

Effects of tetracycline on developmental toxicity and molecular responses in zebrafish (*Danio rerio*) embryos

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Abstract The extensive use of pharmaceuticals has resulted in the intensive contamination of water bodies. Tetracycline is a type of antibiotic and its potential toxicity is causing environmental concern. The effects of developmental toxicity and the mechanisms of tetracycline on fish embryos are not well understood. Zebrafish embryos are used in this study to investigate the developmental toxicity of this compound. Four hour post-fertilization (hpf) zebrafish embryos are exposed to different concentrations of tetracycline until 96 hpf. The larvae display developmental delay phenotypes, including hatching delay, shorter body length, increased yolk sac area and uninflated swim bladder upon exposure to tetracycline. Delayed yolk sac absorption and swim bladder deficiency at 96 hpf are observed in the zebrafish larvae upon exposure to 20 µg/L of tetracycline. To test whether tetracycline causes oxidative damage and the resulting oxidative stress-induced apoptosis, the generation of reactive oxygen species (ROS), Acridine Orange staining and real time polymerase chain reaction have been

performed in this study. The results indicate that tetracycline exposure results in significant increases in ROS production and cell apoptosis, mainly in the tail areas at 96 hpf. The gene expression pattern demonstrates that tetracycline induces ROS which causes apoptosis in the zebrafish larvae, and the results also indicate that caspase-dependent apoptotic pathways may greatly contribute to tetracycline-induced apoptosis in the early-life stages of the zebrafish. In addition, we have investigated the effects of tetracycline on marker genes related to resistance mechanisms and gene regulating drug biotransformation. The results of these gene expression studies indicate that tetracycline could induce zebrafish to resist pharmaceuticals and Cytochrome P450s that are involved in the biotransformation of tetracycline in zebrafish larvae. The overall results indicate that tetracycline can produce oxidative stress and induce apoptosis, which brings about significant developmental delay in zebrafish embryos.

Keywords Tetracycline · Developmental delay · Gene expression · Apoptosis · Zebrafish embryos

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Introduction

Pharmaceuticals have been widely used as medication for humans to treat or prevent diseases, and as veterinary drugs and husbandry growth promoters in agricultural areas (Halling-Sørensen et al. 1998). As a result of wider usage, increased environmental concentrations and highly potent toxicity, pharmaceuticals have been recently identified as one of the major emerging environmental pollutants. Pharmaceuticals have been detected in the natural environment across the world (Kolpin et al. 2002; Ramirez et al. 2009; Yan et al. 2013). Landfill, and animal,

freshwater aquaculture, hospital and industrial wastes as well as domestic waste are the major sources of environmental pharmaceuticals (Eggen et al. 2012; Pal et al. 2010; Lapworth et al. 2012). Although reported concentrations of pharmaceuticals in the environment are generally low, due to continuous emission and their resistance to degradation, many of the commonly found pharmaceuticals are pseudo-persistent in the environment (Monteiro and Boxall 2009; Peck 2006). The prevalence of pharmaceuticals is ubiquitous in water resources. The continuous discharging of pharmaceuticals into the environment might lead to uncertain ecological effects because they may act in an unpredictable manner when mixed with other chemicals (Gilbert 2012; Li 2014). Previous studies have demonstrated that pharmaceuticals can induce negative non-targeting effects onto aquatic organisms at different trophic levels, such as algae, fish and mussels (Wollenberger et al. 2000; Mehinto et al. 2010; Liu et al. 2011; Vannini et al. 2011; Cuklev et al. 2011; Gilbert 2011; Brodin et al. 2013; Ledford 2013).

Antibiotics, as a pharmaceutical product, have been used in both humans and animals for treating diseases, and also in the animal industry as growth promoters (Sapkota et al. 2008). Antibiotics have become more commonly used in the commercial and aquaculture industries, especially in developing countries in recent decades. According to statistics, the consumption of antibiotics can be up to 100,000–200,000 tons every year around the world (Kümmerer 2009). China, as the world's largest market to produce and consume pharmaceutical products (Richardson et al. 2005), consumes more than 25,000 tons of antibiotics each year (Xu et al. 2007). The wide use and increased production of antibiotics has resulted in the frequent appearance of antibiotics in sewage, surface waters, sediments and biota around the world, especially in China (Gulkowska et al. 2007; Jiang et al. 2008; Lin and Tsai 2009; Xu et al. 2009; Zuccato et al. 2010; Rosal et al. 2010; Zhou et al. 2011; Luo et al. 2011; Jiang et al. 2011; Ji et al. 2011; Zou et al. 2011; Shen et al. 2012; Yan et al. 2013). Recent studies have demonstrated that antibiotics are toxic to aquatic organisms (Carlsson et al. 2013; Madureira et al. 2011; Lin et al. 2013). Furthermore, continuous exposure to low concentrations of antibiotics in the environment can induce antibiotic resistance genes (Thomas and Foster 2004; Kim et al. 2007; Zhang and Zhang 2011; Zhu et al. 2013).

Tetracycline antibiotics are a class of broad spectrum antibiotics produced by *Streptomyces*. Tetracycline antibiotics usually bind to 30S ribosomal subunits and prevent ammonia acyl tRNA from combining with ribosome. In recent years, with the rapid development of offshore intensive animal husbandry and aquaculture, tetracycline antibiotics are applied as a veterinary drug and feed additive, and its usage has increased every year. The annual

consumption amount of antibiotics in the European Union (EU) is 5,000 tons, and the annual consumption amount of tetracycline antibiotics is about 2,300 tons, which is about 46 % of the total (Wang and Wei 2013). China produces about 210,000 tons of antibiotics each year, and 97,000 tons of raw materials are used for animal husbandry and aquaculture, which account for 46.1 % of the total consumption (Chen 2013). Tetracycline antibiotics are mainly used in the livestock and poultry breeding industries in China and other parts of the world (Wei et al. 2004; Sarmah et al. 2006).

As most antibiotic drugs used on animals cannot be fully absorbed, they are expelled along with their waste into sewage treatment plants, or directly into the environment (Sarmah et al. 2006). In recent years, scholars have identified the presence of antibiotics in farm wastewater, surface water and groundwater in the Shanghai waters (Jiang et al. 2008, 2011; Ji et al. 2011; Shen et al. 2012; Yan et al. 2013). The flow of antibiotics and their metabolites into surface waters poses a potential risk to aquatic organisms. Several widely reported antibiotics are scored based on their environmental concentrations combined with their LC_{50} values in mice (Zhang et al. 2014). The results are summarized in Table S1, and it indicates that tetracycline may have higher toxicity in China surface waters as compared with other antibiotics. Tetracycline is hence chosen as the target for further examination in this study.

With regard to the potential ecotoxicity of antibiotic contamination to aquatic organisms, there has been much concern in recent years. The toxicity of tetracycline to aquatic organisms has been reported in several studies. The inhibition of protein synthesis and chloroplast formation is the primary negative effects of tetracycline on microalgae (Halling-Sørensen 2000; Ferreira et al. 2007; Xu et al. 2013). Tetracycline can inhibit plant growth via inhibiting chloroplast synthase (Bradel et al. 2000; Kasai et al. 2004). Tetracycline also inhibits the activity of several important enzymes (Lunden et al. 1998), and damage the DNA of exposed fish (Qu et al. 2004; Li et al. 2006). However, little is known about the potential developmental toxicity of tetracycline antibiotics to aquatic organisms.

Fish, as a vertebrate at the top of the food chain, plays a very important role in aquatic ecosystems. Fish is also one of the major food and nutrient sources for humans. Fish embryo assays are considered as pain-free *in vivo* tests and accepted as a replacement of animal experiments (Voelker et al. 2007). Zebrafish is one of the most widely adopted model species for investigating the developmental toxicity of chemical exposure during early life stages. Zebrafish embryo is small in size and transparent, and easy to maintain and handle, and the species has high fecundity, rapid embryogenesis and continuous reproduction. In addition, the zebrafish genome has been sequenced and

genetic information is rapidly accumulating (<http://zfin.org>), which makes it feasible to find the genes and pathways involved in toxicant exposure.

In the present study, zebrafish embryos are employed to investigate the developmental toxicity of tetracycline. Different endpoints, such as survivorship, hatching rates, morphology of the larvae, as well as certain gene expression levels have been selected for this study. However, some endpoints employed in this study (e.g., hatching rates, phenotypes, larvae survival) may provide low sensitivity compared to effects detected during low-level exposure, and these endpoints generally do not provide any information on the mode of action that leads to toxic effects. Alternatively, approaches that use gene expression are likely to indicate long-term detrimental effects (Voelker et al. 2007). Therefore, genes that are involved in apoptosis (p53, AIF, Bax, Apaf-1, Bcl-2, Cyt c, and Caspases-3, 6, 7, 8 and 9), oxidative damage (CAT, SOD), multidrug resistance (ABCC1, ABCC2 and ABCC4) and genes that play critical roles in detoxification (CYP1A) are also investigated. Finally, apoptotic cell staining and reactive oxygen species (ROS) detection and total protein content are studied to elucidate the toxicity mechanism of tetracycline in zebrafish embryos.

Materials and methods

Chemicals and reagents

Tetracycline (purity: 90 %) was purchased from MERYER CO LTD. (Shanghai, China). Tricaine (MS-222) and RNAlater Storage Solution were obtained from Sigma-Aldrich Shanghai Trading Co. Ltd. (China). All other chemicals and reagents utilized in this study are analytical grade.

Data collection strategies

To provide an overall view of antibiotics research in the aquatic environment in Shanghai waters, a systematic literature review was performed by carrying out electronic searches on the ISI Web of Knowledge, PubMed, Elsevier, Springer, and Google[®] Scholar. Literature published in Chinese was retrieved from the China Knowledge Resource Integrated Database and the WANFANG Data of E-Resources for China Studies. Given the large number of studies found in the literature, our study focused on those which were the most relevant to China aquatic environments. We collected the data that were considered to be useful, and calculated the median of the data to reflect the average level of tetracycline antibiotics in different water areas within these years stated in the studies.

Zebrafish maintenance, embryo collection and experimental setup

Wild-type (AB strain) zebrafish were maintained at 28 ± 0.5 °C in a 14:10 h light:dark cycle in a closed flow-through system with charcoal-filtered tap water. The fish were fed *brine shrimp* twice daily. The zebrafish embryos were obtained from spawning adults placed in groups of about 20 males and 10 females in tanks overnight. Spawning was induced in the morning when the light was turned on. At 4 h post-fertilization (hpf), the embryos were examined under a dissection microscope (Motic, Xiamen, China), and embryos that had developed normally and reached the blastula stage were selected for subsequent experiments. Twenty embryos were randomly placed into each well of 24 tissue culture plates which contained 2 mL of solution that comprised 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.75 mM NaHCO_3 and 0.08 mM KCl (ISO 6341-1982). A series of tetracycline hydrochloride concentrations (0, 2, 10, 20, 200, 2000 and 20,000 $\mu\text{g/L}$) were applied for the acute exposure and gene expression studies. Four replicates for each concentration were used. The water was aerated for 24 h prior to the preparation of the treatment solutions. The solutions were changed once every 24 h. All of the embryos or larvae were kept at 28 ± 0.5 °C and subjected to a no light environment.

Measurement of the toxicity endpoints

The development of zebrafish embryos and larvae was monitored at specified time points. Endpoints used for assessing the developmental effects of tetracycline hydrochloride on the zebrafish included survival, hatching success, body length, and other morphological and functional malformations. The hatching was monitored from 48 to 72 hpf, and survival rates were recorded at 24, 48, 72 and 96 hpf. The body length (96 hpf) of each zebrafish was measured by using a dissection microscope (Leica, Wetzlar, Germany) through which embryos and larvae in each replicate were examined for malformation at 96 hpf. After the measurement, the embryos or larvae were returned to the well for subsequent experiments and the dead were discarded. The experimental temperature was maintained at 28 ± 0.5 °C.

Acridine orange staining

Acridine orange (AO) staining is a nucleic acid selective metachromatic stain technique that can identify cell apoptosis (Chan and Cheng 2003; Deng et al. 2009; Zeng et al. 2014; Yuan et al. 2014). After 96 h of exposure to a series of tetracycline hydrochloride concentrations (0, 5, 10, and 20 $\mu\text{g/L}$), five larvae from each replicate ($n = 4$)

were washed twice in 30 % Danieau's solution (58 mM of NaCl, 0.7 mM of KCl, 0.4 mM of MgSO_4 , 0.6 mM of $\text{Ca}(\text{NO}_3)_2$, and 5 mM of HEPES, pH 7.4), and then transferred into 5 $\mu\text{g}/\text{mL}$ of AO dissolved in 30 % Danieau's solution for 20 min at room temperature. The larvae were then washed with 30 % Danieau's solution three times for 5 min each. Before examination, the embryos were anesthetized with 0.016 M tricaine for 3 min. The apoptotic cells were identified with a fluorescence microscope (Leica, Wetzlar, Germany). The apoptotic cells appeared as obvious bright spots.

ROS measurement

The generation of ROS in the embryos exposed to tetracycline hydrochloride (0, 2, 10 and 20 $\mu\text{g}/\text{L}$) at 96 hpf was measured by using dichloro-dihydro-fluorescein diacetate (DCFH-DA; Zeng et al. 2014) obtained from the Beyotime Institute of Biotechnology (Jiangsu, China). Twenty larvae were washed with cold 30 % Danieau's solution three times and then incubated with 20 $\mu\text{M}/\text{L}$ DCFH-DA solution for 1 h. After 1 h incubation, the larvae were washed with 30 % Danieau's solution three times, and then homogenized in 200 μL cold 30 % Danieau's solution. The homogenate was centrifuged at $8,000\times g$ at 4 °C for 4 min, and the supernatant (200 μL) was transferred into a 96-well plate and incubated at room temperature for 5 min. The fluorescence intensity was measured by using a Spectra-Max M5 multi-mode microplate reader (Molecular Device, USA) with excitation and emission at 485 and 530 nm, respectively.

Total protein content assay

Briefly, the embryos or larvae were treated from 4 to 72 hpf before collection for analysis. To evaluate the effect of the deysolving process, the yolk sacs were first ruptured with a fine needle and the deysolved embryos were washed twice with a phosphate buffer saline (PBS; pH 7.4) and then homogenized in an ice-cold protein extraction buffer to extract the proteins. The homogenates were centrifuged for 30 min at $12,000\times g$ and the supernatants were collected. Protein concentrations were determined by using the Bradford method (Bradford 1976).

Total RNA extraction and RT-PCR

The total RNA was extracted from the studied zebrafish embryos or larvae by using the Qiagen kit (Qiagen China Co., Ltd, Shanghai, China). The total RNA contents were determined by measuring the absorbance at 260 nm and the quality was verified by measuring the 260/280 nm ratio.

One percent agarose formaldehyde gel electrophoresis with ethidium bromide staining was used to further verify the quality of the total RNA. First-strand cDNA synthesis was performed by using a commercial kit (Takara, Kyoto, Japan) and following the manufacturer's instructions. Real-time quantitative polymerase chain reaction (PCR) was carried out by using an ABI-7500 detection system (Applied Biosystems, Foster City, CA, USA).

In order to examine whether tetracycline hydrochloride has effects on the oxidative damage of the genes of zebrafish embryos, we conducted a preliminary study on the signaling pathways related to apoptosis. A total of 2 genes related to oxidative damage (CAT, SOD), 11 genes related to apoptosis (p53, AIF, Bax, Apaf-1, Bcl-2, Cyt c, Caspases-3, 6, 7, 8 and 9), 3 genes related to ABC transporter proteins (ABCC1, ABCC2 and ABCC4) and 1 gene related to drug metabolism (Cytochrome P450, CYP1A) were chosen in this study. β -actin was chosen as the internal control to normalize the data. The primers for these genes are listed in Table S2. Amplification was carried out in 96-well PCR plates (Applied Biosystems, Foster City, CA, USA) with a volume of 20 μL , which contained 10 μL of $2\times$ SYBER Green real time PCR master mix from Takara Biotechnology Co Ltd (Dalian), 0.4 μM of each primer and 2 μL of $10\times$ diluted cDNA samples. All of the reactions were carried out in triplicate. The reactions were carried out in the following order: temperature of 95 °C for 1 min (1 cycle) and 95 °C for 3 s, and 34 s at the annealing temperatures (60 °C for β -actin, p53, AIF, Bax, Apaf-1, Bcl-2, Cyt c, Caspases-3, 6, 7, 8 and 9, CAT, SOD, ABCC1, ABCC2, ABCC4 genes; 62 °C for CYP1A), 72 °C for 20 s (40 cycles), followed by a melting curve analysis to determine the specificity of the reaction. The $\Delta\Delta C_T$ method was used to calculate the relative mRNA expression levels of genes with the β -actin gene.

Statistical analysis

All values were presented as the mean \pm standard error (SE) from four separate experiments. The assumptions of homogeneity of variances were checked by Levene's test. The main factor for analysis of variance was treatment. Significant differences between mean values were determined using one-way analysis of variance (ANOVA), and the Dunnett's test was used to determine the significant difference ($p < 0.05$) between tetracycline-treated and control groups. Percentage data were arc-sin square-root-transformed before analysis. The ANOVA was performed using SPSS 17.0 (SPSS, Chicago, IL, USA) and the figures were generated by using Graphpad Prism 5.0. A value of $p < 0.05$ was used as the criterion for statistical significance.

Results

Tetracycline antibiotics in China water bodies

Four kinds of tetracycline antibiotics have been detected in different waters in recent years, as shown in Table 1. Taking both concentration and detection frequency into consideration, tetracycline and oxytetracycline are found to be the two major antibiotics among the tetracycline antibiotics that exist in the different waters of China. Besides that, between the two antibiotics, the concentration of tetracycline is lower than that of oxytetracycline in surface water. However, in the inflow of sewage plants, tetracycline is higher than oxytetracycline. Specific to tetracycline, livestock husbandry wastewater is the main water resource for the aquatic environment. Overall, compared with other waters, the concentrations of all of the tetracycline antibiotics in livestock husbandry wastewater are the highest. Antibiotics lead to high toxicity and environmental risk to aquatic organisms when wastewaters are discharged into natural water. The toxicity data of several major antibiotics in mice is collected, and Table S1 shows that tetracycline poses the greatest potential risk in the aquatic environment.

Effect of tetracycline on zebrafish embryos and larvae

The exposure study showed that tetracycline has significant effects on the growth and development of zebrafish embryos and larvae, including decreased body length, delayed hatching, increased yolk sac area and absence of a swim bladder.

The fitting curve of the dose–response effect of tetracycline on the embryos/larvae is given in Fig. 1. The total effects of tetracycline on the zebrafish embryos/larvae increased in a dose–response manner. The fitting curve equation is shown in Fig. 1. The EC_{10} of tetracycline to zebrafish larvae after 96 h of exposure is 3.16 $\mu\text{g/L}$, which is close to its environmental concentrations (0.26–1 $\mu\text{g/L}$; Shen et al., 2012), while the concentration from wastewater that originated from breeding farms is even higher than the EC_{10} here (Ji et al., 2011). This result suggests that the tetracycline in the aquatic environment has potential ecotoxicological effects on aquatic organisms.

As shown in Fig. 2, compared with the control group (Fig. 2a), the group treated with 20 $\mu\text{g/L}$ of tetracycline shows an increased yolk sac area, uninflated swim bladder and shorter body length. The percentage of major malformation phenotypes in the zebrafish embryos upon exposure to 20 $\mu\text{g/L}$ tetracycline (Fig. 2c), and the correlation of different phenotype effects (Fig. 2d) are recorded at 96 hpf.

The growth in terms of the body length of the larvae was measured at 96 hpf and the results are shown in Fig. 3c. All of the concentrations of tetracycline do not have a significant effect on body length compared to the controls.

Over 98 % of the control zebrafish embryos hatched out of the chorion at 72 hpf (Fig. 3b). Even though there was not a significant dose-dependent decrease in the hatching rate of the tetracycline-treated groups compared with the control groups ($p < 0.05$), with hatching rates of $90.0 \pm 0.2\%$ in 200 $\mu\text{g/L}$, tetracycline could significantly inhibit hatching at higher concentrations ($>200 \mu\text{g/L}$), and no embryo hatched above 500 mg/L (data not shown). At 96 hpf, the hatching rate restored in the treated groups

Table 1 Summary of tetracycline antibiotic concentrations in different waters in China

Sampling location		Concentration ($\mu\text{g/L}$)				Reference
		Tetracycline	Oxytetracycline	Chlorotetracycline	Deoxytetracycline	
Surface water	Huangpujiang River	0.26–1.0	0.1–2.26	– ^c	–	Shen et al. (2012)
	Jiulongjiang River	<LQ ^a –0.1895	0.0112–0.2045	<LQ ^a –1.0365	–	Zhang et al. (2011)
	Nanming River	0.08–6.8	0.1–3.0	0.09–0.14	–	Liu et al. (2009)
	Jiangsu	0.27–0.81	0.22–2.2	0.32–2.42	nd ^b	Wei et al. (2011)
Sewage plant (Inflow)	Guiyang	9.5–11.0	2.1–2.3	0.5–1.1	–	Liu et al. (2009)
	Hong Kong	0.016–1.42	<LQ ^a –0.842	<LQ ^a	–	Leung et al. (2012)
Livestock husbandry wastewater	Shanghai	110.8–129.3	183.2–237.8	–	–	Ji et al. (2011)
	Guangzhou	nd ^b –16.0	nd ^b	–	–	Guo et al. (2011)
	Jiangsu	0.43–10.3	0.914–72.91	0.58–3.67	0.63–39.54	Wei et al. (2011)

^a Limited quantity, ^b not detected, ^c not analyzed

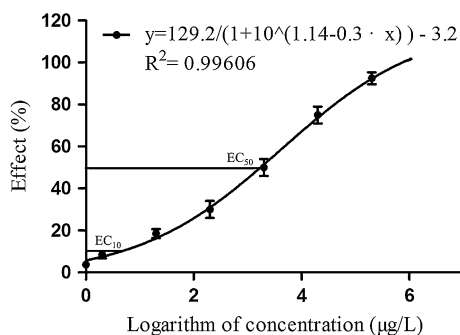


Fig. 1 Cumulative effect of tetracycline in zebrafish embryos upon exposure to different concentrations (0, 2, 20, 200, 2000 and 20,000 µg/L) at 96 hpf. ($n = 80$)

(200 µg/L) (data not shown). However, no embryos treated with more than 500 mg/L of tetracycline successfully hatched out at 96 hpf.

In the most affected groups, the developmental abnormalities included increased yolk sac area and lack of a swim bladder (Fig. 3a, d). Among these, the yolk sac area in the control groups was significantly larger than the groups treated with 2, 20 and 200 µg/L of tetracycline, and also, the groups treated with 20 and 200 µg/L of tetracycline had a swim bladder deficiency.

AO staining

No obvious apoptotic cells were observed in the control embryos, whereas in the groups treated with 5, 10 and 20 µg/L of tetracycline, apoptotic cells appeared, mainly

around the tail area, but in the groups treated with 20 µg/L of tetracycline, some of the apoptotic cells appeared around the heart area in a few of the larvae (Fig. 4).

Effect of tetracycline on production of ROS in zebrafish larvae

The fluorescence intensity of ROS in the zebrafish larvae at 96 hpf is shown in Fig. 5. No significant difference in the level of ROS production can be observed in the larvae treated with 2 µg/L of tetracycline when compared with the control. However, ROS levels in the embryos treated with higher concentrations of tetracycline (10 and 10 µg/L) are significantly higher than that of the control.

Total protein content assay

The total protein content of the zebrafish larvae at 72 hpf is shown in Fig. 6. The results show that there is no significant difference in the larvae treated with all of the concentrations (2, 20, and 200 µg/L) when compared with the control. However, this trend shows that higher tetracycline concentrations may inhibit the protein synthesis of zebrafish embryos or larvae.

Gene expression studies

Environmental pollutants are well-known inducers of ROS, and ROS can be reason for the depletion of antioxidant defenses (Livingstone 2001). The results demonstrated that tetracycline induced up-regulation of two ROS related

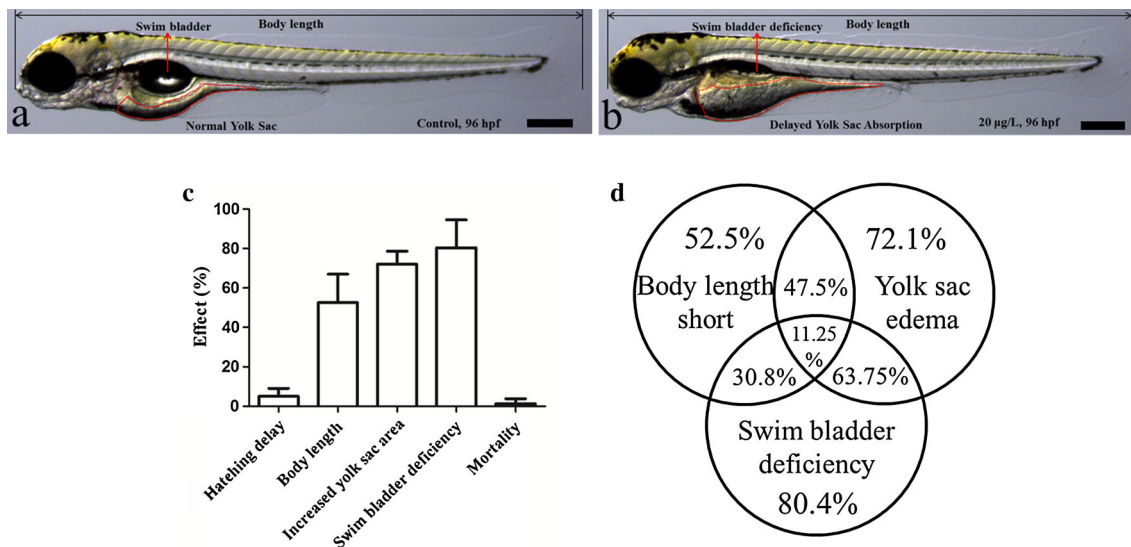
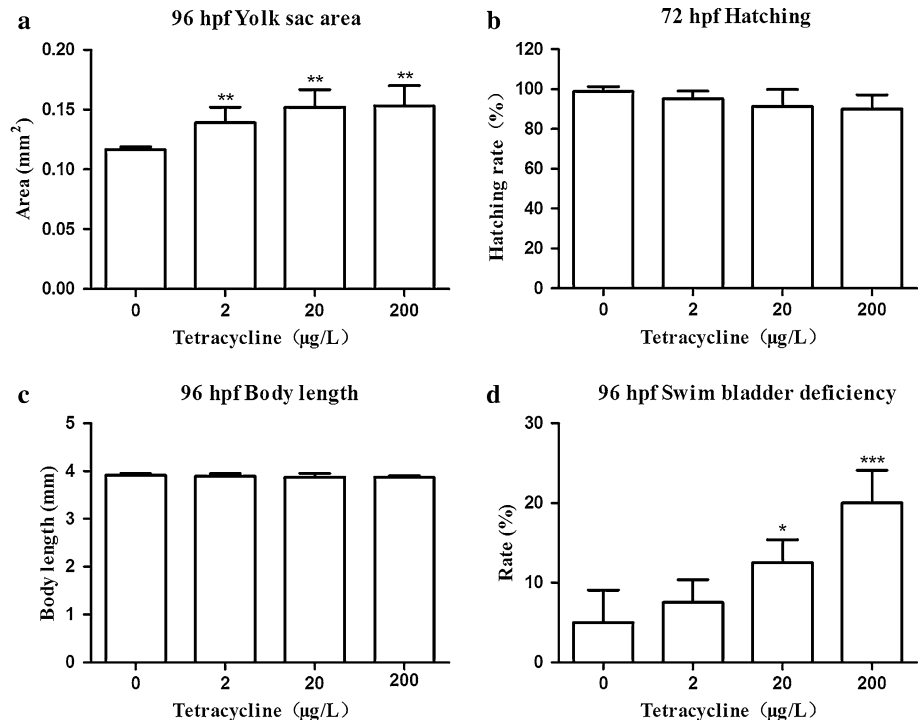


Fig. 2 Representative control (a) and treated zebrafish embryos (20 µg/L, tetracycline) at 96 hpf. Exposure to tetracycline induces serious developmental delays in the treated zebrafish embryos, including uninflated swim bladder (red arrows in a, b), decreased

body length and delayed absorption of the yolk sac. Percentage of major phenotypes in the zebrafish embryos upon exposure to 20 µg/L tetracycline at 96 hpf (c), and correlation of different phenotype effects (d). ($n = 80$) Scar bar = 300 µm (Color figure online)

Fig. 3 Yolk sac area/hatching rate/body length/swim bladder deficiency of zebrafish larvae upon exposure to tetracycline (96 hpf). The values are presented as the mean \pm SE. ($n = 80$) The results of one-way ANOVA are * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$



genes (SOD and CAT) (Table 2) and ROS overproduction in zebrafish embryos at 96 hpf (Fig. 5). The mRNA expressions of p53, Apaf-1, Cyt c, and Caspases-3 and 9 genes from the zebrafish larvae were significantly up-regulated for the group treated with 10 $\mu\text{g/L}$ of tetracycline (96 hpf) (Table 2), and the expression of Bcl-2 was significantly down-regulated for the same group (Table 2). However, the expression of AIF, Bax, and Caspases-3, 7 and 8 did not significantly change in the group treated with 10 $\mu\text{g/L}$ of tetracycline (some data not shown). After 96 hpf exposure, the expression of the ABCC1, ABCC2 and ABCC4 genes significantly changed upon exposure to tetracycline (Table 2). The expression level of ABCC1 was significantly increased by 1.52, 1.83 and 1.64-fold in the groups treated with 10, 20 and 200 $\mu\text{g/L}$ of tetracycline (Table 2). Moreover, the expressions of ABCC2 and ABCC4 were significantly up-regulated in the groups treated with 20 and 10 $\mu\text{g/L}$ of tetracycline respectively (Table 2). The RT-PCR data demonstrated that CYPIA was significantly increased in the larvae treated with 20 and 200 $\mu\text{g/L}$ of tetracycline compared with the control (Table 2).

Discussion

Zebrafish embryo serves as a widely adopted model for studies of mechanism-based developmental toxicity (Shi et al. 2008). Our results showed that tetracycline

significantly affects embryo's early development. The results shown in Figs. 1, 2, 3 indicate that tetracycline affects the development of embryos in a dose-dependent manner. Exposure to tetracycline has significant negative effects on the survival of the treated embryos and larvae. In this study, a significant increase in mortality was observed with exposure to 500 mg/L of tetracycline between 4 and 96 hpf (data not shown), and the LC_{10} and LC_{50} are 551.78 and 759.71 mg/L, respectively. The results indicate that the acute toxicity of tetracycline to zebrafish embryos is low. The growth inhibition in zebrafish embryos might be a secondary effect of tetracycline mediated toxicity caused by the malabsorption of nutrients required for normal development (Hill et al. 2004; Du et al. 2012).

Body length is an important indicator of embryo growth, and the loss of nutrients may induce a shorter body length. In the control group, the larvae were healthy and active with a body length of 3.8 ± 0.04 mm, had an inflated swim bladder, and could absorb nutrients from the yolk sac at 96 hpf. Zebrafish embryos exposed to lower concentrations (2, 20 and 200 $\mu\text{g/L}$) of tetracycline exhibited a significant ($p < 0.05$) reduction in the inflation of their swim bladder and delayed yolk sac absorption (Fig. 3).

Hatching is known to be a critical period of time for zebrafish embryogenesis. Delayed hatching may be due to retarded development or the inability of embryos to break the chorion (Osman et al. 2007). Hatching delay or hatching failure will induce the death of embryos. Hatching

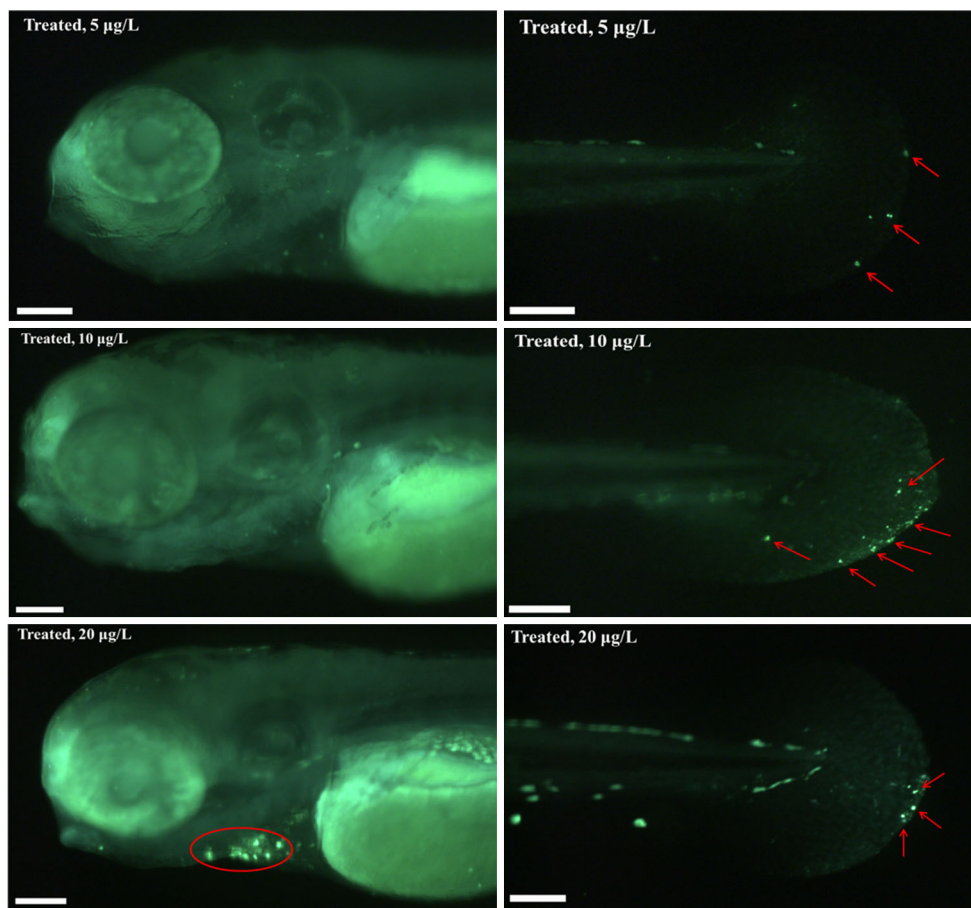


Fig. 4 After exposure to tetracycline for 96 hpf, the zebrafish larvae are stained with Acidine Orange. Apoptotic cells mainly appear in the tail region. Apoptotic cells are indicated by red arrows. ($n = 80$) Scale bar = 100 μm (Color figure online)

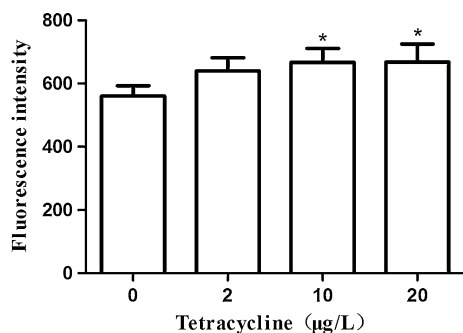


Fig. 5 Effects of different concentrations of tetracycline on reactive oxygen species production at 96 hpf. The values are presented as the mean \pm SE. ($n = 80$) Values are significantly different from the control and the result of one-way ANOVA is $*p < 0.05$

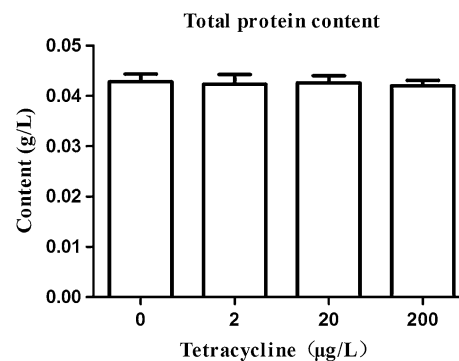


Fig. 6 Effects of different concentrations of tetracycline on total protein synthesis at 96 hpf. The values are presented as the mean \pm SE. ($n = 80$)

is often the result of a combination of biochemical (enzymatic), biophysical (mechanical) and osmotic mechanisms (Yamagami 1981), and so, the observed inhibition may have resulted from the effects of tetracycline on more than one or all of these mechanisms. The marked difference in the hatching rates of the embryos with higher

concentrations of tetracycline compared to the control is likely to be a function of their slower development.

The yolk sac plays an important role during the early developmental stage, because it is the only source of nutrition for embryos, and its physical size will decrease along with the embryonic development. The swim bladder

Table 2 The mRNA expressions of ROS, apoptosis, multidrug resistance, xenobiotic metabolism genes following exposure to tetracycline ($n = 80$)

Genes	Control	2 $\mu\text{g/L}$	10 $\mu\text{g/L}$	20 $\mu\text{g/L}$	200 $\mu\text{g/L}$
ROS					
SOD	1 \pm 0 ^a	0.983 \pm 0.04	0.889 \pm 0.07	1.277 \pm 0.12 ^{**b}	1.358 \pm 0.15 ^{***}
CAT	1 \pm 0	1.10 \pm 0.16	1.505 \pm 0.09 ^{***}	0.837 \pm 0.06	0.159 \pm 0.04 ^{***}
Apoptosis					
p53	1 \pm 0	1.123 \pm 0.09	1.331 \pm 0.12 [*]	1.119 \pm 0.18	1.066 \pm 0.11
AIF	1 \pm 0	1.068 \pm 0.02	0.883 \pm 0.11	1.113 \pm 0.11	0.735 \pm 0.03 ^{***}
Bax	1 \pm 0	0.950 \pm 0.12	1.099 \pm 0.08	0.997 \pm 0.05	1.038 \pm 0.07
Apaf-1	1 \pm 0	1.016 \pm 0.13	1.424 \pm 0.15 ^{**}	1.149 \pm 0.15	1.126 \pm 0.24
Bcl-2	1 \pm 0	0.967 \pm 0.04	0.652 \pm 0.09 [*]	1.314 \pm 0.2 [*]	1.172 \pm 0.17
Cyt c	1 \pm 0	0.855 \pm 0.11	1.289 \pm 0.14 ^{**}	0.879 \pm 0.13	0.884 \pm 0.05
Caspase-9	1 \pm 0	0.858 \pm 0.06	1.311 \pm 0.17 ^{**}	1.056 \pm 0.02	1.181 \pm 0.16
Caspase-8	1 \pm 0	1.527 \pm 0.09 ^{***}	0.446 \pm 0.07 ^{***}	0.723 \pm 0.13 ^{**}	0.537 \pm 0.13 ^{***}
Caspase-7	1 \pm 0	1.014 \pm 0.06	0.983 \pm 0.06	0.451 \pm 0.09 ^{***}	1.252 \pm 0.13 ^{**}
Caspase-6	1 \pm 0	0.973 \pm 0.08	1.046 \pm 0.06	1.068 \pm 0.1	1.196 \pm 0.23
Caspase-3	1 \pm 0	1.099 \pm 0.18	1.287 \pm 0.09 ^{**}	0.579 \pm 0.07 ^{***}	1.026 \pm 0.12
Multidrug resistance					
ABCC1	1 \pm 0	0.945 \pm 0.13	1.518 \pm 0.05 ^{**}	1.826 \pm 0.26 ^{***}	1.618 \pm 0.08 ^{***}
ABCC2	1 \pm 0	0.763 \pm 0.21	1.007 \pm 0.2	1.336 \pm 0.13 [*]	0.553 \pm 0.17 ^{**}
ABCC4	1 \pm 0	0.870 \pm 0.07	1.183 \pm 0.13 [*]	1.047 \pm 0.04	0.973 \pm 0.11
Xenobiotic metabolism					
CYP1A	1 \pm 0	0.532 \pm 0.11	0.596 \pm 0.12	1.566 \pm 0.49 [*]	2.709 \pm 0.16 ^{***}

^a Each value represents the mean \pm SE of four replicates and each replicate with 20 embryos

^b One-way ANOVA was used with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to the control group

is an air-filled sac located dorsally in the abdominal cavity, which helps fish to balance hydrostatic pressure and reduce the energetic cost of swimming (Jönsson et al. 2012). No deformities were noticed in the control group. However, in the treated group, both delayed yolk sac absorption and uninflated swim bladder appeared. These results illustrate that tetracycline can significantly delay the development of zebrafish embryos.

Furthermore, tetracycline exposure results in the superfluous generation of ROS in zebrafish embryos. Studies have demonstrated that embryonic development is especially sensitive to ROS and the resulting oxidative stress-induced cell apoptosis is thought to contribute to abnormal embryonic development (Yamashita 2003; Shi and Zhou 2010). The results of the AO staining are consistent with the speculation.

Apoptosis, also called as programmed cell death (Ulkaya et al. 2011), is an important regulator of growth and development. In the present study, apoptotic cells are observed in the tail of the zebrafish larvae as revealed by AO staining (Fig. 4). Generally, the p53 and caspase pathways are the two main pathways involved in contaminant-triggered apoptosis. Typically, the p53 pathway induces apoptosis by up-regulating the transcription of pro-apoptotic genes, such as p53 and Bax, and down-regulating anti-apoptotic genes, including the Bcl-2. Up-regulation of p53 can lead to apoptosis by the up-regulating expression of Bax and down-regulating expression of Bcl-2, which

was observed in some studies (Li et al. 2011; Zeng et al. 2014). In this study, in the group exposed to 10 $\mu\text{g/L}$ of tetracycline, up-regulation of p53 and down-regulation of Bcl-2 were detected, but did not include the up-regulation of Bax. So, the p53-Bax-Bcl-2 pathway is not the pathway for tetracycline-induced apoptosis.

The caspase pathway is also an important pathway involved in contaminant-triggered apoptosis (Xiong et al. 2009). Caspase-3 is confirmed as a key executor to be activated down-stream in apoptosis pathways (Liu et al. 2007; Deng et al. 2009). Generally, two pathways of caspase activation related to caspase pathways are involved in apoptosis. The first one is cytochrome c, which is released from the mitochondria, promotes caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex which then leads to apoptosis (Gao et al. 2013). Typically, the reduction of Bcl-2 factor expression has relation with activation of caspases 3 and 9 (Morales-Cano et al. 2013). In the latter, the stimulation of Fas, a tumour necrosis factor receptor (TNFR) or TNF-related apoptosis-inducing ligand receptor (TRAILR), results in the activation of the initiator caspase-8 (Fulda 2009). In the present study, the gene transcription of Apaf-1, Cyt c, and caspases-3 and -9 are greatly increased for the 10 $\mu\text{g/L}$ of tetracycline treated groups, and the gene transcription of Bcl-2 is down-regulated for the groups treated with 10 $\mu\text{g/L}$ of tetracycline. Taken together, these results indicate that caspase-dependent apoptotic pathways may

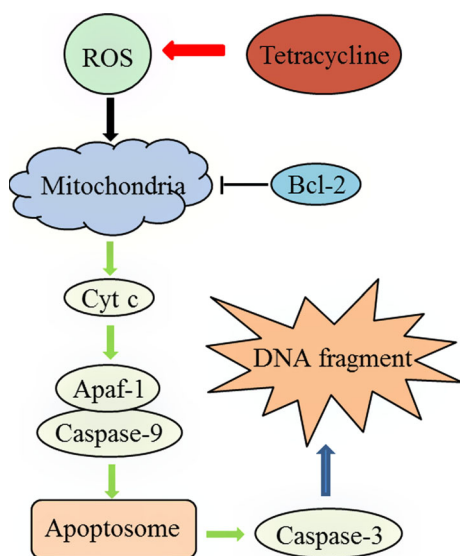


Fig. 7 Proposed apoptotic pathway induced by tetracycline exposure in early-life stages of zebrafish. Cytochrome c promotes caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex which then leads to apoptosis

greatly contribute to tetracycline-induced apoptosis in the early-life stages of zebrafish. The tetracycline-induced apoptosis pathway is shown in Fig. 7.

As an antibiotic, tetracycline can kill bacteria but also induce resistance. In tumor cells, an ATP-dependent decrease in cellular drug accumulation is often attributed to overexpression of certain ABC transporter proteins (Keppler et al. 1999). In addition to their roles in resisting anti-tumor drugs, these efflux pumps have important functions in the transport of a wide variety of compounds across biological membranes (Leslie et al. 2005; Takahashi et al. 2005). In aquatic organisms, the presence of ABC transporters which are involved in multidrug resistance (MDR) has been demonstrated (Kington et al. 2007). It has been demonstrated that heavy metals can induce the overexpression of ABC genes during the early development stage of zebrafish (2–120 hpf) and ABC transporters are involved in the efflux of heavy metals (Long et al. 2011a, b, c, d). In this study, we have investigated the transcriptional response of ABCC1, ABCC2, ABCC3 and ABCC4 cDNA from zebrafish larvae upon exposure to tetracycline. The significant changes of ABCC1, ABCC2 and ABCC4 mRNA expression upon exposure to a certain concentration of tetracycline suggest that zebrafish larvae may transfer tetracycline or degradation products out via multidrug resistance-associated proteins.

In zebrafish, Cytochrome P450s (CYPs) are important xenobiotic metabolizing proteins. CYPs are involved in the biotransformation of many endogenous and exogenous compounds, and have been characterized in eukaryotes

(Otyepka et al. 2007; Seliskar and Rozman 2007, Smith and Wilson 2010), and the majority of zebrafish CYP genes are expressed in embryos (Goldstone et al. 2010). In this study, CYPs are involved in the biotransformation of tetracycline in zebrafish larvae.

In summary, the present study demonstrates that tetracycline has developmental toxicity on zebrafish embryos/larvae, and can significantly affect embryo development. The developmental toxicity may be related to the generation of ROS and the consequent triggering of apoptosis. In addition, gene expression studies shows that caspase-dependent apoptotic pathways may play important roles in tetracycline-induced apoptosis in the treated zebrafish embryos. However, the exact mechanisms still require further study.

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Conflict of interest The authors declare that they have no conflict of interest.

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