Original Research Article

Study on the effect of *salvia miltiorrhiza* on lowering blood lipid based on zebrafish hyperlipidemia model

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Abstract: Objective: This study investigated the lipid-lowering effects of *Salvia miltiorrhiza* by constructing the high blood lipid zebrafish model. **Methods:** The hyperlipidemia model was constructed using egg yolk to feed the zebrafish larval. On this basis, Zebrafish larval with hyperlipidemia were treated with different concentrations of *Salvia miltiorrhiza* extract. Then the light density, length, cholesterol, and triglyceride content were determined. By comparing the above data between the drug treatment group and the blank group, the hypolipidemic efficacy of *Salvia miltiorrhiza* was analyzed. **Results:** The triglyceride (TG) and total cholesterol (TC) contents of the model groups were significantly increased comparing control group, showing that the hyperlipidemia model had been effectively established. After treatment with 0.516 μg/ml, 1.135 μg/ml, and 2.325 μg/ml of *Salvia miltiorrhiza* extract, the lipid reduction in the hyperlipidemia model was 27.11%, 41.58%, and 62.83%, respectively. Furthermore, the TG content was reduced by 32.07%, 53.12%, and 51.46%. The TC content was reduced by 20.29%, 29.55%, and 38.80%, respectively.The data of each group were statistically significant ($P<0.01$). **Conclusion:** *Salvia miltiorrhiza* has a good hypolipidemic effect in the zebrafish hyperlipidemia model. The above data provide a basis for the further development of *Salvia miltiorrhiza* in the field of hypolipidemic.

Keywords: Zebrafish; *Salvia miltiorrhiza*; hypolipidemic;

1 INTRODUCTION

Salvia miltiorrhiza Bunge is a herb of Labiatae, mainly distributed in the central provinces of China.The common medicinal *salvia miltiorrhiza* is the root and rhizome of the *salvia miltiorrhiza* plant. It was first recorded in *the Shennong Classic of Materia Medica* that it tastes bitter and has a cold nature. It is a commonly used traditional Chinese medicine that promotes blood circulation and dissipates blood stasis, smoothes the meridians, relieves pain, clears heat, and calms the nerves. Because of its remarkable effect on promoting blood circulation and removing blood stasis, it is said that "one *salvia miltiorrhiza* root has the same function

UNDER PEER REVIEW

as the four substances"[1]. Some modern medical research finds that its good blood-activating function can improve microcirculation and elevate blood flux[2]. Hence, it is widely used in treating and preventing cardiovascular diseases[3]. In addition, it has anti-tumor[4], anti-bacterial and anti-inflammatory[5-6].

Hyperlipidemia (HLP) is a metabolic disease caused by abnormal lipid metabolism or transport, making one or several lipids in plasma higher than normal. HLP is often manifested as hypercholesterolemia, hypertriglyceridemia or mixed hyperlipidemia.Because most lipids combine with plasma proteins and run throughout the body, hyperlipidemia is often reflected in hyperlipoproteinemia, which is the main cause of atherosclerosis, cardiovascular disease, and fatty liver.Therefore, the prevention and treatment of hyperlipidemia remains a huge challenge for global public health[7].

Although *Salvia miltiorrhiza* has existed in many blood lipid lowering TCM compound preparations, most studies still focus on its application in cardiovascular, not hyperlipidemia. Zhong Li Yun[8] reported that *Salvia miltiorrhiza* played an auxiliary role in hypolipidemic effects using the rat's model.

Compared with other model animals, the zebrafish model is characterized by its small size, high spawning, rapid development, and transparency of embryos and larval fish. They share high homology with the human genome. Hence, increasingly TCM efficacy and toxicity screenings are performed using the zebrafish model, which dramatically shortens the drug research cycle[9-10].

With careful consideration, the zebrafish are selected in this project to establish a blood lipid model, and the lipid-lowering effects of *Salvia miltiorrhiza* are investigated.

2 Material and methods

2.1 Material and reagents

Zebrafish (adult AB line, six months old) and brine shrimp were purchased from Shanghai Fexi Biotechnology Co., Ltd. in September 2021.

Oil Red O Dye reagent, Absolute ethanol, Paraformaldehyde, Propylene glycol, 1-phenyl 2-thiourea (PTU), Methyl blue, and other reagents were purchased from Chengdu Kelong Chemical Reagent Factory. The bacteriostatic agent (oxytetracycline sea salt) was purchased from Shanghai Fexi Biotechnology Co., Ltd. Nitrifying bacteria and water-quality stabilizers were purchased from Shanghai Cunjing Quatic Products Co., Ltd.

Salvia miltiorrhiza was purchased from Yu He Tang Chinese Materia Medica Co., Ltd.

2.2 Methods

2.2.1 Zebrafish husbandry, breeding, and larva incubation

UNDER PEER REVIEW

Zebrafish husbandry: Using the brine shrimp to feed the Zebrafish 2-3 times a day.To ensure water quality, water purification agents and nitrifying bacteria were added to the water.

Breeding and hatching of larval: Male and female fish were put into a special breeding tank in the afternoon or evening, separating with a clapboard. Then the baffle was removed the following day, and the fish eggs were collected after fertilization. Next, larva culture solution (including methyl blue, bacteriostatic element, and PTU) was poured into the culture dish. During fish hatching at 28℃, the culture solution should be changed every morning and evening, and hatching should be completed in 2-3 days [11].

2.2.2 Establishment of zebrafish hyperlipidemia model

One hundred and fifty zebrafish larval around 5 days post fertilization (dpf) were randomly placed in three petri dishes (50 larval per dish). In the model group, the larva was fed in 2mg/ml egg yolk powder solution for 72 hours (water quality needs to be constantly observed and controlled). The other petri dishes were the control group (brine shrimp as food) and the blank group (without food). It judged whether the hyperlipidemia model was successfully established according to the optical density data.

2.2.3 Evaluation of the effect of *Salvia miltiorrhiza* on lowering blood lipid

2.2.3.1 The extraction of *Salvia miltiorrhiza*

The *Salvia miltiorrhiza* was pulverized, dried, and passed through a 60-mesh sieve. 10 g powder of *Salvia miltiorrhiza* was placed in a beaker with ten times the amount of 75% ethanol, ultrasonic frequency 40 Hz, and extraction time 30 min. After filtration, the filtrate was collected and concentrated by rotary evaporation to obtain the extract. Finally, the *Salvia miltiorrhiza* extraction solution was adjusted to 85mg/mL.

2.2.3.2 Determination of the maximum tolerance concentration (MTC)

Salvia miltiorrhiza extracts were separately added to six-well plates（0μl、100μl、200μl、250μl、300μl、400μl). Then, the culture solution was added to 12ml, and 30 zebrafish larval were added to each well. Zebrafish performance and death were observed and recorded after 48h of constant temperature culture at 28℃, and the maximum tolerated dose of the drug to zebrafish pairs was determined.

2.2.3.3 Evaluate the lipid-lowering effect

After establishing the model, 150 zebrafish larval with hyperlipidemia (2mg/ml Egg yolk feeding) were randomly selected and divided into three groups (30 in each group). Within the concentration range of MTC,used 0.516μg/ml、1.135μg/ml, and 2.325μg/ml *Salvia miltiorrhiza* as the sample groups, 0.240μg/ml atorvastatin calcium as the positive control group. In addition, 30 zebrafish larval (blank group) shall be treated nothing. After 48h of treatment, larval fish were taken out, and optical density, triglyceride and cholesterol content, and body length were determined, respectively.

（1）Determination of total cholesterol (TC) and triglyceride (TG)

UNDER PEER REVIEW

After weighing the zebrafish larval, homogenizing medium (95% alcohol) was added, and the solution was smashed with a glass rod. After the homogenization was completed, a small centrifuge 2500r/min was used for centrifugation for 10min. After the centrifugation and filtration, the TC and TG contents of the supernate were detected according to the kit's instructions.

（2）Optical density measurement

The zebrafish larval was sucked into the culture dish and fixed shape. Wrapped and sealed with a black plastic bag and stored in a 4℃ refrigerator. Used a straw to suck out the paraformaldehyde solution and then poured in propylene glycol solution (20%, 50%, 80%) for gradient elution for 15min. After the elution, 80% propylene glycol solution was sucked out and poured into 0.5% oil red O dye solution. Dyed it in a refrigerator at 4℃ away from light for 12h, then sucked out the excess dye solution and used propylene glycol solution (20%, 50%, 80%) to clean the excess dye.

After the oil red O staining, the fat was observed under the light microscope, and the body length was measured. Image-ProPlus 6.0 was performed to analyze the statistical sum of blood lipid optical density (s) of blood vessels in the zebrafish tail. The hypolipidemic effects of *Salvia miltiorrhiza* were evaluated according to the statistical results of the sum of optical density.

3 Results and discussion

3.1 Results of hyperlipidemia model establishment

UNDER PEER REVIEW

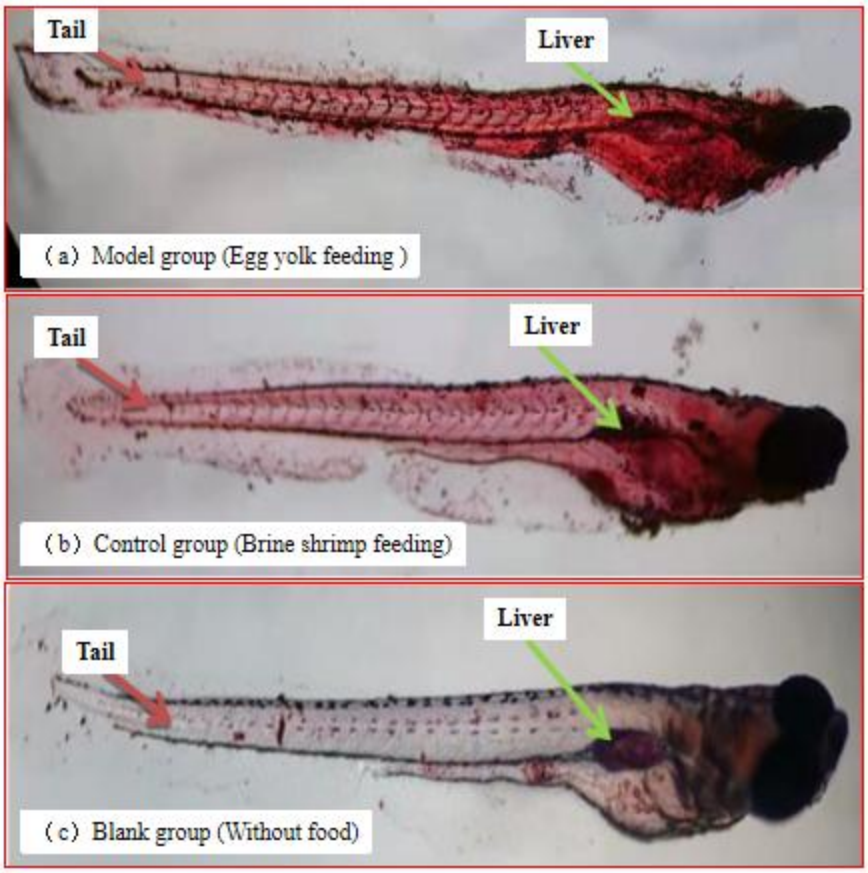

Figure 1 The establishment of hyperlipidemia models

The oil red O staining results (Figure 1) showed that the liver and tail's staining area and color of the model and control group increased relative to the blank group, indicating the fat accumulation in the body after eating. The liver of the zebrafish in the model group was dyed bright red, and the tail blood vessels had prominent fat accumulation lines. In contrast, the larval fish fed with the brine shrimp (control group) had a smaller and lighter staining area. After measuring each group's blood lipid optical density through Image-ProPlus6.0, Table 1 showed that the blood lipid of the brine shrimp-fed group (control group) increased by 248% compared with the blank group. The blood lipid in the yolk-fed group (model group) increased by 723% compared with the blank group.

As shown in Table 1, the TG content (1.035mmol/gprot) of the control group was about 3 times more than that of the blank group, and the TC content (2.809mmol/gprot) was about 2 times more than that of the blank group; Compared with the control group, the content of TG (2.971 mmol/gprot) in the model group was about 3 times of that in the control group. The content of TC (4.948 mmol/gprot) was about 1.5 times that in the control group. It can be concluded that egg yolk feeding can significantly increase the content of TC and TG in zebrafish larval.

In addition, the average length of the control group was typically 3.925mm, which was 6.629% higher than that of the blank group; The average length of the model group with egg yolk was 3.865mm, which was

4.999% longer than the blank group. It can be concluded that egg yolk feeding does not affect the body length growth of zebrafish larval.

The above data showed that using 2mg/ml egg yolk solution effectively establishes a hyperlipidemia model.

Table 1 Blood lipid/TG/TC/length analysis results of zebrafish (n=30)

Group	Feeding of each group	Total optical density of blood lipid in tail vessels (mean±SE)	Blood lipid growth rate (%)	TG content (mmol/gprot) (mean±SE)	TC content (mmol/gprot) (mean±SE)	Length of zebrafish (mm) (mean±SE)	Length variation (%)
Blank group	no feeding	0.7944±0.5627	-	0.250±0.076	1.061±0.461	3.681±0.188	-
Control group	brine shrimp	2.7615±0.8203	248	1.035±0.121	2.809±0.319	3.925±0.175	6.629
Model group	2mg/ml egg yolk solution	6.9050±0.9675	769	2.971±0.248	4.948±0.679	3.865±1.89	4.999

3.2 Research results of the blood lipid-lowering effect of *Salvia miltiorrhiza*

3.2.1 Maximum tolerance concentration (MTC) of *Salvia miltiorrhiza*

As shown in Table 2, when the concentration of *Salvia miltiorrhiza* extract was 1.416-3.540μg/ml, the zebrafish larval didn't die within 48h, and when the concentration was up 5.664μg/ml, 86.7% larval died. Therefore, the maximum tolerance (MTC) of *Salvia miltiorrhiza* against zebrafish was 3.540μg/ml。

Table 2 Maximum tolerance concentration results after *Salvia miltiorrhiza* treatment (n=30)

Solution concentration (μg/ml)	Death of fish within 24h (piece)	Death of fish within 48h (piece)	Mortality (%)
0	0	0	0
1.416	0	0	0
2.832	0	0	0
3.540	0	0	0
4.248	0	6	20%
5.664	6	26	86.7%

3.2.2 Lipid-lowering effect of *Salvia miltiorrhiza*

As shown in Figure 2, the blood vessels of the zebrafish tail in the blank group had many oil-red O attachments, and the whole staining degree was high (Figure 3a). After 0.355μg/ml atorvastatin calcium treatment, the oil red O attachment in the zebrafish's tail was significantly reduced, and the color became pale (Figure 2b). However, after treatment with different concentrations of *Salvia miltiorrhiza*, the staining of the fishtail was also attenuated (Figure 3 c,d,e). Table 3 showed serum lipids reduced by 45.37% after 0.355μg/ml atorvastatin calcium treatment (Positive control group). After 0.516μg/ml, 1.135μg/ml, and 2.325μg/ml *Salvia*

UNDER PEER REVIEW

miltiorrhiza extract treatment, lipid-lowering was 27.11%, 41.58%, 62.83%, respectively, which indicated that *Salvia miltiorrhiza* had lipid-lowering effects similar as atorvastatin calcium. Moreover, the lipid-lowering effects also showed an enhanced trend with increasing concentrations of *Salvia miltiorrhiza*.

Table 3 and Figure 4 also exhibited that the TG and TC content decreased by 42.32% (5.848mmol/gprot) and 27.39% (2.753mmol/gprot) after 0.355μg/ml atorvastatin calcium treatment. *Salvia miltiorrhiza* extract (0.516μg/ml、1.135 μg/ml、2.325μg/ml) made TG levels (6.420 mmol/gprot, 5.675 mmol/gprot, 4.930 mmol/gprot) decreased by 32.07%, 53.12%, 51.46%, respectively. TC content (3.242mmol/gprot, 2.238mmol/gprot, 2.317mmol/gprot) reduced by 20.29%, 29.55%, and 38.80%, respectively. It exhibited that *Salvia miltiorrhiza* could simultaneously reduce the content of TC and TG in zebrafish, and the reduction was higher on TG than on TC. In addition, the body length changed below 1%, showing that *Salvia miltiorrhiza* had no significant effect on the body length of zebrafish.

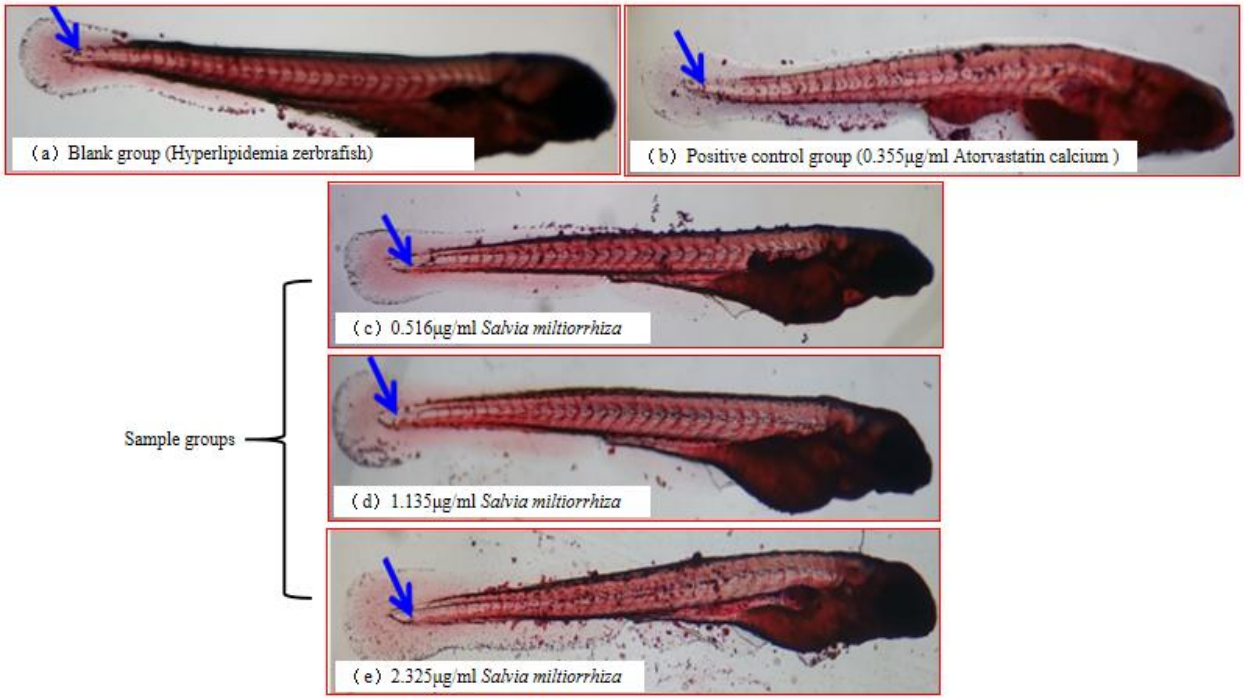

Figure 2 The contrasts of treatments (Blue arrows indicated the fish tail oil red O accumulation)

Table 3 Hypolipidemia analysis results of hyperlipidemia model zebrafish after treatmen (n = 15, mean±SE)

Group	Treatments in each group	Tail vessel blood lipid optical density sum	Lipid growth rate (%)	TG content (mmol/gprot)	TC content (mmol/gprot)	Length of zebrafish	Length variation (%)
Blank group	-	7.9462±0.8412	-	8.054±1.826	4.773±0.215	3.294±0.152	-
Positive control group	0.355μg/ml Atorvastatin calcium	4.3409±0.7589	45.37	5.848±1.230	2.753±0.200	3.295±0.149	0.03

Sample groups	0.516μg/ml	5.7923±0.5268	27.11	6.420±1.132	3.242±0.270	3.221±0.105	0.86
	1.135μg/ml	4.6425±0.8167	41.58	5.675±1.371	2.238±0.193	3.228±0.186	0.65
	2.325μg/ml	2.9536±0.8743	62.83	4.930±1.303	2.317±0.354	3.266±0.248	0.85

4 Conclusions

Our group found *that Salvia miltiorrhiza* has a place in many TCM lipid-lowering formulas. Therefore, we explored the hypolipidemic efficacy of *Salvia miltiorrhiza* based on the zebrafish model and verified its effect by measuring fishtail optical density, TG\TC content, and body length. The results indicated that *Salvia miltiorrhiza* had a strong hypolipidemic effect and that *Salvia miltiorrhiza* could simultaneously reduce the contents of triglycerides and cholesterol, with a particularly prominent effect on triglycerides ($P<0.01$). The results will be valuable for the later development of *Salvia miltiorrhiza*-centered medicines and health products for application in obesity.

References

[1] Shijun Yin, Congpeng Zhao, Guang Hu, et al. Investigation on the Metabolism of Salvianolic acid B, Tanshinone IIA, Ginsenoside Rg1 and Ginsenoside Rb1 from Danshen-Sanqi Herbal Pair in Zebrafish by Liquid Chromatography-Tandem Mass Spectrometry Analysis[J]. Clinical Complementary Medicine and Pharmacology,2021,1(1): 1-8

[2] Yue Chen, Yanting Wang, Juan Guo, et al. Integrated Transcriptomics and Proteomics to Reveal Regulation Mechanism and Evolution of SmWRKY61 on Tanshinone Biosynthesis in *Salvia miltiorrhiza* and Salvia castanea [J]. Frontiers in Plant Science,2022,12.

[3] Ding M, Ye T X, Zhao G R, *et al*. A queous extract of Salvia miltiorrhiza attenuats increased endothelial permeability induced by tumor necrosis factora[J]. International Immunopharmacol, 2005, 5(11): 1641-1651.

[4] Oberkersch Roxana E, Lidonnici Jacopo, Santoro Massimo M. How to Generate a Vascular-Labelled Transgenic Zebrafish Model to Study Tumor Angiogenesis and Extravasation.[J]. Methods in molecular biology,2023,2572.

[5] Larijani Bagher, Hamidpour Shayesteh Kokabi, TayanlooBeik Akram, et al. An Overview of Zebrafish Modeling Methods in Drug Discovery and Development.[M]. Advances in experimental medicine and biology,2021.

[6] Saravanan S, Pari L. Role of thymol on hyperglycemia and hyperlipidemia in high fat diet-induced type 2 diabetic C57BL / 6J mice [J]. European Journal of Pharmacology, 2015, 761: 279-287.

[7] Naziri Mahdyieh, Ghafari Arezoo, Mehrabi Hoda, et al. A Mini-Review of the Anticancer Properties of Cryptotanshinone: A Quinoid Diterpene Extracted From the Root of *Salvia miotiorrhiza* Bunge[J]. Frontiers in

Drug Discovery,2022.

[8] Omer Ibrahim Abdallh Omer. A Review on Zebra Fish as a Preclinical Model for Natural Drug Discovery[J]. Pharmaceutical Science and Technology,2021,5(2): 62-67

[9] Zhou J, Xu YQ, Guo SY, *et al.* Rapid analysis of hypolipidemic drugs in a live zebrafish assay [J]. J Pharmacol Toxicol Methods, 2015, 72: 47-52.

[10] Huang C C, Monte A, Cook J M, *et al*. Zebrafish heart failure models for the evaluation of chemical probes and drugs [J]. Assay and Drug Development Technologies, 2013, 11(9/10): 561-572.

[11] Mhlongo Fikile, Cordero-Maldonado Maria Lorena,Crawford Alexander D., et al.. Evaluation of the wound healing properties of South African medicinal plants using zebrafish and in vitro bioassays[J]. Journal of Ethnopharmacology,2022,286: 114867.

UNDER PEER REVIEW