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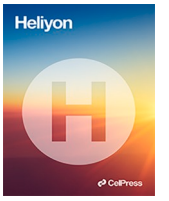

Research article

Lipid regulation of protocatechualdehyde and hydroxysafflor yellow A via AMPK/SREBP2/PCSK9/LDLR signaling pathway in hyperlipidemic zebrafish

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ABSTRACT

The consumption of a high-cholesterol diet is known to cause hyperlipidemia, which is one of the main risk factors for cardiovascular disease. Protocatechualdehyde (PCA) and hydroxysafflor yellow A (HSYA) are the active components of Salvia miltiorrhiza and safflower, respectively. However, their exact mechanism is still unclear. The aim of this study is to investigate its effects on lipid deposition and liver damage in hyperlipidemic zebrafish and its mechanism of anti-hyperlipidemia. The results showed that the use of PCA and HSYA alone and in combination can improve lipid deposition, slow behavior, abnormal blood flow and liver tissue damage, and the combined use is more effective. Further RT-qPCR results showed that PCA + HSYA can regulate the mRNA levels of *PPAR-γ*, *SREBP2*, *SREBP1*, *HMGCR*, *PCSK9*, *mTOR*, *C/EBPα*, *LDLR*, *AMPK*, *HNF-1α* and *FoxO3a*. The PCA + HSYA significantly improves lipid deposition and abnormal liver function in hyperlipidemic zebrafish larvae, which may be related to the AMPK/SREBP2/PCSK9/LDLR signaling pathway.

1. Introduction

There is increasing awareness about high cholesterol diets (HCD) due to their risk of obesity, hyperlipidemia, and other health problems [1]. Cholesterol feeding is often used to study the causes of metabolic disorders related to hyperlipidemia [2]. Among the most common forms of dyslipidemia, hyperlipidemia raises the risk of fatty liver, hypertension, atherosclerosis, thrombosis, and

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; dpf, day post fertilization; HCD, high-cholesterol diet; HDL-C, high-density lipoprotein cholesterol; HSYA, hydroxysafflor yellow A; LDL-C, low-density lipoprotein cholesterol; PCA, Protocatechualdehyde; RT-qPCR, real-time quantitative polymerase chain reaction; SCFAs, short-chain fatty acids; Sim, simvastatin; TCM, traditional Chinese medicine; TG, triglycerides; TC, total cholesterol.

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cerebrovascular disease [3,4]. Currently, hyperlipidemia is prevalent in both low- and middle-income countries [5]. It affects people's health, so finding more safe and cost-effective drugs that may treat hyperlipidemia is necessary.

At present, mild hyperlipidemia is usually treated by introducing lifestyle changes, and drug therapy is suitable for more severe hyperlipidemia cases [6]. However, its exact underlying mechanism is not fully understood. Long-term and high-dose application of single chemical medicine is prone to some side effects, such as the cholesterol synthesis inhibitor simvastatin (Sim), which can cause liver damage, joint pain, memory loss, etc. [7,8]. More and more studies show that traditional Chinese medicine (TCM) has a good curative effect on hyperlipidemia. Safflower (*Flos Carthami*) and Salvia miltiorrhiza (*Radix Salvia Miltiorrhizae*) are traditional food and medicine [9,10]. Multiple clinical and pharmacological effects suggest that Salvia miltiorrhiza can be used to treat vascular diseases, hyperlipidemia, and hepatitis [11,12]. Safflower has been proven to promote blood circulation and clear obstruction, and it is widely used in TCM to treat cerebrovascular and cardiovascular diseases [13,14]. Salvia miltiorrhiza-safflower is a blood-activating drug pair of TCM, which is commonly used in TCM prescriptions for cardiovascular and cerebrovascular diseases, such as modern Chinese medicine preparation Danhong injection, Jingzhiguanxin Tablet, Danhong Huayu Koufuye, etc. It has been proven that protocatechualdehyde (PCA) in Salvia miltiorrhiza protects the cardiovascular system from oxidative stress, and atherosclerosis and reduces cardiac hypertrophy [15], and as a component of safflower, hydroxy safflower yellow A (HSYA) works to reduce blood lipid levels, regulate vasoconstriction, and maintain diastolic function [16], both have been shown to prevent and treat atherosclerosis [17]. When PCA and HSYA are combined in the rat hyperlipidemia model, by accelerating the absorption of each other in vivo, the plasma clearance rate of the two drugs is significantly increased, which has a certain impact on each other. Compared with PCA, it has a greater impact on the absorption of HSYA [18], which also reflects the rationality of the combination of PCA and HSYA.

Zebrafish is a popular animal for studying lipid metabolism [19], such as fatty liver, hyperlipidemia, diabetes, etc. It has the advantages of small size and optical transparency, which can be used to evaluate drug efficacy and toxicity in vivo [20], and there are many similarities between fish and mammals when it comes to lipid metabolism [21], such as lipids in the gut absorption, vascular lipid deposition, etc [22]. Our study used HCD-fed zebrafish as a model of hyperlipidemia, and explored the effects of PCA, HSYA and PCA + HSYA on lipid regulation and liver function damage caused by hyperlipidemia through AMPK/SREBP2/PCSK9/LDLR signal pathway.

2. Materials and methods

2.1. Materials

Protocatechualdehyde (Lot: HS14510S1) and hydroxysafflor yellow A (Lot: HR21714B1) were purchased from Baoji Herbest Bio-Tech (Xi'an, China). Cholesterol (>95 % purity) and propylene glycol were obtained from Macklin Biochemical Co., Ltd (Shanghai, China). Zebrafish feed was obtained from Shanghai FishBio Co., Ltd (Shanghai, China). Oil Red O and Methyl cellulose were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Simvastatin and Egg yolk powder were purchased from Shanghai source leaf Biological Technology Co., Ltd. (Shanghai, China). Dimethyl Sulfoxide (DMSO) was obtained from Solarbio Life Science (Beijing, China).

2.2. Methods

2.2.1. Animals maintenance and high-cholesterol diet

Wild-type (AB) zebrafish were obtained from the Shanghai FishBio Co., Ltd (Shanghai, China). During the experiment, zebrafish were cultured at 28.5 °C with a 14 h light/10 h dark cycle and were fed on a commercial diet twice a day (Shanghai FishBio Co., Ltd). A natural mating method was used to obtain the embryos from AB strain zebrafish, and incubated at 28.5 °C in embryo culture solution (Shanghai FishBio Co., Ltd). 5 days post fertilization (dpf) AB wild-type zebrafish larvae were prepared in 100 × 20 mm petri dishes in 25 mL fish water at pH 7.4 at a maximum density of 30 larvae per dish [23]. We fed the Control group with ordinary feed, while the HCD and drug groups received HCD (4 % weight per weight cholesterol added to egg yolk powder) and corresponding concentrations of Sim, PCA and HSYA for 48 h. In this study, all experiments were performed in compliance with the Ethics Committee's policy on animal use and ethics.

2.2.2. Toxicity Assessment on zebrafish larvae

AB strain zebrafish larvae (5 dpf) were randomly selected under the microscope and given Sim, PCA and HSYA for 48 h respectively, and the Control group (fish water treatment group) was set up. Mortality, body length and morphological abnormalities were assessed, such as spinal curvature, uninflated swim bladder and pericardial edema. In drug treatment experiments, Sim was used at 0.05, 0.1, 0.2, 0.4 and 0.5 μg/mL, PCA and HSYA were tested at 25, 50, 100, 200, 400, 800 and 1000 μg/mL. The no observed adverse effect level (NOAEL) is the highest concentration of the experimental drug that can be used without causing any observable adverse effects in zebrafish. Each group was cultured to 7 dpf at 28.5 °C, and dead zebrafish larvae were recorded and taken out every day. Zebrafish larvae were observed using stereomicroscopes after the experiment to determine their toxic phenotypes, body length, and mortality. After determining the NOAEL of PCA and HSYA respectively, set the concentration of PCA + HSYA group as PCA (NOAEL) + HSYA (NOAEL), PCA (1/2 NOAEL) + HSYA (1/2 NOAEL), PCA (1/4 NOAEL) + HSYA (1/4 NOAEL), PCA (1/8 NOAEL) + HSYA (1/8 NOAEL) and PCA (1/16 NOAEL) + HSYA (1/16 NOAEL).

2.2.3. Oil Red O staining

Zebrafish larvae were washed with 1 × PBS before fixed in 4 % paraformaldehyde overnight, followed by washed three times with

Table 1
RT-qPCR primer sequence.

Gene	Forward Primer	Reverse Primer
LDLR	ACCTACACGGAGGTCAGTTC	TGGAAAGGCGGTTTGGTTCT
SREBP1	CTAACCGACAGCCAAGTGAA	AGACGACAACAACAACAACAAC
SREBP2	AGGAGGAGTGGTGAAGGA	GTTGATGGAGGAGCGGTAG
HMGCR	CTGCTATCTATATTGCTTGTG	TTGAGGAGGAAGGTTAGT
PCSK9	CCGACTTCAACAGAGTGCCT	CCACTGATCACCCCTGCAAT
mTOR	GGGAGAGCGTATGAGAGAGGAGATG	AAACTGGTGAAGGGCGTGATGTG
PPAR-γ	GGAACTGGAGGAGCTGGAGGAC	CGATGCCTGATATGCTGCTGTAGTC
C/EBPα	GCCTACATTGATCCGTCTGCCTTC	CCGTGGTGGTAGTCGTAGTCTCC
AMPK	AGTTATCAGCACACCGACAG	CAGTAATCCACCCCTGAGATG
HNF-1α	CAAGAAGCAGGCAGAGATCAGTCAG	CTCCACCAGTCCCTCCCTTTCC
FoxO3a	TTCCTACGCCGACCTCATCACC	GCGACTATGGAGCGACAGGTTATG
β-Actin	GGCTACAGCTTCACCACCA	TGCTGATCCACATCTGCTG

PBS again, gradient infiltration of propylene glycol solution, dyeing with filtered Oil Red O solution, and then wash away with a gradient of reduced concentration of propylene glycol. The larvae were placed under an anatomical microscope to observe lipid staining and images were obtained with a digital camera for analysis. Integrated optical density (IOD) values were obtained by using Image-Pro Plus (IPP) software to standardize dyeable lipid content in the images.

2.2.4. TG, TC, HDL-C and LDL-C assay

Zebrafish larvae were gently homogenized in absolute ethanol with a homogenizer. Following centrifugation, the supernatant was collected and assayed triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) concentrations using Nanjing Jiancheng Institute of Biological Engineering commercial kits (Nanjing, China).

2.2.5. Behavioral assay

In each experimental group, larvae were randomly selected and analyzed using the DanioVision (Noldus, Wageningen, The Netherlands) instrument. The zebrafish larvae were placed in each well of a 96-well plate filled with 200 μL of fish water individually. The 96-well plate was placed with the larvae in a DanioVision chamber at 28.5 °C for 10 min to acclimatize before starting to record behavior for 1 h EthoVisionXT15 software (Noldus, Wageningen, The Netherlands) was used to analyze and process the motion behavior videos and quantify the total distance moved and mean velocity in an hour.

2.2.6. Hemodynamics analysis

After feeding on HCD and exposure to PCA, HSYA and PCA + HSYA, zebrafish larvae were randomly selected to record video to evaluate blood flow. Blood Flow Activity was assessed by analyzing the movement of erythrocytes in the marked area of the video by DanioScope (version 1.1, Noldus, Wageningen, The Netherlands) software, and the results were compared [24,25].

2.2.7. AST/GOT and ALT/GPT detection

Zebrafish larvae were homogenized and centrifuged to get the supernatant, subsequently, ALT and AST levels were measured using a GPT/ALT kit and GOT/AST kit (Nanjing, China).

2.2.8. H&E staining

Zebrafish larvae were fixed in 4 % paraformaldehyde at 4 °C overnight, dehydrated in gradient ethanol, then embedded in paraffin and sectioned at 4 m lengths. The slices were stained with H&E, then sealed with neutral gum. The zebrafish liver histopathological changes were photographed on the pathological section scanner (NanoZoomer2.0-RS, HAMAMATSU, Japan).

2.2.9. Short-Chain fatty acids Quantification

For SCFAs analysis, 60 zebrafish larvae were collected from each group, homogenized with water and glass beads, then centrifuged to obtain supernatant, then added with 15 % phosphoric acid, 375 μg/mL 4-methyl valerate solution (internal standard) and ether, then centrifuged to obtain supernatant. The Thermo Trace 1310 gas phase system in series Thermo ISQ LT mass spectrometer was tested.

2.2.10. Real-time Quantitative Polymerase Chain Reaction

RT-qPCR detection of the expression levels of sterol regulatory element binding protein 2 (*SREBP2*), CCAAT enhancer binding protein alpha (*C/EBPα*), proprotein convertase subtilisin-kexin type 9 (*PCSK9*), peroxisome proliferator-activated receptor-gamma (*PPAR-γ*), Adenosine monophosphate-activated protein kinase (*AMPK*), low-density lipoprotein receptor (*LDLR*), sterol regulatory element binding protein 1 (*SREBP1*), 3-hydroxy-3-methyl-glutaryl Coenzyme A reductase (*HMGCR*), Mammalian Target of Rapamycin (*mTOR*), hepatocyte nuclear factor 1α (*HNF-1α*) and *forkhead box class O3a* (*FoxO3a*) in zebrafish hyperlipidemia. RT-qPCR primer sequences [26–28] used in this study are shown in Table 1. Each group of zebrafish larvae was homogenized after treatment with HCD

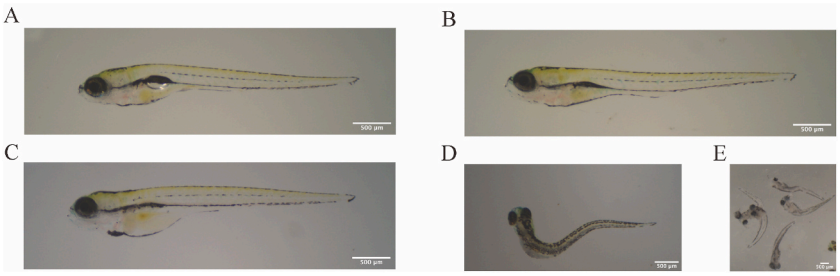

Fig. 1. Toxic phenotype of zebrafish larvae. (A)No obvious abnormality, (B)uninflated swim bladder, (C)pericardial edema, (D)spinal curvature and (E)death. Magnification: (A–C)20 ×, (D)15 × and (E)7 ×.

Table 2
Effects of drugs on mortality, body length and morphology of zebrafish larvae.

Group	Concentration (μg/mL)	Mortality (%)	Spinal curvature (%)	Uninflated swim bladder (%)	Pericardial edema (%)	Body length (μm)
Control	–	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3711.87 ± 139.23
Sim	0.05	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3704.20 ± 133.46
	0.1	61.11 ± 5.09	1.11 ± 1.92	2.22 ± 1.92	0.00 ± 0.00	3712.30 ± 95.63
	0.2	72.22 ± 5.09	7.78 ± 5.09	4.44 ± 1.92	0.00 ± 0.00	3668.00 ± 42.03
	0.4	100.00 ± 0.00	–	–	–	–
	0.5	100.00 ± 0.00	–	–	–	–
PCA	25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3702.74 ± 180.26
	50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3690.15 ± 84.19
	100	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3682.98 ± 105.14
	200	91.11 ± 8.39	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3395.90 ± 167.63
	400	100.00 ± 0.00	–	–	–	–
	800	100.00 ± 0.00	–	–	–	–
	1000	100.00 ± 0.00	–	–	–	–
HSYA	25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3673.37 ± 147.24
	50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3731.32 ± 147.96
	100	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3715.63 ± 144.59
	200	2.22 ± 1.92	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3721.72 ± 139.12
	400	34.44 ± 10.18	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3677.33 ± 123.95
	800	100.00 ± 0.00	–	–	–	–
	1000	100.00 ± 0.00	–	–	–	–
PCA + HSYA	6.25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3707.52 ± 106.86
	12.5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3699.56 ± 133.14
	25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3692.91 ± 115.21
	50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3692.02 ± 128.91
	100	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3691.52 ± 142.04

and drugs, and total RNA was extracted using a Vazyme Kit (Nanjing, China). RNA concentrations were subsequently determined with NanoDropOne (Thermo Fisher Scientific) and RNA was reverse transcribed into cDNA using abm reverse transcription Kit. RT-qPCR was performed using the ABclonal SYBR Green (Wuhan, China) on a QuantStudio 3 applied biosystem. There were three biological and three technological repeats performed in RT-qPCR. The cycling conditions were used: 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. $2^{-\triangle\triangle Ct}$ was used to calculate relative mRNA expression levels of target genes normalized to *β-Actin*.

2.2.11. Statistical analysis methods

We used GraphPad Prism 9.0 to analyze the data, and the results were expressed as $\bar{x}$±SD. In all experiments, *P* less than 0.05 was considered statistically significant when comparing multiple groups using Tukey's multiple comparison test for one-way ANOVA.

3. Results

3.1. Assess mortality, body length and morphology

In order to determine the administration concentrations of Sim, PCA and HSYA, zebrafish were evaluated for administration, and the toxic phenotype was shown in Fig. 1 and Table 2. Loss of heartbeat in zebrafish larvae was defined as death. We treated zebrafish with drugs for 48 h and recorded mortality and toxicity afterward. In this study, in comparison to the untreated Control group, no visible abnormalities were observed at 0.05 μg/mL in the Sim group, 61.11 % and 72.22 % of zebrafish larvae died at 0.1 and 0.2 μg/mL, respectively, and all zebrafish larvae died at 0.4 and 0.5 μg/mL. In comparison to the Control group, the PCA group did not show any lethal effects and no obvious morphological abnormalities at 25, 50 and 100 μg/mL, 91.11 % of zebrafish larvae died at 200 μg/

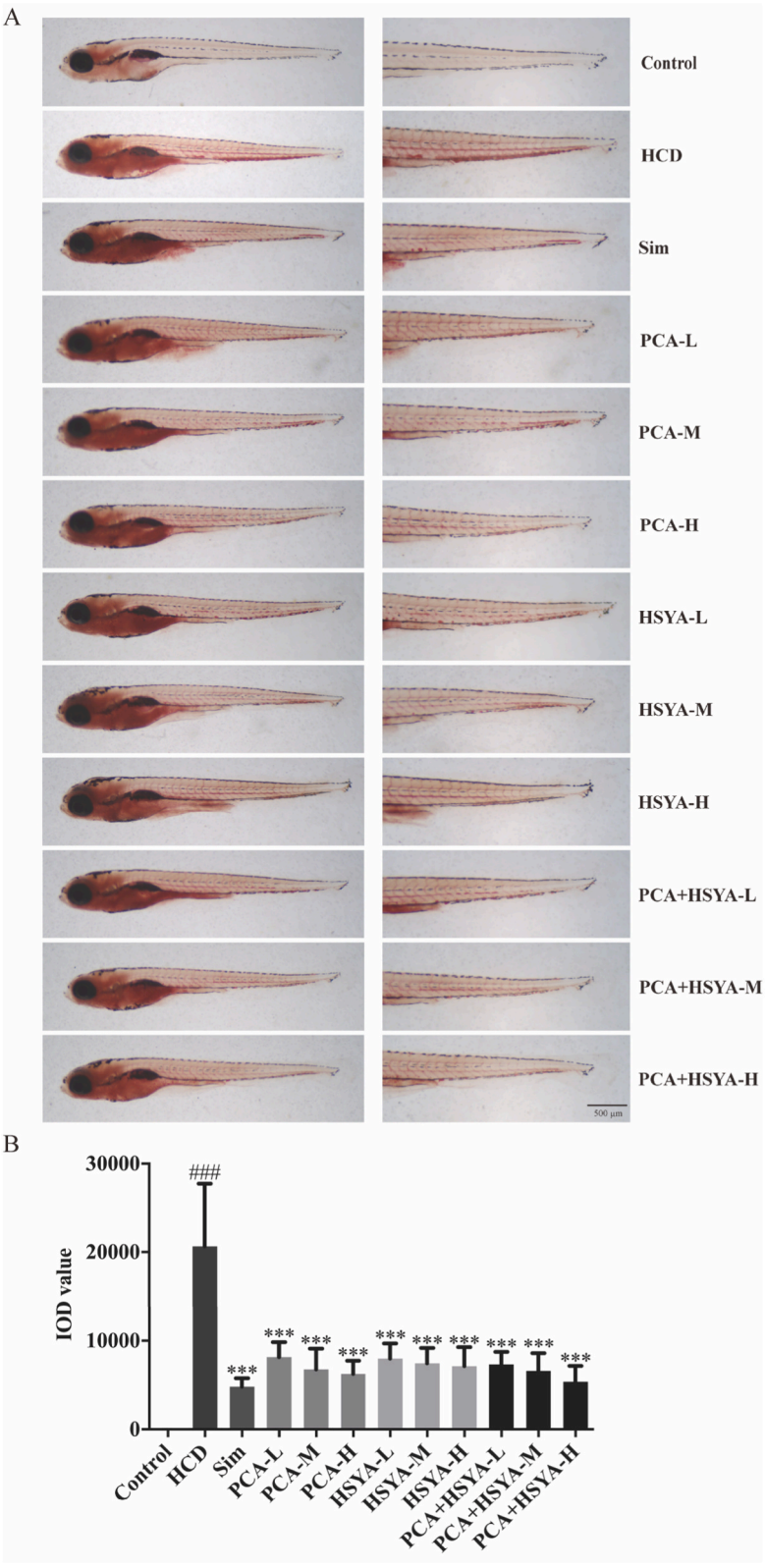

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Fig. 2. Sim, PCA, HSYA and PCA + HSYA all decreased lipid accumulation in the caudal vein. (A) Oil red O staining of zebrafish larvae. (B)IOD values. The Control group; HCD group; PCA-L, HCD + PCA 25 μg/mL; PCA-M, HCD + PCA 50 μg/mL; PCA-H, HCD + PCA 100 μg/mL; HSYA-L, HCD + HSYA 25 μg/mL; HSYA-M, HCD + HSYA 50 μg/mL; HSYA-H, HCD + HSYA 100 μg/mL; PCA + HSYA-L, HCD + PCA 25 μg/mL + HSYA 25 μg/mL; PCA + HSYA-M, HCD + PCA 50 μg/mL + HSYA 50 μg/mL; PCA + HSYA-H, HCD + PCA 100 μg/mL + HSYA 100 μg/mL $^{###}P < 0.001$, in comparison with the Control group. $^{***}P < 0.001$, in comparison with the HCD group, and the error bars represent SD. Magnification: (A) 20 × and 45 × .

mL, and all larvae died at 400, 800 and 1000 μg/mL. Compared with the Control group, zebrafish exposed to 25, 50 and 100 μg/mL HSYA had no obvious morphological abnormalities and death, 200 and 400 μg/mL HSYA caused 2.22 % and 34.44 % death, 800 and 1000 μg/mL HSYA caused all death.

Therefore, the NOAELs for Sim, PCA and HSYA were 0.05 μg/mL, 100 μg/mL and 100 μg/mL, respectively. In subsequent formal experiments, Sim was selected at a concentration of 0.05 μg/mL (NOAEL), PCA and HSYA at selected concentrations of 25 μg/mL (1/4 NOAEL), 50 μg/mL (1/2 NOAEL) and 100 μg/mL (NOAEL).

Therefore, the concentration of PCA + HSYA group can be set to PCA 100 μg/mL (NOAEL) + HSYA 100 μg/mL (NOAEL), PCA 50 μg/mL (1/2 NOAEL) + HSYA 50 μg/mL (1/2 NOAEL), PCA 25 μg/mL (1/4 NOAEL) + HSYA 25 μg/mL (1/4 NOAEL), PCA 12.5 μg/mL (1/8 NOAEL) + HSYA 12.5 μg/mL (1/8 NOAEL) and PCA 6.25 μg/mL (1/16 NOAEL) + HSYA 6.25 μg/mL (1/16 NOAEL) were evaluated for administration. The results showed that PCA + HSYA concentrations of 6.25, 12.5, 25, 50, and 100 μg/mL did not have significant morphological abnormalities or death. Therefore, in the subsequent experiment, the PCA + HSYA-L group was set as PCA 25 μg/mL + HSYA 25 μg/mL; the PCA + HSYA-M group was set as PCA 50 μg/mL + HSYA 50 μg/mL; the PCA + HSYA-H group was set as PCA 100 μg/mL + HSYA 100 μg/mL.

3.2. Lipid-lowering effect on hyperlipidemia zebrafish

In order to determine the effects of PCA and HSYA treatment in zebrafish models of hyperlipidemia, we co-treated zebrafish with HCD and 2 monomers or their combination. Fig. 2 shows the results of Oil Red O staining. While the caudal vein of the Control group

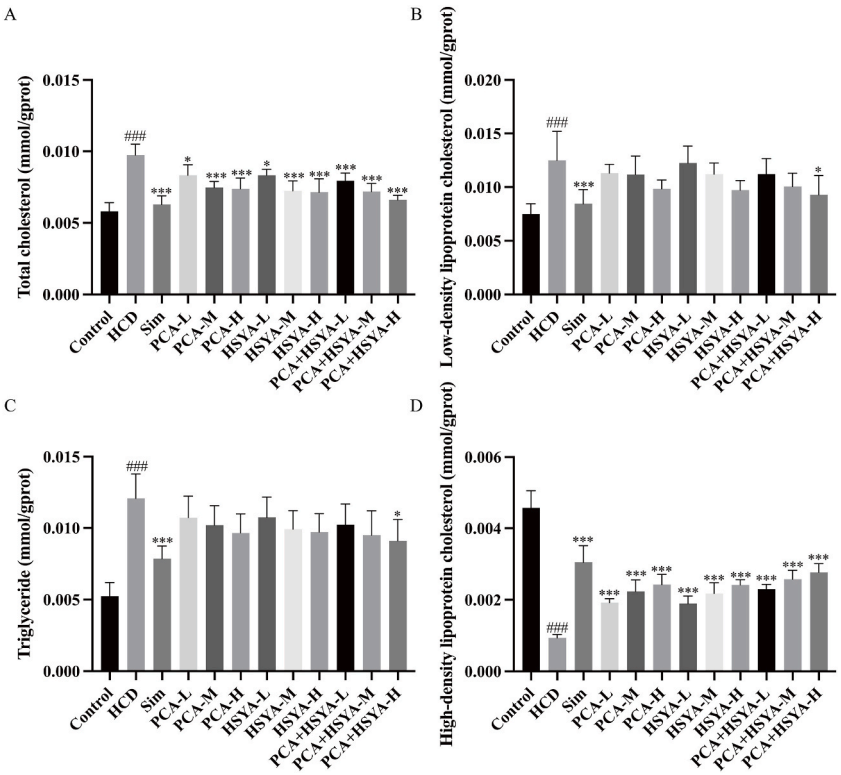

Fig. 3. Effects of PCA, HSYA and PCA + HSYA administration on HCD-induced hyperlipidemia in zebrafish larvae. The Control group; HCD group; PCA-L, HCD + PCA 25 μg/mL; PCA-M, HCD + PCA 50 μg/mL; PCA-H, HCD + PCA 100 μg/mL; HSYA-L, HCD + HSYA 25 μg/mL; HSYA-M, HCD + HSYA 50 μg/mL; HSYA-H, HCD + HSYA 100 μg/mL; PCA + HSYA-L, HCD + PCA 25 μg/mL + HSYA 25 μg/mL; PCA + HSYA-M, HCD + PCA 50 μg/mL + HSYA 50 μg/mL; PCA + HSYA-H, HCD + PCA 100 μg/mL + HSYA 100 μg/mL. Histogram of (A)TC, (B)LDL-C, (C)TG and (D)HDL-C in zebrafish larvae. $^{###}P < 0.001$, in comparison with the Control group. $^{*}P < 0.05$, $^{***}P < 0.001$, compared with the HCD group, and the error bars represent SD.

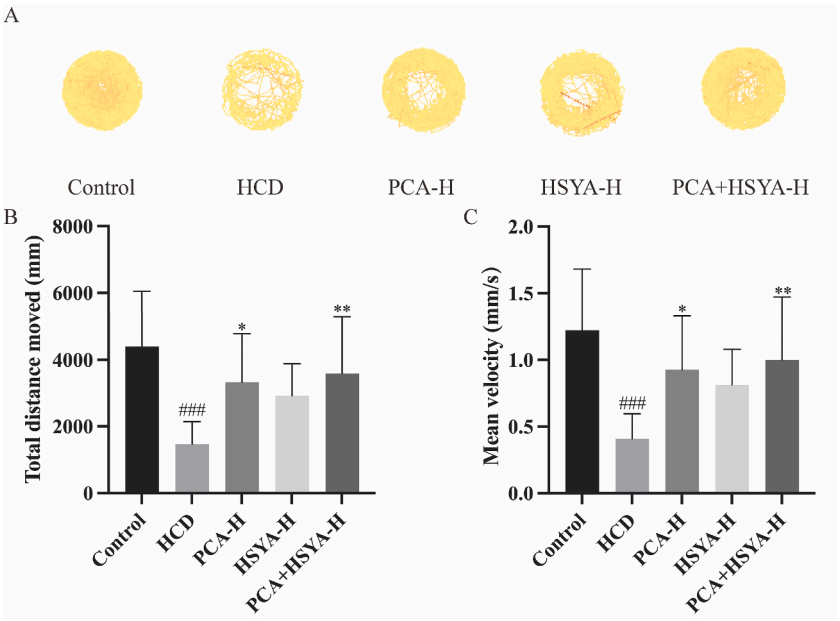

Fig. 4. Behavior evaluation of zebrafish larvae in each group. The Control group; HCD group; PCA-H, HCD + PCA 100 μg/mL; HSYA-H, HCD + HSYA 100 μg/mL; PCA + HSYA-H, HCD + PCA 100 μg/mL + HSYA 100 μg/mL (A)Behavioral trajectories of each group of zebrafish larvae. (B) Graphical representation of total distance moved (mm) of treated larvae in the Control, HCD, PCA-H, HSYA-H and PCA + HSYA-H groups. (C) Graphical representation of the mean velocity (mm/s) of treated larvae in the Control, HCD, PCA-H, HSYA-H and PCA + HSYA-H groups. $^{###}P < 0.001$, in comparison with the Control group. $^{*}P < 0.05$, $^{**}P < 0.01$, in comparison with the HCD group, and the error bars represent SD.

had no obvious lipid deposition, the vein of the HCD group had darker staining, indicating that the hyperlipidemia zebrafish model was successfully established. In contrast to the HCD group, the IOD values of the PCA, HSYA and PCA + HSYA groups showed dose-dependent decreases in blood lipid levels, indicating that each group reduced zebrafish with hyperlipidemia blood lipid levels. Among them, the lipid-lowering efficacy of the PCA group and HSYA group was equivalent, and the lipid-lowering efficacy of the PCA + HSYA group in each concentration was better than that of the PCA group and HSYA group treat alone, the optimal dose was PCA 100 μg/mL + HSYA 100 μg/mL.

3.3. Effects of PCA, HSYA and PCA + HSYA on lipid levels in zebrafish

After 48 h of HCD, as shown in Fig. 3, the HCD group levels of TC, LDL-C and TG in zebrafish larvae were significantly higher than the Control group, while HDL-C value was significantly decreased. According to these results, the hyperlipidemia model has been successfully established in zebrafish. Compared with the HCD group, after 48h of PCA and HSYA treatment, a significant reduction in TC, LDL-C, and TG was noted, but an increase in HDL-C was also seen, and the combined effect was better than that of single use. It suggested that PCA, HSYA and PCA + HSYA had effective lipid-lowering effects on hyperlipidemia zebrafish in a dose-dependent manner.

3.4. Behavioral assay

Fig. 4A showed the trajectory of zebrafish larvae. The total distance moved (4399.3 ± 1649.57 mm) and mean velocity (1.22 ± 0.46 mm/s) by the Control zebrafish larvae were greater than the total distance moved (1469.5 ± 673.41 mm) and mean velocity (0.41 ± 0.19 mm/s) by the larvae treated with HCD, indicating that the distance and velocity of the movement were affected by HCD feeding; the total distance moved and mean velocity (Fig. 4B–C) of larvae fed HCD and supplemented with PCA, HSYA and PCA + HSYA treatment, the former was 3330 ± 1453.98 mm, 2922.3 ± 961.30 mm and 3586.8 ± 1705.30 mm, respectively, and the latter was 0.93 ± 0.40 mm/s, 0.81 ± 0.27 mm/s and 1.00 ± 0.47 mm/s, respectively.

3.5. Hemodynamics analysis

Our study further evaluated the effect of PCA and HSYA on zebrafish hemodynamics. As shown in Fig. 5, the flow activity of HCD zebrafish larvae decreased significantly compared with the Control group, when PCA, HSYA and PCA + HSYA were given, the flow activity increased, and PCA + HSYA was better than when used alone. Our results show that PCA + HSYA can improve the blood hypercoagulation state in zebrafish.

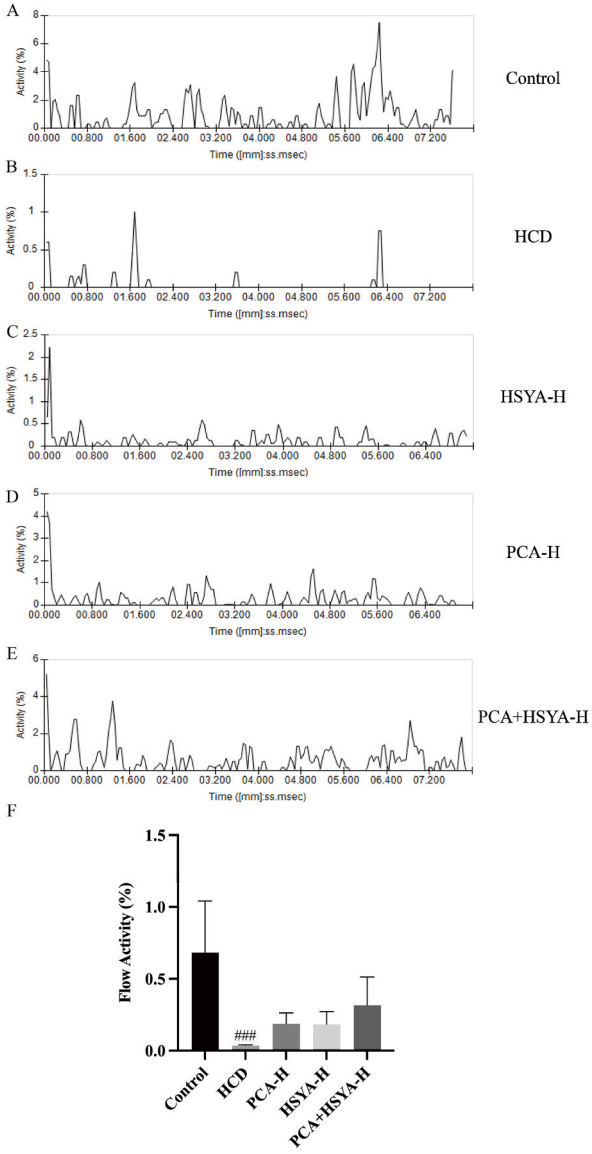

Fig. 5. Hemodynamic evaluation of zebrafish larvae. The Control group; HCD group; PCA-H, HCD + PCA 100 μg/mL; HSYA-H, HCD + HSYA 100 μg/mL; PCA + HSYA-H, HCD + PCA 100 μg/mL + HSYA 100 μg/mL. The flow activity of zebrafish larvae in the (A)Control, (B)HCD, (C)PCA-H, (D) HSYA-H and (E)PCA + HSYA-H groups. (F)Histogram representation of flow activity. $^{###}P < 0.001$, in comparison with the Control group, and the error bars represent SD.

3.6. ALT and AST levels in zebrafish

Determination of biochemical indicators ALT and AST in zebrafish larvae, and liver function investigation, a significant increase in ALT and AST activity was determined in the HCD group, but decreased in Sim group compared with HCD group without statistical difference, while the situation was improved in PCA-H, HSYA-H and PCA + HSYA-H groups (Fig. 6).

3.7. Evaluation of liver Histological damage

So as to further study the effect of HCD in the hepatic steatosis of zebrafish larvae and the improving effect of PCA and HSYA, the histopathological observation of liver tissue was carried out. Histopathological results showed that the liver tissue of the Control group was intact. However, in the HCD and Sim groups, a large number of vacuoles appeared in the liver tissue, and the liver cells were deformed and arranged irregularly. In contrast, treatment with PCA, HSYA and their combination ameliorated liver steatosis and restored liver tissue structure in zebrafish larvae, especially in the PCA + HSYA-H group (Fig. 7). It shows that Sim has no obvious effect on improving liver function, while PCA and HSYA have an obvious effect on improving liver function.

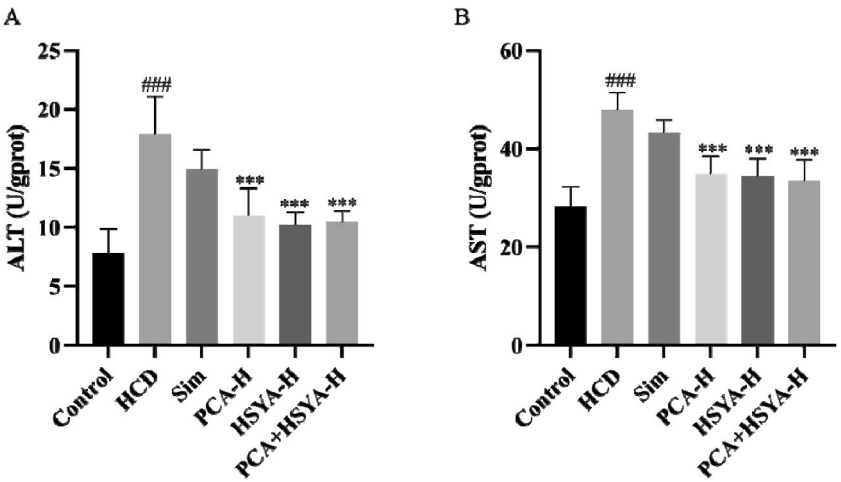

Fig. 6. PCA, HSYA and PCA + HSYA can reduce AST and ALT levels. The Control group; HCD group; PCA-H, HCD + PCA 100 μg/mL; HSYA-H, HCD + HSYA 100 μg/mL; PCA + HSYA-H, HCD + PCA 100 μg/mL + HSYA 100 μg/mL (A-B)Zebrafish larvae fed HCD were given PCA, HSYA and PCA + HSYA, and AST and ALT levels were measured. $^{###}P < 0.001$ in comparison with the Control group; $^{***}P < 0.001$, in comparison with the HCD group, and the error bars represent SD.

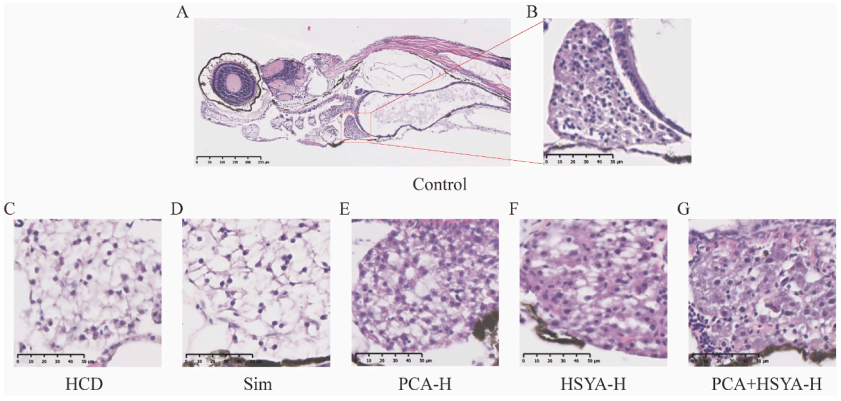

Fig. 7. Pathological observation of the liver in each group of zebrafish larvae. The Control group; HCD group; PCA-H, HCD + PCA 100 μg/mL; HSYA-H, HCD + HSYA 100 μg/mL; PCA + HSYA-H, HCD + PCA 100 μg/mL + HSYA 100 μg/mL (A-B)Control, (C)HCD, (D)Sim, (E)PCA-H, (F)HSYA-H and (G)PCA + HSYA-H groups. Magnification: (A)100 × and (B–G)400 × .

3.8. Short-Chain fatty acids analysis

The results of SCFAs are shown in Fig. 8. In comparison with the Control group, the HCD group had lower contents of butyric acid, acetic acid, isovaleric acid, propionic acid, isobutyric acid, valeric acid and caproic acid in the zebrafish larvae HCD group decreased, but there was no statistical difference. As compared to the HCD group, acetic acid, propionic acid and isobutyric acid in the PCA + HSYA group were down-regulated, while valerate, butyric acid, isovalerate and caproic acid were up-regulated ($P < 0.05$).

3.9. Gene expression

By detecting the mRNA expression levels of *SREBP2*, *SREBP1*, *AMPK*, *PCSK9*, *LDLR*, *HMGCR*, *PPAR-γ*, *C/EBPα*, *mTOR, HNF-1α* and *FoxO3a*, the effect of combined use of PCA and HSYA was further explored. The mRNA expression levels of *PPAR-γ*, *SREBP2*, *SREBP1*, *HMGCR*, *PCSK9*, *mTOR*, *C/EBPα* and *HNF-1α* were significantly up-regulated in the HCD group as compared to the Control group ($P < 0.001$). The mRNA expression levels of *PPAR-γ*, *SREBP2*, *SREBP1*, *HMGCR*, *PCSK9*, *mTOR*, *C/EBPα* and *HNF-1α* were significantly decreased after PCA and HSYA treatment in combination ($P < 0.001$, Fig. 9A–G). In comparison to the Control group, the *LDLR*, *AMPK* and *FoxO3a* mRNA levels of the HCD group were significantly down-regulated ($P < 0.001$). The mRNA expression levels of *LDLR*, *AMPK* and *FoxO3a* were significantly increased after combined PCA and HSYA treatment ($P < 0.001$, Fig. 9H–I).

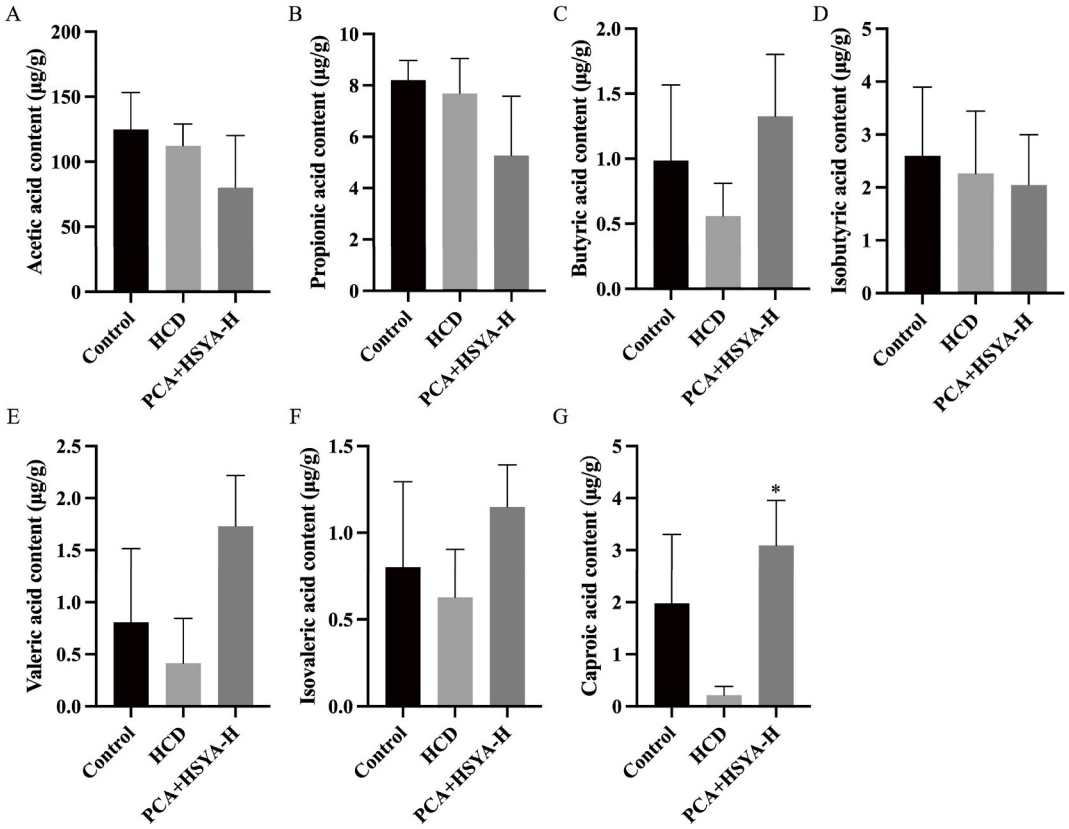

Fig. 8. Contents of SCFAs in zebrafish larvae. The Control group; HCD group; PCA-H, HCD + PCA 100 μg/mL; HSYA-H, HCD + HSYA 100 μg/mL; PCA + HSYA-H, HCD + PCA 100 μg/mL + HSYA 100 μg/mL (A)Acetic acid, (B)propionic acid, (C)butyric acid, (D)isobutyric acid, (E)valeric acid, (F)isovaleric acid and (G)caproic acid. $^{*}P < 0.05$, in comparison with the HCD group, and the error bars represent SD.

4. Discussion

Hyperlipidemia is one of the most common diseases with abnormal blood lipid levels and is one of the major risk factors for the development of cardiovascular disease [29], which can lead to atherosclerosis [30], and it has posed a considerable threat to public health worldwide. Salvia miltiorrhiza and Safflower are both commonly used for promoting blood circulation and removing blood stasis. They are also used as very effective blood rheology agents [31]. The active ingredient of Carthamus tinctorius, hydroxysafflor yellow A, has a good blood-activating effect [32]. Protocatechualdehyde has been confirmed to be the main bioactive component of Salvia miltiorrhiza in the treatment of cardiovascular ischemic injury [33]. In the present study, we investigated the effects of PCA and HSYA on lipid deposition in zebrafish fed the HCD diet and found that PCA and HSYA may regulate lipids through the AMPK/S-REBP2/PCSK9/LDLR signaling pathway.

Studies have shown that the characteristic of dyslipidemia is a systemic abnormality in the blood lipid profile, accompanied by an increase in TC, TG, and LDL-C levels or a decrease in HDL-C level [34,35]. Anil Zechariah et al. [36] showed that VEGF-induced angiogenesis is affected by hyperlipidemia, which translates into subsequent stroke injury, disruption of the metabolic penumbra and loss of hemodynamic improvement after cerebral infarction. As the most important organ in lipid metabolism, the liver can accumulate excess hepatic fat and hepatocyte fat when feeding a high-fat diet for a long period of time [37]. We, therefore, established a hyperlipidemia zebrafish model with HCD feeding in this study. The advantage of zebrafish for studying lipid metabolism and with lipid abnormalities is the optical transparency and importantly the genes involved in the metabolism of lipid and lipoprotein (such as APOB, APOE, APOA1, LDLR, etc.) are conserved from zebrafish to humans [38]. According to this study, we found that hyperlipidemic zebrafish, compared to the Control group of zebrafish, had higher TC, TG and LDL-C levels, reduced blood flow and improved liver function. In this hyperlipidemic model, PCA and HSYA supplementation improved lipid distribution of HDL-C, LDL-C, TG and TC and increased blood flow. Similar to our results, treatment with DHI in hyperlipidemic rats showed a dose-dependent decrease in TC, TG and LDL-C levels and an increase in HDL-C levels in serum or liver tissue [39]. The results of Myeongjoo Son et al. [40] showed that Pyrogallol-Phloroglucinol-6,6′-Bieckol is a valuable active compound in isolated phloroglucinol that improves blood circulation in mouse models of diet-induced obesity and diet-induced hypertension. Our results showed that PCA and HSYA improved the lipid profile of zebrafish fed HCD diet and that PCA + HSYA was superior to HCD diet alone, suggesting that it facilitates the improvement of hyperlipidemia. The SCFAs affect TC and LDL-C, adipose tissue thermogenesis, Browning, and food intake, all of which affect

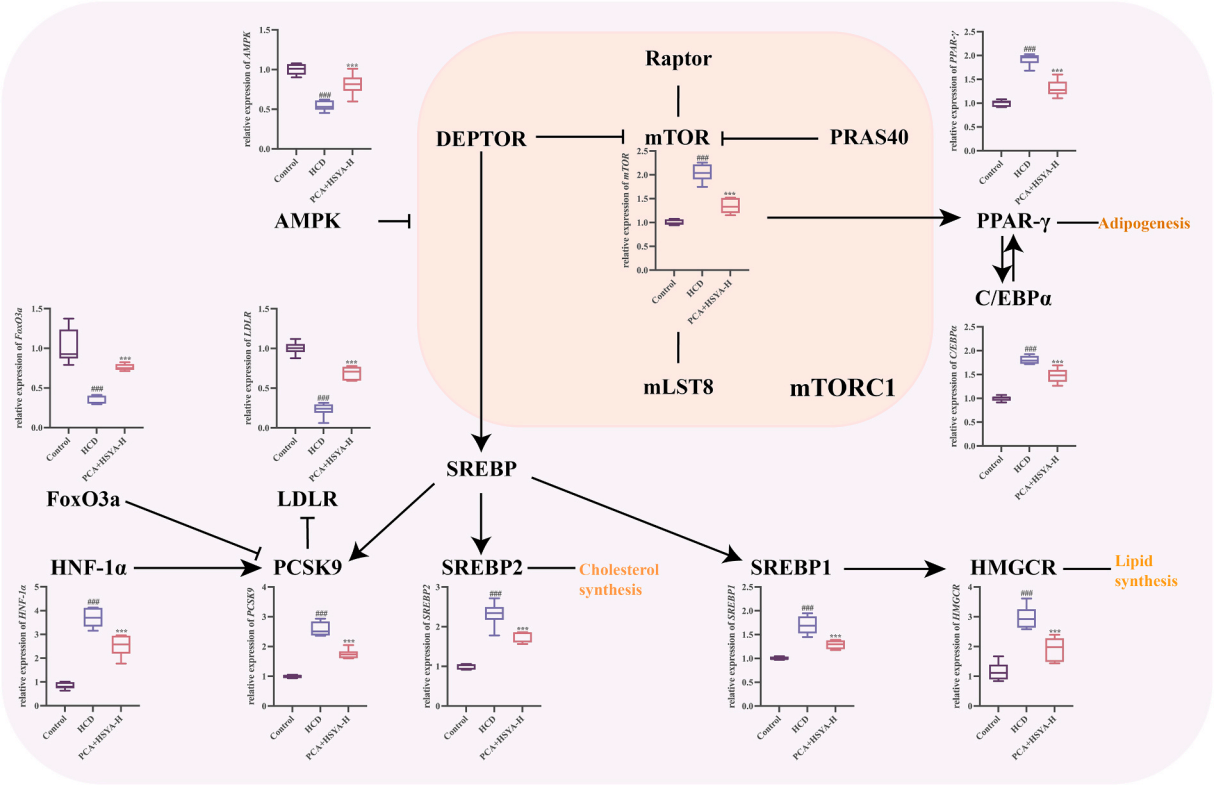

Fig. 9. Relative mRNA expression in zebrafish groups. The Control group; HCD group; PCA-H, HCD + PCA 100 μg/mL; HSYA-H, HCD + HSYA 100 μg/mL; PCA + HSYA-H, HCD + PCA 100 μg/mL + HSYA 100 μg/mL. Relative mRNA expression of *SREBP2*, *SREBP1*, *AMPK*, *PCSK9*, *LDLR*, *HMGCR*, *PPAR-γ*, *C/EBPα*, *mTOR*, *HNF-1α* and *FoxO3a*. $^{###}P < 0.001$, in comparison to the Control group. $^{***}P < 0.001$, in comparison to the HCD group.

cholesterol homeostasis [41]. The results of this study showed that PCA combined with HSYA increased the production of valerate, butyric acid, isovalerate and caproic acid.

It was found that the downstream SREBP-2/PCSK9/LDLR pathway can be regulated by AMPK inhibition of mTORC1 thereby reducing lipid levels [42]. LDLR deficiency is a major cause of familial hypercholesterolemia in humans, and the expression of LDLR, as well as the expression of HMGCR proteins involved in cholesterol biosynthesis, is regulated by the transcription factor SREBP-2, which SREBP-2 is regulated by intracellular cholesterol levels and also regulates cholesterol synthesis [28,43,44]. mTOR signaling pathway specifically regulates PPAR-γ activity, which is critical to the C/EBP-α expression positive feedback Control and the adipose gene expression program and is therefore critical to start and maintain adipogenesis [45]. SREBP1 is a physiologically related high-density lipoprotein receptor, which plays an important role in the cholesterol transport of high-density lipoprotein. It helps to transport excess cholesterol in the form of cholesterol esters to the liver and excrete it into bile acids and feces, thus reducing the incidence of recurrent hypercholesterolemia and cardiovascular events [46]. PCSK9 expression is also controlled by the activity of SREBP-2 as well as a specific transcriptional activator, HNF-1α, a liver-rich transcription factor that regulates many target genes in the liver and intestine [47]. HNF-1α is a transcription factor that has been shown to play a role in lipid metabolism in the liver, where PCSK9 expression is regulated by HNF-1α [48]. Studies have shown [49] that FoxO3a is an important transcription factor of PCSK9, and its binding with PCSK9 can significantly down-regulate the expression of PCSK9. HNF-1α and FoxO3a partially overlap in the promoter binding region of PCSK9, and there is a competitive relationship. Studies have shown [50] that epigallocatechingallate (EGCG) inhibits PCSK9 production by promoting nuclear FoxO3a and reducing nuclear HNF-1α, leading to upregulation of LDLR expression and low-density lipoprotein uptake. This inhibits PCSK9 levels in the liver and circulation and ultimately lowers LDL-C levels. Pinostrobin significantly increased the level of nuclear FoxO3a protein, enhanced the formation of FoxO3a/PCSK9 promoter complex, and weakened the promoter binding capacity of nuclear HNF-1α [51]. In this study, we detected the expression of genes associated with cholesterol metabolism, with *SREBP2*, *SREBP1*, *AMPK*, *PCSK9*, *LDLR*, *HMGCR*, *PPAR-γ*, *C/EBPα*, *mTOR*, *HNF-1α* and *FoxO3a*. PCA + HSYA down-regulated *PPAR-γ*, *SREBP2*, *SREBP1*, *HMGCR*, *PCSK9*, *mTOR*, *C/EBPα* and *HNF-1α* expression, but up-regulated *LDLR*, *AMPK* and *FoxO3a* expression. In this paper, the combined concentration of PCA and HSYA is relatively simple, and more possible exploration can be made on the combined concentration of PCA and HSYA.

5. Conclusion

In conclusion, PCA and HSYA can improve HCD-induced lipid deposition, liver function and slow blood flow, and the combined effect is better. Based on our experiments, the protective mechanism of PCA and HSYA against hyperlipidemia may be achieved by regulating the AMPK/SREBP2/PCSK9/LDLR signaling pathway. These results may provide new ideas to study the therapeutic mechanism of PCA and HSYA to improve hyperlipidemia and develop related drugs for future clinical use in hyperlipidemia.

Data and code availability

Raw data from Figures were deposited on Mendeley at [https://data.mendeley.com/preview/n9phzvs5pd?a=f3ed7075-78aa-4ea0-8637-eb4f85171197].

Notes

The authors declare no competing financial interest.

Ethics approval

Not applicable.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Bingying Lin: Methodology. **Haofang Wan:** Resources. **Jiehong Yang:** Conceptualization. **Li Yu:** Supervision. **Huifen Zhou:** Writing – original draft. **Haitong Wan:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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