

# Model design for screening effective Antihyperlipidemic drugs using zebrafish system

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**Abstract:** The purpose of this paper was to explore a new method for screening lipid-lowering drugs in zebrafish models. The suitable drug concentrations of atorvastatin (ATV), fenofibrate (FEF) and ezetimibe (EZE) were first determined. Then, the serum cholesterol and triglyceride levels were detected in high-fat diet (HFD)-fed zebrafish. The HFD zebrafish models were constructed and the effects of drugs on them were observed by Oil red O staining and fluorescence labeling. Statistical analyses among groups were conducted using SPSS software. The lowest drug concentration (LDC) and the highest (HDC) of ATV, FEF and EZE were 0.3  $\mu$ M/37.0 $\mu$ M, 1.2 $\mu$ M/3.5 $\mu$ M, and 6.3  $\mu$ M/26.4 $\mu$ M, respectively, while, the intermediate (IDC) was, in order, 18.5 $\mu$ M, 1.8 $\mu$ M, 13.2 $\mu$ M. The cholesterol and triglyceride levels in HFD-fed zebrafish were increased after 7 weeks fat feeding ( $p<0.05$ ). Moreover, the levels of triglyceride were significantly decreased after LDC of ATV and FEF treated ( $p<0.05$ ), but not that of EZE. While, the cholesterol levels were reduced in three groups ( $p<0.05$ ). Moreover, the 5 dpf high-fat zebrafish model was established successfully and maintained stably for 24h. ATV produced effects in a concentration-dependent manner, while only IDC and HDC of FEF and EZE made effects on this model. Intravascular cholesterol levels were significantly increased after HCD treatment and decreased after drug treated. The high-fat zebrafish model induced by HFD-fed was available and successful, besides, the Oil red O staining may be an available and rapid method for screening lipid-lowering drugs.

**Keywords:** zebrafish; hyperlipidemia; lipid-lowering drug; cholesterol; triglyceride

## INTRODUCTION

Hyperlipidemia, a most common dyslipidemia disease, is characterized by an abnormally elevated level of lipids or lipoproteins such as cholesterol concentration and triglycerides in blood (Luskey and Luo 2001; Yuan *et al.* 2007). Moreover, hyperlipidemia is one of the leading risk factors for development of cardiovascular diseases, which can lead to atherosclerosis (Ross and Harker 1976), coronary heart disease (Shepherd *et al.* 1995) and myocardial infarction (Hazzard *et al.* 1973). It has posed a considerable threats to public health all over the world.

Currently, several drugs are approved by Food and Drug Administration in the treatment of hyperlipidemia, such as atorvastatin (ATV) (Athyros *et al.* 2002), fenofibrate (FEF) (Ellen BSc and McPherson 1998), ezetimibe (EZE) (McKenney *et al.* 2006), fluvastatin (Derosa *et al.* 2004), pravastatin (Dangas *et al.* 1999), lovastatin (Arad *et al.* 1990) and simvastatin (Grundy *et al.* 2005). All of these drugs can reduce the lipid levels to some extent. However, adverse effects are presented as a thorny problem (Nelson 2013), besides, blood lipids in patients are still not well controlled in some situations, such as contraindication or tolerance. Additionally, the determination of their lipid-lowering effects is complicated and can be a laborious processes. Thus, it is essential to develop a new, simple

and rapid method for screening lipid-lowering drugs for the treatment of hyperlipidemia.

Currently, many animal models with high fat diet (HFD)- or high cholesterol diet (HCD)-fed have been used to understand the processes of hyperlipidemia. For example, rat models have been used in hyperlipidemia research and for screening hypolipidemic drugs (Schurr *et al.* 1972). In addition to rat model, zebrafish (*Danio rerio*) model of hyperlipidemia has been developed (Clifton *et al.* 2010; Baek *et al.* 2012). As a vertebrate, zebrafish resembles mammals in the development and metabolic processes (Dooley and Zon 2000). The body of zebrafish is small and translucent, and it has a strong propagation behavior. What's more, it is relatively a low cost for maintenance and rapid development of zebrafish (Streisinger *et al.* 1981). Based on these characteristics, zebrafish has become a popular animal model for biomedical research in developmental biology, genetics and neurobiology (Lieschke and Currie 2007). Interestingly, the developing organs and biological processes in vivo can be visualized non-intrusively due to the translucent feature (Holttä-Vuori *et al.* 2010). It has been indicated that zebrafish is a good vertebrate model for fluorescent imaging (Ko *et al.* 2011; Cross *et al.* 2003; Blackburn *et al.* 2011). It has been also used to analyze the expression of intestinal fatty acid-binding proteins (Andre *et al.* 2000) and observe the lipid metabolism (Walters *et al.* 2012). Therefore, we speculate that the zebrafish model may contribute to the study of

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hyperlipidemia. However, rare studies focus on the high-fat zebrafish model in the screen of effective lipid-lowering drugs for hyperlipidemia.

In this study, three lipid-lowering drugs (ATV, FEF and EZE) were chosen to determine their hypolipidemic effects and suitable concentrations on zebrafish. High-fat zebrafish model was constructed. Then, Oil red O staining and fluorescence labeling were applied to zebrafish. In addition, the feasibility of this method was verified with ATV, FEF and EZE as controls. The purpose of this study was to explore a new, simple and rapid method for screening lipid-lowering drugs with zebrafish model.

## MATERIALS AND METHODS

### Animals

Zebrafish (Casper strain) were obtained from Shanghai Research Center for Model Organisms (Shanghai, China) and the experiment was conducted at Shanghai Ninth People's Hospital Affiliated Shanghai Jiaotong University. All zebrafish were raised and maintained at  $28\pm1^{\circ}\text{C}$ , pH=7.0 reverse osmosis-purified water (fish water) under 14-h light/10-h dark cycles, and accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Approval was obtained from the Animal Care and Use Committee of Shanghai Ninth People's Hospital Affiliated to Shanghai Jiaotong University.

### Preparation of high-cholesterol diet (HCD)- and high-fat diet (HFD)-feed

A HCD for zebrafish was made as follows: conventional fish feed containing 3% crude fat (Slack laboratory animal co., LTD, Shanghai, China) was dissolved in cholesterol (Slack laboratory animal co., LTD, Shanghai, China)-chloroform (Shanghai yuanye Bio-Technology Co., Ltd, Shanghai, China) solution. After chloroform was completely evaporated, HCD with 4% (w/w) cholesterol was prepared.

In the same way, 1/100000 (g/g) fluorescent probe Chol Esteryl BODIPY 542/563 C11 (Invitrogen, Carlsbad, CA, USA) was added to fish feed and the HFD (Andre *et al.* 2000) was prepared according the previous method with some modifications (Clifton *et al.* 2010) using egg yolk powder (0.1%, g/100 ml) (Shanghai yuanye Bio-Technology Co., Ltd, Shanghai, China).

Importantly, the HFD and HCD were alternated in the current study. The reasons were as follows: HFD was made up of egg yolk powder, and its fat content was very high, but the proportion of cholesterol which was used to identify lipid-lowering drug and to calculate the lipid-lowering rate (LLR) via the Oil red O staining was not; for HCD, cholesterol was added to the feeding, and HCD was applied to verify the effects of lipid-lowering drugs

using CholEsteryl BODIPY 542/563 C11 fluorescent probes. In addition, the HFD in the detection of blood lipid included not only cholesterol but also egg yolk power which fat content was very high but the proportion of cholesterol was not, while the HCD was only the former.

### Determination of antihyperlipidemic drug concentration

ATV (Pfizer, Groton, NY, USA), FEF (Laboratoires Fournier SA, Daix, France) and EZE (Schering-Plough Research Institute, Kenilworth, New Jersey, USA) were dissolved in dimethyl sulphoxide (DMSO) (Sangon Biotech, Shanghai, China), respectively. Subsequently, DMSO solution was added to fish water to the final concentration of 0.5% (v/v). The three drugs were prepared in a concentration gradient of 0.1 $\mu\text{M}$ , 0.2 $\mu\text{M}$ , 0.4 $\mu\text{M}$ , 0.6 $\mu\text{M}$ , 0.8 $\mu\text{M}$ , 1 $\mu\text{M}$ , 2 $\mu\text{M}$ , 4 $\mu\text{M}$ , 6 $\mu\text{M}$ , 8 $\mu\text{M}$ , 10 $\mu\text{M}$ , 20 $\mu\text{M}$ , 40 $\mu\text{M}$ , 60 $\mu\text{M}$ , 80 $\mu\text{M}$ , 100 $\mu\text{M}$ , 200 $\mu\text{M}$ , 400 $\mu\text{M}$ , 600 $\mu\text{M}$ , 800 $\mu\text{M}$ , 1000 $\mu\text{M}$ , separately. The 0.5% DMSO (v/v) solution without drugs was regarded as the control.

One day post fertilization (1 dpf) zebrafish and 7 dpf larvae (n=30, each concentration) were placed in drug solution with different concentrations (n=30, each concentration). After 24h and 72h treatment, the mortality of 4 dpf larvae and 8dpf zebrafish was recorded, respectively. Zebrafish with severe complications, such as obvious deformity and cerebral hemorrhage, were classified as death. Median lethal concentration ( $\text{LC}_{50}$ ) and 1% lethal concentration ( $\text{LC}_1$ ) of 4 dpf and 8 dpf were calculated using SPSS software (version 11.5, SPSS Inc., Chicago, IL), respectively.  $\text{LC}_1$  of 4 dpf was defined as the lowest drug concentration (LDC).  $\text{LC}_1$  of 8 dpf was defined as the highest drug concentration (HDC) and 50%  $\text{LC}_1$  of 8 dpf was defined as the intermediate drug concentration (IDC). Each experiment was repeated for three times.

### Detection of serum cholesterol and triglyceride in high fat model after intervened by lowering lipid drugs

Male zebrafish (3 months old) were randomly divided into 5 groups (n=50, each group). Zebrafish in Group 1 and 2 were kept in fish water with 0.5% DMSO, while zebrafish in Group 3, 4 and 5 were maintained with LDC of ATV, FEF and EZE in fish water, respectively. What's more, fish of Group 1 (control) were also fed with conventional fish feed twice a day and fish in Group 2-5 were fed with excess HFD once a day alternatively with cholesterol and egg yolk power. The feed residuals was removed 30 min after feeding.

After HFD feeding for 7 weeks, 30 zebrafish were randomly selected from each group for measuring the serum levels of cholesterol and triglyceride. Zebrafish was anesthetized with 200 parts per million (ppm) MS-222 (Sigma, St Louis, MO, USA), and 5 $\mu\text{l}$  aorta blood was collected from each fish. Serum samples of 5

zebrafish in the same group were mixed and were then diluted 20-fold in physiological saline. The serum levels of cholesterol and triglyceride were measured by Siemens ADVIA2400 chemistry system (Siemens Healthcare, Camberley, UK).

### **High fat model construction for a rapid screening method for lipid-lowering drugs**

A total of 10 larvae (5 dpf) were kept in fish water as the control and other 40 zebrafish were maintained on HFD feeding with 0.1% (g/100ml) egg yolk suspension for 48 h. The egg yolk suspension was exchanged every 4h. After 48 h HFD feeding, zebrafish (7 dpf, n=10) were transferred to fish water and fasted for 72h. The lipid level of zebrafish in control and HFD groups (n=10, each time point) were evaluated by Oil red O staining at 0, 24, 48 and 72h fasting time points, which were named 0h post HFD (0h p HFD), 24h p HFD, 48h p HFD and 72h p HFD, respectively.

The Oil red O staining was performed according to a previous method (Schlombs *et al.* 2003). Zebrafish was anaesthetized and immersed in 4% para formaldehyde (Sigma, Louis, USA) for 12h at 4°C. Next, samples were washed with 10mM phosphate-buffered saline (PBS) 3 times and immersed in 60% isopropanol for 30 min. Then, zebrafish was stained in fresh filtered 0.3% Oil red O solution (Sigma, Louis, USA) for 3 h.

After staining, zebrafish was examined by NikonSMZ1500 microscope using NikonACT-1 imaging software (Nikon Corporation, Tokyo, Japan). Images of zebrafish intestine end at the left border were captured at a magnification of ×100 under the same condition. Only the lipids in the blood vessels were stained by Oil red O staining. The integrated optical density (IOD) was analyzed by Image Pro Plus (IPP, Media Cybernetics Inc., Rockville, MD, USA) version 6.0 software.

### **A rapid screening method for lipid-lowering drugs by calculating the lipid-lowering rate**

The effects of ATV, FEF and EZE on zebrafish were evaluated after the high fat zebrafish model was successfully constructed. After 48h HFD feeding, the zebrafish were randomly divided into 11 groups (n = 30, each group): zebrafish in two groups were cultured in fish water with or without 0.5% DMSO for 24h; another nine groups kept in fish water containing LDC, IDC and HDC of ATV, FEF and EZE for 24h, respectively. Later, 10 zebrafish were randomly selected from each group for Oil red O staining. In addition, the LLR was calculated using the following formula:

$$\text{LLR (\%)} = (1 - \text{IOD (experimental)} / \text{IOD (control)}) \times 100\%$$

### **Verification of the effects of lipid-lowering drugs on cholesterol control**

In order to test and verify the rapid screening method for

lipid-lowering drugs, further research was carried out to analyze the cholesterol-lowering effects of ATV, FEF and EZE (as positive controls) on HCD-fed zebrafish models. The 5 dpf zebrafish were randomly divided into 5 groups: ND group, HCD group, HCD + ATV group, HCD + FEF group and HCD + EZE group. Zebrafish in the ND group (n = 30) was fed with normal diet, and zebrafish in other groups (n=30, each group) were fed with HCD twice a day (feed residuals removed 30 min after feeding). Subsequently, all of them were supplemented with fluorescent analogue of cholesteryl (cholesteryl BODIPY 542/563-C11, 10 µg/g) (Invitrogen) (Stoletov *et al.* 2009). ND group and HCD group were maintained in fish water with 0.5% DMSO, while other groups were cultured in fish water containing LDC of drugs, respectively.

After continuous feeding for 10 days, the fluorescence intensity of cholesteryl BODIPY 542/563 C11 in blood vessels were analyzed by fluorescence microscopy using NIS-Elements BR 3.1 software (Nikon, Melville, NY, USA).

## **STATISTICS ANALYSIS**

Data were presented as mean ± standard deviations (SD). All statistical analyses were carried out using SPSS software. The comparison among groups was performed by one-way analysis of variance (ANOVA) and the difference between two groups was calculated by the least-significant difference (LSD) method. The comparison of the rates was performed by chi-square test. A  $p < 0.05$  was considered statistically significant.

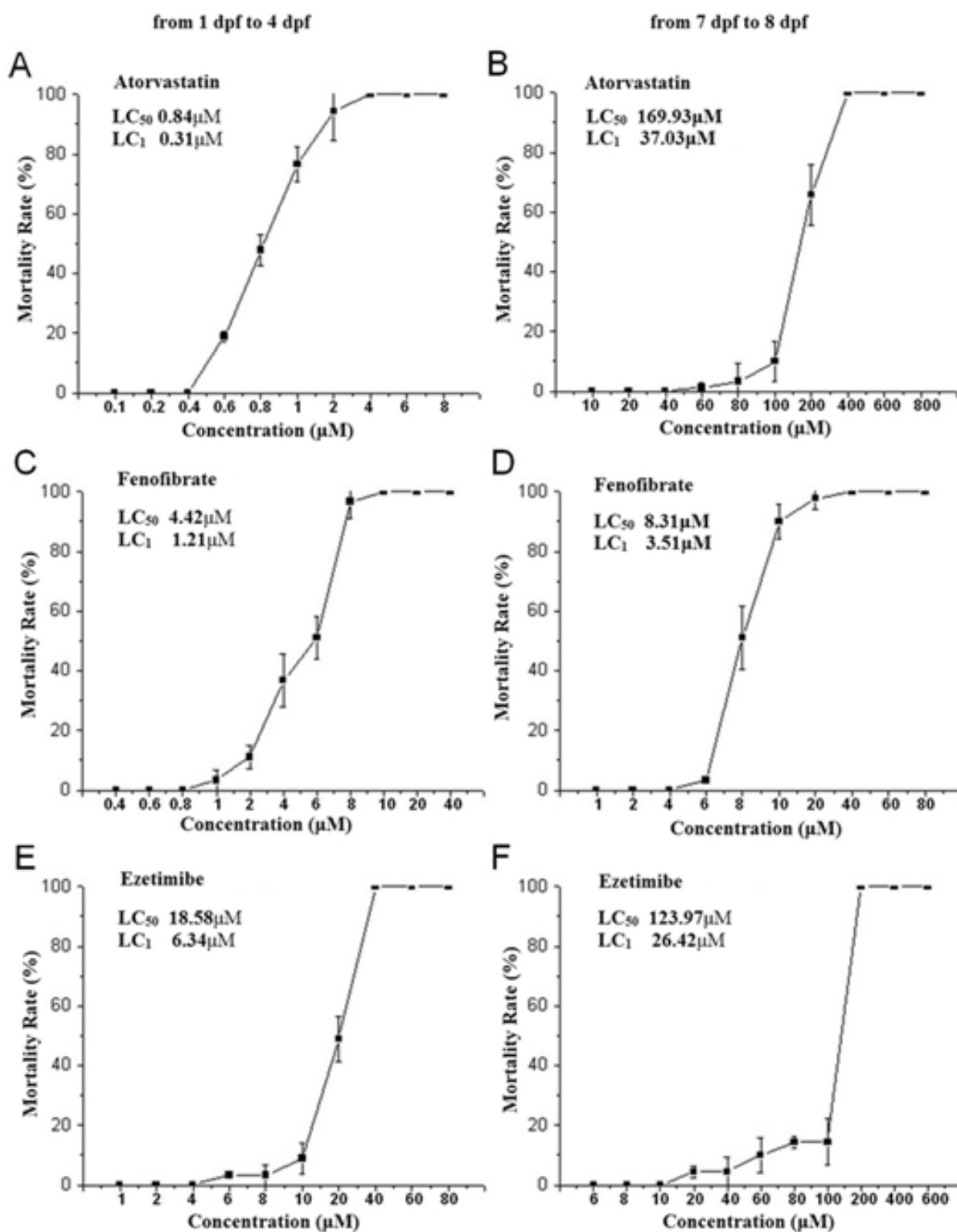
## **RESULTS**

### **The suitable concentration of lipid-lowering drugs on zebrafish**

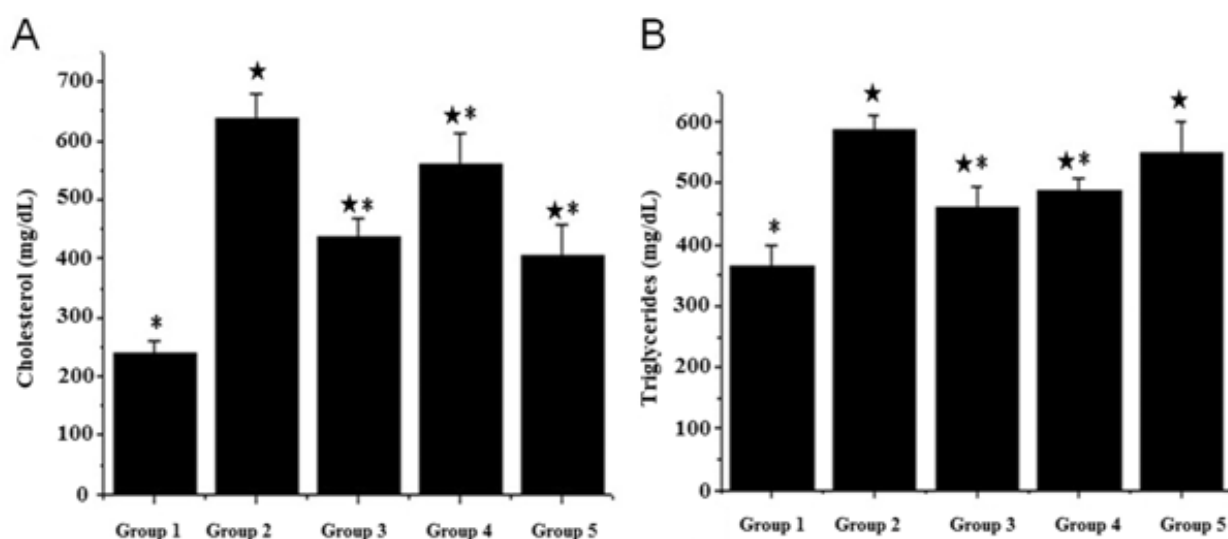
The LC<sub>50</sub> and LC<sub>1</sub> for zebrafish treated with lipid-lowering drugs were shown in fig. 1. The LDC and HDC of ATV, FEF and EZE available for zebrafish were 0.3µM (fig. 1A)/37.0µM (fig. 1B), 1.2µM (fig. 1C)/3.5µM (fig. 1D) and 6.3µM (fig. 1E)/26.4µM (fig. 1F), respectively. In addition, the IDC was calculated to be 18.5µM, 1.8µM, 13.2µM for ATV, FEF and EZE, respectively.

### **Effects of lipid-lowering drugs on serum cholesterol and triglyceride**

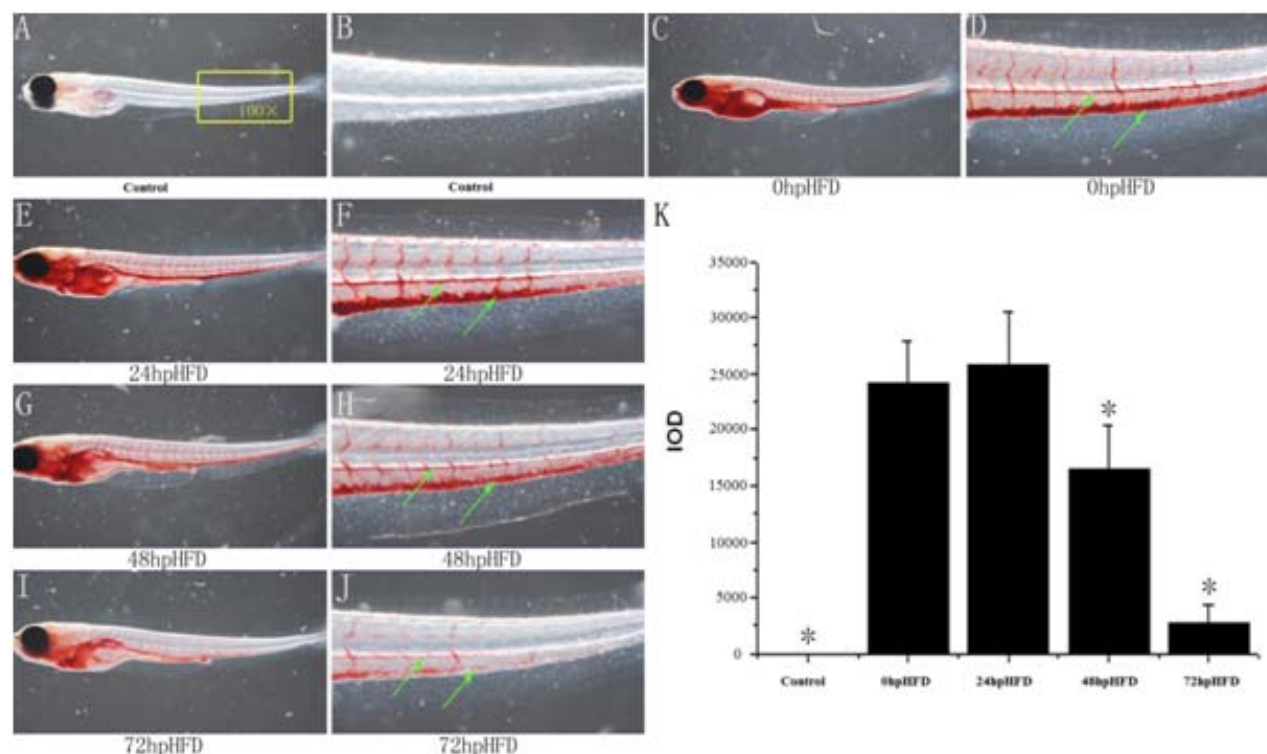
No death was found in the 5 groups after 7 weeks of feeding. As shown in fig. 2A, the serum cholesterol levels in high-fat groups (Group 2-5) were significantly higher than that in control group ( $p < 0.05$ ). While the serum cholesterol levels in Group 3, 4 and 5 were significantly reduced compared with that of Group 2 ( $p < 0.05$ ).



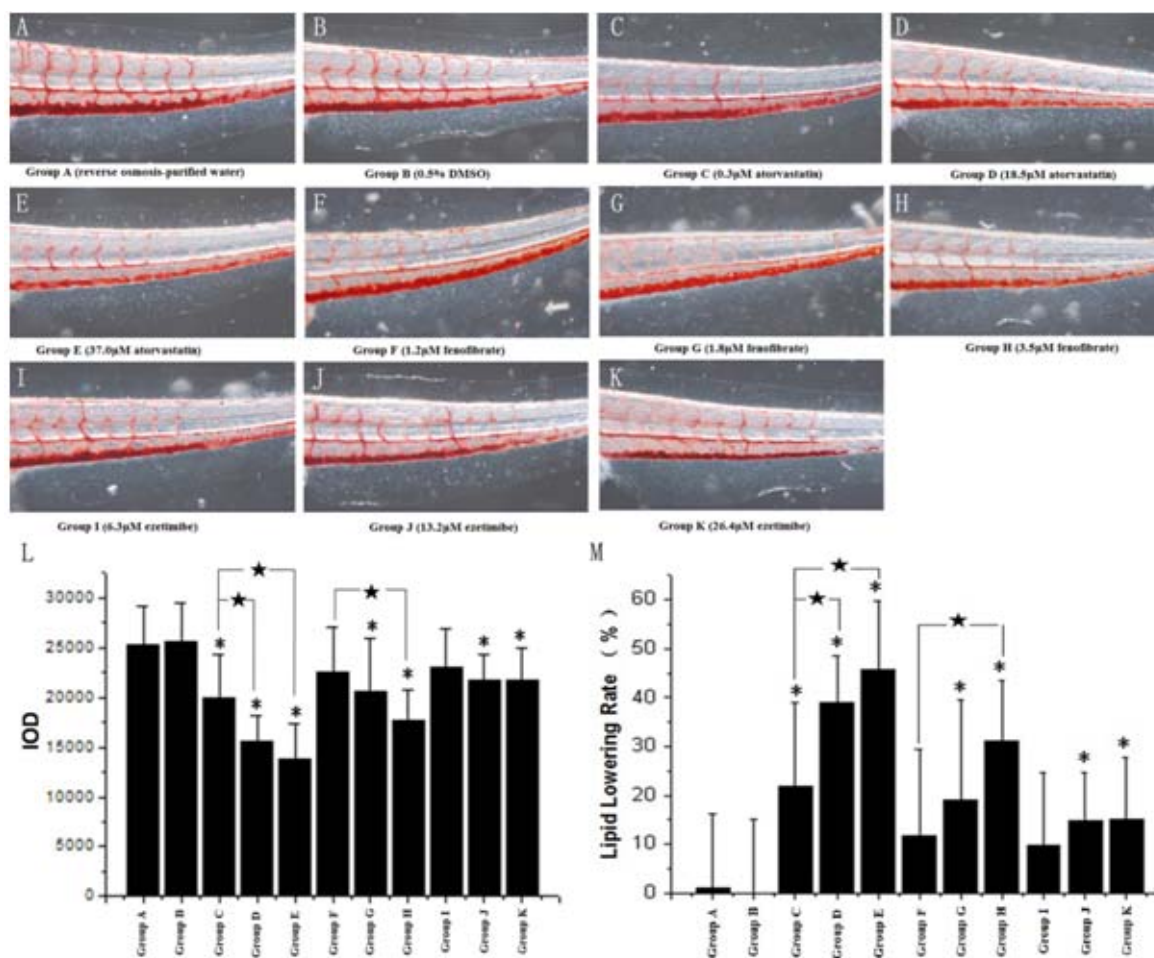
**Fig. 1:** Concentrations of atorvastatin, fenofibrate and ezetimibe in zebra fish. A, C and E: 1 day post fertilization (dpf) zebra fish in atorvastatin, fenofibrate and ezetimibe, respectively, for 72h; B, D and F: 7 dpf zebra fish in atorvastatin, fenofibrate and ezetimibe, respectively, for 24h. LC<sub>50</sub> is the median lethal concentration and LC<sub>1</sub> is the 1% lethal concentration.



**Fig. 2:** The serum levels of cholesterol (A) and triglyceride (B) in zebra fish. Group 1-5: The cholesterol level of zebra fish in normal, dimethyl sulphoxide (DMSO) control, 0.3 $\mu$ M atorvastatin, 1.2 $\mu$ M fenofibrate and 6.3 $\mu$ M ezetimibe groups, respectively. The zebra fish in Group1 were fed with conventional fish diet, and others were fed with high-fat diet (HFD). \*:  $p < 0.05$  vs. Group 2; \*\*:  $p < 0.05$  vs. Group 1.



**Fig. 3:** Effects of high-fat diet (HFD) on zebra fish detected by Oil red O staining. A and B: 7 day post fertilization (dpf) zebra fish without feeding (control); C and D: 5 dpf zebra fish with HFD-fed for 48h (0h p HFD); then, fasting for 24h (E and F), 48h (G and H) and 72h (I and J) after HFD-fed, respectively. K: integrated optical density (IOD) value. Hp HFD: hours post high fat diet. A, C, E, G, I:  $\times 30$ ; B, D, F, H, J:  $\times 100$ . \*:  $p < 0.05$  vs. 0h p HFD group. Green arrow: lipids stained with Oil red O.



**Fig. 4:** Effects of atorvastatin, fenofibrate and ezetimibe on zebra fish with 48h high-fat diet (HFD) feeding ( $\times 100$ ). Zebra fish in groups of normal (A) and DMSO control (B), 0.3 $\mu$ M (C), 18.5 $\mu$ M (D) and 37.0 $\mu$ M (E) atorvastatin, 1.2  $\mu$ M (F), 1.8 $\mu$ M (G) and 3.5 $\mu$ M (H) fenofibrate, 6.3 $\mu$ M (I), 13.2 $\mu$ M (J) and 26.4 $\mu$ M (K) ezetimibe, 24h. L: integrated optical density (IOD) value; M: lipid-lowering rate (LLR, %). \*:  $p < 0.05$  vs. Group B; \*:  $p < 0.05$  vs. groups with same drug at different concentration.

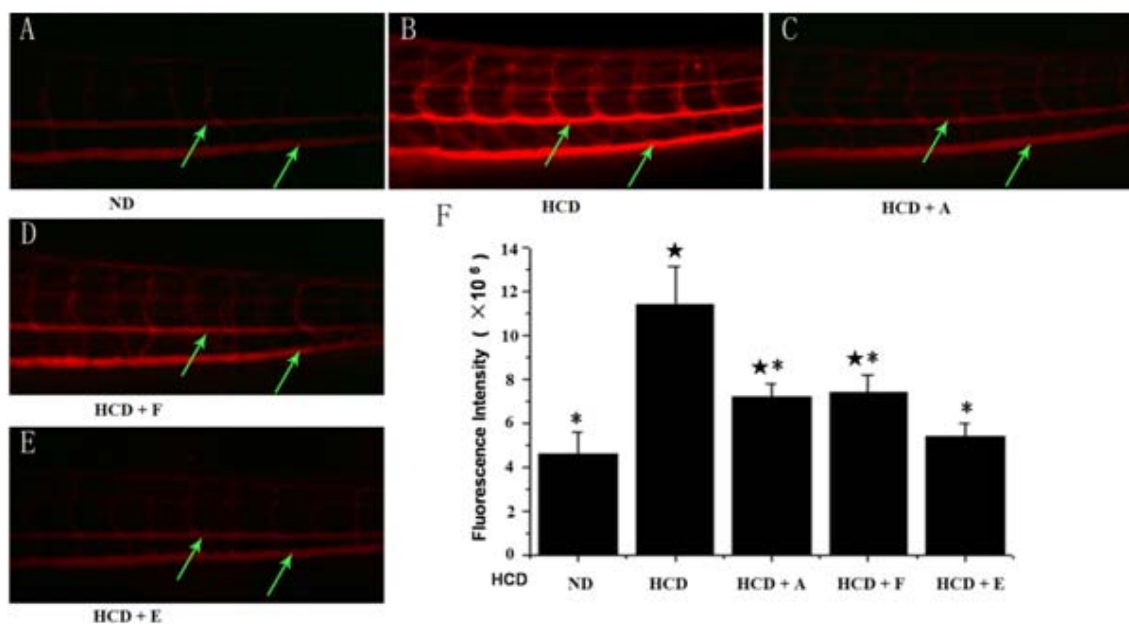
**Table 1:** The serum levels of cholesterol and triglyceride after treatment with atorvastatin, fenofibrate and ezetimibe

| Group | Cholesterol concentration (mg/dL) | Triglyceride concentration (mg/dL) |
|-------|-----------------------------------|------------------------------------|
| 1     | 239.7 $\pm$ 19.2 <sup>#</sup>     | 363.7 $\pm$ 35.3 <sup>#</sup>      |
| 2     | 636.7 $\pm$ 42.1 <sup>*</sup>     | 585.7 $\pm$ 26.7 <sup>*</sup>      |
| 3     | 438.3 $\pm$ 31.3 <sup>*#</sup>    | 459.3 $\pm$ 34.6 <sup>*#</sup>     |
| 4     | 559.5 $\pm$ 52.5 <sup>*#</sup>    | 487.8 $\pm$ 20.1 <sup>*#</sup>     |
| 5     | 406.2 $\pm$ 50.5 <sup>*#</sup>    | 548.7 $\pm$ 53.2 <sup>*</sup>      |

Note: Data were presented as means  $\pm$  SD (standard deviations). n = 30. \*:  $P < 0.05$  vs. Group 1; #:  $P < 0.05$  vs. Group 2.

The serum level of triglyceride in Group 2 was significantly increased compared with that of Group 1 (fig. 2B). In addition, the serum level of triglyceride in zebrafish treated with ATV and FEF was significantly declined compared with that of Group 2 ( $p < 0.05$ ). However, there was no significant difference in the serum levels of triglyceride between Group 5 and Group 2 ( $p > 0.05$ ).

As shown in Table 1, the cholesterol and triglyceride levels of HFD-fed groups were 636.7 $\pm$ 42.1mg/dL and 585.7 $\pm$ 26.7mg/dL, respectively. While, the cholesterol levels of ATV, FEF and EZE-treated groups were 438.3 $\pm$ 31.3mg/dL, 559.5 $\pm$ 52.5mg/dL and 406.2 $\pm$ 50.5 mg/dL, respectively. What's more, treatment with drugs decreased the triglyceride level of them were 459.3 $\pm$ 34.6 mg/dL, 487.8 $\pm$ 20.1mg/dL and 548.7 $\pm$ 53.2mg/dL, respectively.



**Fig. 5:** Effects of atorvastatin, fenofibrate and ezetimibe on zebrafish with 10 days high-fat diet (HCD) feeding ( $\times 100$ ). Zebrafish treated with conventional fish diet (ND) (A), HCD (B), HCD with  $0.3\mu\text{M}$  atorvastatin (HCD+A) (C), HCD with  $1.2\mu\text{M}$  fenofibrate (HCD+F) (D) and HCD with  $6.3\mu\text{M}$  ezetimibe (HCD+E) (E). F: fluorescence intensity. Green arrow: cholesterol stained by fluorescent probe. \*:  $p < 0.05$  vs. HCD group; \*:  $p < 0.05$  vs. ND group.

#### High-fat model construction for screening lipid-lowering drugs

As shown in fig. 3, no red staining of 7 dpf zebrafish without feeding in control group was observed (fig. 3A and 3B). Compared with the control group, the lipid level in 0h p HFD group was significantly increased ( $p < 0.05$ , fig. 3C and 3D), and remained stable in 24h p HFD group (fig. 3E and 3F). The lipid levels were significantly decreased after 48h fasting ( $p < 0.05$ , fig. 3G and 3H) and continuously declined after 72h fasting (fig. 3I and 3J). The statistical differences of IOD among different groups were shown in fig. 3K. These results indicated that the 5 dpf high-fat zebrafish model was established after 48h high-fat diet (0.1% egg yolk), and maintained stably for 24h.

#### A rapid screening method for lipid-lowering drugs

Red staining was observed in all groups after 48 h HFD feeding (fig. 4A-4K). The results showed that there was no significant difference in lipid level of zebrafish between normal and DMSO control ( $p > 0.05$ , fig. 4A and 4B). Compared with that of fig. 4B, the lipid level was significantly decreased in fig. 4C, D and E ( $p < 0.05$ ). Similarly, FEF decreased the lipid level in fig. 4F, G, H. Likewise, the lipid level was also reduced in fig. 4I, J, K after the administration of EZE. However, there was no significant difference in lipid level between fig. 4I and 4B ( $p > 0.05$ ). The lipid level was significantly decreased in fig. 4J and K compared with that of fig. 4B ( $p < 0.05$ ). The IOD values among different groups were shown in fig. 4L.

According to fig. 4M, the LLR levels of ATV group was increased in a concentration-dependent manner. Besides, the LLR levels of FEF and EZE groups showed significant increases after IDC and HDC treatment than that of DMSO control ( $p < 0.05$ ).

#### Verification of the effects of lipid-lowering drugs on cholesterol control

After continuous feeding for 10 days, 3, 3, 4, 6 and 6 zebrafish were dead in ND, HCD, HCD + ATV, HCD + FEF and HCD + EZE groups, respectively. However, there was no significant difference in the mortality rate among these groups. As shown in fig. 5, intravascular cholesterol level was significantly increased in HCD group with intense red fluorescence compared with that in ND group ( $p < 0.05$ , fig. 5A and 5B). Besides, the cholesterol level in lipid-lowering drug groups was significantly decreased compared with that in HCD group ( $p < 0.05$ , fig. 5C-E). The statistical differences of fluorescence intensity among different groups were shown in fig. 5F.

#### DISCUSSION

Hyperlipidemia, which is characterized by abnormal elevated levels of lipids in blood, is the most common dyslipidemia disease (Owens et al. 2014). The primary treatment for hyperlipidemia patients is the dietary treatment combined with drug interventions (Anderson et al. 2000). However, there is no effective anti-hyperlipidemia drug or drug screening method widely

approved nowadays. In this study, we aimed to explore a rapid screening method for effective lipid-lowering drugs screening using high-fat zebrafish model and to measure the feasibility of it. The results showed that the lipid levels of HFD-fed zebrafish were significantly increased and ATV, FEF and EZE had lipid-lowering effects to some extent. Therefore, the rapid method for screening lipid-lowering drugs with zebrafish model would be available and successful.

According to the blood lipid, the levels of both cholesterol and triglycerides were significantly higher in Group 2. Our results indicated that HFD-fed included egg yolk power and cholesterol could induce the high-fat zebrafish, which was consistent with a previous study of Yoon *et al.* (Yoon *et al.* 2013) who reported that HCD induced the vascular lipid accumulation, hypercholesterolemia and atherosclerotic changes in the early stage of zebrafish. In addition, ATV, FEF and EZE showed lipid-lowering effects on zebrafish. In this study, ATV treatment led to a significant decrease in cholesterol and triglycerides levels, while FEF mainly declined the level of triglycerides and EZE only reduced the level of cholesterol significantly. These results were consistent with previous studies (Nawrocki *et al.* 1995; Krause and Newton 1995; Forcheron *et al.* 2002; Knopp *et al.* 2003). It has been reported that ATV could reduce not only level of cholesterol (Nawrocki *et al.* 1995) but also that of triglyceride (Krause and Newton 1995). Forcheron *et al.* (Forcheron *et al.* 2002) found that FEF therapy showed significant effect on the decline of serum triglycerides in patients with hypertriglyceridemia, but not the serum total cholesterol level. EZE, as a cholesterol absorption inhibitor, can significantly decrease the level of cholesterol in patients with primary hypertriglyceridemia (Knopp *et al.* 2003). Additionally, Clifton *et al.* (Clifton *et al.* 2010) have demonstrated that EZE inhibited the lipid absorption in zebrafish. Similarly, Baek *et al.* (Baek *et al.* 2012) have indicated that EZE effectively decreased the cholesterol level in HCD-fed zebrafish larvae, but this research was conducted in zebrafish homogenate for the cholesterol level in the body fluids, and that in blood was not clear. Moreover, no fish died in the 7-week drug treatment. All these results demonstrated that the concentrations of drugs used for high-fat zebrafish model were feasible and these treatment can be used as positive controls in further studies.

In this study, the blood lipid levels of zebrafish were significantly elevated after 48 h high-fat diet. Furthermore, the lipid level was significantly higher in 0h p HFD group than that of control group, which suggesting that the high-fat zebrafish model which was in accordance with the above findings was successful. Additionally, the lipid level in 24h p HFD group was not significantly different from that of 0h p HFD, while the lipid levels in 48h p HFD and 72h p HFD groups were significantly

decreased. These results suggested that the lipid level could remain high within 24 h. There fore, we speculated that the high-fat zebrafish model induced by HFD-fed was successful and could be used to screen the lipid-lowering drugs within 24 h.

Simple and effective approaches are necessary for antihyperlipidemic drug screening in zebrafish. Oil Red O is a low-cost, neutral and reproducible fat-staining reagent (Mehlem *et al.* 2013). In the current study, to evaluate the feasibility of the method of Oil red O staining in screening lipid-lowering drugs, ATV, FEF and EZE as positive controls were used. The Oil red O staining results of HFD-fed and drugs treatment showed significant effect on lowering the blood lipids in high-fat zebrafish model. These results suggested that Oil red O staining with image analysis on high lipid model could accurately exhibit the effects of drugs on the blood lipid, which were agreed with a previous study (Ramirez-Zacarias *et al.* 1992). Ramirez-Zacarias *et al.* (Ramirez-Zacarias *et al.* 1992) reported that Oil red O staining was a better quantitative method for triglycerides and lipid level. Our results also showed that the greater the drug dosage, the higher the LLR value. The changes of LLR value in zebrafish treated with ATV was greatest, which was in line with previous study that the values of the serum total cholesterol and triglycerides were decreased significantly in patients administered with ATV (Sasaki *et al.* 2002). Therefore, Oil red O staining may be an available, simple and rapid method for detecting the lipid levels after drug intervention.

Apart from total lipid level, cholesterol levels were closely associated with hyperlipidemia (Wang *et al.* 2013). Therefore, the effects of lipid-lowering drugs on cholesterol level were analyzed. Importantly, a former study has demonstrated that zebrafish model was successfully used for studying vascular lipid accumulation, lipoprotein oxidation and macrophage lipid uptake in hypercholesterolemic by fluorescence probe (Stoletov *et al.* 2009). In this study, fluorescence probe Chol Esteryl BODIPY 542/563 C11 was used to label cholesterol in zebrafish. The data of fluorescence intensity revealed that the cholesterol level in zebrafish was significantly increased after HCD treatment. While the cholesterol levels in HCD zebrafish treated with ATV, FEF and EZE were significantly reduced, which revealed the lipid-lowering effect of them. Therefore, these results suggested that the fluorescent-labeled cholesterol could intuitively reflect the cholesterol level in zebrafish blood. However, this experimental procedure took up a long period (about 10 days). The work of Fang *et al.* (Fang *et al.* 2011) reported that the fluorescent-labeled HCD in zebrafish larvae were sustained as long as 14 days. The cholesterol level in this processing may maintain in a more stable level. Unfortunately, a finding suggested that the intravascular cholesterol was markedly increased in

zebrafish older than 7dpf fed with HCD for  $\geq 10$  d (Stoletov *et al.* 2009). Worryingly, long period of incubation containing BODIPY 542/563 C11 led to both blood vessels and other areas in the zebrafish emitting fluorescence, which made the quantification of intravascular fluorescence intensity imprecise. In addition, the fluorescence was decreased by drug treatment. Therefore, it suggested that the method of fluorescent-labeled cholesterol might be unsuitable for rapid drug screening, but it may be an available method for further study on the specific effect of a lipid-lowering drug.

Although construction of high-fat model and Oil red O staining method was available and simple to screen the lipid-lowering drugs, there still remained some limitations in this study. The mortality was recorded to determine the concentrations of drugs used in the subsequent studies, yet the extent of morbidity of zebrafish was not analyzed. There was no exact quantification of food intake of each zebrafish during HFD- and HCD-fed treatment. Moreover, we didn't evaluate the method of fluorescent-labeled HCD in zebrafish in a short operational time period. Additionally, alterations in expression of genes known to be influenced by drugs, or changes in lipid levels in body fluids in larvae after removing abdomen from them were not verified. Therefore, further studies are needed to confirm these results.

## CONCLUSION

In conclusion, the rapid method for screening lipid-lowering drugs in zebrafish model was available and successful. The high-fat zebrafish model was successfully conducted with 48h-HFD. ATV, FEF and EZE could make lipid-lowering effects on high-fat zebrafish model to some extent. The Oil red O staining might be a rapid, available and simple method for detecting lipid levels after drug intervention.

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