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Toxicity Assessment of Combined Fluoroquinolone and Tetracycline Exposure in Zebrafish (*Danio rerio*)

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ABSTRACT: Fluoroquinolones (FQs) and tetracyclines (TCs), the two β -diketone antibiotics (DKAs), are two frequently detected pollutants in the environment; however, little data are available on their combined toxicity to zebrafish (*Danio rerio*). This study reports that toxicologic effects of combined DKA (FQs–TCs) exposure on zebrafish were comparable with or slightly less than those of TCs alone, showing that TCs played a major toxicologic role in the mixtures. The effects of FQs, TCs, and DKAs on malformation rates of zebrafish were dose dependent, with EC₅₀ values of 481.3, 16.4, and 135.1 mg/L, respectively. According to the combined effects of DKAs on zebrafish hatching, mortality, and malformation rates, the interaction between FQs and TCs was shown to be antagonistic based on three assessment methods: Toxic Unit, Additional Index, and Mixture Toxic Index. The 1.56 mg/L TC and 9.38 mg/L DKA treatments resulted in higher zebrafish basal swimming rate compared with the control group at 120 hours postfertilization (hpf), in both light and light-to-dark photoperiod experiments. Under conditions of no obvious abnormality in cardiac development, the heart beats were decreased significantly because of DKA exposure, such as decreasing by ~20% at 150 mg/L DKAs. Transmission electron microscopy observation of myocytes from DKA-exposed hearts displayed prominent interruptions and myofibrillar disorganization of the normal parallel alignment of thick and thin filaments, and partial edematous and dissolved membranes of cell nuclear tissues. At 90 mg/L DKAs, the transcriptional levels of the *acta1a*, *myl7*, and *gle1b* genes, related to heart development and skeletal muscle formation, were significantly changed. This is consistent with the swimming behavior and histopathologic results obtained by transmission electron microscopy. In summary, the toxicity of the combined DKAs to zebrafish was comparable with or less than that of TCs

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alone and had the ability to impair individual behaviors that are of great importance in the assessment of their ecologic fitness. © 2014 Wiley Periodicals, Inc. *Environ Toxicol* 31: 736–750, 2016.

Keywords: combined exposure; behavioral toxicity; malformation rate; histopathologic analysis; gene transcriptional level

INTRODUCTION

Fluoroquinolones (FQs) and tetracyclines (TCs) are the two β -diketone antibiotics (DKAs) widely used to prevent and treat a large variety of infectious diseases in humans and veterinary practices with broad-spectrum activity against bacteria (Lanza and Sellergren, 1999). For example, a large portion of the antibiotics administered in fish farms (70%–80%) has been reported to reach the environment, and over 8000 ton of DKAs are used as feed additives in China each year (Ben et al., 2008). Because they are weakly absorbed and not completely metabolized in the animal's gut, the majority of DKAs are excreted intact in feces and urine (Shi et al., 2012). In recent years, FQs were detected in many environments with concentrations from 2.3 to 40 $\mu\text{g/L}$ measured in China (Xu et al., 2007), USA (Kolpin et al., 2002), and European countries (Tamtam et al., 2008). In addition, ciprofloxacin was detected at a concentration level of 0.101 mg/L in untreated hospital sewages (Lindberg et al., 2004; Martins et al., 2008). What is noteworthy is the relatively high concentration (up to 0.355 mg/L) of ofloxacin found in hospital and residential effluent in New Mexico (Brown et al., 2006). In the hospital sewage water, large temporal variations in the analyte concentration were observed during the course of the sampling period by Lindberg et al. (2004). Analyte concentrations were in the range of 3.6–101.0 mg/L for ciprofloxacin, 0.2–7.6 mg/L for ofloxacin, and 0.6–6.7 mg/L for doxycycline. In Norway, oxytetracycline was detected at concentration levels of 0.1–4.9 mg/kg dry matter (Boxall et al., 2004). Because the activities of DKAs and their main metabolites persist in aqueous environments, great concern arises about the adverse effects of the use and disposal of DKAs on human and ecologic health (Burka et al., 2005; Khetan and Collins, 2007).

In comparison with other antibiotic classes, DKAs have the highest risk of causing colonization with methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* (Tacconelli et al., 2007). Chlortetracycline had a significant toxic effect on population growth and chlorophyll-a accumulation in *Microcystis aeruginosa* and *Scenedesmus obliquus* (Guo and Chen, 2012). Robinson et al. (2005) reported toxicities of seven FQs to a variety of nontarget species, such as *Pseudokirchneriella subcapitata*, *Lemna minor*, *Daphnia magna*, and *Pimephales promelas*. Acetylcholinesterase activity can be severely depressed in muscle tissue when catfish are exposed to enorxacin, suggesting a potential neurotoxicologic effect of enorxacin in fish (Wang

et al., 2009). Please provide details of the citation “Wang et al., 2009” in the reference list or delete the text citation. Similarly, Hall et al. (2011) found that FQs were associated with a wide array of musculoskeletal complications involving tendon, cartilage, bone, and muscle. Further, Ding et al. (2010) showed that lidamycin could inhibit angiogenesis of zebrafish embryo by down-regulation of VEGF expression. TCs facilitate a resultant fatty liver by affecting the gene expression associated with lipid metabolism by increasing triglycerides and cholesterol biosynthesis and decreasing β -oxidation of fatty acids (Yin et al., 2006). Previous studies showed that DKAs have strong gene and genetic toxicity, such as DNA damage, adduct formation, DNA hypermethylation of kidney cell in *Carassius auratus*, and teratogenic effects on *Cyprinus carpio* (Li et al., 2010; Khadra et al., 2012). Previous research has mainly focused on toxicity and its associated mechanisms for a single DKA compound (Li et al., 2010; Khadra et al., 2012; Wang et al., 2014). However, little data are available on the aquatic toxicity of DKA mixtures using zebrafish (*Danio rerio*) as a model organism. Owing to the limitations of both traditional mammalian models and cell-based assay approaches, zebrafish are rapidly gaining acceptance as a promising animal model for toxicology studies (Bowman and Zon, 2010). Several studies have confirmed strikingly similar mammalian and zebrafish toxicity profiles (Berghmans et al., 2008; Eimon and Rubinstein, 2009). Compared with using mice as a model organism, zebrafish provide real-time tracking of pathogen infection, a better understanding of the pathogenesis, and possess innate and adaptive immunity to pathogen infection making high-throughput screening possible (Mukhopadhyay and Peterson, 2006). Unlike many other model organisms, one of the major advantages of zebrafish is that the embryos develop externally and are optically transparent. This allows for simple noninvasive microscopic observation of individual cells and heart rhythms, as well as assessment of vasculature and circulation *in vivo* across a broad range of developmental stages. Zebrafish embryos develop more rapidly than mammals, and thus using zebrafish embryos as a model organism is more efficient for toxicologic studies (Wang et al., 2014). As researchers have started to recognize the benefits of zebrafish for elucidating molecular pathways in normal and diseased states, the use of zebrafish in scientific research has markedly increased in recent years (Heijne et al., 2005; Lieschke and Currie, 2007).

In real-world environments, thousands of DKAs may enter surface waters, and thus they may interact with each other, resulting in changes in their combined toxicity

potential. Toxicologic tests for individual and mixtures of DKAs are very important, because these data can be used to simulate environmental concentration effects and to further analyze their interactions. Although the individual concentrations of DKA antibiotics in aquatic environments may be low, interactions among mixtures of DKAs could result in significant toxicity to aquatic organisms. Because chemicals in a mixture may or may not interact synergistically or antagonistically, it is essential to investigate the potential interactions of antibiotic mixtures for several different classes (Teuschler, 2007; Rodea-Palomares et al., 2010).

Our research previously examined the effects of DKAs on the development of embryo-larval zebrafish (*D. rerio*). We found that DKAs have much lower toxicity compared with other persistent pollutants: the estimated 120 hours postfertilization (hpf) nominal no-observed-effect concentration and lowest-observed-effect concentration for DKAs were 18.75 and 37.50 mg/L, respectively (Wang et al., 2014). However, these previous studies of combined exposure to DKAs did not evaluate combined toxicity effects related to synergistic, antagonistic, or additive effects, or the related toxicologic mechanisms.

In this study, six representative DKAs (four FQs and two TCs) were selected to evaluate their combined developmental effects on the embryo-larval stage of zebrafish (*D. rerio*). After a short-term exposure (6–120 h), a series of fitness indicators, including mortality, hatching rate, malformation, heart rate (HR), and behavior, were examined. Histopathologic analyses of cardiac and muscular tissues were evaluated for evidence of tissue damage. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to compare transcriptional profiles for pooled individual RNA samples of adult zebrafish. The expressions of myosin, light polypeptide 7, regulatory (*myl7*, with synonyms of *cmlc2*) gene and *Gle1* RNA export mediator homolog (yeast) (*Gle1*) gene related to cardiac function were compared with the actin, alpha 1, skeletal muscle (*acta1*) gene involved in skeletal muscle tissue (Kendirgi et al., 2003; Folkmann et al., 2011; Ceyhan and Beggs, 2013; Huttner et al., 2013).

MATERIALS AND METHODS

Chemicals

Four FQs (ciprofloxacin, ofloxacin, norfloxacin, and enrofloxacin) and two TCs (chlortetracycline and doxycycline) were selected for study (Fig. 1 and Table I). All DKAs were sourced from Amresco (Solon, OH) and used as received.

Fish Husbandry and Embryo Collection

Wild-type zebrafish (AB strain) were raised in an aquarium receiving circulated and aerated tap water at 28°C with a 14:10-h light–dark cycle (lights on at 8:00 a.m.) according to

standard zebrafish breeding protocols (Westerfield, 1995). Water supplied to the system was dechlorinated and filtered by reverse osmosis (pH 6.5–7.5), and instant ocean salt was added to the water to raise the conductivity to 450–1000 µS/cm. Zebrafish were fed twice daily with live *Artemia* (Jiahong Feed Co., Tianjin, China) and dry flake diet (Zeigler, Aquatic Habitats, Apopka, FL). The developmental status of zebrafish embryos and larvae was observed with an inverted microscope (×8–50, Nikon, SMZ1500, Japan).

Zebrafish embryos used for chemical exposure were obtained from spawning adults in tanks overnight with a sex ratio of 1:1. Embryos were collected within 1 h of light exposure and rinsed in embryo medium (EM). The fertilized and normal embryos were inspected and staged for subsequent experiments under a stereomicroscope according to Kimmel et al. (1995).

Exposure Protocols and Microscopic Observation

The zebrafish embryo-larval toxicity test was performed according to previous studies with minor modifications (Stehr et al., 2006). After a series of preliminary experiments, the exposure concentration, time interval, and toxicologic testing endpoint of zebrafish for each compound were determined (Table II). Co-exposure of six antibiotics (DKAs) was carried out by mixing equal weight concentrations and volumes of each DKA stock solution in EM. Each treatment was conducted in triplicate and contained 30 embryos, incubated in a light-controlled incubator at 28°C and renewed with 100% fresh solution each day. To assure the accuracy and reproducibility of results, the minimum survival and hatching rate percentages were set to 95% for the control group.

To determine the median effect concentration (EC₅₀) values, normal embryos were exposed to a series of FQs (0, 25, 50, 100, 200, 300, 400, and 600 mg/L) and TCs (0, 1.56, 3.13, 6.25, 12.5, 25, and 50 mg/L) from 6 to 120 hpf. EC₅₀ value was computed by the following Boltzmann equation (Eq. 1):

$$Y = A_2 + (A_1 - A_2) / (1 + \exp((X - X_0)/dX)) \quad (1)$$

where, X was concentration of a mixture or a given contaminant, and Y denoted malformation effect that a mixture or a given contaminant has on the test organism (Zhang et al., 2012a).

Embryos were kept in sterile 96-well plates with one embryo per well containing 200 µL EM, and the plates were covered with sealing film to prevent evaporation and contamination. After observing and recording zebrafish malformation at 120 hpf, the data were plotted for estimating EC₅₀ values for each DKA compound. EC₅₀ values for zebrafish exposed to combined DKA compounds (0, 18.75, 37.5, 75, 150, 300, and 450 mg/L) were determined as for individual

Fig. 1. The chemical structures of the investigated β -diketone antibiotics.

DKA trials. For each batch of zebrafish embryos, the hatching rate was measured at 72 hpf, and body length, mortality, and malformation were determined at 120 hpf using an inverted dissecting microscope. In addition, adult zebrafish (2-month old, 1:1 male:female ratio) were observed after exposure to DKAs in different concentrations (30, 60, and 90 mg/L) for 2 months, with 100% replacement of fresh solution each day.

Joint Toxicity Effect of DKAs

In the combined acute toxicity experiments, we took the 120-hpf malformation EC_{50} of DKAs as the standard con-

centration, and took each FQ and TC as the basic concentration. To characterize the type of joint action between FQs and TCs, three methods were used to assess the combined toxicity: Toxic Unit (TU) method (An et al., 2004), Additional Index (AI) method (Meng and Xiao, 2004) and Mixture Toxic Index (MTI) method (Montvydiene and Marciulioniene, 2004). To clarify the difference between the toxicity of a certain compound “*i*” in the joint exposure and that in the single exposure, EC_{50i} was introduced to present EC_{50} values for compound *i* in the joint exposure. With combined exposure, joint toxicity of compounds with concentrations at their respective EC_{50i} value will contribute a toxicity strength having a 50% chance of causing malformation.

TABLE I. Basic information for the selected DKAs used in this study

DKAs	Antibiotic	Source	CAS No.	Purity (%)	Molecular Weight (g/mol)	pKa
FQs	Ciprofloxacin	Amresco	86393-32-0	>98	331.3	5.6 (pKa1); 8.9 (pKa2)
	Ofloxacin	Amresco	82419-36-1	>99	361.4	6.2
	Norfloxacin	Amresco	70458-96-7	>99	319.3	6.6
	Enrofloxacin	Amresco	93106-60-6	>99.9	359.4	5.6 (pKa1); 8.0 (pKa2)
TCs	Chlortetracycline	Amresco	64-72-2	>95	478.8	6.0
	Doxycycline	Amresco	24390-14-5	>98	444.4	6.5

TABLE II. The experimental scheme used in toxicity study

DKAs	Exposure Concentrations (mg/L)	Exposure Period	Toxicologic Endpoints	Results
FQs	0, 25, 50, 100, 200, 300, 400, 600	6–120 hpf	Hatching rate (72 hpf); mortality (120 hpf);	Figs. 2–4
TCs	0, 1.56, 3.13, 6.25, 12.5, 25, 50		malformation (120 hpf)	
FQs + TCs	0, 18.75, 37.5, 75, 150, 300, 450			
FQs	0, 12.5, 25, 50	6–96 hpf	Swimming speed (light stimulation test)	Fig. 5
TCs	0, 1.56, 3.13, 6.25		(144 hpf)	
FQs + TCs	0, 4.69, 9.38, 18.75			
FQs + TCs	0, 9.38, 18.75, 37.5, 75, 150	6–72 hpf	Heart beat (60 s, 48 hpf, 60 hpf, 72 hpf)	Fig. 6
FQs + TCs	0, 45, 60, 95	2~4 month old	Histopathologic observation	Figs. 7 and 8

Thus, the EC_{50i} of FQs and TCs in combined exposure can be calculated according to the relationship between the concentration of FQs or TCs in the mixture and the malformation rate. Therefore, the TU value of a component i with the concentration of EC_{50i} is calculated by the following Eqs. 2–5:

$$TU_i = C_i / EC_{50i} \quad (2)$$

$$M = \sum_{i=0}^n TU_i \quad (3)$$

$$M_0 = M / TU_{i, \max} \quad (4)$$

$$MT_i = 1 - (\log M) / \log M_0 \quad (5)$$

where C_i is the concentration of component i in the mixture; EC_{50i} is the EC_{50} of component i ; and $TU_{i, \max}$ is the maximum toxicity unit in the mixtures. When $M = 1$, the joint action is additive; when $M > M_0$, the joint action is antagonistic; when $M < 1$, the joint action is synergistic; when $M = M_0$, the joint action is independent; and when $M_0 > M > 1$, the joint action is partly additive.

For the calculation of AI, $AI = M - 1$, when $M = 1$; $AI = 1/M - 1$ when $M < 1$; and $AI = (-1) \times M + 1$ when $M > 1$. When $AI > 0$, the joint action is synergistic; when $AI < 0$, the joint action is antagonistic; and when $AI = 0$, the joint action is additive.

For the joint action judgment of MTI, it is antagonistic when $M_{Ti} < 0$; independent when $M_{Ti} = 0$; partly additive when $M_{Ti} = 0-1$; concentration-dependent additive when $M_{Ti} = 1$; and super additive when $M_{Ti} > 0$ (Montvydiene and Marciulioniene, 2004).

Behavior Assessment

According to the EC_{50} values, normal embryos were exposed to FQs (0, 12.5, 25, and 50 mg/L), TCs (0, 1.56, 3.13, and 6.25 mg/L), or combined DKAs (0, 4.69, 9.38, and 18.75 mg/L) from 6 to 96 hpf and subjected to behavior assessment at 120 hpf. At 96 hpf, morphologically normal larvae were individually loaded into each well of a 24-well plate with 2 mL EM. Two sets of observations were made after a 10-min adaptation. Larvae locomotion was first observed in visible light for 20 min, and then the larvae's

swimming speed was measured during the 50-min light-to-dark (10 min for each period) transition stimulation. Larval behavior was assessed in a ZebraLab behavior monitoring station (Version 3.5 with background subtraction, Viewpoint Life Science, France) based on a prior study with some modifications (Winter et al., 2008). The optimized instrumental parameters were as follows: background pels, 18–24 pixels; duration time, 20 min; movement threshold, inactive/small (3.0 mm/s) or small/large (6.0 mm/s); and light sequence duration time, 10 min.

Heart Beat and Morphology Observation

Phenylthiourea (100 μ M) was added to 20-hpf larval EM to increase the transparency of zebrafish larvae, and the heart rhythms and circulation were observed without the need for intervention (Craig et al., 2006). After normal embryos (6 hpf) were exposed to the control and DKA treatments (9.38, 18.75, 37.5, 75, and 150 mg/L), the 60-s heartbeat number for zebrafish embryos was determined at 48, 60, and 72 hpf. Simultaneously, morphologic changes in the heart were recorded in detail, and the distance between the sinus and artery ball were measured using microscopy. All phenotypes were judged objectively with comparison with a normal phenotype. The counts at each time point were made separately by two independent observers and averaged (Mitchell et al., 2010). Three to five larvae for each concentration group were selected randomly to count the mean number of heartbeats over 15 s by viewing under a microscope, which were multiplied by 4 to yield beats per minute (bpm).

Preparation of Biological Samples for TEM

Histopathologic evidence of tissue damage was examined using transmission electron microscopy (TEM 10; Zeiss, Jena, Germany). Zebrafish samples were taken from tanks after exposure to DKAs for 2 months, washed with distilled water to remove surface dunghills and surface dried with filter paper. After examining the anatomy of zebrafish in control and treatment groups, the heart was removed, cut into 1 mm³ tissue blocks and fixed in glutaraldehyde at 4°C. A series of pretreatment procedures, such as poaching, fixing, dehydrating, embedding, and sectioning, were performed to

TABLE III. Primers used in qRT-PCR analysis

Gene Name	Forward Primer Sequence	Reverse Primer Sequence	Amplified Fragment Length
<i>myl7</i>	ATGGCACAGACCCAGAGG	GGGCAGCAGTTACAGACAGA	451 bp
<i>acta1a</i>	ACGAGCAGAGGCAGAACC	ACCAGCCAAGTCCAAACG	577 bp
<i>gle1b</i>	AGAAGCTGAGAGAGGAACAC	CTCCTGTTCTTTCTTCTTCTTC	413 bp
β -actin	AGCCTTCCTTCCTGGGTA	AGGGCAAAGTGGTAAACG	575 bp

prepare tissues for TEM observation according to Sendzik et al. (2010).

qRT-PCR Analysis of Candidate Genes

Three candidate genes (*acta1a*, *myl7*, and *gle1b*), which are closely related to heart development and swimming behavior, were chosen for verification of changes in their transcriptional level using qRT-PCR (Kendirgi et al., 2003; Folkmann et al., 2011; Ceyhan and Beggs, 2013; Huttner et al., 2013). The candidate gene sequences were obtained from the zebrafish genome (*D. rerio*, version zv 9.0) (available at: http://asia.ensembl.org/Danio_rerio/Info/Index). Total RNA was extracted from the corresponding tissues as reported by Fernandes et al. (2006). Assessment of RNA quality was determined by agarose gel electrophoresis on a 1.5% (w/v) agarose gel stained with SYBR safe (Invitrogen, Carlsbad, CA), and the RNA was then quantified with a Nanodrop spectrophotometer (Nanodrop Technologies/Saven Werner, Kristiansand, Norway). The 260:280 nm absorbance ratios were greater than 1.9, indicating high-purity RNA. Five micrograms of total RNA was reverse transcribed to cDNA for PCR (Applied Biosystems, Grand Island, NY). Primer sets were designed using Premier 5.0 software and synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). The primer pairs were designed to span introns to prevent amplification of any contaminating genomic DNA. The details of these fragments are summarized in Table III. The qRT-PCR was performed on a Light Cycler480 Software Setup (Roche, San Francisco, CA) with Applied Biosystems SybrGreen PCR Master Mix (2 $\times$). Samples (20 μ L final volume) contained 2 $\times$ SybrGreen PCR Master Mix (Roche) and 10 μ M of each primer. Cycling parameters were as follows: an initial step of 3 min at 95°C, followed by 15 s at 95°C, and 40 s at 60°C for 40 cycles. Each sample in each group was measured in triplicate, and each experiment was repeated at least three times. Because the expression of β -actin does not vary with the change of environmental conditions, it is often used as a stable reference gene (Piorkowski et al., 2014; Yin et al., 2014). The quantification was normalized to endogenous control β -actin (levels of this transcript were consistent across all samples). Gene expression was calculated as previously described by Nesan et al. (2012), and procedural blanks were conducted for each treatment.

Statistical Analysis

The experimental results were reported as mean $\pm$ standard deviation ($n = 3$). Outliers were removed from each set of measured values, which were defined as values falling outside the range of mean ± 2 standard deviation. The concentration–response curves to determine EC₅₀ values were computed using Origin 8.0 Software (OriginLab, Northampton, MA). One-way analysis of variance was performed to calculate statistical significance followed by Dunnett's test to independently compare each exposure group with the control group, whereas a Student's *t*-test was used to determine treatment differences at each time point. All statistical analyses were conducted using SPSS 18.0 software (SPSS, Chicago, IL) using a $p < 0.05$ significance level, unless otherwise stated.

RESULTS

Combined Effects of FQs and TCs on Zebrafish Hatching and Mortality Rates

For the 72-hpf FQ-exposure treatments, more than 90% of embryos were hatched with no significant difference between treatment and control groups. In comparison, there was a concentration-dependent decreasing trend in hatching rate for the 72-hpf TC-exposure treatments, and a significant difference ($p < 0.05$) was observed between control and treatment groups (Fig. 2). When embryos were exposed to combined DKAs, the 72-hpf hatching rate decreased with increasing exposure concentrations, whereas the decreasing trend was slightly weaker than that of the TC-exposure group. There were statistically significant differences ($p < 0.01$) between the single TCs (25 mg/L) and the combined DKA exposure (75 mg/L) treatment. It can be inferred that FQs have an inhibiting effect on toxicity of TCs, showing an antagonistic action under the experimental conditions. The inhibiting effects of the combined DKAs were comparable with or slightly less than those of TCs, which could possibly be explained by TCs playing an important toxicologic role in the combined DKAs.

A positive dose–response relationship was also observed in mortality rate for 120-hpf in the DKA-exposure group. The mortality rates under the combined exposure of FQs and

Fig. 2. Effect of exposure concentration on zebrafish hatching rate at 72 hpf ($n = 30$). Significant difference from control at $*p < 0.05$ and $**p < 0.01$. Values represent the mean $\pm$ standard error of three replicates. Same meaning of asterisk mean values in the following figures.

TCs were significantly different from that of the FQ-exposure group, but similar to that of the TC-exposure group. After exposure, the mortality rates for the DKA-exposure treatments < 75 mg/L were close to that of the FQ-exposure treatments. However, the mortality rates at 300 mg/L exposure to DKAs were significantly higher than those of FQs, whereas lower than those of TCs (Fig. 3). However, most notable was that the total dose of the combined DKA exposure group was approximately twice as much as that of the single treatment group (FQs or TCs). Therefore, it can be concluded that there was antagonism between FQs and TCs, and this antagonistic effect became more pronounced with increase in exposure time.

Combined Effects of FQs and TCs on Zebrafish Malformation Rate at 120 hpf

When zebrafish were exposed to FQs, TCs, and mixed DKAs from 6 to 120 hpf, the hatching delay phenomena occurred predominantly in the TC-exposure treatments, whereas uninflated swim bladder and bent spine were often observed in the FQ-exposure treatments (Fig. 4). With an increase in exposure concentration, the malformation rates for single and mixed DKA exposure showed an increasing trend. The EC_{50} value of FQs and TCs for embryos was calculated to be 481.3 and 16.41 mg/L, respectively, whereas that of mixed DKAs was 135.1 mg/L at 120 hpf. Based on the EC_{50} values, three assessment methods were adopted to judge the combined effects: Toxic Unit (M), Additional Index (AI), and Mixture Toxic Index (MTI). The results demonstrated that FQs and TCs mainly had antagonistic actions for deformative effects at 120 hpf (Table IV).

TCs are one of the most toxic groups of DKAs in ecosystems, and the 96-h LC_{50} value of TCs was about 406.0 and 322.8 mg/L to zebrafish and carp, respectively (Zhang et al., 2008). Some toxicity tests showed that TC and chlortetracycline could inhibit the activity of *D. magna*, zebrafish (*D. rerio*), and carp (*C. auratus*). After a 48-h exposure, EC_{50} of TCs to *D. magna* was determined to be 617.2 mg/L, whereas the EC_{50} of chlortetracycline was 137.6 mg/L (Dong et al., 2012). In this investigation, the 120-hpf EC_{50} values for malformation rate of zebrafish was 481.3 mg/L in FQs and 16.41 mg/L in TCs, which suggested that the toxicity of TCs was much higher than that of FQs. Therefore, the main toxicity in the combined DKA-exposed treatments resulted from TCs. In real-world environments, FQs and TCs can not only interact with each other, but also with other chemicals or metals. Zhang et al. (2012b) found that oxytetracycline and ciprofloxacin could bind with three heavy metals to produce a complex comprising various speciations. In antibiotic-metal mixtures, antibiotics, metals, and their complexes primarily present concentration addition effects, and the complex is highly toxic and plays a predominating role in toxicity of the mixture (Zhang et al., 2012b). However, the joint toxicity of spiramycin and amoxicillin to *M. aeruginosa* was found to be a synergistic interaction (Liu et al., 2012). Similar to this study, the interaction between TC and chlortetracycline showed an antagonism in soil on the earthworm *Eisenia fetida*.

Effects of DKAs on Swimming Behavior of Zebrafish

Figure 5 shows the effects of FQ, TC, and mixed DKA exposure on the average swimming speed (ASS) of zebrafish larvae at 120 hpf. For the 25 mg/L FQ treatment, the ASS (2.10 mm/s) was slightly higher than that of the control (1.81 mm/s). However, it sharply decreased to 1.60 mm/s for

Fig. 3. Effect of exposure concentration on zebrafish mortality rate at 120 hpf ($n = 30$).

Fig. 4. Left panel shows the malformation patterns of zebrafish after exposure to β -diketone antibiotic compounds at 120 hpf ($n = 30$). (A), bent spine; (B), hatching delay; (C), normal; (D, E, and F), uninflated swim bladder. Right panel shows the effect of exposure concentration on zebrafish malformation at 120 hpf ($n = 30$ embryos). (a), fluoroquinolones; (b), tetracyclines; and (c), β -diketone antibiotics. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the 50 mg/L FQ treatment, which was significantly different ($p < 0.01$) from that of the control [Fig. 5(A)]. Similar results were found for the TC treatments at 120 hpf [Fig. 5(A)]; however, the TC treatments exhibited no significant differences in swimming speed (control = 1.81 mm/s *versus* treatment = 1.75 mm/s) compared with the control group. For the 18.75 mg/L DKA treatment, the ASS (1.71 mm/s) showed a significant effect ($p < 0.01$) with slower ASS than that of the control. In contrast, the ASS in 4.69 and 9.38 mg/L DKA treatments (1.89 and 1.91 mm/s) were faster than that of the control group.

Larvae without visible developmental malformation were used to evaluate the effect of DKAs on photomotor behavior. As shown in Figure 5(B–D), the rapid transition from light to dark resulted in a similar, brief burst of swimming behavior in both control and treatment groups. The 25 mg/L treatment of FQs had slightly higher basal swimming rate than that of the control group ($p < 0.01$) at 120 hpf in both light and light-to-dark photoperiod experiments. In contrast, a lower basal swimming rate was observed in 50 mg/L FQs ($p < 0.05$). Similarly, the 1.56 mg/L TC and 9.38 mg/L DKA groups had higher basal swimming rate than that of the control group at 120 hpf in both light and light-to-dark photoperiod experiments ($p < 0.05$). Subsequently, a lower

basal swimming rate was observed in the FQ, 6.25 mg/L TC, and 18.75 mg/L mixed DKA treatments as compared with the control. These results demonstrated that zebrafish larvae were sensitive to sudden darkness and DKA exposure stress. These trends for the different treatments were in general agreement with those of locomotor activity assays.

TABLE IV. Assessment of combined toxicologic effects of FQs and TCs on zebrafish embryos

Exposure Pattern	Toxicologic Endpoints		
	72-hpf EC ₅₀ for Hatching Rate	120-hpf LC ₅₀ for Mortality Rate	120-hpf EC ₅₀ for Malformation Rate
FQs	—	—	481.3
TCs	13.8	18.31	16.41
DKAs	130.3	149.8	135.1
Judging methods	M	M_0	AI
	2.933	1.068	−1.933
Judging basis	$M < M_0$	AI < 0	$M_{Ti} < 0$
Results	Antagonism	Antagonism	Antagonism

Fig. 5. The swimming speed of zebrafish larvae exposed to various β -diketone antibiotic concentrations from 6 to 96 hpf when subjected to a 20-min light period (A) or a 50-min dark-to-light photoperiod (B–D) at 120 hpf: (B), fluoroquinolones; (C), tetracyclines; (D), β -diketone antibiotics. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Effects of DKAs on Cardiac Development and HR

Zebrafish can serve as an excellent model for assessing drug-induced cardiotoxicity, although its heart structure differs to some extent from that of mammals (Ali et al., 2011). As can be seen from Figure 6, the HRs for embryos were different among the three observation periods (48, 60, and 72 hpf), and there was an obvious concentration-dependent decreasing trend for HRs. At 48 hpf with exposure to DKAs, the treatment groups 9.38, 18.75, 37.5, 75, and 150 mg/L had 172.6, 163.0, 158.8, 154.1, and 154.1 bpm, respectively, compared with 178.1 bpm in the control group ($p < 0.05$ or $p < 0.01$). At 60 hpf, the HR decreased to 157.5 bpm for the 150 mg/L DKA treatment compared with 170.1 ± 2.4 bpm for the control ($p < 0.05$). At 72 hpf, the HRs decreased from 175.5 ± 2.4 bpm in the control to 140.7 ± 1.2 bpm in the 150 mg/L DKA treatment ($p < 0.01$). Under conditions of no obvious abnormality of cardiac development, the

Fig. 6. Effect of fluoroquinolones, tetracyclines, and β -diketone antibiotics concentrations on 60-s HRs of zebrafish embryos at 48, 60, and 72 hpf, respectively ($n = 30$).

Fig. 7. Transmission electron microscopy images of zebrafish skeletal muscle thin sections. (A–D), severe edema in sarcoplasmic reticulum; (E–H), melted muscle fiber; and (I–L), edema in mitochondria. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

above results demonstrated that the HRs were decreased significantly because of DKA exposure ($\sim 20\%$ at 150 mg/L DKAs).

Histopathologic Analysis for Heart and Skeletal Muscle

As discussed earlier, DKA exposure had a significant effect on swimming behavior and HR of zebrafish. These functional changes resulted from changes in tissue and cell activities or structure. Therefore, 2-month-old zebrafish were selected to investigate their related histopathologic changes after exposure to different DKA concentrations (30, 60, and 90, mg/L) for 2 months. Ultrastructural analysis revealed defects in the sarcomeric assembly characterized by disorganized sarcomere structure with blurry Z-bands and wider gaps between Z-bands in DKA-exposed adult zebrafish when compared with the control. In addition, there were necrotic areas and a large number of edema in the cytoplasm (Fig. 7). We also observed white zones in the longitudinal

muscle fibers. As the muscle fibers melt, part of the mitochondria gathered and was distributed in the muscle fibers in a disordered arrangement. Vacuolization or edema in organelles like mitochondria was present in skeletal muscle cells of the treatment groups. Meanwhile, mitochondria swelling, reduction of mitochondrial cristae, broken cristae with disordered arrangement, and vacuous matrix were observed in treatment groups (Fig. 7).

As for cardiac tissues, myocytes from DKA-exposed hearts displayed prominent interruptions and myofibrillar disorganization of the normal parallel alignment of thick and thin filaments, and the overall distance between Z-bands was greater than that in wild-type specimens. There were also several areas in impaired hearts where the organization and alignment of Z-bands between sarcomeres was not conserved. In addition, some membranes of the cell nuclear tissues were edematous and partially dissolved. The observed disruption of myofibrillar organization with DKA exposure leads to reduced myocyte contractility and cardiac function that may cause death (Fig. 8). In addition, the number of

Fig. 8. Ultrastructural analysis of cardiomyocyte tissues by transmission electron microscopy. (A–D), disordered arrangement of muscle fibers; (E–H), melted muscle fiber; (I–L), partial edematous membrane of cell nuclear materials; (M–P), edema in mitochondria; and (Q–T), abnormal mitochondria. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mitochondria was increased, and some diffuent mitochondria and vacuolization or edema were observed, which formed a large electronic transparent area (Fig. 8).

Changes in Transcriptional Levels of *acta1a*, *myl7*, and *gle1b* Genes

Some genes are closely involved in heart development and formation of skeletal muscle, such as *acta1a*, *myl7*, and *gle1b*. On injecting the mutant *acta1a* mRNA into zebrafish embryos, the zebrafish display muscle weakness as evidenced by their thin bodies and curved tails at 2 days post-fertilization and reduced motility in touch-evoked escape response assay at 5 days postfertilization (Ceyhan and Beggs, 2013). These genes were selected to investigate changes in transcriptional levels under DKA-exposure using qRT-PCR techniques. As shown in Figure 9, the transcriptional levels of the *acta1a*, *myl7*, and *gle1b* genes were significantly changed at 90 mg/L DKAs compared with the control. The expression of *acta1a*, which is related to skeletal muscle, was significantly up-regulated ($p < 0.01$) in the 45 mg/L DKA treatment. However, with further increases in DKA-exposure concentrations, the expression of *acta1a* decreased significantly, i.e., only accounting for ~50% of the control group in the 90 mg/L DKA treatment. These results were consistent with the swimming behavior and histopathologic results provided by TEM.

The expression of *myl7* is related to heart development, and its mutations can lead to nemaline myopathy type 3, similar to that of *acta1a*. When compared with the control group, the level of *myl7* mRNA was first induced at the 45 mg/L DKA-exposure level and was significantly suppressed with further increases in exposure concentration. Especially at 90 mg/L DKAs, the expression level of *myl7* was severely inhibited ($p < 0.01$), only accounting for ~60% of the control group.

Gle1 plays an important role in early zebrafish heart development. The transcriptional levels of *Gle1* in all DKA-exposed groups were low in comparison with the control group, although no statistical difference was observed with exposure to 45 and 60 mg/L DKAs. However, there was a significant difference in the expression of the *Gle1* gene between the 90 mg/L DKA treatment group and the control group ($p < 0.05$).

DISCUSSION

Behavioral analysis often serves as a sensitive tool for detecting sublethal chemical effects (Kane et al., 2004), and motor neurons and muscle fiber are involved in the overall locomotive behavior (Drapeau et al., 1999; Flanagan-Steet et al., 2005; Levin et al., 2009). Zebrafish displayed a bio-rhythm that larvae became active after exposure to sudden darkness and then slowdown, which was consistent with literature reports (MacPhail et al., 2009). Additionally, there

Fig. 9. Relative expression of *acta1a*, *myl7*, and *gle1b* in adult zebrafish exposed to β -diketone antibiotics at concentrations of 0, 45, 60, and 90 mg/L.

were significant differences in response to dark–light stimulation for zebrafish between the DKA-exposed treatment groups and control groups. Deng et al. (2005) reported that FQs can cause both neurotoxicity and muscle toxicity. Based on the behavioral effects and muscle tissue pathologic analysis, we conclude that DKAs had a prominent toxicologic effect on nerve and muscle tissues. However, the exact mechanism(s) for neuro or muscle toxicity are not fully understood; for example, whether the long-term exposure to DKAs at low or sublethal doses can result in hyperactivity and autism of F1 is not known. Therefore, further study is required to elucidate the mechanistic DKA-related behavioral responses in zebrafish larvae.

Gene analysis revealed that development and maturity of the heart depended on the heart function (Glickman and Yelon, 2002), and the change in HR can be considered as a state of functional defect (Milan et al., 2003). The development of the zebrafish heart is a complex process, which involves cell proliferation, migration, differentiation, and the interaction between cells of different origins and also associated with a variety of gene expression and regulation. The heart is the earliest formed functional organ in the process of embryonic development, and the circulation function plays a vital role for normal development of embryos. HR is an important index to measure cardiac function, and tends to be stable after 48 hpf for zebrafish. This study found that DKAs can result in significantly lower HR in zebrafish embryos, presenting a dose-dependent response under the conditions of no obvious abnormalities in cardiac shapes.

The transcriptional levels of two heart development-based genes, *myl7* and *Gle1b*, were significantly decreased under DKA-exposed conditions, suggesting that long-term exposure to DKAs induces a cardiac toxicity to zebrafish. Transcriptome analysis is of crucial importance to understand the molecular basis of embryogenesis and the causes

of developmental defects (Smith and Greenfield, 2003). Gene ontology annotations related to *myl7* include ATPase activity and calcium ion binding. Myosins are a large family of motor proteins that share the common features of ATP hydrolysis, actin binding and potential for kinetic energy transduction (available at: www.genecards.org). The *Gle1* gene shows an interesting characteristic through the perturbation of left–right patterning involving the spaw and ins, thus leading to a defective heart. *Gle1* was first identified as an essential mRNA export factor in both *Saccharomyces cerevisiae* and human cells (Kendirci et al., 2003). In addition to mRNA export, Bolger et al. (2008) recently discovered a novel role for *Gle1* in mediating translation initiation and termination. This work, together with substantial biochemical and cell biological analysis of *Gle1* function, reveals that *Gle1* is a key regulator of multiple post-transcriptional gene expression steps (Folkman et al., 2011). In this investigation, there were no obvious abnormalities of cardiac shape after short-term exposure to DKAs; however, the HR was lower. As for long-term exposure (2-month-old zebrafish exposed to a sublethal dose for 2 months), obvious pathologic changes in heart tissue were observed. Furthermore, the transcriptional levels of *myl7* and *Gle1b* were inhibited prominently when exposed to relatively high concentrations (>60 mg/L). As a consequence, long-term exposure to a sublethal DKA dose might have contributed to the observed cardiac abnormalities.

TEM analysis showed clear muscle fibrous and mitochondria structures in the control group. In DKA treatments, there were sparse mussy muscle fiber and an increased number of mitochondria. Part of the fibers was mussy, diffuent, and disappeared, and also vacuolization or edema in organelles like mitochondria existed both in heart and skeletal muscle. Mitochondria are very efficient energy producers in the presence of oxygen. Although cells have other means of producing energy if oxygen is unavailable, no other process is as efficient as the mitochondrion's method. Because of the critical role of mitochondria in breaking down food sources and efficiently converting these raw materials into energy packets, any flaws in the mitochondria can have a serious impact on organism's health. Fent and Meier (1992) found out that if there was damage in mitochondria caused by chemicals, edema would result because of the obstacle of metabolism or osmotic pressure regulating function. If mitochondria in muscle tissue is too little or of an abnormal form, organisms cannot produce enough energy to maintain the contraction of muscle activity; thus, a weakness of muscles can appear. When zebrafish were exposed to DKAs (60 and 90 mg/L) for 2–4 months, the expression of *acta1a* was significantly decreased in comparison with the control group. Mutations in this gene cause nemaline myopathy type 3, congenital myopathy with excess of thin myofilaments, congenital myopathy with cores, and congenital myopathy with fiber-type disproportion; these conditions lead to muscle fiber defects. Consequently, we conclude that the abnormalities or

damage to the mitochondria resulted in supply obstacles for myocytes and even myocyte injury.

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