Emodin alleviated hyperlipidemia and hepatic lipid metabolism in zebrafish larvae fed a high-cholesterol diet via AMPK/SREBP-2/PCSK9/LDLR signaling pathway

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Research

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Abstract

Background

Emodin (EM) is one of bioactive components extracted from *Rheum palmatum* L. (Dahuang), which possesses numerous pharmacological activities including hypolipidemic effect. However, the potential action of EM on hyperlipidemia (HLP) remains unclear. Here, the therapeutic effect of EM against HLP were investigated.

Methods

In this study, the hypolipidemic properties of EM were evaluated using high-cholesterol diet (HCD)-stimulated zebrafish larvae model. The body weight, body length and body mass index (BMI) was measured. The total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) as well as the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were detected by corresponding assay kits. Tg (*flil: eGFP*) zebrafish were utilized to observe vascular cholesterol accumulation and Tg (*mpx: eGFP*) zebrafish to visualize and quantify neutrophil inflammation. The hepatic lipid deposition and hepatic histopathology were analyzed by Oil red O staining and H&E staining, respectively. Finally, the underlying mechanism of EM were investigated using real-time quantitative PCR (RT-qPCR) analysis to assess the gene levels of adenosine monophosphate-activated protein kinase alpha (AMPKα), sterol regulatory element binding protein 2 (SREBP-2), proprotein convertase subtilisin kexin 9 (PCSK9), low-density lipoprotein receptor (LDLR), 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), adenosine triphosphate binding cassette transporter A1 (ABCA1) and adenosine triphosphate binding cassette transporter G1 (ABCG1).

Results

Our data indicated that EM reduced obesity of zebrafish as evidenced by the decrease in body weight, body length and BMI. EM significantly reduced TC, TG, and LDL-C, and increased HDL-C contents. Moreover, it displayed a prominent inhibitory effect on blood cholesterol accumulation, hepatic lipid accumulation, and neutrophil inflammation in vascular site. Additionally, EM improved the liver function through decreasing ALT and AST levels of zebrafish with HCD-induced hepatosteatosis. Further investigation showed that EM treatment attenuated lipid accumulation via upregulating the expression of AMPKα, LDLR, ABCA1 and ABCG1, and downregulating the expression of SREBP-2, PCSK9 and HMGCR.

Conclusion

To conclude, EM alleviated lipid metabolism disorder symptoms caused by HCD via modulating AMPK/SREBP-2/PCSK9/LDLR pathway in larvae, suggesting that EM may be developed into hypolipidmic agent for treating lipid metabolism related diseases.
With the improvement of life quality and dietary standards, hyperlipidemia (HLP) and other metabolic syndrome are becoming more prevalent (1). HLP is a pathological condition in which there are abnormalities in the levels of lipids in the blood as a result of lipid metabolism dysfunction, which represents the predictive risk factor for cardiovascular and hepatic diseases (2). Substantial evidence has demonstrated that HLP plays a crucial role in driving the development of atherosclerosis, because excessive lipids accumulate in the arterial intima, of which cholesterol exerts the greatest impact (3–5). The liver constitutes the central organ of lipid metabolism involved in lipid digestion, absorption, transportation, decomposition and synthesis. It was been reported that the deposition of lipids in the liver may lead to hepatotoxicity and inflammatory response, thereby exacerbating lipid metabolism disorder (6, 7). Currently, the hypocholesterolemic drug statins are the mainstay for treating HLP, but long-term administration of statins may cause adverse effects, such as gastrointestinal events and musculoskeletal pain (3, 8). Therefore, seeking safe, economical and alternative agents is necessary for the treatment of HLP.

*Rheum palmatum* L. (Dahuang), a common Chinese herb, is skilled in promoting blood circulation and removing blood stasis achieving significant therapeutic effects on atherosclerotic cardiovascular disease and chronic liver diseases (9, 10). Emodin (EM, Fig. 1a), a major bioactive component in Dahuang, exhibits numerous biological activities including anti-inflammatory, antioxidant, lipid-lowering and hepatoprotective effects (11–13). EM attenuated atherosclerotic lesions through enhancing antioxidant capacity and modulating sphingomyelin synthesis in rabbits (14). Another study showed that EM reversed the abnormal total cholesterol (TC), triglyceride (TG) and hepatic steatosis in zebrafish with nonalcoholic fatty liver disease (NAFLD) induced by egg powder (15). Moreover, EM administration inhibited 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), a key enzyme in TC synthesis, in *vitro* experiment (16). However, the effect and underlying mechanism of EM on HLP in *vivo* experiments are still limited.

Compared with mammalian models, the zebrafish (*Danio rerio*), a small freshwater fish, is considered as an emerging and promising tool for drug screening and development due to its unique strengths, including small sizes, transparent embryo, low feeding cost and short experimental period (17). Intriguingly, zebrafish and mammals share similarities in hepatocellular composition, function as well as genetics (18). Moreover, zebrafish is also resemble to human in lipid metabolism, such as lipid absorption in intestine and cholesterol transport mediated by lipoproteins (19, 20). These strengths make them an excellent model utilized to study lipid metabolism-related diseases, including HLP, NAFLD and atherosclerosis (15, 21). Therefore, this study aimed to explore the effect of EM on lipid metabolism in HLP zebrafish fed with high-cholesterol diet (HCD), which provided a theoretical basis for the research on the therapeutic mechanism of HLP and the development and utilization of EM.

**Materials And Methods**

**Chemical and reagents**
EM (98.73% pure) was purchased from Chengdu Must Biotechnology Co., Ltd (Chengdu, China). Cholesterol (purity ≥ 99.0%) and Oil red O dyeing solution were obtained from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). Cholesteryl BODIPY® 542/563-C11 was bought from Thermo Fisher Scientific Co., Ltd (MA, USA). Animal Total RNA Isolation Kit was provided from Foregene Co., Ltd (Chengdu, China). BCA Protein Quantification Kit was provided from Wuhan Servicebio technology Co., Ltd (Wuhan, China). 5×All-In-One RT MasterMix with AccuRT and EvaGreen 2×qPCR MasterMix-No Dye were purchased from Applied Biological Materials Inc (Richomnd, BC, Canada).

**Preparation of high cholesterol diet (HCD) and drug solutions**

Ordinary larval zebrafish feed (Azoo 9 in 1 Artificial Rotifera) was purchased from Taikong Corp. (Taiwan, China) as the normal feed. The feed mainly contained 41.6% crude protein and 5.6% crude fat. The HCD was prepared by mixing a diethyl ether solution of cholesterol with normal feed to get 4% (w/w) cholesterol in the diet after diethyl ether evaporation (22, 23). For the aim of analyzing vascular cholesterol accumulation in zebrafish larvae, both HCD and normal feed were additionally added with 10 µg/g of Cholesteryl BODIPY® 542/563-C11 in the dark (22). Due to the low solubility of EM in water, the drug was solved with DMSO to 1mg/mL and diluted with cultured zebrafish water (less than 0.1% DMSO v/v).

**Animal and experimental groups**

The wild-type AB-line zebrafish, Tg (flil: eGFP) zebrafish with vascular endothelial cells expressing green fluorescent protein (GFP) and Tg (mpx: eGFP) zebrafish with neutrophils expressing GFP were provided by zebrafish laboratory of Pharmacy College, Chengdu University of TCM. All adult zebrafish were raised and maintained in the zebrafish breeding system (circulated water temperature 27.5 ± 1°C, pH 7.2–7.6, electrical conductivity 500–550 microns/cm and a 14 h light/10 h dark cycle). Zebrafish embryos were obtained by naturally mating and cultured at 28°C in a constant temperature incubator. wild-type AB-line or transgenic zebrafish larvae at 5 days post fertilization (dpf) were randomly divided into five groups in 6-well plate. The five groups were prepared as follows: Normal feed as the control group, 4% HCD as the HCD group, 4% HCD plus 0.125 µg/mL EM as the EM-L group, 4% HCD plus 0.25 µg/mL EM as the EM-M group, 4% HCD plus 0.5 µg/mL EM as the EM-H group. All groups were fed equal mass feed 30 mg/day (twice daily for 10 days) followed the schedule (Fig. 1b) and the residual food was removed 1 h after feeding. All research involving larval zebrafish was conducted under the approval of the Institutional Animal Care and Use Committee of Chengdu University of TCM (No. SYXK (CHUAN) 2014 – 128).

**Measurement of growth parameters**

After EM administration for 10 days, body weight and length of anesthetized larval zebrafish with tricaine were analyzed. Body length of zebrafish larvae from the front end of the mouth to the end of tail showing in (Fig. 2a) was photographed under leica M165-FC microscope and measured by Image Pro Plus software. Body mass index (BMI) was calculated as body weight (g) divided by the square of body length (cm).
Measurement of biochemical parameters

30 euthanized zebrafish larvae were washed 3 times by precooled phosphate buffered saline (PBS) and then homogenized with 270 ul normal saline. After centrifugation at 4°C, 2500 rpm for 10 min, the separated supernatant was used to determine the levels of TC, TG, low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) as well as the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by specific commercial assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Confocal image analysis

In order to observe vascular cholesterol accumulation, Tg (flil: eGFP) zebrafish larvae were fed with normal fed or 4% HCD for 10 days. The fluorescent images were captured under Olympus FV-OSR confocal microscope. Vascular vessels in the green channel and fluorescent cholesteryl ester in the red channel were excited at 559 nm and 488 nm respectively. Mean fluorescence intensity (MFI) of caudal arteries were analyzed by OLYMPUS Stream software.

Oil red O staining

Before staining, zebrafish larvae were euthanized with high concentration of tricaine and then fixed in 4% paraformaldehyde (PFA) at 4°C for 12 h. Briefly for oil red O staining, larvae were washed 3 times with PBS and orderly submerged in 25, 50, 75 and 100% 1, 2-propanediol at room temperature for 15 min, this was followed by 6 h incubation in freshly prepared 0.5% Oil red O dye solution in the dark. Subsequently, the samples were sequentially rinsed in 100, 75, 50 and 25% 1,2-propanediol for approximately 30 min and washed twice with PBS. Eventually, the images were photographed under leica M165-FC microscope and analyzed for lipid deposition in the liver. Integrated optical density (IOD) value of liver, a relative quantitative index of lipid deposition in liver, was measured by Image Pro Plus software.

Hematoxylin and eosin (H&E) staining

After fixed with 4 % PFA overnight, larvae was processed in accordance with standard procedures of H&E staining. The whole fish was embedded in paraffin, sectioned and the histopathological changes of zebrafish liver was observed under leica M165-FC microscope.

Determination of inflammatory level

Studies have shown that fluorescent neutrophils in Tg (mpx: eGFP) zebrafish larvae can be used to track the inflammatory response (24, 25). Therefore, we use Tg (mpx: eGFP) zebrafish to visualize and quantify neutrophil inflammation. Images were collected by microscope and the average number of neutrophils in the blood vessels of tail were counted by Image Pro Plus software.

RNA Extraction and Real-time quantitative PCR (RT-qPCR) analysis
Total RNA of 40 larvae in each group was extracted using Animal Total RNA Isolation kit and then dissolved in 65 µL RNase-free ddH$_2$O. The total RNA purity was analyzed by measuring the OD$_{260/280}$ value using a nucleic acid/protein analyzer. The RNA was reverse-transcribed by 5×All-In-One RT MasterMix with AccuRT for the synthesis of cDNA. The resultant cDNA was applied as template for qPCR analyses with EvaGreen 2×qPCR MasterMix-No Dye on the StepOnePlus Real-time fluorescent quantitative PCR system. The reaction conditions were as follows: 95°C for 10 min, 95°C for 15 s and 60°C for 60 s (40 cycles). The calculated mRNA expression data were expressed as relative expression ratio normalized to GAPDH by 2$^{-\Delta\Delta C_t}$ method. The designed sets of gene primer sequences for RT-qPCR were synthesized by TSINGKE Biological Technology (Chengdu, China) listed in Table 1.

| Gene      | Forward primer (5’→3’)     | Reverse primer (5’→3’)     |
|-----------|----------------------------|----------------------------|
| AMPKa     | AGTTATCAGCACACCGCAGAC      | CAGTAATCCACCCCTGAGATG      |
| SREBP-2   | CTGAGCTGATTGTCGGT          | GGTCGCTTTATCTCTCGCA        |
| PCSK9     | CCGACTTCAACAGAGTGCCT       | CCACGTGATCACCCTGCAAT       |
| HMGCR     | CCACGTAGAGGTCTCCAGT        | TGCCCTGCTTAGTGCGATGTC      |
| LDLR      | ACCTACACCGAGTGCAGT        | TGGAAAGGCGGTTGTTGCTT       |
| ABCA1     | CAGTATGGCATCCTCGACC       | TCCATCACCATTCTCTCCG        |
| ABCG1     | ACACCACTGTTCCAGCACA       | TCCGAGGCTGGATGAGAAC        |
| GAPDH     | GGATCTGACAGTCCGTCTTGAGAA  | CCATTGAAAGTTCAGTGACCAACCC  |

### Statistical analysis

Statistical analysis was performed using SPSS version 20.0 software. All experimental data were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare differences among multiple groups and Least significant difference (LSD) was used for comparison between the two groups. When p < 0.05 level, the results were considered to be statistically significant. Graphpad Prism 8.0 software was used to generate graphs.

### Results

#### Effect of EM on the growth of zebrafish larvae with HLP

After 10 days of feeding, the growth parameters including body weight and length of each group were determined. Our data showed that HCD increased zebrafish BMI (p < 0.05) and promoted obesity (Fig. 2b-d). However, compared with HCD group, Body weight, length and BMI were decreased significantly in EM-M and EM-H group (p < 0.05), but not EM-L group.
EM improved lipid profiles and liver function

The levels of TC, TG, LDL-C and HDL-C were measured to confirm the lipid-lowering of EM. The results indicated that the levels of TC, TG and LDL-C in HCD group were markedly increased and the HDL-C level was low as compared to control group (p < 0.01) (Fig. 3a-d). Interestingly, EM reduced the concentration of TC and LDL-C and increased HDL-C level in a dose-dependent manner. However, TG had no significant effect at all doses of EM except EM-H group (p < 0.01). The biochemical indexes ALT and AST were measured to investigate liver function (Fig. 3e, f). There was a increase in ALT and AST activities in HCD group compared to control group, whereas the intervention of EM significantly decreased the activities of ALT and ALT (p < 0.01).

EM reduced vascular cholesterol accumulation

Fluorescence images were shown in Fig. 4a, b. Cholesterol tagged with red fluorescent cholestery ester accumulated in the green blood vessel including caudal artery (arrows in Fig. 4a). The extent of vascular cholesterol accumulation of larvae from trunk to tail, including caudal artery in each group were observed (Fig. 4b). The fluorescence level of vascular red cholesterol was significantly higher (p < 0.001) in larvae fed with HCD than in larvae fed with normal feed, but this increase was suppressed significantly when EM administrated (Fig. 4c). Among which, EM-M and EM-H groups obviously reduced blood lipid in zebrafish fed with HCD.

EM alleviated hepatic lipid accumulation and liver histological damage

The results of Oil red O staining were shown in Fig. 5a, there was no obvious lipid deposition in the liver of control group, whereas the HCD group showed darker staining. Compared with HCD group, the lipid droplets dyed red in the EM treatment groups reduced markedly, which consistent with IOD (p < 0.001) (Fig. 5b). Among which, EM-M and EM-H treatment groups showed obvious attenuation of hepatic lipid staining. Subsequently, liver histopathological observation was performed. H&E staining results (Fig. 5c, d) suggested that the liver tissues were intact and the hepatocytes were closely arranged in control group. However, it was found that there were a large quantity of vacuoles in the liver, and deformed and irregularly arranged hepatocytes in HCD group. In contrast, the vacuoles in the liver tissue reduced and the arrangement of hepatocytes was tended to be regular after treating with EM, especially in EM-H group.

EM ameliorated inflammatory response

To detect the inflammatory response in the process of hyperlipidemia, Tg (mpx: eGFP) zebrafish larvae were selected to be examined under a microscope. The fluorescent images of inflammatory response in larvae fed with normal feed, HCD and HCD added with EM were shown in Fig. 6a. Compared with control group, the green fluorescent-labeled neutrophils at the vascular site obviously increased in HCD group.
larvae. No difference in neutrophil number was found between HCD group and EM-L group (p > 0.05). However, neutrophil number diminished significantly at the vascular site in EM-M and EM-H group (p < 0.001) compared with HCD group (Fig. 6b). These results demonstrated that EM effectively inhibited the migration and recruitment of neutrophil in vascular site, thereby ameliorating vascular inflammatory response in HLP zebrafish.

**Effect of EM on mRNA expression of lipid metabolism relevant molecules**

To determine the underlying mechanism of EM on alleviating hyperlipidemia in HCD-induced zebrafish larvae, the genes expression of lipid metabolism relevant molecules including AMPKα, SREBP-2, PCSK9, LDLR, HMGCR, ABCA1 and ABCG1 were detected by RT-qPCR. The results (Fig. 7a) showed that, compared to control group, the genes expression of AMPKα, LDLR, ABCA1 and ABCG1 in HCD-induced zebrafish were significantly decreased, while SREBP-2, PCSK9 and HMGCR were increased (p < 0.001). After 10 days treatment with EM, the mRNA expression levels of AMPKα, LDLR, ABCA1 and ABCG1 were markedly increased, while SREBP-2, PCSK9 and HMGCR were lower in a dose-dependent manner.

**Discussion**

HLP is a metabolic syndrome due to lipid metabolism imbalance, and usually represented as elevated TC and/or TG, as well as a reduction in HDL-C. HLP results in cardiovascular disease and liver injury, which seriously threatens human life (2). Lipid metabolism disorder may attributed to the interaction between genetics and environmental factors, such as irrational dietary habits. For instance, excessive consumption of HCD and high-fat diet (HFD) are potent risk factors for dyslipidemia and atherosclerosis (26, 27).

In the present study, we used HCD to induce HLP disease to evaluate the effect of EM on lipid metabolism disorder. Our results indicated that HLP zebrafish larvae model was successfully established by consuming HCD for 10 days, which was consistent with the previous study (21). As can be seen from the results, HCD promoted obesity of zebrafish larvae, as evidenced by elevated body weight, length and BMI (Fig. 2b-d). However, after EM treatment, this situation were effectively alleviated. The translucency of larval zebrafish until 30 dpf permits observation of vascular lipid accumulation and deposition in real time, which is a unique advantage different from the HLP rodent model. It was reported that excessive lipid particularly cholesterol in the blood vessel may predispose to atherosclerosis (28). Recent evidence suggests that the accumulated lipid in the vascular wall of HLP zebrafish model is similar to the composition of early atherosclerotic plaque of humans (29, 30). Compared with the control group, it was observed that large amounts of red cholesterol tagged with fluorescent cholesteryl ester accumulated in the green blood vessel (including the caudal artery) in the HCD group (Fig. 4a-c). Intriguingly, EM remarkably improved cholesterol deposition in the area, showing its hypocholesterolemic effect. In addition, neutrophil-regulated inflammatory reaction is essential for early atherosclerosis, and neutrophils, an important immune cell, are recruited in large numbers to the injured sites of endothelial cell layer.
driving early atherosclerosis and plaque destabilization (31). Our results (Fig. 6a, b) found abundant neutrophils were recruited in the blood vessel of tail in HLP zebrafish larvae, while EM reversed this change. Together, this results suggested that EM reduced vascular cholesterol deposition and improved vascular inflammatory response to inhibit the occurrence of early atherosclerosis in zebrafish larave fed with HCD.

The liver is a center organ for lipid metabolism. When the generated lipid cannot be released into the blood, they are usually accumulated and deposited in hepatocytes in the form of lipid droplets. In the case of hypercholesterolemia, elevated hepatic lipid accumulation contributes to NAFLD and liver injury (32). Transaminases is sensitive to the damage of hepatocytes. ALT and AST are crucial indicators for evaluating liver damage. In this study, compared to the control group, HCD-induced larvae showed that severe lipid accumulation in the liver, hepatic steatosis and abnormal morphology of hepatocytes (Fig. 5a-d). Whereas, the administration of EM prevented the HCD-induced abnormalities in TC, TG, LDL-C and HDL-C levels, as well as the increased activities of ALT and AST (Fig. 3a-f). These results demonstrated that EM could attenuate hepatic lipid accumulation and improve liver function of HLP zebrafish.

Adenosine monophosphate-activated protein kinase (AMPK) is considered as the key molecule that regulates biological energy metabolism and thus has become potential therapeutic target for metabolic diseases. Viollet et al. demonstrated that the improvement of lipid metabolism disorder may be attributed to AMPK activation and its downstream target genes sterol regulatory element binding protein 2 (SREBP-2) (33). The SREBP-2/PCSK9/LDLR signalling pathway is an important and effective pathway that regulates lipid metabolism (34). SREBP-2, a member of SREBPs transcription factors family, regulates the expression of genes required for cholesterol synthesis and uptake (35). Studies have shown that overexpression of SREBP-2 induced by HFD stimulation can dramatically increase genes expression involved in lipid synthesis and metabolism, which may be the cause of diet-induced lipid metabolism disorder (36). LDLR on the surface of hepatocytes is the mediator for uptaking LDL-C in the circulation, leading to decrease LDL-C level. PCSK9 is a serine protease and mainly synthesized in the liver, which is regulated by SREBP-2 (37). It is reported that PCSK9 can triggers LDLR intracellular degradation via attaching to LDLR surface and translocating to lysosomes, resulting in increase both circulating LDL-C and the risk of cardiovascular disease (37). In addition, HMGCR is the key enzyme regulated by the activity of SREBP-2 for cholesterol biosynthesis and an effective target of statins for HLP prevention and treatment (38). Thus, we determined whether EM attenuates lipid accumulation in HCD-induced HLP zebrafish larvae via modulating the AMPK/SREBP-2/PCSK9/LDLR signalling pathway.

In this study, our data (Fig. 7a) showed that EM significantly prevented the HCD-induced decrease of AMPK mRNA in zebrafish. Previous studies have shown that EM activated AMPK signaling pathway and promoted fatty acid oxidation, thereby exerting protective effect in NAFLD zebrafish fed with egg powder (15). Moreover, our results (Fig. 7a) indicated that the expression of SREBP-2 and its target genes involved in PCSK9 and HMGCR was significantly increased, while that of LDLR was decreased in zebrafish with HLP induced by HCD. However, EM reversed the increased expression of SREBP-2, HMGCR
and PCSK9, but upregulated the expression of LDLR, which might be the reason of decrease of cholesterol endogenous synthesis and circulated LDL-C content. Reverse cholesterol transport (RCT) process is an effective method to reduce cholesterol in extrahepatic tissues and has been demonstrated to reduce HLP (39). Adenosine triphosphate binding cassette transporter A1 (ABCA1) and adenosine triphosphate binding cassette transporter G1 (ABCDE) both participate in regulating the secretion of cholesterol, which is beneficial to RCT (40). Our research found that EM effectively upregulated the expression of ABCA1 and ABCG1, and promoted the elimination of excess cholesterol.

Collectively, present study revealed that EM can improve obesity, reduce lipid deposition, alleviate inflammation and liver injury on HLP zebrafish larvae fed with HCD through regulating genes expression of lipid metabolism relevant factors and redistributing of lipid in the blood and liver.

**Conclusion**

In summary, EM can improved metabolic profiles, liver function and lipid accumulation caused by HCD. According to our work, the protective mechanism of EM against HLP was probably achieved by modulating the AMPK/SREBP2/PCSK9/LDLR signaling pathway (Fig. 7b). Therefore, EM could be a potential drug for the treatment of lipid metabolism related diseases.

**Abbreviations**

HLP, hyperlipidemia; EM, emodin; NAFLD, nonalcoholic fatty liver disease; HCD, high cholesterol diet; dpf, days post fertilization; BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AMPK, adenosine monophosphate-activated protein kinase; SREBP-2, sterol regulatory element binding protein 2; PCSK9, proprotein convertase subtilisin kexin 9; LDLR, low-density lipoprotein receptor; ABCA1, adenosine triphosphate binding cassette transporter A1; ABCG1, adenosine triphosphate binding cassette transporter G1; HMGCR, 3-hydroxy-3-methyl-glutaryl CoA reductase.

**Declarations**

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**Author contributions**

L. H., and Y. L., conceived and designed the experiments. L. H., Y. L., C. W., Y. Z., C. G., and Y. W. conducted biological experiments and analyzed the data. L. H., and Y. L. wrote and reviewed the manuscript. All authors have read and approved the final manuscript.
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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The experiments involving larval zebrafish were performed under the approval of the Institutional Animal Care and Use Committee of Chengdu University of Traditional Chinese Medicine (No. SYXK (CHUAN) 2014−128).

Consent for publication

We declare that the Publisher has the Author's permission to publish the relevant contribution.

Competing interests

The authors declare that they have no conflicts of interest.

References

1. Karr S. Epidemiology and management of hyperlipidemia. Am J Manag Care. 2017;23(9 Suppl):139-s48.
2. Sozen E, Ozer NK. Impact of high cholesterol and endoplasmic reticulum stress on metabolic diseases: An updated mini-review. Redox Biol. 2017;12:456–61.
3. Rocha VZ, Libby P. Obesity, inflammation, and atherosclerosis. Nature reviews Cardiology. 2009;6(6):399–409.
4. Ference BA, Ginsberg HN, Graham I, Ray KK, Packard CJ, Bruckert E, et al. Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. European heart journal. 2017;38(32):2459–72.
5. Yang XY, Zhong DY, Wang GL, Zhang RG, Zhang YL. Effect of Walnut Meal Peptides on Hyperlipidemia and Hepatic Lipid Metabolism in Rats Fed a High-Fat Diet. Nutrients. 2021;13(5).
6. AlSharari SD, Al-Rejaie SS, Abuohashish HM, Ahmed MM, Hafez MM. Rutin Attenuates Hepatotoxicity in High-Cholesterol-Diet-Fed Rats. Oxid Med Cell Longev. 2016;2016:5436745.
7. Li L, Li L, Chen L, Lin X, Xu Y, Ren J, et al. Effect of oleylethanolamide on diet-induced nonalcoholic fatty liver in rats. J Pharmacol Sci. 2015;127(3):244–50.
8. Zhang X, Hu D. [Comments for 2013 ACC/AHA Guideline on the Treatment of Blood Cholesterol to Reduce Atherosclerotic Cardiovascular Risk in Adults]. Zhonghua nei ke za zhi. 2015;54(1):9–12.

9. Qin LS, Zhao HP, Zhao YL, Ma ZJ, Zeng LN, Zhang YM, et al. [Protection and bidirectional effect of rhubarb anthraquinone and tannins for rats’ liver]. Zhongguo zhong yi jie he za zhi Zhongguo zhongxiyi jiehe zazhi = Chinese. journal of integrated traditional Western medicine. 2014;34(6):698–703.

10. Lin X, Liu T, Li P, He Z, Zhong Y, Cui H, et al. iTRAQ-Based Proteomics Analysis Reveals the Effect of Rhubarb in Rats with Ischemic Stroke. Biomed Res Int. 2018;2018:6920213.

11. Shrimali D, Shanmugam MK, Kumar AP, Zhang J, Tan BK, Ahn KS, et al. Targeted abrogation of diverse signal transduction cascades by emodin for the treatment of inflammatory disorders and cancer. Cancer letters. 2013;341(2):139–49.

12. Wang J, Ji J, Song Z, Zhang W, He X, Li F, et al. Hypocholesterolemic effect of emodin by simultaneous determination of in vitro and in vivo bile salts binding. Fitoterapia. 2016;110:116–22.

13. Luo N, Fang J, Wei L, Sahebkar A, Little PJ, Xu S, et al. Emodin in atherosclerosis prevention: Pharmacological actions and therapeutic potential. Eur J Pharmacol. 2021;890:173617.

14. Hei ZQ, Huang HQ, Tan HM, Liu PQ, Zhao LZ, Chen SR, et al. Emodin inhibits dietary induced atherosclerosis by antioxidation and regulation of the sphingomyelin pathway in rabbits. Chin Med J. 2006;119(10):868–70.

15. Yu L, Gong L, Wang C, Hu N, Tang Y, Zheng L, et al. Radix Polygoni Multiori and Its Main Component Emodin Attenuate Non-Alcoholic Fatty Liver Disease in Zebrafish by Regulation of AMPK Signaling Pathway. Drug Des Devel Ther. 2020;14:1493–506.

16. Wang W, He Y, Lin P, Li Y, Sun R, Gu W, et al. In vitro effects of active components of Polygonum Multiflorum Radix on enzymes involved in the lipid metabolism. J Ethnopharmacol. 2014;153(3):763–70.

17. Vedder VL, Aherrahrou Z, Erdmann J. Dare to Compare. Development of Atherosclerotic Lesions in Human, Mouse, and Zebrafish. Front Cardiovasc Med. 2020;7:109.

18. Goessling W, Sadler KC. Zebrafish: an important tool for liver disease research. Gastroenterology. 2015;149(6):1361–77.

19. Henderson RJ, Tocher DR. The lipid composition and biochemistry of freshwater fish. Prog Lipid Res. 1987;26(4):281–347.

20. Hölttä-Vuori M, Salo VT, Nyberg L, Brackmann C, Enejder A, Panula P, et al. Zebrafish: gaining popularity in lipid research. Biochem J. 2010;429(2):235–42.

21. Stoletov K, Fang L, Choi SH, Hartvigsen K, Hansen LF, Hall C, et al. Vascular lipid accumulation, lipoprotein oxidation, and macrophage lipid uptake in hypercholesterolemic zebrafish. Circ Res. 2009;104(8):952–60.

22. Stoletov K, Fang L, Choi S, Hartvigsen K, Hansen L, Hall C, et al. Vascular lipid accumulation, lipoprotein oxidation, and macrophage lipid uptake in hypercholesterolemic zebrafish. Circulation research. 2009;104(8):952–60.
23. Meguro S, Hasumura T, Hase T. Coffee polyphenols exert hypocholesterolemic effects in zebrafish fed a high-cholesterol diet. Nutrition metabolism. 2013;10(1):61.
24. Renshaw S, Loynes C, Trushell D, Elworthy S, Ingham P, Whyte M. A transgenic zebrafish model of neutrophilic inflammation. Blood. 2006;108(13):3976–8.
25. Zhang W, Liu X, Piao L. Chlorogenic acid-enriched extract of Ilex kudingcha C.J. Tseng tea inhibits neutrophil recruitment in injured zebrafish by promoting reverse migration via the focal adhesion pathway. J Food Biochem. 2020;44(8):e13279.
26. Ma Y, Wang W, Zhang J, Lu Y, Wu W, Yan H, et al. Hyperlipidemia and atherosclerotic lesion development in Ldlr-deficient mice on a long-term high-fat diet. PloS one. 2012;7(4):e35835.
27. Bin-Jumah M. Monolluma quadrangula Protects against Oxidative Stress and Modulates LDL Receptor and Fatty Acid Synthase Gene Expression in Hypercholesterolemic Rats. Oxidative medicine and cellular longevity. 2018;2018:3914384.
28. Mozaffarian D, Benjamin E, Go A, Arnett D, Blaha M, Cushman M, et al. Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. Circulation. 2016;133(4):e38–60.
29. Fan X, Han J, Zhu L, Chen Z, Li J, Gu Y, et al. Dendrobium huoshanenseProtective Activities of C. Z. Tang et S. J. Cheng Polysaccharide against High-Cholesterol Diet-Induced Atherosclerosis in Zebrafish. Oxidative medicine and cellular longevity. 2020;2020:8365056.
30. Fang L, Green S, Baek J, Lee S, Ellett F, Deer E, et al. In vivo visualization and attenuation of oxidized lipid accumulation in hypercholesterolemic zebrafish. J Clin Investig. 2011;121(12):4861–9.
31. Soehnlein O. Multiple roles for neutrophils in atherosclerosis. Circulation research. 2012;110(6):875–88.
32. Lee KS, Chun SY, Kwon YS, Kim S, Nam KS. Deep sea water improves hypercholesterolemia and hepatic lipid accumulation through the regulation of hepatic lipid metabolic gene expression. Mol Med Rep. 2017;15(5):2814–22.
33. Viollet B, Andreelli F. AMP-activated protein kinase and metabolic control. Handbook of experimental pharmacology. 2011(203):303–30.
34. Chae H, You B, Kim D, Lee H, Ko H, Ko H, et al. Sauchinone controls hepatic cholesterol homeostasis by the negative regulation of PCSK9 transcriptional network. Scientific reports. 2018;8(1):6737.
35. Madison B. Srebp2: A master regulator of sterol and fatty acid synthesis. Journal of lipid research. 2016;57(3):333–5.
36. Li Q, Wang H, Zhang C, Tong R, Chen H, Qie R. Ethyl acetate extract of sappanwood alleviates experimental atherosclerosis in rats through changes in FGF21 and SREBP-2 expression. Int J Clin Exp Pathol. 2020;13(2):220–9.
37. Catapano AL, Pirillo A, Norata GD. New Pharmacological Approaches to Target PCSK9. Curr Atheroscler Rep. 2020;22(7):24.
38. Chung J, Cho S, Oh E, Lee D, Lim L, Jang S, et al. Effect of HMGCR variant alleles on low-density lipoprotein cholesterol-lowering response to atorvastatin in healthy Korean subjects. Journal of clinical pharmacology. 2012;52(3):339–46.

39. Fisher E, Feig J, Hewing B, Hazen S, Smith J. High-density lipoprotein function, dysfunction, and reverse cholesterol transport. Arteriosclerosis, thrombosis, and vascular biology. 2012;32(12):2813–20.

40. Phillips M. Molecular mechanisms of cellular cholesterol efflux. J Biol Chem. 2014;289(35):24020–9.

Figures

Figure 1

Experimental outline of the feeding. (a) The chemical structure of emodin (EM). (b) Experiment protocol and grouping.
Figure 2

Effect of EM on the growth of zebrafish larvae. (a) Standard diagram of body length of larval zebrafish (x represents the body length from the front end of the mouth to the end of tail). (b) Body length of larval zebrafish (n=30 zebrafish). (d) Average body weight of larval zebrafish (n=30 zebrafish). (d) Effect of EM on BMI in larval zebrafish (n=30 zebrafish). Values are expressed as mean ± SD in each group. #P < 0.05, ###P < 0.001 compared with control group; *P < 0.05, **P < 0.01, ***P < 0.001 compared with HCD group; n.s. indicates no significant.
Figure 3

EM improved lipid profiles and liver function. (a) TC levels of zebrafish larvae in each group. (b) TG levels of zebrafish larvae in each group. (c) LDL-C levels of zebrafish larvae in each group. (d) HDL-C levels of zebrafish larvae in each group. (e) ALT values of zebrafish larvae in each group. (f) AST values of zebrafish larvae in each group. Values are expressed as mean ± SD in each group. ##P < 0.01, ###P < 0.001 compared with control group; *P < 0.05, **P < 0.01, ***P < 0.001 compared with HCD group; n.s. indicates no significant.
Figure 4

EM reduced vascular cholesterol accumulation. Tg (flil: eGFP) larval zebrafish at 5 dpf were fed a control diet, a HCD, or EM diets for 10 days; all diets additionally added 10 μg/g of Cholesteryl BODIPY® 542/563-C11. (a) The fluorescence image from trunk to tail of Tg (flil: eGFP) zebrafish larvae (× 100). Yellow arrows: caudal artery. Blood vessel is shown green fluorescence, while lipid (cholesterol) is in red. Green channel: 488 nm; Red: 559 nm. (b) EM reduced vascular cholesterol accumulation of zebrafish fed with HCD. (c) Mean fluorescence intensities (MFI) of the caudal arteries of fish (n=8 zebrafish). Values are expressed as mean ± SD in each group. ###P < 0.001 compared with control group; **P < 0.01, ***P < 0.001 compared with HCD group.
Figure 5

EM alleviated hepatic lipid accumulation and liver histological damage. (a) The whole-mount Oil red O staining of zebrafish larvae (yellow arrows point to lipids in the liver). (b) The value of integrated optical density (IOD) was calculated by fluorescence intensity of liver (n=9 zebrafish). (c) Schematic diagram of H&E staining section, the red circles represents the liver of larvae. (d) Histopathological changes of zebrafish liver by H&E staining (× 400). Values are expressed as mean ± SD in each group. ##P < 0.01,
###P < 0.001 compared with control group; *P < 0.05, **P < 0.01, ***P < 0.001 compared with HCD group; n.s. indicates no significant.

**Figure 6**

EM ameliorated inflammatory response. Tg (mpx:eGFP) zebrafish larvae induced by 4% HCD was used to assess the anti-inflammation effect of EM. (a) Aggregation of neutrophils of zebrafish in each group. The yellow squares mark the caudal vascular area of the zebrafish larvae. (b) The average number of neutrophils counted in the marked area of the zebrafish larvae in each group (n=15 zebrafish). Values are expressed as mean ± SD in each group. ###P < 0.001 compared with control group; ***P < 0.001 compared with HCD group; n.s. indicates no significant.
Figure 7

mRNA expression of EM on lipid metabolism related pathway of zebrafish larvae. (a) Lipid metabolism related molecules (n=40 zebrafish). (b) Effect of EM on the lipid metabolism relevant pathway. Values are expressed as mean ± SD in each group. ###P < 0.001 compared with control group; *P < 0.05, **P < 0.01, ***P < 0.001 compared with HCD group; n.s. indicates no significant.
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