Food & Medicine Homology

ISSN 3006-6867 (print); 3006-4252 (online)
https://doi.org/10.26599/FMH.2026.9420096

Research Article

Lipid-clearing tea aqueous extract alleviates dyslipidaemia *in vivo* and *in vitro* by activating the AMPK pathway

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Received: 20 October 2024 / **Revised:** 2 December 2024 / **Accepted:** 4 December 2024

Abstract: Diseases such as dyslipidemia caused by lipid accumulation have gradually become one of the most damaging underlying threats to human health. Lipid Clearing Tea (LCT) is a kind of functional food formulated with four homology of medicine and food, and it includes hawthorn, cassia seed, chrysanthemum, and green tea, containing a variety of natural hypolipidemic functional and active ingredients. The purpose of this study is to investigate the effect of Lipid-clearing Tea Aqueous Extract (LCTAE) on alleviating dyslipidemia in an *in vivo* rat model induced by high-fat diet (HFD), and in an *in vitro* HepG2 cell model induced by oleic acid (OA) through examining biochemical indices, pathologic features, and protein expressions. The result of experiments *in vivo* showed that LCTAE ameliorated the abnormalities of biochemical indices in the serum, the accumulation of lipids in liver tissues, and reversed the expression of AMPK/FAS pathway proteins in rats. The results of *in vitro* experiments showed that LCTAE could significantly reduce the levels of TC and TG in OA-induced HepG2 cells compared with the OA group ($P < 0.01$), decrease intracellular lipid accumulation, and activate the activity of phosphorylated AMPK, which could regulate the AMPK/SREBP1c/FAS/ACC pathway and improve dyslipidemia. The above results indicate that LCTAE is beneficial to the improvement of dyslipidemia; thus, providing a theoretical basis for the development of functional food formulated with LCT and extending possibilities for the prevention and treatment of dyslipidemia and related metabolic diseases.

Keywords: lipid-clearing tea; aqueous extract; dyslipidemia; AMPK pathway; *in vivo* and *in vitro*

Citation: Ren W. J., Liu Z. X., Wang T. Q., et al. Lipid-clearing tea aqueous extract alleviates dyslipidaemia *in vivo* and *in vitro* by activating the AMPK pathway. *Food & Medicine Homology*, 2026, 3: 9420096. https://doi.org/10.26599/FMH.2026.9420096

1 Introduction

With progressing social and economic development, people's living standards are becoming increasingly higher. With this, people's lifestyle of consuming fat-rich food, sitting long hours at home and in the office, and not exercising, subsequently destroys the balance of lipid metabolism and causes lipid metabolism disorders in body systems[1-3]. Lipid metabolism disorders could lead to a series of chronic diseases such as hyperlipidemia, hypertension, nephropathy, obesity, diabetes mellitus, and atherosclerosis[4-8]. The current indication of dyslipidemia in Chinese people is mainly hyperlipidemia, and it is one of the most common disorders of lipid metabolism, mainly characterized by increased levels of triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and decreased levels of high-density lipoprotein cholesterol (HDL-C)[9, 10]. In recent years, hyperlipidemia has also been listed as one of the most alarming risk factors for coronary heart disease (CHD) and other cardiovascular diseases (CVD). As one of the major causes of high mortality rates in the world, CVD is extremely harmful to human health and has become a non-negligible health problem[11-14]. Therefore, the prevention and treatment of hyperlipidemia has become critical in China and throughout the world.

Lipid-clearing tea is a functional food made from hawthorn (*Crataegus pinnatifida* Bunge), cassia (*Cassia semen*), chrysanthemum (*Chrysanthemum morifolium* Ramat.), and green tea through the processes of pulverization, mixing, granulation, and drying. Hawthorn, a deciduous tree plant of the genus Hawthorn in the family Rosaceae, is a kind of traditional medicine and food homologous plant, which is mainly distributed in Northeast China, Inner Mongolia, Shaanxi, Jiangsu, and other provinces. The plant mainly contains chemical compounds such as flavonoids, terpenoids and organic acid compounds[15-17]. According to the records of "*The Classic of Materia Medica*" and the Pharmacopoeia of the People's Republic of China, hawthorn has efficacy in digesting food and strengthening the stomach, promoting circulation of qi and dispersing blood stasis, lowering blood lipids, and preventing atherosclerosis[18-21]. Hawthorn has

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been commonly used for dyspepsia caused by inadequate diet since as early as the 13th century. As a natural herb, it is widely used in the protection and treatment of CVD with its multiple bioactive properties[22]. Based on previous studies, the efficacy of hawthorn and its aqueous extracts in regulating lipid metabolism and improving dyslipidemia has been widely investigated[23-26]. Cassia seed, the dried mature seeds of the Cassia obtusifolia (*Senna obtusifolia* L.) or Cassia tora (*Senna tora* L.), is mainly produced in Anhui, Jiangsu, Guangxi, Sichuan, Zhejiang, and Guangdong in China. It contains chemical constituents such as anthraquinones, naphthopyranones and fatty acids, and has been widely used in food and traditional Chinese medicine in China[27,28]. Furthermore, cassia seed is a kind of Chinese herbal medicine widely used in clinical practice, clearing heat, brightening eyes, moistening intestine, and purging, etc. It also has the pharmacological properties of lowering blood pressure and blood lipids, thus preventing diabetes[29-31]. Several studies have also reported that the extract of cassia seed can improve dyslipidemia in the body and alleviate obesity and other related diseases caused by HFD[28,32,33]. Aurantio-obtusin (AO) is an active ingredient in the aqueous extract of cassia seed, and it has been reported that AO may regulate the expression of proteins involved in lipid metabolism and reduce the accumulation of lipids by activating the AMPK and insulin signaling pathway[34,35]. Chrysanthemum, a perennial herb of the genus Chrysanthemum in the family of Asteraceae, native to China and now found in various countries around the world, is one of the ten most famous traditional Chinese flowers. Chrysanthemum has the efficacy of dispersing wind and clearing heat, calming the liver, and brightening the eyes, as well as antimicrobial, antitumor, and cardiovascular system protecting properties[36,37]. In China, in addition to its high ornamental value, chrysanthemum is often used for food, tea and medicine. The kind of chrysanthemum used for nutritional health care is called "functional chrysanthemum", which is rich in flavonoids, chlorogenic acid, and a variety of amino acids and other chemical components. These nutrients have anti-inflammatory, antioxidant, blood glucose lowering and blood lipids regulating effects, and it is clinically effective in hypertension, hyperlipidemia, coronary heart disease, and other diseases[38-41]. Both hot water and ethanol extracts of Jinsi Huangju (Golden Royal Chrysanthemum) have been shown to have hypolipidemic activity, reducing lipid accumulation and ameliorating non-alcoholic fatty liver disease (NAFLD) associated liver injury and steatosis through activation of the AMPK signaling pathway[42]. Imperial Chrysanthemum (IC) was found to have a variety of natural active ingredients that help to regulate dyslipidemia and antioxidant status in humans after a HFD meal[43]. Green tea, a shrub or small tree plant of the genus Camellia in the family Camelliaceae, is native to China and has extremely rich varieties and economic value. As a type of unfermented tea, it greatly retains the natural substances in the fresh leaves. The main pharmacological components include tea polyphenols (especially catechins), caffeine, lipopolysaccharides, and theanine[44,45]. Reports have indicated that green tea may positively affect chronic diseases such as CVD, cancer, diabetes, hyperlipidemia, and other chronic diseases due to its anti-oxidant, anticancer, anticardiovascular disease, antihyperglycemic, and lipid metabolism regulating properties[46-48]. Studies have shown that *in vivo* and *in vitro*, green tea can regulate glucose-lipid metabolism to varying degrees, improve the health of the body, and can be used to assist in the prevention and treatment of diabetes mellitus, hyperlipidemia, and CVD[49-52].

For a long time, specialists have gradually realized the complex pathogenesis of CVD and other chronic diseases, including hyperlipidemia, since active drugs with a single composition are clinically much less effective and has risks of drug tolerance and potential side effects[53]. In contrast, the synergistic effect between multiple herbs or ingredients in a Chinese medicine formulae or compositions of Chinese medicines have been used in China for thousands of years and have gradually become popular and accepted in Western medicine. As of now, the effectiveness of this modality has been extensively demonstrated by *in vivo* experiments and clinical studies[54]. The synergistic or complementary effect of two or more herbs or drugs formulated together is a principle followed in Chinese medicine, in other words, maximizing the therapeutic effect while minimizing the adverse effects[55]. According to existing reports, the four components of LCT have different efficacy in hypolipidemic or regulating dyslipidemia. To improve the therapeutic effect of dyslipidemia and reduce drug resistance, hawthorn, cassia seeds, chrysanthemums, and green tea were formulated together according to the principles of Chinese traditional medicine to investigate the effect on blood lipids. The prevention and treatment of dyslipidemia by natural functional foods such as this one, which formulates traditional Chinese herbs and medicinal foods together, is poorly studied, and the potential mechanisms linking natural functional foods to lipid metabolism have not been clearly discussed. Therefore, the aim of this study was to investigate the role and potential mechanisms of LCT aqueous extract (LCTAE) in ameliorating dyslipidemia *in vitro* and *in vivo*.

In our study, hot water extraction of LCT particles was performed to investigate the role of LCTAE in improving dyslipidemia using an *in vivo* SD rat hyperlipidemia model and an *in vitro* HepG2 cell lipid accumulation model through detecting lipid levels *in vitro* and *in vivo*, evaluating pathological changes in tissues and cells, and verifying the expression levels of relevant lipid metabolism at the protein level. This study provides a theoretical basis for the prevention and treatment of dyslipidemia and related metabolic diseases using functional food prepared by LCT.

2 Materials and Methods

2.1 Materials

LCT granules were supplied by Beijing Tongrentang Technology Development Co., Ltd. (Beijing, China, Lot No. 21247388). Oleic acid was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China, Lot#K2208593). Basal medium/DMEM/F12, penicillin-streptomycin, PBS, Trypsin 1:250, dimethyl sulfoxide (DMSO), 4% histiocyte fixative solution, and BCA protein assay kit were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). Human hepatocellular carcinoma cell line HepG2 cells were obtained from the laboratory of Prof. Junxia Guo at Beijing Union University (Beijing, China). TG and TC detection kits were purchased from Nanjing Jiancheng Bioengineering Research Institute Co., Ltd. (Nanjing, China). Fetal bovine serum was purchased from Zhejiang Tianhang Biotechnology Co., Ltd. (Zhejiang, China). Western and IP Cell lysate and (ORO) staining kits were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Primary antibodies anti-AMPKα antibody (1:1000, #5831), anti-Phospho-AMPKα antibody (1:1000, #2535), anti-ACC antibody (1:1000, #3676), anti-

FAS antibody (1:1000, #3661), and anti-β-Actin antibody (1:1000, #4970) were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). Primary antibody anti-SREBP1c antibody (1:2000, #25h1508) was purchased from Affinity Biosciences Ltd. (Jiangsu, China). All analytical grade chemical reagents were purchased from Tianjin Zhiyuan Chemical Reagent Co., Ltd. (Tianjin, China).

2.2 Preparation of aqueous extract of LCT

The specification of LCT granules was 2 g/bag. 50 bags of LCT granules were taken and 10 times the volume of sterile water was added and immersed for 30 min at 80 °C atmospheric pressure. After immersing and extracting twice, the combined extracts mixture was concentrated to 100 mL where 1 mL was equivalent to 1 g of bagged tea contents, and stored at 4 °C.

2.3 Experimental Animal Design

60 SPF-grade male SD rats (weight 1401 ~ 1160 g) purchased from Beijing HFK Bioscience Co., Ltd. were kept in the animal room of the Health Food Functional Testing Center, College of Applied Arts and Sciences, Beijing Union University, with the temperature of the rearing room at (20 ± 2) °C, the relative humidity at 45% ~ 60%, and the 12 h day/night cycle. After 1 week of adaptive feeding, they were randomly divided into 5 groups (n = 12): normal control group (Con), HFD group, LCTAE low-dose group (LCTAE-L, 0.25 g/kgBW), LCTAE medium-dose group (LCTAE-M, 0.50 g/kgBW), and LCTAE high-dose group (LCTAE-H, 1.50 g/kgBW). Con group was given normal feed and HFD, LCTAE-L, LCTAE-M, and LCTAE-H were given HFD feeds (20% sucrose, 15% lard, 1.2% cholesterol, and 0.2% sodium cholate added to the normal feed). All feeds were provided by Beijing HFK Bioscience Co., Ltd. (Beijing, China, SCXK (Beijing) 2019-0008). All rats had free access to food and water, and equal volumes of sterile water were given by gavage in the Con and HFD groups. At the end of the experiment, rats were anesthetized by intraperitoneal injection of 2% sodium pentobarbital (2.5 mL/kg BW). Blood was obtained from the abdominal aorta, and liver tissues were obtained by dissection for subsequent pathological analysis. All experimental protocols were in accordance with the program Beijing Key Laboratory of Bioactive Substances and Functional Foods, Beijing Union University (SYXK (Beijing) 2017-0038).

2.4 Determination of biochemical indicators

The whole blood was allowed to stand for 30 min, in which the serum was separated by centrifugation at 3,500 r/min for 15 min. The serum levels of TG, TC, HDL-C, and LDL-C were detected using a BECKMAN AU480 automatic biochemistry analyzer (Beckman Coulter Ltd., Brea, California, USA) according to the manufacturer's instructions.

2.5 Histopathological status of liver was observed by H&E staining

Liver tissues fixed in neutral universal tissue fixative solution (Wuhan Servicebio Technology Co., Ltd., Wuhan, China) were stained with hematoxylin-eosin (H&E). Observations were taken using a Nikon TE2000-U microscope (Nikon Instruments Inc., Shanghai, China).

2.6 ORO staining of liver tissue

The liver tissues in the fixative solution were sectioned using a frozen microtome and stained with ORO. The sections were photographed using a Nikon TE2000-U microscope and analyzed using Image-Pro Plus 6.0.

2.7 Immunohistochemistry

The liver tissue in the fixative solution was removed to make a wax block, which was then subjected to immunohistochemistry. The wax blocks were sliced for about 5 μm thick, baked for 60 min, deparaffinized with xylene and ethanol, followed by thermal repair of the antigen using an antigen repair solution. The endogenous peroxidase was removed using 3% H_2O_2, the slices were closed with 5% BSA in the circles drawn by immunohistochemistry pens and incubated with primary and secondary antibodies. The color was developed using a DAB kit (Aibixin Biotechnology Co., Ltd, Shanghai, China), and the nuclei were stained with hematoxylin. The slices were dehydrated with ethanol and xylene and sealed.

2.8 Cell culture and treatment

2.8.1 Cell culture

HepG2 cells were cultured in DMEM medium, containing 10% fetal bovine serum, and 1% dual antibody. Logarithmic growth phase cells were taken for experiments.

2.8.2 Preparation of reagent

Preparation of oleic acid: Using a pipette, 100 μL oleic acid was dissolved in 1.475 mL anhydrous ethanol. Through vortexing and shaking, a fully dissolved and mixed 200 mmol/L reserve solution was obtained. It was stored at −20 °C away from light and diluted to the required concentration according to the experimental needs.

Preparation of LCTAE: The LCTAE concentrate was freeze-dried (Box freeze-dryer, Jinan Junde Instrument Co., Ltd, Shandong, China) to obtain LCTAE solids. The LCTAE solids were prepared into a 1 mg/mL LCTAE stock solution and stored at 4 °C. According to the experimental needs, stock solution was diluted to required concentration.

2.8.3 Determination of cell viability

The cell viability was measured by MTT assay. HepG2 cells in the logarithmic stage were re-inoculated into 96-well plates with 100 μL per well and 10,000 cells per well. 100 μL sterile PBS was added to each well at the edge, and the cells were placed in 5% CO_2 in an incubator at 37 °C for 12 h. (SANYO Electric Co., Ltd., Osaka, Japan). Different concentrations of reagents were added to the corresponding wells and placed in the incubator for 24 h. Subsequently, 10 μL of prepared 0.5 mg/mL MTT solution was added to each well and placed in the incubator for 4 h. Centrifugation was performed at 4 °C, 1,000 r/min for 10 min and the supernatant was discarded. Finally, 100 μL of DMSO was added to each well and shaken at low speed for 10 min. The OD values were measured at 570 nm using a microplate reader with a reference wavelength of 630 nm and calculated with the formula:

Cell survival rate (%) = (experimental group OD/control group OD) × 100%

2.8.4 Cell treatment

HepG2 cells were inoculated in 6-well plates for culturing. The oleic acid stock solution was diluted to a final concentration of 0.003,125, 0.006,25, 0.012,5, 0.025, 0.05 mmol/L. The accumulation of intracellular lipid droplets was observed by incubation for 6 h, 12 h, 24 h, and 48 h, respectively. The LCTAE stock solution was diluted to final concentrations of 5, 15, 45

μg/mL, and incubated for 12 h, 24 h, and 48 h to observe the improvement of intracellular droplet accumulation.

2.9 Determination of TG and TC levels in HepG2 cells

At the end of the 6-well plate culture, the medium was discarded, and the cells were collected by trypsin digestion. Centrifugation was performed at 1,000 r/min for 10 min at 4 °C. Cells were lysed by adding isopropanol and using an ultrasonic cell crusher (Ningbo Scientz Biotechnology Co., Ltd, Zhejiang, China). After cooling on ice for 30 min, the levels of TG and TC were determined according to the instructions of the corresponding kits.

2.10 ORO staining of HepG2 cell

For treated cells, the medium was discarded, washed with pre-cooled PBS, and fixed using 4% tissue cell fixative solution. The HepG2 cells were stained according to the instructions of ORO kit and finally observed and photographed under the microscope.

2.11 Western blot

Tissue: Took the appropriate size of liver tissue, added 10 times the volume of lysate, and broke the tissue at 4 °C by using high-speed and low-temperature tissue grinding instrument (Wuhan Servicebio Technology Co., Ltd., Wuhan, China). After cooling on ice for 30 min and centrifuge at 4 °C, 12,000 r/min for 20 min, the supernatant was collected, and the protein concentration was determined using a BCA kit.

Cells: The medium was discarded and washed with pre-cooled PBS. After an appropriate amount of lysate was added, the cells were cooled on ice for 20 min, collected with a cell scraper, transferred to a centrifuge tube, and ultrasonically crushed. Centrifugation was performed at 4 °C, 12,000 r/min for 20 min. The supernatant was collected, protein concentration was determined using a BCA kit. After the samples were tested on SDS-PAGE gels, it was transferred onto PVDF membranes and were respectively incubated with the corresponding primary antibodies at 4 °C overnight, and with Horseradish peroxidase labeled Goat anti-Rabbit IgG (Beyotime Biotechnology Co., Ltd., Shanghai, China) for 2 h at room temperature.

2.12 Statistical Analysis

The experimental data were analyzed with analysis of variance (ANOVA) and paired t-tests with SPSS 26.0 software and plotted for graphing with GraphPad Prism 9.4.1. All data are expressed as mean ± standard deviation. A significant difference is indicated at $P < 0.05$ and a highly significant difference at $P < 0.01$, indicating statistical significance.

3 Results

3.1 Effect of LCTAE on body weight in rats

Following the administration of LCTAE by gavage, the differences in body weight were compared between the different groups (Table 1). After 14 days of administration of HFD, the rats were randomly divided into HFD, LCTAE-L, LCTAE-M, and LCTAE-H groups based on the level of serum total cholesterol, and there was no significant difference in body weight between the groups. After 35 days of LCTAE administration by gavage, the body weight of rats in the HFD group increased significantly ($P < 0.01$) compared to the Con group. However, there was no significant difference between the LCTAE dose groups compared to the HFD group.

Table 1 The weight of rats

Rat Group	Body	
	Before LCTAE Weight (g)	After LCTAE Weight (g)
Con	336.9 ± 24.2	449.0 ± 18.5
HFD	354.9 ± 23.3	477.2 ± 23.5$^{\#\#}$
LCTAE-L	343.3 ± 22.4	472.0 ± 25.2
LCTAE-M	337.0 ± 28.2	462.1 ± 31.8
LCTAE-H	352.0 ± 17.9	480.4 ± 21.8

Note: Compared with the Con group, $^{\#\#}P < 0.01$.

3.2 Effects of LCTAE on HFD-induced dyslipidemia in rats

To determine the effect of LCTAE on blood lipids in HFD-induced model rats, serum levels of TC, TG, HDL-C, and LDL-C were determined, and the pathology of liver tissue was observed using H&E staining (Fig. 1). At this point, as shown in Figs. 1A, C, G, the serum levels of TC, TG, and LDL-C in rats induced by HFD all significantly increased ($P < 0.01$) by 1.84, 1.48, and 5.5-fold, and the level of HDL-C was reduced by 32.73% compared with the Con group prior to gavage administration of LCTAE (Fig. 1E), indicating the success of modeling as it is consistent with the results of previous studies[9,10]. In addition, Figs. 1B, D, H showed a significant improvement ($P < 0.01$) in the blood lipid status of the LCTAE-H group compared to the HFD, with a reduction of 22.47% in the level of TC, 18.85% in the level of TG, and 33.76% in the level of LDL-C. However, serum HDL-C levels did not lower significantly in any of the L, M, or H dose LCTAE groups compared to the HFD group. It is noted that the blood lipid status of rats in the LCTAE-H group was significantly reversed. The histological evaluation of the liver demonstrated (Fig. 1I) that the Con group showed normal liver tissue condition. HFD group had obvious hepatic histopathology, such as massive hepatocellular steatosis and enlargement, which was also accompanied by balloon-like degeneration and blurred cell borders. However, after the administration of LCTAE by gavage, the lesions of liver tissues improved significantly with increasing dose, especially the hepatocytes in the LCTAE-H group showed a better condition with more complete round hepatocyte morphology. This reveals that the ameliorative effect of LCTAE on hepatic steatosis is enhanced with increasing dose.

3.3 Effects of LCTAE on HFD-induced lipid accumulation in rat liver

ORO staining of liver tissue (Fig. 2). As shown in Fig. 2A, the liver tissue cells in the Con group were in a normal morphology with almost no red lipid droplets present. The success of the modeling is also illustrated by the presence of large red lipid droplets in the HFD group induced by a HFD. The positive area was significantly increased ($P < 0.01$) in the HFD group compared to the Con group (Fig. 2B). Different from the HFD group, the positive area was reduced in the LCTAE-L ($P < 0.05$), LCTAE-M ($P < 0.01$) and LCTAE-H ($P < 0.01$) groups. Overall, the data demonstrates that LCTAE significantly improves lipid accumulation in liver tissue as the dose increases.

3.4 Effects of LCTAE on the expression of lipid metabolism-related proteins in rat liver tissue

Western blotting and immunohistochemical techniques were

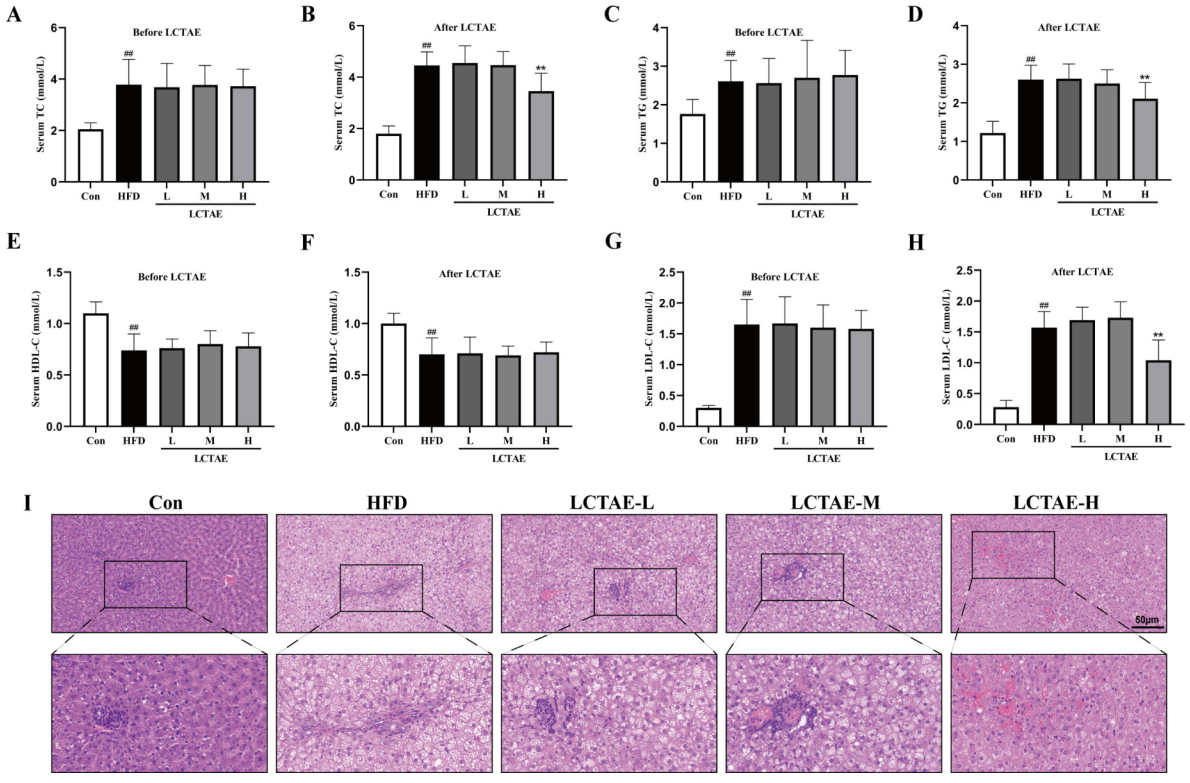

Figure 1 Effect of LCTAE on HFD-induced serum lipid levels in rats. (A) Serum TC levels before LCTAE. (B) Serum TC levels after LCTAE. (C) Serum TG levels before LCTAE. (D) Serum TG levels after LCTAE. (E) Serum HDL-C levels before LCTAE. (F) Serum HDL-C levels after LCTAE. (G) Serum LDL-C levels before LCTAE. (H) Serum LDL-C levels after LCTAE. (I) Representative images of H&E staining sections of liver tissue (scale bar 50 μm). Compared with the Con group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with the HFD group, $^{*}P < 0.05$, $^{**}P < 0.01$.

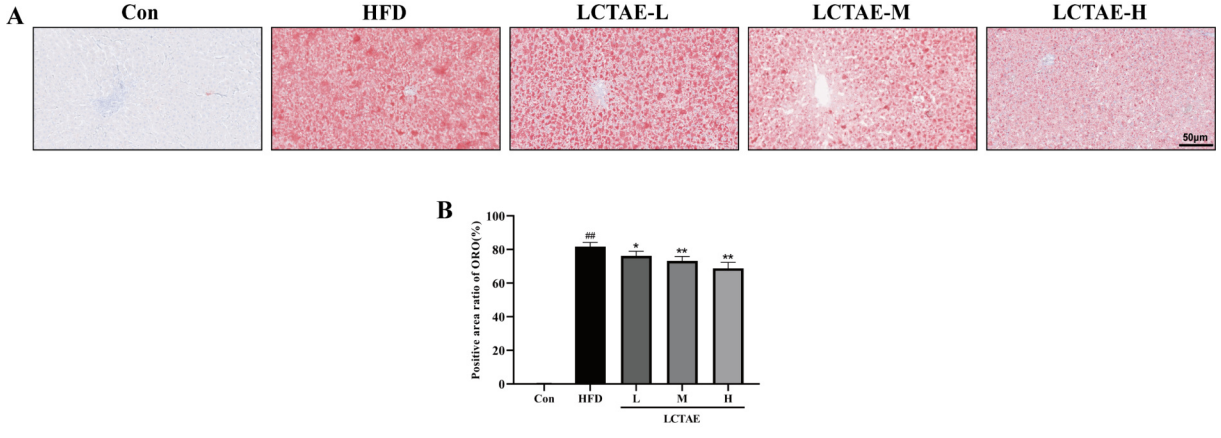

Figure 2 Effect of LCTAE on HFD-induced lipid accumulation in rats. (A) Representative images of ORO staining sections of liver tissue (scale bar 50 μm). (B) Positive area ratio of ORO staining. Compared with the Con group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with the HFD group, $^{*}P < 0.05$, $^{**}P < 0.01$.

used to analyze the regulation of lipid metabolism-related proteins in liver tissue by LCTAE (Fig. 3). As shown in Fig. 3A, HFD feeding significantly decreased the level of phosphorylated AMPK and increased the expression level of FAS in the liver ($P < 0.05$, $P < 0.01$). It was evident that different concentrations of LCTAE activated phosphorylated AMPK and increased the expression level of phosphorylated AMPK, especially in the LCTAE-M and LCTAE-H groups, which significantly increased by 3.84-fold and 19.76-fold ($P < 0.01$). The LCTAE-H group significantly reduced the expression level of FAS ($P < 0.05$). Adipogenesis-related protein (SREBP1c) was increased by HFD feeding as shown in Fig. 3B. With the increase of LCTAE concentration, the generation of SREBP1c was inhibited and the expression level was downregulated. These results suggest that LCTAE exerts beneficial metabolic regulation of dyslipidemia by regulating the expression of AMPK, SREBP1c, FAS.

3.5 Effects of OA and LCTAE treatments on the survival of HepG2 cells

MTT assay was used to detect the effect of OA and LCTAE treatments on HepG2 cell survival (Fig. 4). As shown in Fig. 4A, the effect of OA on cell survival was examined. The survival of HepG2 cells was not only unaffected when the concentration of OA was less than 0.025 mmol/L but also promoted a certain degree of cell proliferation. However, when the OA concentration reached 0.05 mmol/L, the activity of the cells was significantly inhibited ($P < 0.01$). The effect of anhydrous ethanol on cell viability at the maximum viability concentration was tested and

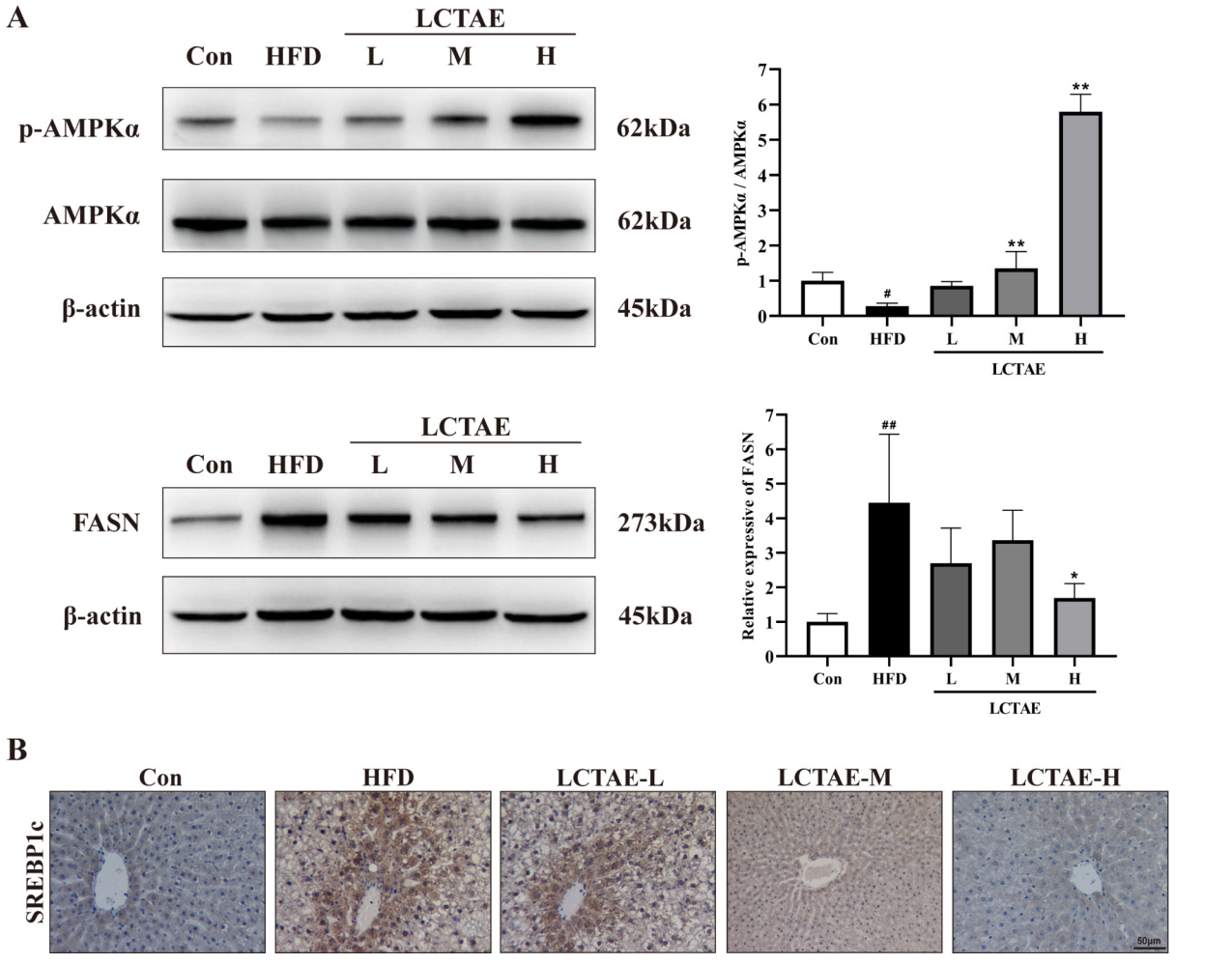

Figure 3 Effects of LCTAE on protein expression related to lipid metabolism in liver. (A) Relative protein expression of p-AMPKα / AMPKα, FASN. (B) Representative image of immunohistochemistry of SREBP1c in liver sections (scale bar 50 μm). Compared with the Con group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with the HFD group, $^{*}P < 0.05$, $^{**}P < 0.01$.

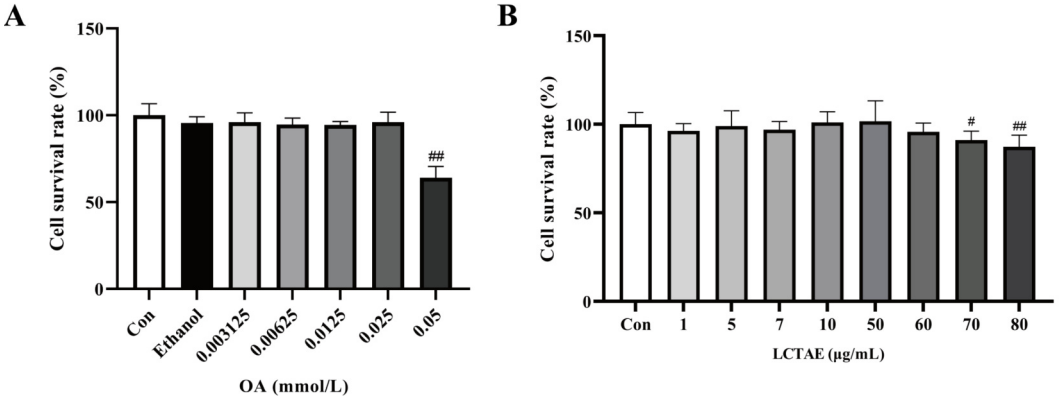

Figure 4 The survival rate of HepG2 cells treated with OA and LCTAE. (A) The survival rate of HepG2 cells treated with OA. (B) The survival rate of HepG2 cells treated with LCTAE. Compared with the Con group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

the results show no statistical difference ($P > 0.05$). Therefore, follow-up experiments using the concentrations above. Illustrated in Fig. 4B, the effect of LCTAE on HepG2 cell survival, it is visible that there was an insignificant decrease in cell survival when the concentration of LCTAE was 60 μg/mL ($P > 0.05$). Cell survival was inhibited by both 70 μg/mL ($P < 0.05$) and 80 μg/mL ($P < 0.01$). For subsequent experiments, the concentration of LCTAE was designed to be 5, 15, 45 μg/mL.

3.6 Effects of LCTAE on OA-induced lipid accumulation in HepG2 cells

The results of the assay according to the kit instructions are shown in Fig. 5. The levels of TC and TG in HepG2 cells were

determined to evaluate the role of OA and LCTAE in lipid accumulation. As shown in Figs. 5A, B, the levels of TC and TG in HepG2 cells were increasing with time when the OA concentration did not exceed 0.025 mmol/L (especially at the 24 h and 48 h time points, $P < 0.05$, $P < 0.01$). It is noted that the TG level of 0.025 mmol/L at 48 h was rather reduced compared with 24 h, and the TC level at 48 h was not significantly increased, which could be due to cell senescence caused by the long periods of culturing. In addition, consistent with the results of MTT, 0.05 mmol/L limited the survival rate of cells and affected the increase of TC and, especially, TG levels. Therefore, according to the above results, HepG2 cells that were treated with 0.025 mmol/L OA for 24 h set the condition for establishing follow-up experimental models. Figs. 5C, D showed that the concentration of 5, 15 μg/mL of LCTAE decreased with time, but with little difference in TC levels ($P > 0.05$). At 24 h, the level of TG decreased significantly ($P < 0.01$). The 45 μg/mL treatment of HepG2 cells resulted in a significant reversal of intracellular lipid accumulation ($P < 0.01$). Since there was no significant difference in both TC and TG levels at 48 h compared to 24 h, an 24 h 45 μg/mL treatment was used for subsequent experiments. As shown by the results of ORO staining of HepG2 cells (Figs. 5E, F), a small number of red lipid droplets appeared in the Con group, and the red lipid droplets in the cells increased significantly after OA treatment ($P < 0.01$), which further proved the successful establishment of the cell model. There was a significant improvement after treatment with 45 μg/mL of LCTAE, which significantly reduced intracellular lipid droplets ($P < 0.01$). These results indicate that LCTAE treatment notably reversed OA-induced lipid accumulation in HepG2 cells.

3.7 Effects of LCTAE on OA-induced expression of lipid metabolism-related proteins in HepG2 cells

LCTAE on the expression of HepG2 cell related lipid metabolism proteins (Fig. 6). HepG2 cells treated with OA significantly downregulated the expression of phosphorylated

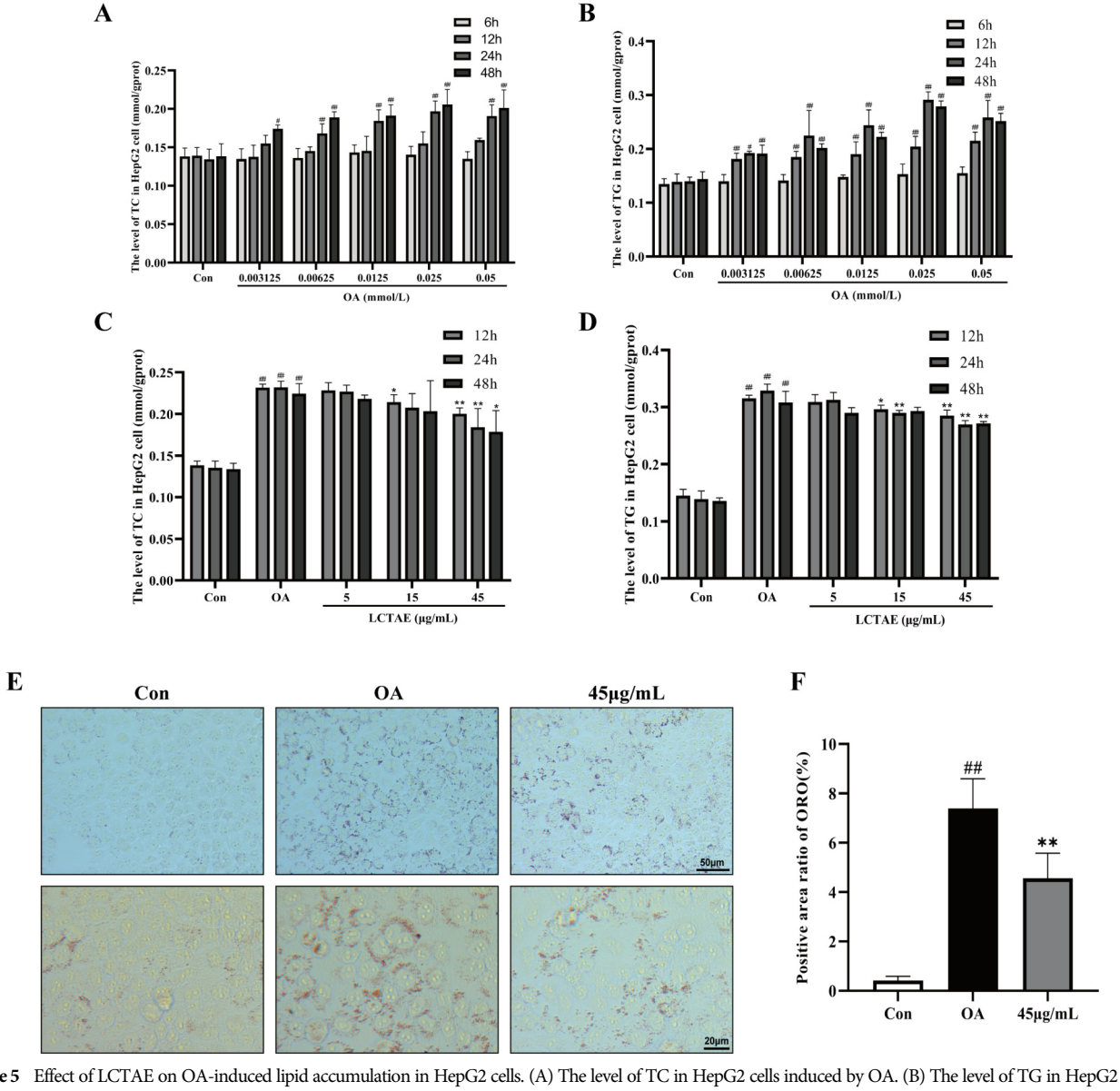

Figure 5 Effect of LCTAE on OA-induced lipid accumulation in HepG2 cells. (A) The level of TC in HepG2 cells induced by OA. (B) The level of TG in HepG2 cells induced by OA. (C) TC level in OA-induced HepG2 cells treated with LCTAE. (D) TG level in OA-induced HepG2 cells treated with LCTAE. (E) ORO staining of HepG2 cells induced by OA (scale bar, 50 μm and 20 μm). (F) Intracellular lipid accumulation induced by OA. Compared with the Con group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with the OA group, $^{*}P < 0.05$, $^{**}P < 0.01$.

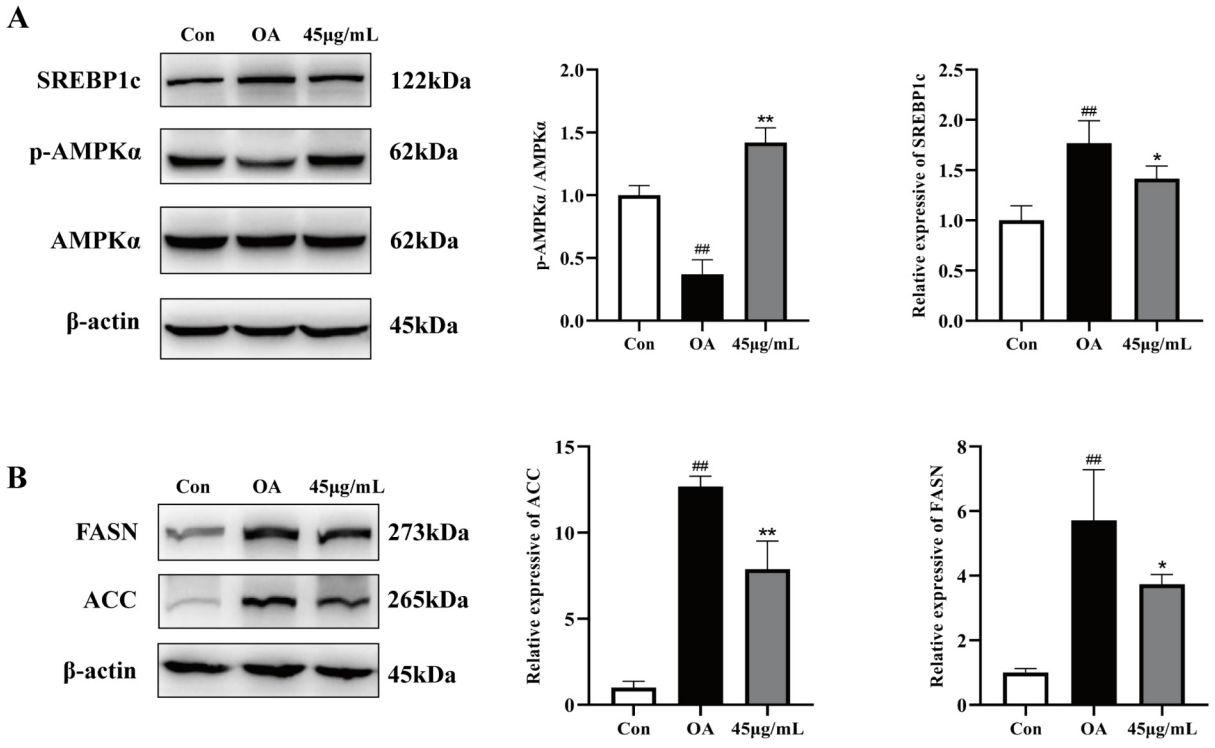

Figure 6 Effect of LCTAE on the expression of related lipid metabolism proteins in HepG2 cells. (A) Relative protein expression of p-AMPKα / AMPKα, SREBP1c. (B) Relative protein expression of ACC, FASN. Compared with the Con group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; Compared with the OA group, $^{*}P < 0.05$, $^{**}P < 0.01$.

AMPK ($P < 0.01$) and significantly upregulated the expression of SREBP1c, ACC, and FAS ($P < 0.01$). However, protein expression levels were reversed after 45 μg/mL LCTAE treatment, and the expression of phosphorylated AMPK was up-regulated 2.86-fold, whereas the levels of SREBP1c, ACC, and FAS were down-regulated by 19.82%, 37.8%, and 34.62%, respectively. The data were statistically different compared to the OA group. The above results indicate that LCTAE could reverse OA-induced expression of lipid metabolism-related proteins in HepG2 cells and play a beneficial role in lipid metabolism.

4 Discussion

Metabolic disorders caused by dyslipidemia could lead to the development of hyperlipidemia, which, based on available clinical studies, could directly or indirectly affect the function of the heart, thereby inducing CVD[56]. Interestingly, extant clinical medications for dyslipidemia are often accompanied by drug resistance and side effects. To minimize this effect, due to the complexity of the ingredients and that they are rarely used in isolation, the formulation of substances from the homology of medicine and food as a novel functional food has been widely used in China and is becoming a trend around the world. In addition, the functional improvement of dyslipidemia or disorders of lipid metabolism formed by the homology of medicine and food ingredients has been confirmed by studies. The formulation of hawthorn, wolfberry, bitter melon and silymarin has provided strong evidence in both *in vivo* and *in vitro* experiments demonstrating the potential of the formulation to treat hyperlipidemia[57]. However, there was no statistically significant difference in the effect of the four-ingredient formulation on fat and plasma TG levels in mice, which indicates the limited effect of the formulation. In the present work, the potential of LCTAE to alleviate dyslipidemia was tested by making LCT granules from a mixture of four ingredients, including hawthorn, cassia seed, chrysanthemum, and green tea, which were subjected to hot water extraction. As is known, each of these four ingredients has been shown in studies to alleviate dyslipidemia or disorders of lipid metabolism. There are few studies with potential mechanisms for improving dyslipidemia by formulating these four ingredients. The result of the present study suggests that LCTAE not only reduces lipid accumulation *in vitro* and *in vivo*, but also reduces the expression of SREBP1c, a key transcription factor for fatty acid synthesis, possibly by activating the phosphorylation of AMPK and, thus, inhibiting lipid synthesis and ameliorating dyslipidemia.

In recent decades, growing evidence has linked pathological lipid accumulation to various metabolic diseases and CVD, such as dyslipidemia[11]. HFD feeding significantly increased the body weight of rats compared to the Con group, but there was no statistically significant difference between the LCTAE dose group and the HFD group. Excessive intake of saturated fatty acids from the diet, increased free fatty acid production from catabolism, decreased hepatic β-oxidation or up-regulation of fat synthesis from scratch are all thought to cause lipid accumulation, which can lead to hepatic steatosis. Alterations in serum levels of TG, TC, LDL-C, and HDL-C serve as the main features of dyslipidemia and are one of the main entry points to improve and alleviate dyslipidemia nowadays[58]. It has been reported that extracts of hawthorn and cassia seeds either alone or in combination with lotus leaves attenuated hepatic lipid accumulation and steatosis. However, the level of TG in rat serum may not be significantly altered under the use of a mixture of the three ingredients[59]. Oleic acid, as a monounsaturated fatty acid, is readily absorbed by cells and results in the accumulation of lipid droplets, leading to the increased levels of TC and TG. The OA-induced HepG2 cell model is a suitable *in vitro* model for conventional hepatic steatosis, showing the pathological features of fatty liver[60]. In the present study, TC and TG levels significantly reduced in OA-

induced HepG2 cells, indicating that LCTAE is beneficial for the reduction of lipid accumulation. Moreover, LCTAE reduced lipid accumulation in the serum and liver, alleviating hepatic steatosis in the HFD-induced rat model.

The liver is a central organ for complex signaling and lipid metabolism[61]. According to available studies, HFD can cause lipid accumulation, exacerbate histopathology and lipid metabolism disorders in the liver, and ultimately lead to dyslipidemia and other related metabolic diseases[62]. Therefore, in the present study, to further confirm the role of LCTAE in lipid metabolism, Western blot and immunohistochemistry were used to analyze and quantify the expression of lipid metabolism-related proteins in liver and cells. AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that exists as a heterotrimeric complex consisting of an alpha-catalytic subunit, a beta-regulatory subunit, and a gamma-regulatory subunit. AMPK is a key molecule in the regulation of energy metabolism and can be activated by increased intracellular AMP/ATP, which regulates gene transcription in tissues such as liver and adipose to modulate lipid metabolism[63]. It was confirmed that activation of AMPK effectively inhibits lipid accumulation and suppresses SREBP1c-mediated hepatic lipogenesis[64]. Sterol regulatory element binding proteins (SREBPs) are key transcriptional regulators of lipid metabolism, inducing the expression of genes involved in cholesterol synthesis and maintenance of lipid homeostasis. There are three subtypes of SREBPs protein in mammalian cells: SREBP-1a, -1c, and -2, of which SREBP1c mainly controls fatty acid synthesis. The protein also regulates the expression of key enzymes of fatty acid synthesis in its downstream target genes, including fatty acid synthase (FAS) and acetyl coenzyme A carboxylase (ACC). As expected, HFD induction and oleic acid induction significantly reduced the expression of phosphorylated AMPK and inhibited the activity of phosphorylated AMPK, which increased the expression of SREBP1c, resulting in lipid accumulation *in vitro* and *in vivo*. However, LCTAE reduced the level of the transcription factor SREBP1c by activating the overexpression of phosphorylated AMPK and alleviated the dyslipidemia caused by lipid accumulation. ACC is the rate-limiting enzyme for fatty acid synthesis from scratch and is responsible for catalyzing the formation of malonyl coenzyme A from acetyl coenzyme A, which is then used as a substrate for fatty acid synthesis. FAS is also responsible for catalyzing the synthesis of fatty acids. Many studies have shown that activation of the AMPK signaling pathway can down-regulate adipogenic genes (SREBP1c, FAS, ACC) to inhibit fatty acid and cholesterol synthesis, thereby reducing lipid accumulation and alleviating or ameliorating diseases such as dyslipidemia in the body[65]. In the present study, LCTAE improved dyslipidemia by reducing lipid accumulation through the AMPK/SREBP1c/FAS pathway in the HFD-induced rat model. In addition, in the OA-induced HepG2 cell model, LCTAE not only exhibited the same underlying mechanism of the AMPK/FAS signaling pathway as the animal model, but also found that the high expression of phosphorylated AMPK suppressed the expression level of ACC. The above results confirmed our previous speculation that LCTAE activated the AMPK signaling pathway and exerted an ameliorative effect on dyslipidemia through AMPK/SREBP1c/FAS.

In order to avoid the adverse effects of long-term use of conventional drugs for chronic diseases such as dyslipidemia, the use of natural products as alternatives for the prevention and treatment of related metabolic diseases is crucial. In this study, the ameliorative effects, and potential mechanisms of LCTAE formulated according to the concepts of traditional Chinese medicine on dyslipidemia *in vitro* and *in vivo* were investigated at multiple levels using animal and cellular models. In detail, high doses of LCTAE exerted optimal effects in both *in vivo* and *in vitro* experiments. This study reveals for the first time the preventive and therapeutic effects of the LCTAE formulated from hawthorn, cassia seed, chrysanthemum, and green tea on dyslipidemia, providing a new perspective on HFD-induced dyslipidemia and the associated metabolic disorders. However, the mechanisms by which LCTAE ameliorates dyslipidemia or lowers blood lipids in the gut-hepatic axis need to be further elucidated and the specific targets where the active ingredients in LCTAE act and the relationship between gut flora-hepatic lipid metabolism in terms of possible interactions need to be explored.

5 Conclusion

In conclusion, the present study demonstrated that LCTAE, a formulation of hawthorn, cassia, chrysanthemum, and green tea, exerted an ameliorative effect on dyslipidemia. LCTAE modulated the levels of biochemical indices in serum, reduced lipid accumulation and steatosis in liver tissue, and regulated the expression levels of AMPK/FAS pathway-related proteins in the liver in the HFD-induced rat model through a dose-dependent effect. LCTAE reduced the levels of TC and TG in the OA-induced HepG2 cell model, as well as intracellular lipid accumulation, and reversed the expression levels of intracellular AMPK/SREBP1c/FAS/ACC lipid metabolism pathway proteins. As such, this finding suggests that LCT formed from the formulation of homology of medicine and food could become a promising future development as a novel functional food for the prevention and treatment of dyslipidemia and related metabolic diseases.

Institutional Review Board Statement

All experimental protocols were approved by the Experimental Animal Ethics Committee of the Health Food Function Testing Center, College of Applied Arts and Sciences, Beijing Union University (20221103).

Author Contributions

Formal analysis, methodology, visualization, writing—original draft preparation, W.R.; methodology and preparation of materials, T.W.; conceptualization, J.Z.; writing—editing, Y.H.; data curation and methodology, X.D.; formal analysis and investigation, Y.L.; supervision and funding acquisition, Q.H.; project administration and funding acquisition, S.Z.; writing—review and editing and supervision, Y.S. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 11975048), the National Key R&D Program-Special Project on Modernization of Traditional Chinese Medicine (No. 2017YFC1702604).

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