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Original article

Rapid analysis of hypolipidemic drugs in a live zebrafish assay

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ABSTRACT

Introduction: Hyperlipidemia is the most common form of dyslipidemia, which is the key risk factor for cardiovascular disease and stroke. The development of effective and safe drug treatments for hyperlipidemia has been proven challenging. **Methods:** In this study, taking advantage of the transparency of larval zebrafish, we developed a zebrafish hyperlipidemia model for drug screening and efficacy assessment. Zebrafish at 5 d.p.f (days post fertilization) were fed with 0.1% egg yolk for 48 h (hours), followed by drug treatment for 24 h or 48 h. Tested drugs were administered into the zebrafish by direct soaking. Drug effect was evaluated based on quantitative analysis of Oil Red O (ORO) in zebrafish vena caudalis. **Results:** All 5 human hypolipidemic drugs (simvastatin, lovastatin, ezetimibe, bezafibrate and hyodesoxycholic acid) showed significant hypolipidemic effects ($p < 0.01$) in a dose-dependent manner in the zebrafish hyperlipidemia model. 'We also found a well-known Chinese tea Pu-erh tea significantly reduced lipids in this model ($p < 0.001$ and $p < 0.01$). **Discussion:** Our results demonstrate that the zebrafish hyperlipidemia model developed and validated in this study could be used for *in vivo* hyperlipidemia studies and drug screening and for assessing hypolipidemic drugs with different mechanisms.

1. Introduction

Hyperlipidemia involves abnormally elevated levels of any or all lipids and/or lipoproteins in the blood. It is the most common form of dyslipidemia, which is the key risk factor for cardiovascular disease and stroke. The number of diseases associated with hyperlipidemia is rapidly increasing. Current experimental studies of hyperlipidemia often use genetically modified mice, rabbit and hamster fed high-fat, high-cholesterol diets, which rapidly induce extreme hyperlipidemia and lipid accumulation in the artery wall. In addition, mammalian hyperlipidemia models are often time-consuming, labor-intensive and expensive (Xiangdong et al., 2011). Drug screening in cell culture models and in other *in vitro* systems has been carried out, but due to lacking organ structures, extrapolation of these results to the whole organism is often challenging. An *in vivo* live animal model that allows a detailed analysis of lipid metabolism would be highly valuable for lipid metabolism studies and for lipid-lowering drug screening.

Zebrafish *Danio rerio* is emerging as a predictive vertebrate animal model for *in vivo* assessment of drug efficacy, toxicity and safety (Li, Q3
Luo, Awerman, & McGrath, 2011; Li, Luo, & McGrath, 2011; McGrath & Q4
Li, 2008). An important advantage of the zebrafish animal model is that the morphological and molecular basis of tissues and organs is either identical or similar to other vertebrates, including humans (Granato & Nüsslein-Volhard, 1996). The sequence and presumed function of many genes that are important for vertebrates are conserved in the zebrafish (Howe, Clark, Torroja, et al., 2013). Oil Red O (ORO) staining was initially used by Koopman R to quantify lipids in skeletal muscle sections of rats (Koopman, S. G., & Hesselink, 2001) and Schlombs, Q5
Wagner, and Scheel (2003) first adapted ORO to stain whole zebrafish embryonic lipids. Subsequently, researchers monitored neutral lipid by staining fixed zebrafish larval with ORO to track endotrophic lipid consumption in whole-larval zebrafish (Schlegel & D.Y.R.S., 2006). Q6
Work from the Babin laboratory also used the ORO method to study Fibrate-induced embryonic malabsorption syndrome in zebrafish during the early stages of vertebrate development (Raldua, Andre, &

Abbreviations: d.p.f, days post fertilization; ORO, Oil Red O; MNLC, maximum non-lethal concentration; DMSO, dimethyl sulfoxide; IOD, integrated option density; GI, gastrointestinal.

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Babin, 2008). Egg yolk powder was applied to study zebrafish lipid metabolism-associated gene expression and functions (Carten, Bradford, & Farber, 2011; Marza et al., 2005). Other investigators in this field have used this conventional method for assessing global lipid stores and gene functions in zebrafish (Avraham-Davidi, Ely, et al., 2012; Clifton et al., 2010; Cruz-Garcia & Schlegel, 2014; Sadler, Amsterdam, Soroka, Boyer, & Hopkins, 2005; Stoletov et al., 2009). In the present study, we developed a zebrafish hyperlipidemia model that utilized egg yolk powder with ORO staining to assess vascular lipid change with lipid–lowering drugs (simvastatin, lovastatin, ezetimibe, bezafibrate and hyodesoxycholic acid). Our results indicate that the zebrafish hyperlipidemia model developed in this study is convenient and predictive for rapid *in vivo* screening and efficacy assessment of lipid-lowering drugs.

China is an outstanding hometown of teas and a birthplace of tea culture. Chinese teas have been discovered and used for over forty-five thousand years and Pu-erh tea is one of well-known Chinese teas. Using zebrafish hyperlipidemia model developed in this research, we confirmed the therapeutic effects of Pu-erh tea extracts on hyperlipidemia, which was consistent with earlier reports (Deka & Vita, 2011; Yang & Koo, 1997). Our results from zebrafish hyperlipidemia model in combination with literature support Pu-erh tea as an ideal beverage in retarding the development of hyperlipidemia.

2. Materials and methods

2.1. Zebrafish handling

Adult AL strain zebrafish were housed in a light- and temperature-controlled aquaculture facility with a standard 14: 10 h light/dark photoperiod and fed with live brine shrimp twice daily and dry flake once a day. Four to five pairs of zebrafish were set up for nature mating every time. On average, 200–300 embryos were generated. Embryos were maintained at 28 °C in fish water (0.2% Instant Ocean Salt in deionized water, pH 6.9–7.2, conductivity 480–510 mS cm^{-1} and hardness 53.7–71.6 mg l^{-1} $CaCO_3$). The embryos were washed and staged at 6 and 24 h.p.f (hours post fertilization) (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). The zebrafish facility at Hunter Biotechnology, Inc. is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAA LAC) International.

2.2. Egg yolk and tested drugs

Egg yolk was bought from Tianyuan company of Beijing (Lot 20120612), which was a high-cholesterol diet. Larvae was fed with 0.1% egg yolk to develop hyperlipidemia model. Human hypolipidemic drugs simvastatin, lovastatin, ezetimibe, bezafibrate and hyodesoxycholic acid were selected for the validation of the zebrafish hyperlipidemia model. All the tested drugs were purchased from Sigma-Aldrich (St. Louis, USA) excepted ezetimibe and hyodesoxycholic acid that were from Aladdin (Shanghai, China). Pu-erh tea extracts (Lot N: 2010 F01), a dark brown powder was a gift from Yunnan Tasly Deepure Biological Tea Group Co., LTD. Drug stock solutions were prepared in either 100% dimethyl sulfoxide (DMSO) or ultrapure water, and serial dilutions were made before each experiment. Zebrafish treated with 1.0% DMSO were used as vehicle controls. Untreated zebrafish were used to confirm that the vehicle solvent did not have an adverse effect on the zebrafish. Ammonia concentration was measured at the end of experiments and no ammonia accumulation was detected in fish water. Dissolved oxygen concentration in fish water was kept >80% during the experiments.

2.3. Determination of zebrafish appearance and clearance times of stainable lipid

Zebrafish exhibit a functional GI tract with spontaneous and rhythmic muscular contractions and exogenous feeding starting by 5 d.p.f (Holmberg, Schwerte, & Fritsche, 2003; Holmberg, Olsson, & Hennig, 2007; Kuhlman & Eisen, 2007). Consequently, we chose 5 d.p.f zebrafish as an optimal stage for the model development. Zebrafish were placed in a beaker at a density of 100 zebrafish in 100 ml of fish water, 1% egg yolk was added to the beaker by a dilution at 1:100 (v/v), and zebrafish were incubated at 28 °C. After adding egg yolk for 24 h and 48 h, zebrafish were fixed, and ORO staining and imaging were performed under a dissecting stereomicroscope (Olympus Co., Tokyo, Japan). ORO was quantified to determine zebrafish appearance and clearance times of stainable lipid.

2.4. Oil Red O staining and quantification

In order to compare the levels of blood lipids, ORO staining was conducted. At the end of experiment, zebrafish were fixed in 4% paraformaldehyde overnight at room temperature. Zebrafish were washed two times with phosphate-buffered saline (PBS) and dehydrated by immersing in 25%, 50%, 75% and 100% methanol in PBT to permeabilize. Zebrafish were stained in 0.5% ORO for 24 h, then rehydrated stepwise to 100% PBT (Schlombs et al., 2003). After ORO labeling, lipids are easily visualized in the blood, liver, gut, brain and so on under a dissecting stereomicroscope. Utilizing the Image-Pro Plus 6.0 (Media Cybernetics. Inc, Washington Street, USA), ORO was quantified from color images using the level of excess red intensity in the red channel in comparison to the blue and green channels which reflects triacylglycerol and cholesterol concentrations (O'Rourke, Soukas, Carr, & Ruvkun, 2009).

2.5. Determination of maximum non-lethal concentration (MNLC)

To determine MNLC of a testing drug, 7 d.p.f zebrafish were treated with a testing drug for 48 h and mortality was recorded at the end of treatment. Dead zebrafish was defined as the absence of heartbeat under a dissecting stereomicroscope. In the initial tests, 5 concentrations (0.1, 1, 10, 100 and 500 μM) were used for each drug. If an MNLC could not be found from the initial tests, additional concentrations within the range of 0.01 to 2000 μM were tested. Mortality curves were generated using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego CA) and MNLC was determined with logistic regression.

2.6. Assessment of drug effect on zebrafish blood lipids

Five known human hypolipidemic drugs (simvastatin, lovastatin, ezetimibe, bezafibrate and hyodesoxycholic acid) were selected for the validation of zebrafish hyperlipidemia model. Additionally, we also verified the therapeutic effects of Pu-erh tea in this model. Zebrafish were fed with egg yolk for 48 h, followed by drug treatment for 24 h and 48 h at 3 concentrations (1/10 MNLC, 1/3 MNLC, MNLC). At the end of treatment, ORO was used to stain the lipids of zebrafish. Fifteen to twenty zebrafish from each group were randomly chose for ORO image acquisition. Zebrafish were immobilized in 3% methyl cellulose and images were acquired in the identical lighting intensity at a 56× magnification under a dissecting stereomicroscope installed with a high-speed video camera (JVC, Japan). When viewed dorso-laterally, the blood vessel of a 8 or 9 d.p.f zebrafish was situated posterior to cloacal pore and predominantly anterior to the tail fin. Quantitative image analysis of ORO was performed and Integrated Option Density (IOD) data were expressed as mean ± SEM. The effect of a test drug or tea abstracts was calculated based on the following formula:

$$\text{Drug effect on lipids lowering}(\%) = [1\text{- IOD(compound)}/\text{IOD(vehicle)}] \times 100\%$$

A positive percentage means that a tested drug could reduce lipids and a negative percentage suggests that the tested drug had no lipid-lowering effect in the zebrafish hyperlipidemia model.

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