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RESEARCH ARTICLE

Toxic effects of celastrol on embryonic development of zebrafish (*Danio rerio*)

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Abstract

Celastrol is a terpenoid purified from *Tripterygium wilfordii* Hook F. As a natural product with pharmacological activities, this compound is a promising candidate for drug development. To provide more information about its toxicity for clinical trials, toxicity assessment of celastrol was conducted with zebrafish model *in vivo*. 1hour post-fertilization (hpf) embryos were treated with various concentrations of celastrol for 120h. Developmental phenotypes were observed and survival rates were recorded. The results showed that the hatching rates of embryos treated with 1.0μM or higher celastrol were significantly lower. Embryos exposed to 1.0μM celastrol had no blood flow in trunk vessels at 48hpf with a median effect concentration (EC_{50}) of 0.94μM. At 72hpf serious edema in pericardial sac was observed in the surviving larvae (hatched from embryos treated with 1.5μM celastrol). Bent tails or hook-like tails were seen as 0.5μM celastrol and the EC_{50} for tail malformation was 0.66 μM at 72hpf. The lethal effect of celastrol on zebrafish embryos was dose-dependent and the LC_{50} values of celastrol on 1hpf embryos were approximately 1.40μM. These results indicate that celastrol affects the normal development of zebrafish embryo in μM concentrations.

Keywords: *Celastrol; zebrafish; embryo; developmental toxicity*

Introduction

Celastrol is a natural product purified from the Chinese herb, *Tripterygium wilfordii* Hook F. (thunder god vine), which has been traditionally used as folk medicine in China (Setty and Sigal, 2005). In recent studies, multiple pharmacological activities have been found. Due to its antioxidant and -inflammatory activities, celastrol may be useful in treating neurodegenerative diseases accompanied by inflammation, such as Alzheimer's disease (Allison et al., 2001). Celastrol was also found to suppress human prostate cancer growth as a potent proteasome inhibitor and inhibit the growth of human glioma xenografts through suppressing vascular endothelial growth factor receptor (VEGF-R) expression in nude mice (Yang et al., 2006; Huang et al., 2003). Therefore, celastrol may be a promising drug for the treatment of related human diseases, including cancer, and its toxicity assessment is necessary to avoid adverse effects in clinical application.

Toxicity assessments of a drug prior to clinical trials are accomplished customarily through laboratory rodent studies, but these studies are expensive, time consuming, and are becoming restricted by law. Toxicological scientists have searched for an alternative animal to partially or completely substitute higher animals. Zebrafish, a tropical fish, may be such a suitable model. In recent years, zebrafish has been found to be a valuable model for toxicity assessments in drug toxicology (Parng, 2005; Rubinstein, 2006). Advantages of zebrafish as a toxicological model species include small size, ease of husbandry and breeding, high fecundity (a single spawning produces 100–200 eggs every 5–7 days), fertilization, and development *in vitro*, transparent embryos, and larvae at early stages. With zebrafish embryo or larvae as an animal model, only a small amount (i.e., on the microgram scale) of drug is required, and the drug is absorbed from the surrounding water solution. The transparency of embryo or larvae facilitates the observation of toxic effects of drug on internal

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tissues or organs with a light microscope. Further, at the molecular level, genes involving embryo development in the early stages of all vertebrates are highly conserved. So, embryos and larvae of zebrafish are a valuable tool in drug toxicology.

Toxic effects of celastrol on development of zebrafish embryos were investigated in the present study. The aims of this study of celastrol were to investigate the mechanism of toxicity and provide valuable information for further assessments prior to clinical trials.

Materials and methods

Chemical

Celastrol ($C_{29}H_{38}O_4$; purity, >98%) was purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). This compound was dissolved in dimethyl sulfoxide (DMSO) to make a 10-mM stock solution. The treatment solutions of celastrol for toxicity tests were obtained by the dilution of the stock solution with embryo medium (Westerfield, 1995). The final concentration of DMSO, as a cosolvent in all treatment solutions, was 0.1% (v/v).

Zebrafish embryos and larvae

The AB line zebrafish used in this study were obtained from Dr. Jingwei Xiong of Harvard Medical School (Boston, Massachusetts, USA) and maintained following the standard procedures (Westerfield, 1995). The night before breeding, adult male and female zebrafish were set up in a breeding tank separated by a mesh screen. After the light was turned on the next morning, embryos were generated by natural mating and then collected within 30 minutes after spawning. After being rinsed three times, the clean embryos were moved to Petri dishes or a larger container with embryo medium and cultured at 28.5°C.

Chemical treatment and phenotype observation in toxicity test

The normal embryos were selected under a stereomicroscope (Olympus SZX16; Tokyo, Japan) and transferred into a multiwell microplate with 1 embryo per well in a 300-μL treatment solution. Thirty embryos at 1 hour postfertilization (hpf) were exposed to various concentrations of celastrol, and controls were incubated in embryo medium containing 0.1% DMSO. The developmental phenotypes of the experimental embryos were observed every 24 for 120 hours and were photographed with a charge-coupled device (CCD) camera. The survival rates of embryos were recorded, and death was defined as no visual heart beat. All tests were repeated three times and were conducted in accord with national and institutional guidelines for the protection of human subjects and animal welfare.

Statistical analysis

Data analysis was performed with SPSS 17.0 for windows (SPSS, Inc., Chicago, Illinois, USA). Comparisons between groups were carried out by one-way analysis of variance (ANOVA) and Dunnett's *t*-test. *P*-values less than 0.05 were considered significant. The median concentrations for lethal effect (LC_{50}) and sublethal effects (EC_{50}) were obtained through the method of probit analysis.

Results

Sublethal effects of celastrol on development of zebrafish embryos

The embryos of the control group developed normally in embryo medium, and hatching began at 48 hpf and was completed at 72 hpf (Figure 1A–1D). The hatching rates of embryos treated with 1.0-μM or higher concentrations of celastrol were significantly lower than that of the control (Figure 2). The median effect concentration (EC_{50}) for delayed hatching was 1.02 μM.

Embryos treated with 0.5-μM or higher concentrations of celastrol also displayed several developmental abnormalities, including no blood flow, edema in pericardial sac, and tail malformation. Embryos exposed to 1.0 μM of celastrol had no blood flow in trunk vessels at 48 hpf with an EC_{50} of 0.94 μM (Figure 3), but still had a heart beat. At 72 hpf, serious edema in the pericardial sac was observed in the surviving embryos treated with 1.5 μM of celastrol (Figure 1E), which was not induced by 1.0-μM or lower concentrations of celastrol. Tail malformation was the most marked toxic effect of celastrol on the development of zebrafish embryo (Figure 1E–1G). Bent tails or hook-like tails were obviously caused by 0.5 μM of celastrol and were observed in greater than 90% of embryos (72 hpf) treated with 1.0 μM of celastrol (Figure 4). The EC_{50} was 0.66 μM for tail malformation. Further, the 72-hpf embryo with the abnormal tail displayed irregular cell arrangement in the tail bud at 10 hpf (Figure 1H and 1I).

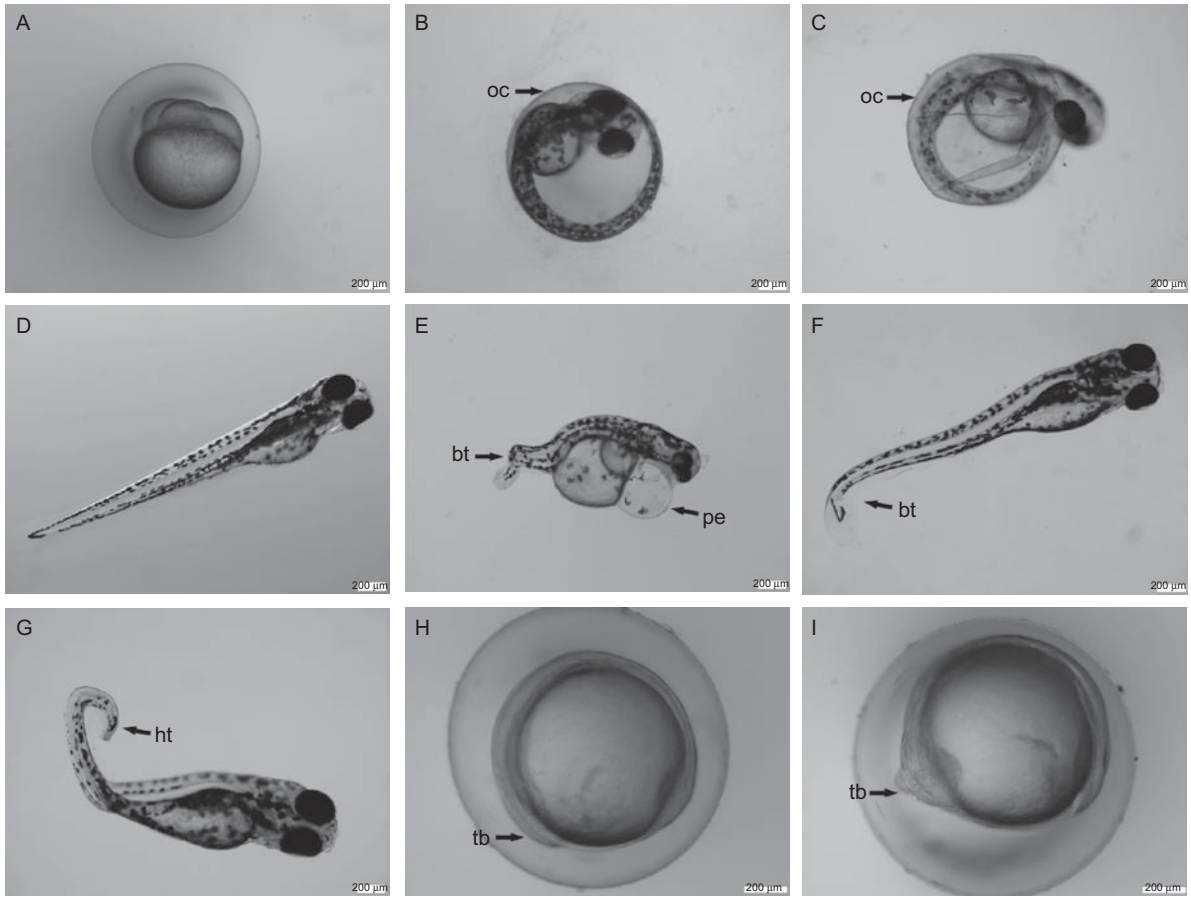

Figure 1. Toxic effects of celastrol on the development of zebrafish embryos. (A) A 1-hpf embryo at the 4-cell stage for the developmental toxicity test. (B) A 48-hpf embryo before hatching. (C) A 48-hpf embryo on hatching. (D) A 72-hpf embryo (hatched larva). (E) The 72-hpf larva with pericardial edema and bent tail hatched from embryo incubated with 1.5 µM of celastrol. (F) The 72-hpf larva with bent tail hatched from embryo treated with 0.5 µM of celastrol. (G) The 72-hpf larva with hook-like tail hatched from embryo incubated with 1.0 µM of celastrol. (H) Normal embryo at the tail-bud stage. (I) Celastrol-treated embryo at the tail-bud stage. Oc, outer chorion; bt, bent tail; pe, pericardial edema; ht, hook-like tail; tb, tail bud.

Lethal effect of celastrol on zebrafish embryos

The survival rates of zebrafish embryos treated with various concentrations of celastrol for 120 hours are shown in Figure 5. All embryos died when treated with 2.0 µM of celastrol for 24 hours. The lethal effect of celastrol was dose dependent, and the LC_{50} value (after treatment for 24 hours) of celastrol on 1-hpf embryos was approximately 1.40 µM. After 96 hours of exposure, none of the embryos survived in 1.5 µM of celastrol, and the survival of embryos incubated in 1.0 µM of celastrol also decreased significantly. However, all embryos incubated in 0.75-µM or lower concentrations of celastrol survived for 120 hours.

Discussion

Preclinical toxicity assessment is a crucial part of drug development to avoid side effects in clinical trials. Previous studies suggest that chemicals or drugs can have similar toxic effects in zebrafish embryos and humans (Nagel, 2002; Zhang et al., 2003; Milan et al., 2003; Lam et al., 2005). The methods of using zebrafish embryos or larvae as an animal model to assess embryonic and teratogenic effects of chemicals or drugs have been developed (Fraysse et al., 2006; Selderslaghs et al., 2009; Yang et al., 2009). These studies support and accelerate the application of zebrafish embryos or larvae to predict the toxicity of compounds of potential value.

In this study, the developmental toxicity of celastrol was investigated in zebrafish embryos. Developmental abnormalities were caused by celastrol, including no blood flow in trunk vessels, edema in the pericardial sac, and tail malformation. No blood flow may result from the cardiotoxicity of celastrol. In a previous study, we have found that celastrol is cardiotoxic to zebrafish embryos or larvae and may induce a significant decrease of heart rate (Wang et al., 2009). In this study,

it was also observed that the heart beat of embryos or larvae with no blood flow was weaker. Moreover, edema in the pericardial sac was observed in zebrafish embryos treated with celastrol. On the other hand, no blood flow in trunk vessels may also arise from the antiangiogenic activity of celastrol. It was reported that three terpenoids isolated from *Tripterygium wilfordii* (including celastrol) could inhibit the growth of intersegmental vessels in zebrafish embryos (He et al., 2009). If the growth of blood vessels were inhibited, blood flow could not occur.

Tail malformation in zebrafish embryo at 72 hpf is one of the toxicological end-points for evaluating the teratogenicity of chemicals. For example, bent and hook-like tails were observed in 72-hpf embryos treated with 5 μM of curcumin (Wu et al., 2007). In the present study, this effect was also seen with celastrol. Moreover, it was found that abnormal cell arrangement in the tail buds of embryos at 10 hpf resulted in the tail malformation. Further investigation to the molecular mechanism of this toxic effect will be conducted in our laboratory.

The hatching of embryos was affected by celastrol, with the hatching rate decreasing with the concentration increasing. One reason for the delay or failure of hatching may be the above-described developmental abnormalities, which may partially or completely limit the ability of developing embryos or larvae to break outer chorion and hatch out. The other reason may be the inhibition of celastrol to enzymes involved in hatching (Strmac et al., 2002; Haendel et al., 2004). Exposure of zebrafish embryos to other compounds also resulted in similar disturbance to hatching (Strmac and Braunbeck, 1999; David and Pancharatna, 2009).

In this study, we found that celastrol was lethal to zebrafish embryos in a low μM range. Further, 2 μM of celastrol had an acute lethal effect on zebrafish embryos within 24 hours. Both sublethal and lethal

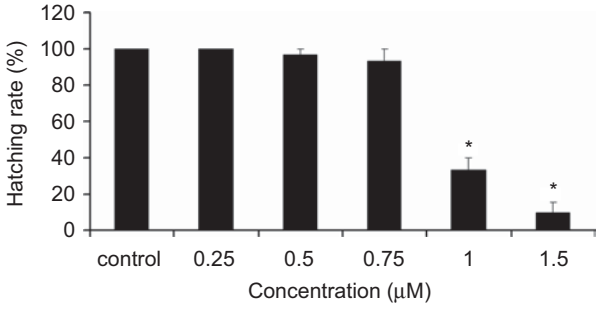

Figure 2. Hatching rates of 72-hpf zebrafish embryos during celastrol incubation. All the control embryos incubated with embryo medium hatched normally at 72 hpf. The hatching rates of embryos treated with 1 and 1.5 μM of celastrol were significantly lower than that of the control. Comparisons between groups were carried out by one-way ANOVA and Dunnett's *t*-test. *P*-values less than 0.05 were considered significant. *Represents significantly different from the control.

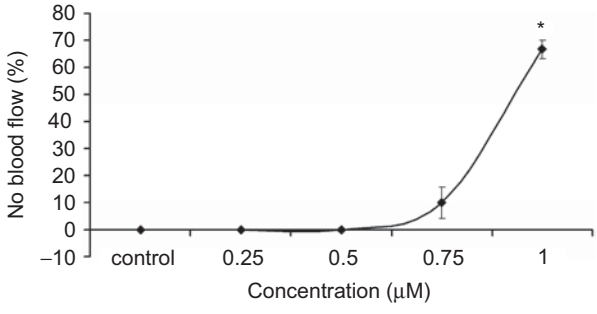

Figure 3. Rates of no blood flow in 48-hpf zebrafish embryos during celastrol incubation. Embryos exposed to 1.0 μM of celastrol had no blood flow in trunk vessels at 48 hpf. Comparisons between groups were carried out by one-way ANOVA and Dunnett's *t*-test. *P*-values less than 0.05 were considered significant. *Represents significantly different from the control.

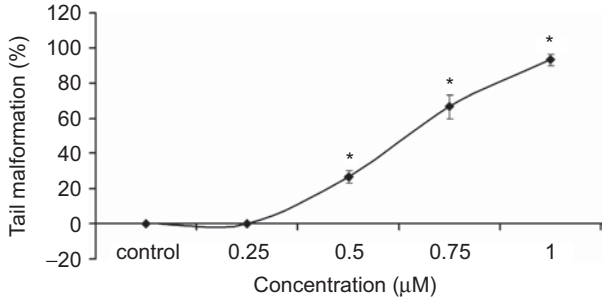

Figure 4. Rates of tail malformation in zebrafish embryos during celastrol incubation. Bent tails or hook-like tails were obviously caused by 0.5 μM of celastrol and were observed in greater than 90% of embryos (72-hpf) treated with 1.0 μM of celastrol. Comparisons between groups were carried out by one-way ANOVA and Dunnett's *t*-test. *P*-values less than 0.05 were considered significant. *Represents significantly different from the control.

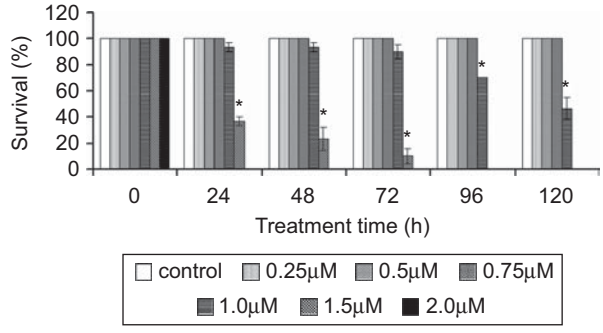

Figure 5. Survival rates of zebrafish embryos during celastrol incubation. Embryos (1-hpf) were incubated in various concentrations of celastrol, and the survival rates were recorded every 24 hours for 120 hours. The death of an embryo was defined as no visual heart beat. Comparisons between groups were carried out by one-way ANOVA and Dunnett's *t*-test. *P*-values less than 0.05 were considered significant. *Represents significantly different from the control.

effects were observed in embryos treated with 1.5 and 1.0 μM of celastrol for 120 hours. Some embryos with no blood flow or pericardial edema observed at 48 or 72 hpf died after 120 hours of incubation. Therefore, these developmental abnormalities may have been reasons for the lethality of celastrol. However, when incubated in 0.75 or 0.5 μM of celastrol, embryos with malformed tails survived for 120 hours, which indicates this teratogenic effect may not be the primary cause of embryo death.

Conclusions

Celastrol is toxic to the development of zebrafish embryos, and side effects of this chemical should be examined in preclinical or clinical trials.

Declaration of interest

The authors are grateful to other members of the Research and Development Platform of Drug Screening of Shandong Academy of Sciences for raising and breeding zebrafish and have no potential conflicts of interest to report. This work was supported by Shandong Province Natural Science Foundation (No. Y2008C178) and Key Science and Technology Project (No. 2006GG3202038).

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