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Protective effect of oligomeric proanthocyanidins against alcohol-induced liver steatosis and injury in mice

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ABSTRACT

The long-term consumption of alcohol has been associated with multiple pathologies at all levels, such as alcoholism, chronic pancreatitis, malnutrition, alcoholic liver disease (ALD) and cancer. In the current study, we investigated the protective effect of oligomeric proanthocyanidins (OPC) against alcohol-induced liver steatosis and injury and the possible mechanisms using ethanol-induced chronic liver damage mouse models. The results showed that OPC significantly improved alcohol-induced dyslipidemia and alleviated liver steatosis by reducing levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total triglyceride (TG), total cholesterol (TC), low-density cholesterol (LDL-c) and liver malondialdehyde (MDA), and increasing levels of serum high-density lipoprotein (HDL-c), liver superoxide dismutase (SOD). Further investigation indicated that OPC markedly decreased the expressions of lipid synthesis genes and inflammation genes such as sterol regulatory element-binding protein-1c (*Srebp-1c*), protein-2 (*Srebp2*), interleukin *IL-1β*, *IL-6* and *TNF-α*. Furthermore, AML-12 cells line was used to investigate the possible mechanisms which indicated that OPC might alleviate liver steatosis and damage through AMP-activated protein kinase (AMPK) activation involving oxidative stress. In conclusion, our study demonstrated excellent protective effect of OPC against alcohol-induced liver steatosis and injury, which could a potential drug for the treatment of alcohol-induced liver injury in the future.

1. Introduction

Alcohol consumption is a well-known risk factor for liver damage, which represents a major cause of liver disease worldwide. Chronic ingestion of alcohol over a certain limit inevitably leads to different levels of alcoholic liver disease (ALD), an extremely common disease with high mortality, ranging from steatosis, steatohepatitis, fibrosis, to life-threatening cirrhosis [1]. The pathogenesis of ALD appears to involve the side effects of alcohol metabolism and multiple cellular injury mechanisms [2]. The metabolism of alcohol is associated with multiple factors including fat accumulation, oxidative stress, cellular redox state and reactive oxygen species (ROS) [3]. Thus, reduced fat accumulation and the formation of ROS in the liver upon alcohol exposure may be a potential target of the treatment of ALD.

The inhibition of hepatic AMPK signaling pathway by ethanol plays a key role in the development of steatosis induced by chronic alcohol consumption [4]. AMPK is a sensor that regulates cellular metabolism and oxidative stress [5,12,26]. It is well-known that classic AMPK activation stimulates ATP-producing catabolic pathways, such as fatty acid oxidation, and inhibits ATP-consuming anabolic pathways, such as lipogenesis [6]. Previous findings suggested that the effect of ethanol on SREBP-regulated promoter activation was partially mediated through AMPK inhibition [7].

Alcohol intake increases free radicals and reactive ROS production, increasing oxidative stress by attenuating the antioxidant defence system [8]. The highly reactive ROS produced during alcohol exposure can damage proteins, lipids, and DNA [9]. In addition, oxidative stress can greatly improve the activity of the

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Table 1
Sequence of the primer used in real-time PCR.

Gene	Forward primer (5′ to 3′)	Reverse primer (5'to 3′)
TNF-γ	GACAGTGACCTGGACTGTGG	TGAGACAGAGGCAACCTGAC
IL-1β	GAAGAAGAGCCCATCCTCTG	TCATCTCGGAGCCTGTAGTG
IL-6	GGACCAAGACCATCCAATTC	ACCACAGTGAGGAATGTCCA
SREBP1c	TTGTGGAGCTCAAAGACCTG	TGCAAGAAGCGGATGTAGTC
SREBP2	GCGTTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAAACAAATCA
HMGCS	GCCGTGAACTGGGTCGAA	GCATATATAGCAATGTCTCCTGCAA
PPAR-α	GGGTACCACTACGGAGTTCACG	CAGACAGGCACTTGTGAAAACG
HMGCR	CTTGTGGAATGCCTTGTGATTG	AGCCGAAGCAGCACATGAT
LDLR	AGGCTGTGGGCTCCATAGG	TGCGGTCCAGGGTCATCT
β-actin	GATCATTGCTCCTCCTGAGC	ACTCCGCTTGCTGATCCAC

AMPK signaling system, which leads to increased expressions of inflammatory cytokines [5,12]. All these previous studies suggested that AMPK may be a possible therapeutic target in the particular case of alcohol-induced liver disease.

Oligomeric proanthocyanidins (OPC), a class of flavonoid compounds, are widely present in many vegetables, fruits, and seeds with a broad spectrum of biological, pharmacological and therapeutic activities [10]. OPC functions as powerful antioxidants and could exert anti-inflammatory activities [11]. In-vitro experiments showed that OPC had different effects on inflammation [12], which was probably due to the different structural characteristics of the molecules tested and the different experimental designs used. Adequate reports over decades revealed potent anti-inflammatory properties of OPC on experimental inflammation in rats and mice [13,14]. However, their anti-inflammatory mechanisms are poorly understood, which has been reported to be related to oxygen free radical scavenging, inhibition of the formation of inflammatory cytokines, and anti-lipid peroxidation [15,16]. Furthermore, to our best knowledge, there is no study reporting the protective effects of OPC against alcohol-induced liver steatosis and injury.

In this study, we established an alcohol-induced chronic liver damage mouse model to investigate the protective effect of OPC against alcohol-induced liver steatosis and injury through pathological staining and measuring the level of serum lipids and the expressions level of lipid synthesis genes and inflammation genes. The possible protective mechanisms of OPC were also explored based on existing evidence.

2. Materials and methods

2.1. Reagents and antibodies

OPC (purity ≥98%) was purchased from Zelang biological technology Co. Ltd (Nanjing, China). Assay kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT) were purchased from Chaoyan biological Technology Co. Ltd (Shanghai, China). Other assay kits, total cholesterol (TC), LDL cholesterol (LDL-c), and HDL cholesterol (HDL-c), were purchased from Yinggong Industrial Co. Ltd (Shanghai, China). Lieber-Decarli liquid food was purchased from Dyets, Inc (Bethlehem, U.S.). The antibodies, AMPK-α,

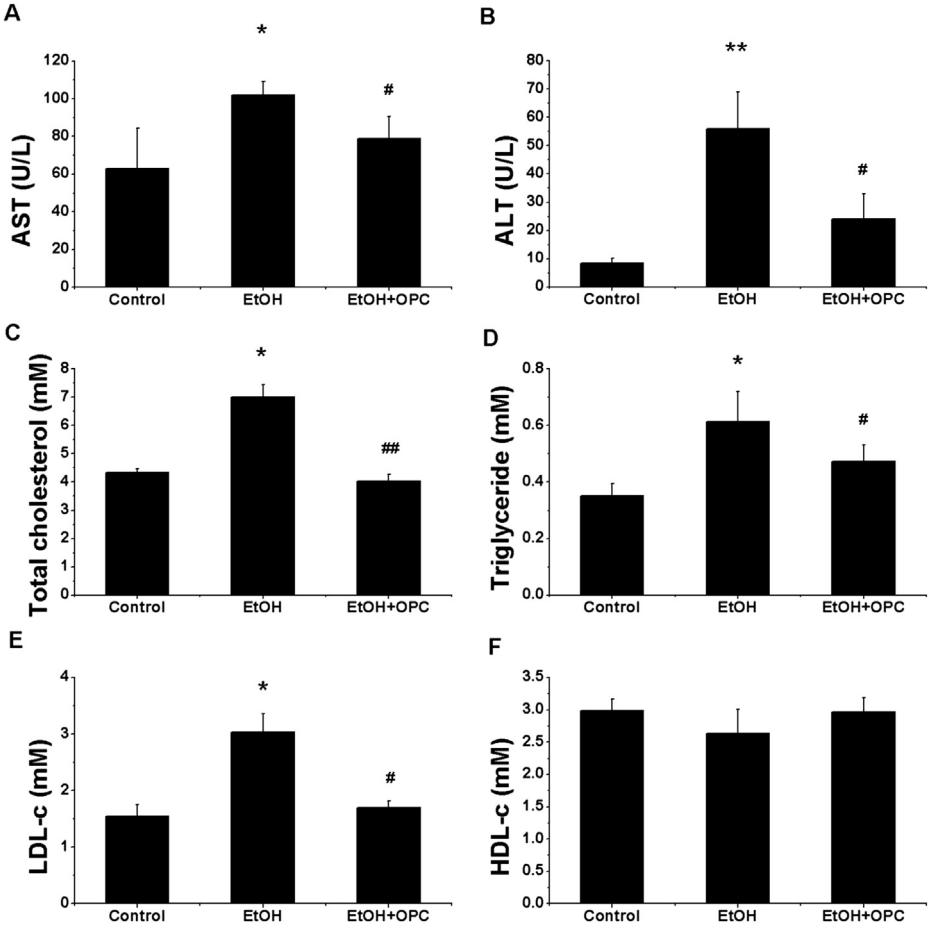

Fig. 1. Effects of oligomeric proanthocyanidins on serum AST (A), ALT (B), TC (C), TG(D), LDL-c(E), and HDL-c (F) in C57BL/6 mice. Values are means ± SE; n = 9. $^{*}P < 0.05$, $^{**}P < 0.01$ (*vs* control group); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (*vs* EtOH group). Control, control diet; EtOH, ethanol diet; EtOH + OPC, ethanol diet plus OPC administration.

phosphorylated AMPK-α and phosphorylated SREBP-1c were from Cell Signaling Ltd. and GAPDH was purchased from Sigma.

2.2. Cell culture and treatments

Mouse AML-12 hepatocyte cells were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 3.7 g/L $NaHCO_3$ at 37 °C. Cells were treated with alcohol and/or OPC (50 μM) dissolved in phosphate-buffered saline (PBS) and/or AMPK blocking agent compound C (10 μM) dissolved in PBS for 24 h. After the treatment, cells were harvested with lysis buffer after washing by PBS for three times.

2.3. Mouse models establishment

Female mice C57BL/6 (six-week-old, weight at 20–24 g) were purchased from animal center of Nanjing Medical University and housed in animal quarters at 22 °C with a 12 h light/dark cycle (light from 8 a.m. to 8 p.m.). After adaptation to the environment with free access to liquid diet (Lieber-Dcarli diet), the mice were randomly divided to three groups: control group (n = 9), EtOH group (n = 9) and EtOH + OPC group (n = 9). In the first 3 days, the concentrations of alcohol in mice diet for the EtOH and EtOH + OPC groups were 1%, 2%, 4% (v/v), respectively, followed by 5% (v/v) for the remaining 9 days. OPC (50 mg/kg) was given to the EtOH + OPC group by oral gavage, while PBS was given to the control and EtOH groups. On the last day, mice were killed under anesthesia, 9 h after a single dose of 30% alcohol gavage. The mouse tissues were snap-frozen in liquid nitrogen immediately after resection and stored at −80 °C until further analysis.

2.4. Biochemical analysis

Serum ALT, AST, total glyceride (TG), TC, and LDL-c concentrations were determined by colorimetric methods according to provided procedures. The activity of superoxide dismutase (SOD) and level of malondialdehyde (MDA) in liver tissues were measured by kits according to the manufacturers' instructions.

2.5. Quantitative real time PCR

RT-PCR was performed in a thermal cycler using RNA PCR Kit (TaKaRa). The intensity of the PCR products visualized by ethidium bromide staining was quantified with Gelworks LABWORK4.0 Analysis Software. Primers used for each gene were listed in Table 1.

2.6. Hepatic pathological assay

The liver tissues were fixed with 4% paraformaldehyde and paraffin embedded sections were stained with hematoxylin & eosin (H&E), then observed using light microscopy. Hepatic steatosis was determined by staining of 10 μm thick frozen sections with Oil-Red-O (Sigma–Aldrich).

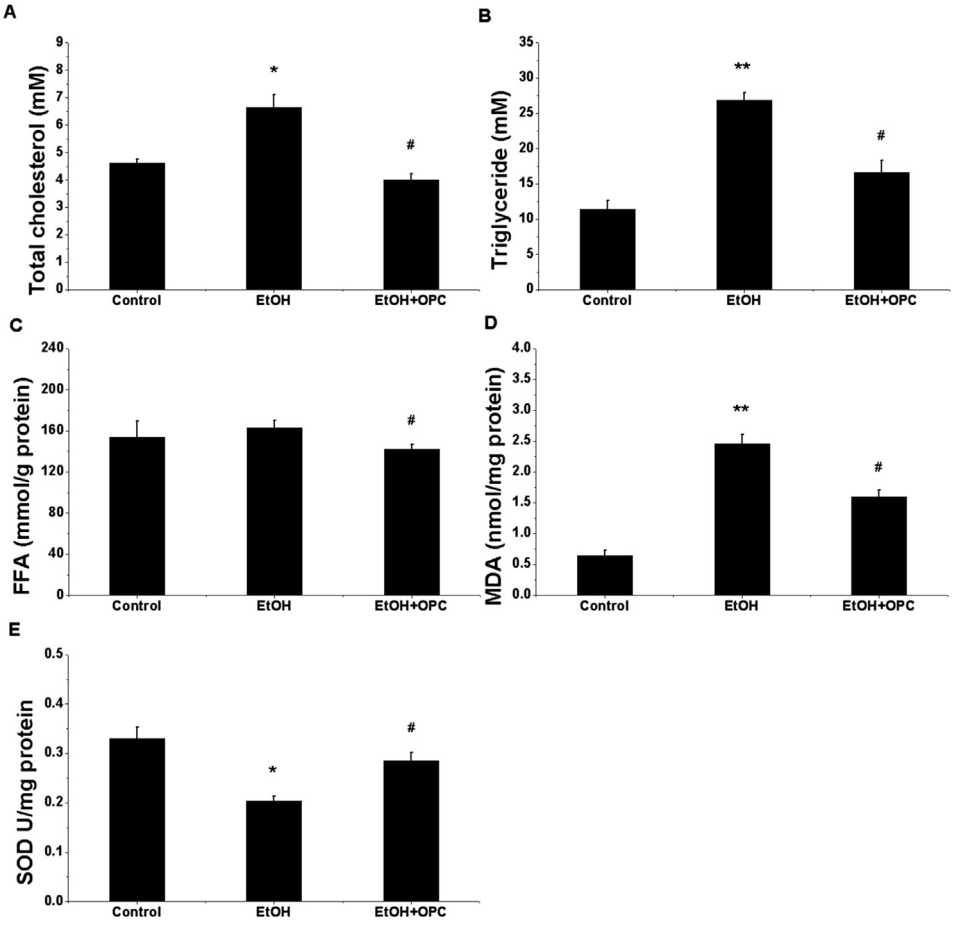

Fig. 2. Effects of oligomeric proanthocyanidins on hepatic concentrations of TC (A), TG (B), FFA(C), MDA (D), SOD (E). Values are means ± SE; n = 9. $^{*}P < 0.05$, $^{**}P < 0.01$ (*vs* control group); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (*vs* EtOH group). Control, control diet; EtOH, ethanol diet; EtOH + OPC, ethanol diet plus OPC administration.

2.7. Western blotting

Cell lysates of AML-12 were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in Tris buffered saline (TBS) containing non-fat dry milk (5% w/v) and thereafter incubated with the relevant primary antibody at 4 °C overnight. Antibodies used were AMPK-α, phosphorylated AMPK-α, phosphorylated SREBP-1c and GAPDH.

2.8. Statistical analysis

All results are presented as the mean ± standard deviation (SD). Differences among groups were analyzed by one-way ANOVA and Duncan's multiple range test (DMRT). A value of $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. OPC improved alcohol-induced dyslipidemia

The effects of OPC on the serum levels of AST, ALT, TC, TG, LDL-c and HDL-c in mice models were shown in Fig. 1. Compared with the EtOH group, the levels of AST and ALT (Fig. 1A, B) were significantly decreased in the EtOH + OPC group. Alcohol consumption also induced dyslipidemia in the mice, demonstrated by increased serum TG, TC, LDL-c in the EtOH group compared with the control group, whereas these variables in the EtOH + OPC group were attenuated (Fig. 1C–E). On the contrary, there was a decrease in serum level of HDL-c in the EtOH group, while these changes in the EtOH + OPC group were not significantly different from the control (Fig. 1F). Collectively, these results indicated that OPC could effectively improve alcohol-induced dyslipidemia in-vivo.

3.2. OPC alleviated alcohol-induced fatty liver and exhibited remarkable antioxidant effects

The liver contents of TC, TG were significantly increased in the EtOH group, compared with the control group (Fig. 2A, B), which were significantly decreased in the EtOH + OPC group. Meanwhile, in the EtOH group, the hepatic FFA level was increased and decreased by OPC in EtOH + OPC group (Fig. 2C). Taken together, these results suggested that OPC played an important role in the alleviation of hepatic steatosis. In the development of liver injury, oxidative stress is considered to be an important process. Alcohol consumption caused severe oxidative stress in the liver tissues of mice, as evidenced by the increased MDA level, along with reduced SOD activity in the EtOH group (Fig. 2D, E). OPC treatment significantly decreased the level of MDA and increased SOD activity. These findings exhibited remarkable antioxidant effects of OPC.

3.3. OPC relieved alcohol-induced overexpression of genes involved in lipid and inflammation synthesis

The overexpression of genes related lipid synthesis was detected. Alcohol intake increased the expression of Srebp-1c and Srebp-2, which were markedly inhibited by OPC (Fig. 3A). In addition, the

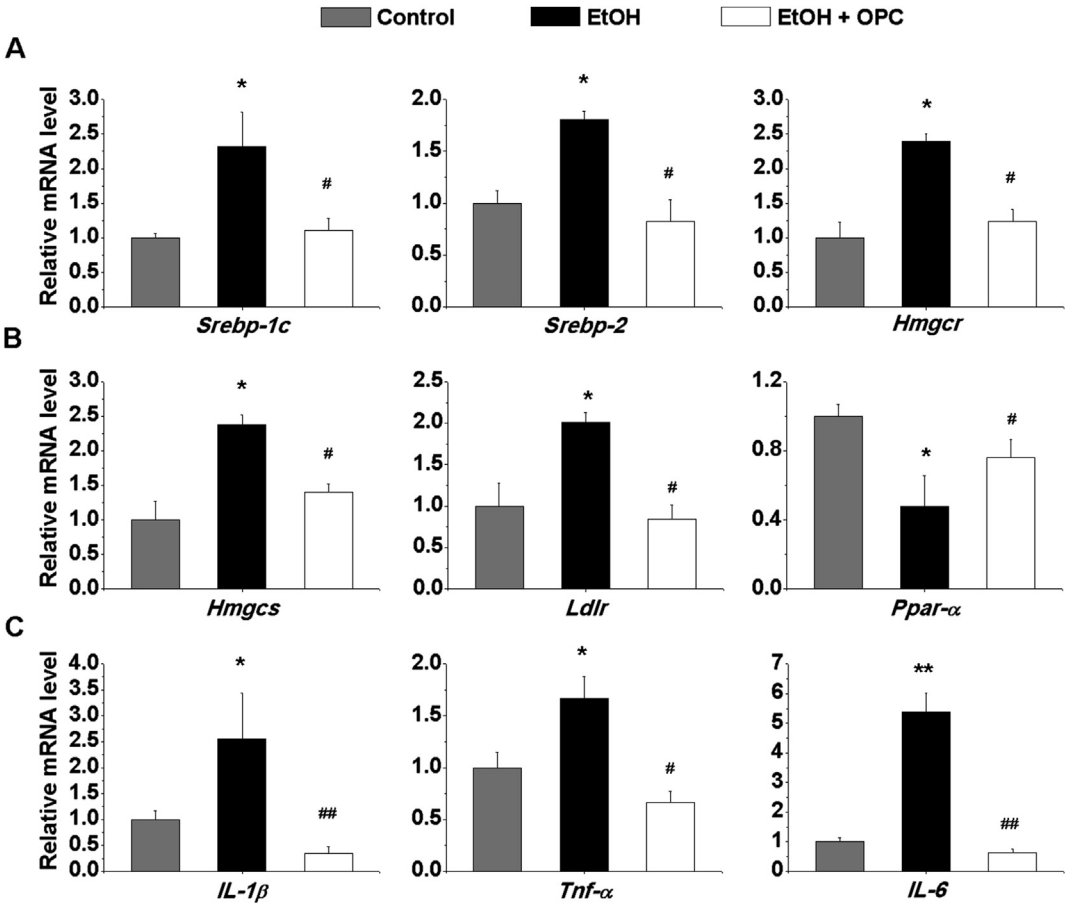

Fig. 3. Effects of oligomeric proanthocyanidins on hepatic mRNA abundances of multiple genes in C57BL/6 mice, including genes of lipid synthesis (A, B) and inflammation (C). Values are means ± SE; n = 9. $^{*}P < 0.05$, $^{**}P < 0.01$ (*vs* control group); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (*vs* EtOH group). Control, control diet; EtOH, ethanol diet; EtOH + OPC, ethanol diet plus OPC administration.

expression of HMGCR, HMGCS and LDL were noticeably enhanced by alcohol consumption and depressed by co-administration of OPC (Fig. 3A, B). Reversely, the expression of PPAR-α was depressed by alcohol which could be abrogated by OPC (Fig. 3B). Briefly, OPC played a key role in attenuating the formation of fatty liver by regulating expression of genes related to hepatic lipid synthesis and consumption.

The expressions of genes related to inflammation and liver injury including *IL-1β*, *IL-6* and *TNF-α* were also analyzed [17]. We found that alcohol exposure significantly elevated the expression of those genes (Fig. 3C). Meanwhile, OPC treatment reversed these ethanol-induced alterations by reducing the genes expression of *IL-1β*, *IL-6* and *TNF-α* (Fig. 3C). Based on the above results, we could conclude that OPC may alleviate the alcohol-induced liver injury by inhibiting the expression of inflammation related genes.

3.4. OPC alleviated alcohol-induced lipid accumulation by regulating related signal pathways

The histological manifestation of liver samples was analyzed to confirm the inhibitory effects of OPC on alcohol-induced fat accumulation. H&E staining result revealed that alcohol consumption clearly resulted in formation of lipid vacuoles in hepatocytes, while such alterations were relieved by OPC treatment (Fig. 4A). Oil-Red-O staining also demonstrated that lipid accumulation was markedly enhanced by alcohol consumption and reduced by OPC treatment (Fig. 4B). Collectively, these histological analyses indicated that OPC could effectively alleviate alcohol-induced liver steatosis.

To further explore the effects of OPC on lipid accumulation in liver cells, we utilized AML-12 cells line to investigate the potential molecular mechanism. It was reported that SREBP gene, a master factor, was down-regulated by the AMPK signaling pathway [16,18–20]. As shown in Fig. 4C, the phosphorylation of AMPK was inhibited by alcohol and reversely enhanced by OPC. Similarly, the phosphorylation of SREBP-1c was decreased by alcohol, which was reversed by OPC and AMPK blocking agent. From these results, we could infer that OPC may attenuate the phosphorylation and activity of SREBP-1c by enhancing the activity of AMPK. Collectively, these results indicated that OPC could alleviate alcohol-induced fat accumulation by reducing SREBP-1c phosphorylation through regulating the AMPK pathway.

4. Discussion

The major metabolism route of alcohol in the liver includes the action of alcohol dehydrogenase (ADH), cell Cytochrome P4502E1 (CYP2E1) and mitochondrial enzyme catalytic. Many of these factors often appear together, which make the incidence and development of fatty liver more complicated. Nowadays, since there is still no effective drug to cure the fatty liver disease, it is an urgent need to develop novel agents for its prevention and treatment.

The purpose of this study was to explore the effects of the natural product OPC on fatty liver disease of mice. The present results showed that OPC supplementation (50 mg/kg) for 12 days had beneficial effects on alcoholic fatty liver syndrome and liver injury in mice with chronic alcohol consumption. For specific

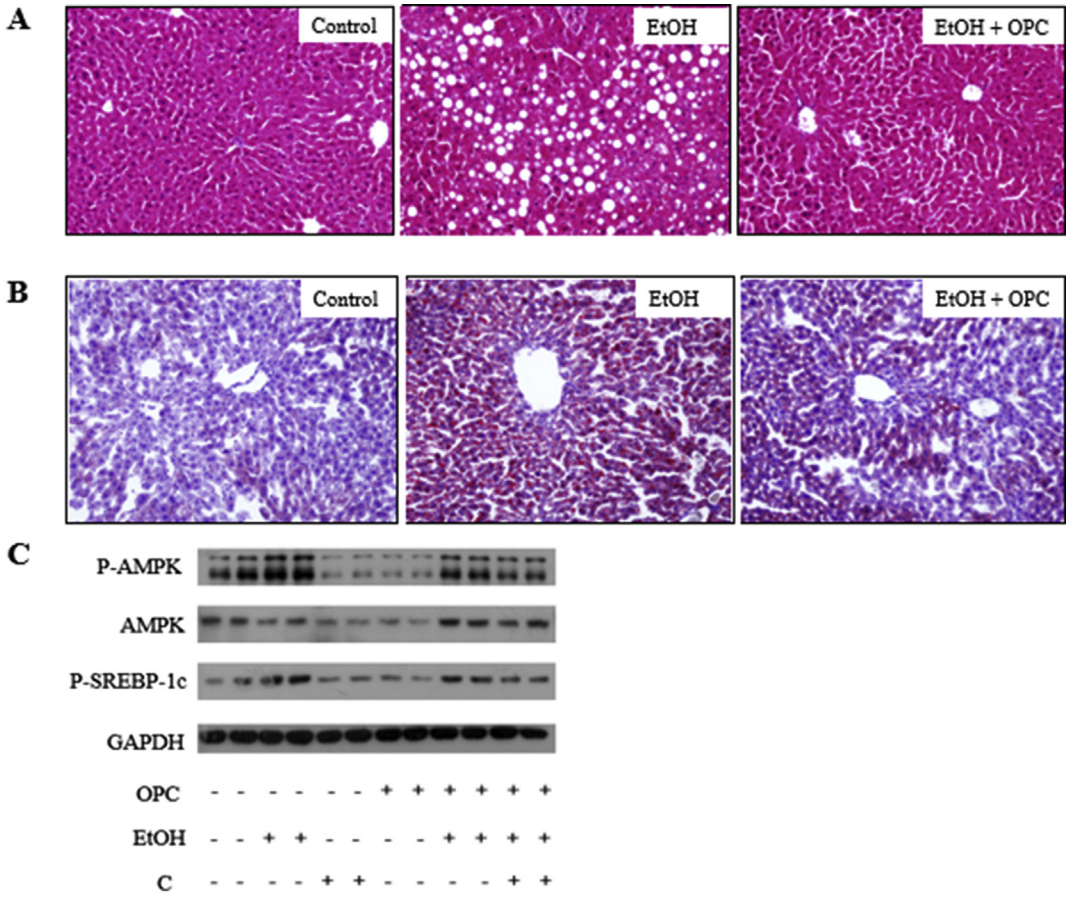

Fig. 4. Representative images of liver H&E staining (A) and Oil-Red-O staining (B). Images are shown in 200×. Effects of OPC abrogates alcohol on the phosphorylation of AMPK and SREBP-1c in AML-12 cells (C). Control, control diet; EtOH, ethanol diet; EtOH + OPC, ethanol diet plus OPC administration.

performance, OPC could markedly reduce the alcohol-induced increases of serum ALT, serum AST, serum TG, serum TC, serum LDL-c (Fig. 1), liver TG, liver TC (Fig. 2), and the expression of genes involved in lipid synthesis (Fig. 3A, B), as well as lipid accumulation in the liver as revealed by Oil-Red-O staining (Fig. 4B). In contrast, the serum HDL-c level was depressed by alcohol, while OPC significantly alleviated such alteration (Fig. 1F).

Alcohol abuse stimulates over-production of ROS, lowers cellular antioxidant levels, and leads to ALD via mechanism involving oxidative stress [24]. It is well-known that antioxidant enzymes, such as SOD and MDA, provide protection against oxidative stress, but they are easily inactivated by excessive lipid peroxides or other ROS resulting from acute alcohol-induced liver damage [21]. Our results demonstrated that alcohol consumption resulted in severe oxidative stress in the liver tissues of mice, as evidenced by the increased MDA level, along with reduced SOD activity. OPCs treatments markedly reversed the effects of alcohol and reduced the oxidative stress in the liver tissues (Fig. 2D, E).

Inflammation also plays an important role in the pathogenesis of ALD [22]. Alcohol can awaken Kupffer cells to be sensitized by lipopolysaccharides and promote the production of *TNF-α, IL-1β,* and *IL-6* [23]. These inflammatory mediators contribute to hepatocyte dysfunction, apoptosis, necrosis, and characteristic fibrosis [24]. Consistently, our study showed that ethanol could significantly induce the expression of inflammatory cytokines such as *TNF-α, IL-1β* and *IL-6* (Fig. 3C). Interestingly, OPC treatment could abrogate the ethanol-induced overexpression of *TNF-α, IL-1β* and *IL-6* (Fig. 3C), indicating that OPC may alleviate alcohol-induced liver injury through reduction of inflammation in the liver.

AMPK is known to act as a key metabolic “master switch” by phosphorylating the target enzymes involved in lipid metabolism in many tissues including liver [25,26]. AMPK protein kinase regulates the activity of the lipid metabolizing enzymes directly, and it also modulates SREBP-1 activity [27]. We noticed that the protein level of phospho-AMPK was significantly increased in the liver of ethanol group mice, which indicated the activation of AMPK, while OPC could reverse such effect (Fig. 4C). Meanwhile, phosphorylation of SREBP-1c was inhibited by alcohol treatment and such effect was also abrogated by OPC administration (Fig. 4C). Taken together, these results suggested that OPC attenuated fatty liver through AMPK activation by blunting the SREBP-1 activation. Furthermore, oxidative stress can greatly improve the activity of the AMPK signaling system. Our studies showed that OPC up-regulated ethanol-induced levels of AMPK phosphorylation. However, we cannot confirm that OPC alleviated liver damage through AMPK activation by inducing oxidative stress.

In conclusion, OPC exhibited an excellent protective effect against alcohol-induced liver injury and fatty liver disease, with such shielding action accomplished via reducing the oxidative stress, inflammatory cytokine production and liver steatosis. OPC should be considered to be developed as a new candidate for the treatment of alcohol-induced liver injury in the future. Of course, its mechanisms, targets and clinical applications need to be further explored in future investigations.

Disclosure statement

The authors have nothing to disclose.

Conflict of interest

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.153.

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