

## Original Article

# Application of chrysophanol in zebrafish to reduce dietary introduced lipid and its possible mechanism

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**Abstract:** Purpose: To explore the therapeutic potential and mechanism of chrysophanol on lipid-lowering function. Methods: Zebrafish or larvae were employed to evaluate the effect of chrysophanol on lipid-lowering. Zebrafish of 5 day post fertilization (dpf, larva) and 13-week old were fed with high-cholesterol diet or high-fat to investigate the influence of chrysophanol comparing with atorvastatin and co-administration of chrysophanol and atorvastatin on subsistent blood lipid using the fluorescence microscope and lipid panel screen. Thereafter, we enrolled zebrafish of 7 dpf fed with high-fat diet to explore the lipid-lowering mechanism of chrysophanol basing on the frequency of peristalsis and the residual on the digestive wall. Results: Chrysophanol could significantly lower cholesterol both in zebrafish and larvae ( $P < 0.05$ ), and the co-administration of chrysophanol and atorvastatin had the best performance in reducing cholesterol ( $P < 0.05$ ). Chrysophanol and atorvastatin could also significantly lower triglyceride. Moreover, we found that chrysophanol attached on the digestive wall for a long time and enhanced the frequency of peristalsis. Conclusions: Chrysophanol has lipid-lowering effect both in zebrafish and larvae which may be attributed to the effect on the frequency of peristalsis and fat absorption, and co-administration with atorvastatin performs better lipid-lowering effect in zebrafish.

**Keywords:** Chrysophanol, atorvastatin, zebrafish, lipid-lowering effect

## Introduction

Atherosclerosis is an inflammatory disease with intense immunological activity, and increasingly threatens public health worldwide [1, 2]. Hyperlipidaemia, especially hypercholesterolemia, caused by high dietary fat and cholesterol is a primary risk factor in atherosclerosis formation and will further induce other serious diseases such as coronary heart disease and cerebral infarction [3, 4]. As typical lipid-lowering drugs, statins are demonstrated to be generally efficient in hyperlipidaemia, hypercholesterolemia and atherosclerosis therapy. However, there are a large number of patients who are unsatisfactory with their lipid-lowering functions and adverse effects [5, 6]. Therefore, an efficient medicine with less adverse effects is in great need.

Chinese rhubarb, a traditional Chinese drug since ancient time, has been frequently used in

hyperlipidaemia and coronary diseases because of the hypercholesterolemic lowering activities [7]. Rhubarb has been reported to be useful in stabilizing intravascular plaque in a mouse study [8]. Chrysophanol, one kind of the anthraquinone compounds, naturally occurs in the rhizome of rhubarb [9]. Recent reports have indicated that chrysophanol has the capacity of anti-inflammatory, antitumor, and anticoagulant [10, 11]. However, as an extractive from rhubarb, little attention is paid to the lipid-lowering effect of chrysophanol.

Zebrafish showed an ideal genetic system for application as a vertebrate model for modeling human diseases [12]. Moreover, many similarities on lipid metabolism between human and zebrafish have been investigated, such as lipid-binding/transfer proteins [13] and lipid-related diseases [14]. Zebrafish embryo has been widely used as an important vertebrate model for assessment of drug effects and studies of lipid

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metabolism diseases because of the unique characteristics [15, 16]. Particularly, the transparent body of zebrafish larva enables intuitive observations of intravascular lipid metabolism by proper fluorescent indicators and staining even in a live animal.

In the present study, to explore the lipid-lowering function and possible mechanisms, we studied the effect of chrysophanol on lipid-lowering of zebrafish vessel walls with artificial high lipid diet. Meanwhile, we also conducted experiments to compare distinctions between chrysophanol and clinical lipid-lowering drugs (atorvastatin). Finally, we tried to explore the potential mechanism of lipid-lowering function in zebrafish through observing the frequency of peristalsis and the residual on the digestive wall.

### Materials and methods

#### *Zebrafish husbandry*

All zebrafish (*Danio rerio*) and zygotes used in this study were obtained from the Shanghai Research Center for Model Organisms, and facilities housing these animals were AAALAC-accredited. Zebrafish (Casper strain) were raised and maintained in reverse-osmosis-purified water (pH 7.0) at 28°C under a 14:10 light/dark photoperiod. Water conditions of environmental quality were calibrated: tank material is made of polycarbonate; the tank was equipped with filtration units; the translucent lid of the tank leaves a hole (no larger than 1 cm). Embryos were raised on 15 cm petri dishes, adolescent fish and adult fish were housed in 2 L tanks with 10 fish per tank.

Moreover, all animal studies were approved by the Committee of Animal Care of Shanghai Jiaotong University and were conducted under the Guidelines in accordance with the Principles of Laboratory Animal Care.

#### *Storage and preparation of drugs*

Chrysophanol (Purity:  $\geq 98.0\%$  (HPLC); Sigma, St. Louis, MO, USA) and atorvastatin (Trade Name: Lipitor, Pfizer, Groton, CT, USA) were selected in our experiment. All the medicines were firstly dissolved in dimethyl sulfoxide (DMSO) with a high concentration and stored at  $-20^{\circ}\text{C}$  before used. The medicines solubilized in DMSO were then added into water and

adjusted to suitable final concentrations (1:200 (v/v)) at the start of experiment.

We used 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222, Fluka, Buchs, Switzerland) as fish anaesthetic [17]. The solution was freshly prepared through dissolving MS-222 powder in system water to the final concentration of 200 mg/L.

#### *Fluorescence probe preparation*

CholEsteryl BODIPY® 542/563 C11 (Invitrogen, Carlsbad, CA, USA) was used as the fluorescence probe to label the cholesterol in vivo or in the fodder. The power was dissolved in chloroform and stored in  $-20^{\circ}\text{C}$ . The fodder was immersed in chloroform containing the probe at the percentage of 1:100,000 (Wt/Wt) before using. Once the chloroform volatilized completely, the probe would combine on the fodder.

#### *High-cholesterol diet preparation*

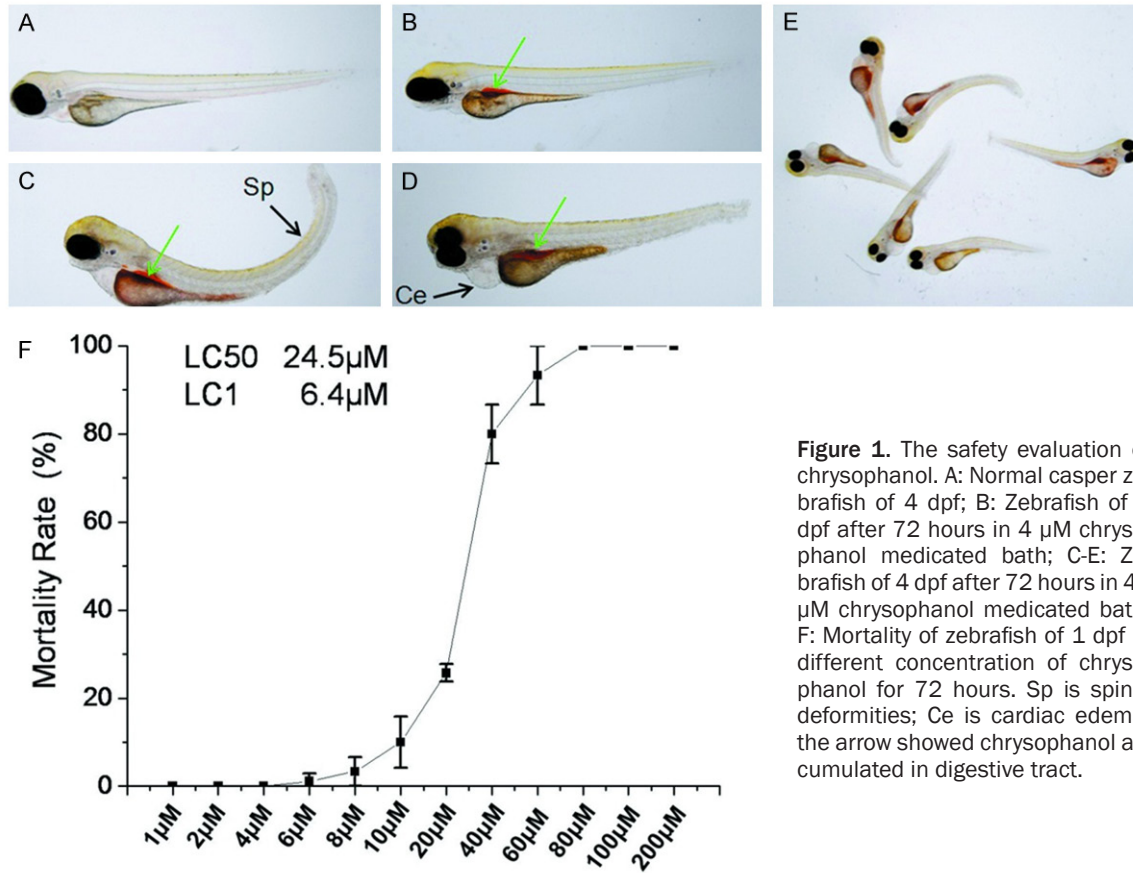
Conventional dried fodder (Slyke, Shanghai, China) for zebrafish, containing about 3% (w/w) crude fat, was ground into powder. The high-cholesterol diet for zebrafish was then made by soaking the powder above in a chloroform solution of cholesterol to achieve a content of 4% (w/w) cholesterol in the fodder. After chloroform evaporation, the cholesterol could be bonded to the surface of fodder.

High lipid feed was prepared by dissolving egg yolk powder (Yuanye, Shanghai, China) into aquaculture water at the final concentration of 0.1 g/100 ml.

#### *Optimization of drug concentration conditions*

In our current experiment, the following pre-experiment trials were conducted with zebrafish embryos to determine the lethal concentration that killed 50% of the zebrafish ( $\text{LC}_{50}$ ) and the appropriate concentration for chrysophanol and atorvastatin: to calculate the  $\text{LC}_{50}$  of chrysophanol, various concentrations of chrysophanol: 1000, 800, 600, 400, 200, 100, 80, 60, 40, 20, 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.4, 0.2  $\mu\text{M}$  and 0.1  $\mu\text{M}$  were prepared for 1 day post fertilization (dpf) zebrafish, and the system water with 0.5% DMSO (V/V) was used as control treatment. For each concentration, 30 fish were randomly and equally distributed and cul-

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**Figure 1.** The safety evaluation of chrysophanol. A: Normal casper zebrafish of 4 dpf; B: Zebrafish of 4 dpf after 72 hours in 4 µM chrysophanol medicated bath; C-E: Zebrafish of 4 dpf after 72 hours in 40 µM chrysophanol medicated bath; F: Mortality of zebrafish of 1 dpf in different concentration of chrysophanol for 72 hours. Sp is spinal deformities; Ce is cardiac edema; the arrow showed chrysophanol accumulated in digestive tract.

tured in 6 wells of 24-well plates for 72 h. During this period, the dead embryos were picked out and recorded every 2 h. Each sample was repeated for 3 times, and the numbers of dead and abnormal embryos of every concentration were analyzed by the statistic software to determine the  $LC_{50}$  and LC1.

The LC1 of chrysophanol, as the highest concentration which did not cause significant difference from the control in aberration rate, was chosen as the highest experiment concentration to explore the possible lipid-lowering mechanism of chrysophanol. In addition, given the damage introduced by long time chrysophanol medicated bath (the longest duration was 7 weeks); we chose 10% of LC1 as the concentration to assess the influence of chrysophanol on intravascular lipid of zebrafish.

### *Groups and detection of intravascular lipid of zebrafish larvae*

To investigate the influence of chrysophanol on intravascular cholesterol of juvenile fish, 150 zebrafish of 5 dpf were randomly distributed into 5 groups with 30 zebrafish in each group:

conventional diet was classified as group 1; high cholesterol diet was classified as group 2; high cholesterol diet combined with chrysophanol bath was classified as group 3; high cholesterol diet combined with atorvastatin bath was classified as group 4; high cholesterol diet treated by co-administration of chrysophanol and atorvastatin was classified as group 5.

All the 150 zebrafish were fed 2 times one day. In order to guarantee sufficient diet for each zebrafish, we have controlled the similar basic physical situation to ensure the similar food intake of zebrafish. Enough feeding time (30 min) and sufficient quality food were both controlled to further ensure each zebrafish could take in enough food. After 10 days, 10 zebrafish were selected randomly to quantify intravascular fluorescence intensity by fluorescence microscope.

### *Groups and detection of intravascular lipid of adult zebrafish*

Totally, 250 13-week old casper zebrafish were divided into 5 groups, just like the above group-

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ing method, to investigate the influence of chrysophanol on intravascular cholesterol. Zebrafish in Group 1 were fed conventional diet 2 times one day, and zebrafish in groups 2-5 was fed high cholesterol or high lipid feed once a day. After 7 weeks, 30 fish per group were selected randomly to test blood lipid.

About 5  $\mu$ l blood of each zebrafish was obtained through dorsal aorta of the fish after anesthetizing by MS-222. Then, 1  $\mu$ l serum from 5  $\mu$ l blood would be obtained by centrifugal separation. To reduce error induced by sample dilution (the least sample amount for biochemical analyzer is 100  $\mu$ l), blood from 5 zebrafish were mixed together and diluted with 0.9% NaCl at the ration of 1:20. SIEMENS advia 2400 Biochemical analyzer (Siemens, Berlin, Germany) was used to detect total cholesterol and triglyceride of the serum as the instruction book. Each experiment was repeated three times, and the average value would be conducted as the further evaluation value.

### *Detection of the frequency of peristalsis*

A total of 60 zebrafish of 7 dpf were fed 0.1% egg yolk, after 30 minutes, transferred into 0.5% DMSO water (control group) or chrysophanol medicated bath (concentration at LC1 and 10% LC1) respectively. Ten fish were selected from each group after 2 hours, 4 hours and 6 hours to collect the frequency of peristalsis in zebrafish, and the influence of chrysophanol on bowel movements were assessed basing on these data.

### *Detection of the residual on the digestive wall*

Additionally, 20 fish of 7 dpf were enrolled to detect the residual on the digestive wall. After feeding 12 hours in medicated bath of chrysophanol, zebrafish were transferred into conventional culture water (contained 0.5% DMSO). The residual fish were examined under the microscope every 12 hours for 2 days.

### *Microscopy and fluorescence intensity detection*

Nikon SMZ1500 (Nikon, Japan) was used to observe the zebrafish and take photos. Selected zebrafish were anesthetized with MS-222 and lay in methylcellulose keeping head to the left. The whole fish images were magnified by 30 times, and group photos were magnified by 7.5 times. Additionally, other images would be magnified by 100 times,

including part of the zebrafish, photos needed to assay the fluorescence signal intensity and images used to comparison.

Fluorescence intensity within the blood vessels was analyzed by NIS-Elements BR 3.1 with software of the Nikon ECLIPSE (Nikon, Shinjuku, Tokyo, Japan).

### *Statistical analysis*

Continuous data were expressed as the mean  $\pm$  standard deviation (SD), whereas categorical variables were presented as number and percentage. Statistical analysis was performed with SPSS11.5 software. One-way ANOVA was used to evaluate the statistical differences involving three or more experimental groups, while the Student's t-test was used for statistical comparisons of inter-groups. Chi-square test or Fisher's Exact Test were used for comparison of rate. A *P* value less than 0.05 was considered statistically significant.

## **Results**

### *Selection of experimental concentration of chrysophanol in zebrafish*

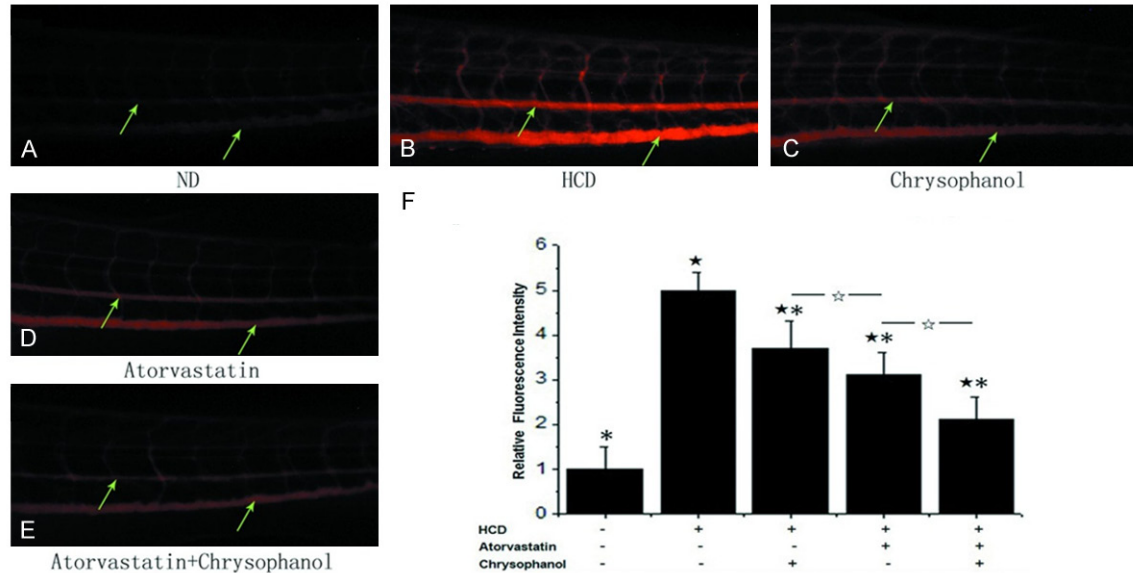
In order to select the appropriate concentration of chrysophanol in zebrafish, quantities of deaths and abnormal performances, characterized by spinal deformities (Sp) and cardiac edema (CE), were observed in experimental fish (**Figure 1A-E**). We could find chrysophanol deposition in zebrafish both in 4  $\mu$ M and 40  $\mu$ M chrysophanol medicated bath comparing with normal casper zebrafish. Moreover, Sp and CE could obviously be observed in the zebrafish in 40  $\mu$ M chrysophanol medicated bath (**Figure 1C-E**).

As shown in **Figure 1F**, LC50 and LC1 of chrysophanol in zebrafish were 24.5  $\mu$ M and 6.4  $\mu$ M, separately. Thus, 6.4  $\mu$ M was chosen as the highest experiment concentration to explore the possible lipid-lowering mechanism of chrysophanol. In addition, 0.6  $\mu$ M was selected as the concentration to assess the influence of chrysophanol on intravascular lipid of zebrafish.

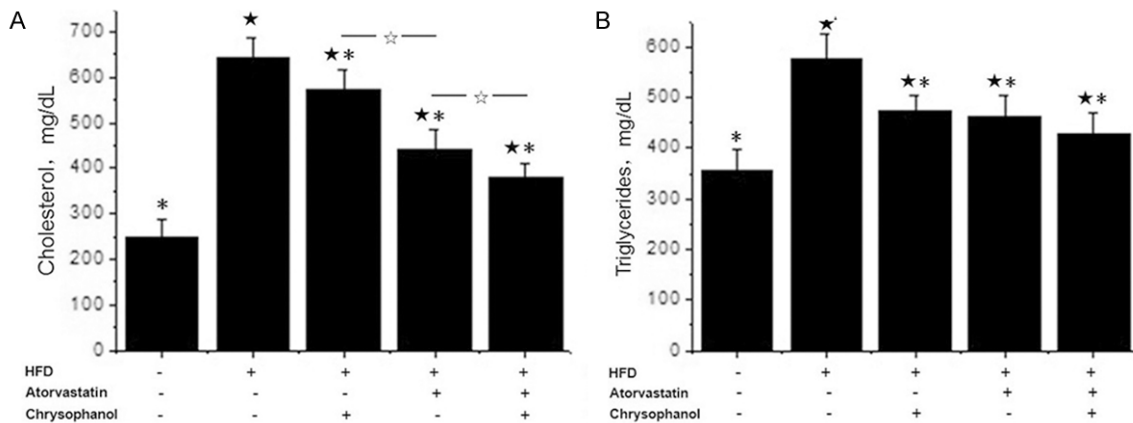
### *Influence of chrysophanol on intravascular cholesterol of zebrafish larvae*

According to **Figure 2A-E**, atorvastatin could significantly decrease the intravascular choles-

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**Figure 2.** Influence of chrysophanol on lowering intravascular cholesterol levels. A: General diet; B: High cholesterol diet; C: High cholesterol diet treated with 0.6  $\mu\text{M}$  chrysophanol medicated bath; D: High cholesterol diet treated with 0.6  $\mu\text{M}$  atorvastatin medicated bath; E: High cholesterol diet treated with co-administration of 0.6  $\mu\text{M}$  atorvastatin and 0.6  $\mu\text{M}$  chrysophanol medicated bath; F: Comparison of relative fluorescence intensity in intravascular among groups. ND is normal diet; HCD is high cholesterol diet. The arrow showed chrysophanol accumulated in intravascular.  $\star$  Comparison with general diet (group A),  $P < 0.05$ ;  $\ast\ast$  Comparison with high cholesterol diet,  $P < 0.05$ ;  $\star\ast$  Comparison between the two groups,  $P < 0.05$ .



**Figure 3.** Influence on blood fat of adult zebrafish. A: Influence of chrysophanol on total cholesterol of adult zebrafish after high fat diet; B: Influence of chrysophanol on triglyceride of adult zebrafish after high fat diet. HFD is high fat diet.  $\star$  Comparison with general diet (group 1),  $P < 0.05$ .  $\ast$  Comparison with high fat diet (group 2),  $P < 0.05$ .  $\star\ast$  Comparison between the two groups,  $P < 0.05$ .

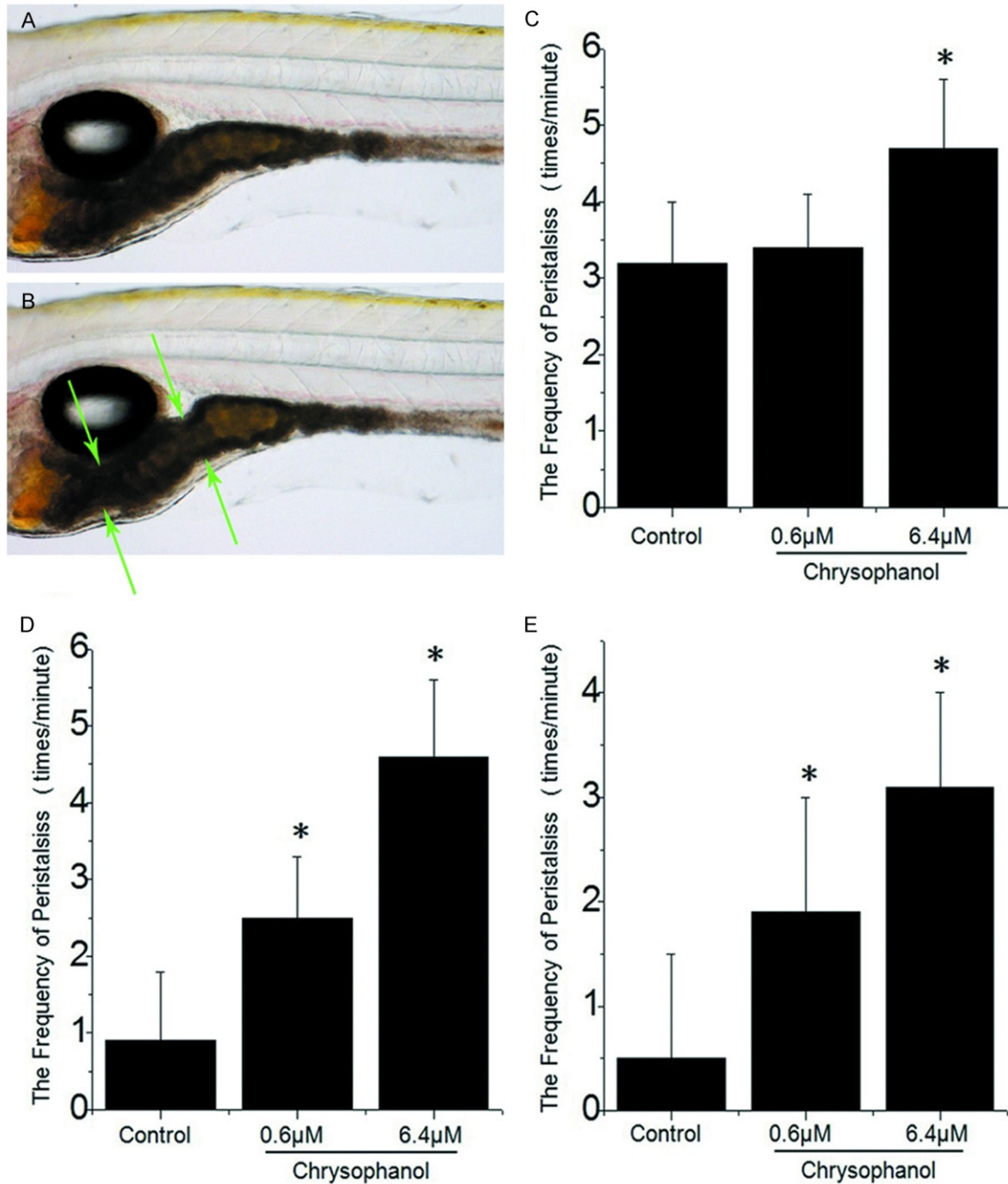
terol (red fluorescent,  $P < 0.05$ ), whereas chrysophanol with the same concentration had weaker function on lowering cholesterol ( $P < 0.05$ ) comparing with atorvastatin. Notably, chrysophanol at 0.6  $\mu\text{M}$  could significantly reduce dietary introduced lipid comparing with high cholesterol diet without treatment. Moreover, among the groups, the co-adminis-

tration of atorvastatin and chrysophanol performed best in lipid-lowering ( $P < 0.05$ ).

### *The influence of chrysophanol on intravascular lipid of adult zebrafish*

Thirteen-week old zebrafish from all 5 groups were all alive after culturing 7 weeks. According

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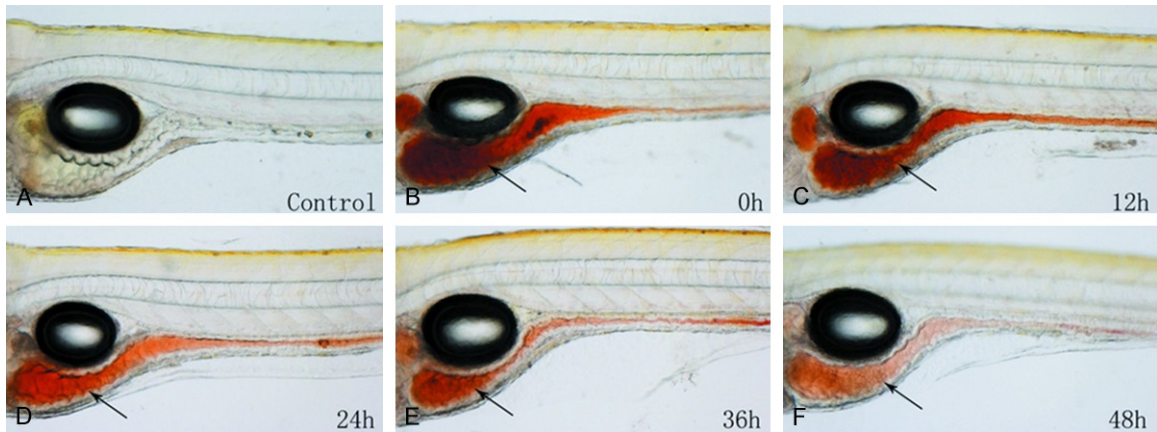


**Figure 4.** Influence on frequency of peristalsis of zebrafish. (A, B) peristalsis of zebrafish; (A) Diastole of intestine; (B) systole of intestine, (the arrow showed the part of intestinal contractions); (C-E) frequency of peristalsis at the 2nd (C), 4th (D), 6th (E) hours (30 minutes after feeding, and 7 dpf zebrafish were raised in aquaculture water or 0.6 μM, 6.4 μM chrysophanol medicated bath). \*Comparison with control group,  $P < 0.05$ .

to the **Figure 3**, total cholesterol and triglycerides from zebrafish in groups fed high fat fodder were higher than zebrafish fed ordinary fodder, chrysophanol and atorvastatin at 0.6 μM could reduce the level of total cholesterol in serum. Moreover, as for total cholesterol lower-

ing effect, the cooperation of the two kinds of drugs had better effect than single action with significant difference (**Figure 3A**). Similar capacities were also found in lowering the triglyceride, but the effects of the chrysophanol, atorvastatin and co-administration of chryso-

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**Figure 5.** Adhesive attraction of chrysophanol on intestinal of zebrafish. (A) zebrafish of 7 dpf without fodder; (B-F) adhesion condition of chrysophanol on intestinal wall after 6.4  $\mu\text{M}$  chrysophanol medicated bath and transferred to aquaculture water lasting for 0 hour (B), 12 hours (C), 24 hours (D), 36 hours (E) and 48 hours (F). The arrow showed adhesion of chrysophanol on the intestinal wall.

phanol and atorvastatin treatment had no significant difference (**Figure 3B**).

### *Influences on the frequency of peristalsis*

The frequency of peristalsis at the 2nd, 4th, 6th hours after 7 dpf zebrafish were raised in aquaculture water or 0.6  $\mu\text{M}$ , 6.4  $\mu\text{M}$  chrysophanol medicated bath were conducted. As shown in **Figure 4**, the frequency of peristalsis was significantly increased after 6.4  $\mu\text{M}$  chrysophanol medicated bath comparing with control group at all the three time points ( $P < 0.05$ ). In addition, the frequency of peristalsis significantly slowed down over time, while the frequency of peristalsis in 6.4  $\mu\text{M}$  chrysophanol group did not slow down over time. **Figure 4C-E** show the frequency of peristalsis at 6.4  $\mu\text{M}$  chrysophanol medicated bath is more powerful than that at 0.6  $\mu\text{M}$ .

### *Residuals of chrysophanol on the digestive wall*

**Figure 5** shows the adhesion condition of chrysophanol on intestinal wall after 6.4  $\mu\text{M}$  chrysophanol medicated bath and transferred to aquaculture water lasting for 0 hour, 12 hours, 24 hours, 36 hours, and 48 hours. As shown in **Figure 5A**, after 12 hours in chrysophanol medicated bath, visible rhubarb phenol could be seen on the digestive wall. Moreover, quality of chrysophanol still adhered to the digestive wall after 48 hours post-exposure in chrysophanol medicated bath (**Figure 5F**).

## Discussion

To our knowledge, this is the first study to assess the function of chrysophanol on lowering blood lipid in the adult and larval zebrafish model. We found that experimental concentrations of chrysophanol could significantly inhibit cholesterol and triglyceride in zebrafish with high fat/cholesterol diet. Moreover, chrysophanol could significantly increase the frequency of peristalsis and keep adhering to the digestive wall for a long time. These findings indicate that chrysophanol has an excellent lipid-lowering function, especially on cholesterol, which might be beneficial for hyperlipidaemia, hypercholesterolemia and atherosclerosis treatment in clinic. Therefore, the discovery of lipid-lowering effect performed by chrysophanol gives us new possibility on developing into new drug that treat lipid metabolic disorders.

Cholesterol-fed zebrafish widely used in researching pathogenesis of human atherosclerosis because of the obvious advantages of zebrafish. Therefore, we copied the hypercholesterolemic zebrafish model using microscopy and fluorescence intensity to study the effect of chrysophanol on lipid level. Similarly, the same lipid-lowering activity of chrysophanol from rhubarb was also proved on high fat diet-induced dyslipidemic rats in 2013 [18]. Based on the conclusion, the lipid-lowering mechanism experiments proved that chrysophanol could not only significantly increase the frequency of peristalsis but also keep adhering to the diges-

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tive wall for a long time. We suggest the phenomenon that chrysophanol adhering to the digestive wall may interfere with the absorption of fat. Moreover, inhibition of dietary lipid absorption has been recognized as a developed strategy to treat disorders of lipid metabolism after cardiovascular [19]. Although no previous study has reported on the association between drug adhesive intestinal wall and lipid absorption, our zebrafish data imply the positive lipid-lowering role of chrysophanol might be related with the disturbed digestion and absorption of dietary lipid. Of course, further fluorescence localization study is needed to verify the topic.

Atorvastatin is a developed hepatic hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase inhibitor which is efficacious in reducing both triglyceride and cholesterol in humans [20]. Whereas chrysophanol performed its lipid-lowering effect in a different way comparing with atorvastatin. The results in the current study suggest that chrysophanol could speed up the emptying and decreased absorption at the source to perform its lipid-lowering effect, which is significantly different from that of Atorvastatin. Therefore, the co-administration of Atorvastatin and chrysophanol performed the best lipid-lowering effect in our study. Ezetimibe is also a kind of lipid-lowering medicines designed to affect the initial absorption of lipid from digestive tract [21]. However, it is still unclear whether ezetimibe and chrysophanol perform their lipid-lowering effect by following the same mechanism. The research by Lee and his colleagues proved that chrysophanol had mild cytotoxicity and anti-diabetic properties and could play metabolic roles in the insulin-stimulated glucose transport pathway [22], which reminds that the lipid-lowering function of chrysophanol may also act through other pathways related with the lipogenesis. Therefore, further studies to investigate the detailed mechanisms of chrysophanol to reduce lipid are in great need, and chrysophanol might be a better combined treatment to enhance the efficacy of some drugs.

There are some limitations should be noted in the research. Firstly, although we have stated that chrysophanol is good in lowering cholesterol for zebrafish and lipid metabolism in zebrafish were similar to humans. However, one

study suggested that there was no significant difference in leukocyte accumulation in vessels between normal and high cholesterol diet fed zebrafish [23]. Hence, a new atherosclerosis model should also be contributed if we want to explore the effect of chrysophanol on atherosclerosis. Secondly, although the food intake has been controlled basing on similar food requirements and enough food supply, the food intake was not controlled strictly in various experimental groups.

In conclusion, chrysophanol could significantly lower total cholesterol and triglyceride, which may be attributed to the promotive effect on digestion and decreased absorption of dietary lipid. Given the limited macroscopic mechanism research of chrysophanol, further studies should be considered to explore the lipid-lowering mechanism.

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### Disclosure of conflict of interest

None.

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