diet [28, 69]. Therefore, the aim of this study was first to determine the effects of SCH A supplementation on hepatic steatosis and related lipid metabolism in mice fed high-fat diet and high-cholesterol diet.

The present study showed that supplementation of SCH A (0.05%, w/w) for 15 weeks did not lead to significant changes in body weight, food intake and food efficiency ratio as well as WAT weight in mice fed a high-fat diet and high-cholesterol diet. However, we observed that SCH A supplementation suppressed hepatic lipid accumulation in mice fed a high-fat diet and high-cholesterol diet. Accordingly, levels of hepatic lipids and plasma FFA as well as hapatic lipid droplet accumulation were significantly lower in SCH A group than in CON group. SCH A supplementation also markedly increased fecal lipids excretion in mice fed a high-fat diet and high-cholesterol diet. These results suggest that SCH A intake may inhibit lipid absorption in mice, although we need more study to elucidate effect of dietary SCH A on lipid absorption.

Disturbances in fatty acid oxidation account for excess lipid storage in the liver [40]. So, we investigated whether SCH A supplementation could affect the activities of hepatic enzymes related to lipid metabolism. We first observed that SCH A supplementation decreased the activities of FAS and PAP in the liver. These are key enzymes in the regulation of *de novo* fatty acid synthesis and triglyceride synthesis, respectively. We also observed that activity of CPT1 as well as β-oxidation in liver was elevated by SCH A supplementation. Furthermore, the enhanced fatty acid oxidation in SCH A-supplemented mice was accompanied by the up-regulated mRNA expression of genes involved in fatty acid oxidation such as *CPT1a* and *PPARa* in liver. The CPT1 is a major rate-limiting enzyme for fatty acid oxidation [68]. It has been reported that the CPT1 is closely related with the

pathogenesis of NAFLD, because CPT1 maintains balance of lipid by regulating fatty acid metabolism in the mithochondrial membrane [70]. This enzyme was encoded by *CPT1a* [71]. In addition, as a transcription factor, *PPARa* governs biological processes by altering the expression of numerous target genes involved in fatty acid oxidation such as *CPT1a* [41]. In particular, *PPARa* in liver is crucial sensor to recognize and respond to fatty acid influx. When recognizing fatty acid influx, *PPARa* induces the activities of fatty acid oxidation systems to prevent hepatic steatosis [40]. Therefore, these results suggest that SCH A supplementation have a protective role in the development of NAFLD.

Dietary cholesterol as an important determinant of the severity of phenotypic changes that occur with progression of NAFLD [6]. Therefore, dietary cholesterol appears to be an important risk factor for NAFLD in animal models [6].

Bile acids have crucial regulatory functions in cholesterol metabolism, because conversion into bile acids represents an important route to remove excess cholesterol from the body [72]. It is reported that a high-fat and high cholesterol diet decreases expression of genes regulating hepatic bile acid metabolism such as *CYP7A1* [46]. In the present study, we observed that SCH A supplementation significantly increased mRNA expression of *CYP7A1*, a key gene of rate-limiting steps for the bile acid synthesis in the liver. Thus, SCH A supplementation may improve hepatic cholesterol accumulation through the conversion of cholesterol to bile acids in the liver. In addition, we also observed that SCH A supplementation markedly increased mRNA expression of *ABCA1* which plays a key role in the initial lipidation of apo A1, the major protein component of HDL in plasma [70]. Although *ABCA1* was up-regulated by SCH A in the present study, SCH A had little effect on level of plasma HDL-cholesterol. So, additional studies are needed to more fully understand the role of SCH A in the ABCA1-mediated efflux of hepatic cholesterol to plasma HDL. However, our results suggest that SCH A may be effective in alleviating NAFLD by removing cholesterol in liver.

4-2. Effect of Schizandrin A on oxidative stress

Lipid peroxidation is main outcomes of free radical-mediated tissue injury that directly damages membranes and, in turn, generates a number of secondary products, such as MDA [32]. An increase in lipid peroxidation products in the liver of patients or animals with NAFLD suggests that oxidative stress is involved in the pathogenesis of NAFLD [58].

Antioxidant enzymes such as SOD, GPX and CAT ameliorate the damaging effects of reactive oxygen species. SOD converts superoxide radicals into hydrogen peroxide, which is then further metabolized by CAT and GPX. CAT and GPX catalyze the destruction of hydrogen peroxide and lipid hydroperoxide [58]. These enzymes are major antioxidant enzymes, which protect cells against damage by free radicals and inhibit lipid peroxidation in cell membranes [23]. We observed that the supplementation of SCH A significantly increased the activities of SOD, CAT and GPX in liver. Also, SCH A supplementation decreased level of hepatic TBARS, which are formed as a byproduct of lipid peroxidation. The increased antioxidant enzymes activities and decreased TBARS level in SCH A group suggest that SCH A may help to cope with oxidative stress, contributing to the reduced risk of NAFLD.

Ⅴ. CONCLUSION

The present study showed that the supplementation of SCH A decreased plasma FFA and hepatic lipids levels in mice fed a high-fat and high-cholesterol diet. Interestingly, we demonstrated that the supplementation with SCH A not only decreased hepatic FAS and PAP activity related to triglyceride synthesis but also increased hepatic CPT1 and β-oxidation activity related to fatty acid oxidation. SCH A supplementation also led to a significant increase in the mRNA expression related to lipid metabolism, such as *CPT1a*, *PPARa, CYP7A1* and *ABCA1,* in the liver of high-fat diet and high-cholesterol diet mice. Moreover, SCH A supplementation promoted fecal lipid excretion in mice, suggesting that dietary SCH A may inhibit lipid absorption. In addition, this study demonstrated that SCH A supplementation not only increased activities of antioxidant enzymes such as SOD, CAT and GPX but also decreased TBARS, a biomarker of lipid peroxidation, in the liver. Taken together, our data suggest that SCH A may have a protective role against high-fat and high-cholesterol diet-induced NAFLD by inhibiting hepatic lipid accumulation and oxidative stress.

In conclusion, SCH A protects the liver against NAFLD through the regulation of activities of enzymes and mRNA expression of genes involved in lipid metabolism, the increase in fecal lipids excretion, and the improvement of oxidative stress. Therefore, SCH A may be useful in the treatment of NAFLD induced by a high-fat and high-cholesterol diet. However, further research is required to elucidate the efficacy of SCH A in subjects with NAFLD.

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