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Effects of β-diketone antibiotics on F1-zebrafish (*Danio rerio*) based on high throughput miRNA sequencing under exposure to parents

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HIGHLIGHTS

- Toxicity of DKAs to zebrafish was assessed by miRNA-seq and bioinformatics analyses.
- 193 mature miRNAs were differentially expressed in three comparison groups.
- A potential network was plotted between 11 positive miRNAs and their target genes.
- Expression of miR-124 and -499 in W-ISH was consistent with qRT-PCR and miRNA-seq.
- DKA exposure induced severe histopathological changes in F0-zebrafish ovary tissue.

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ABSTRACT

The toxicity of β-diketone antibiotics (DKAs), a class of "pseudo-persistent" environmental pollutants, to F0-zebrafish (*Danio rerio*) was investigated using 7-dpf F1-zebrafish miRNA sequencing and bioinformatics analyses. Based on relative expression, 47, 134 and 118 of 193 mature miRNAs were differentially expressed between control *vs* 6.25 mg/L, control *vs* 12.5 mg/L and 6.25 *vs* 12.5 mg/L treatments, respectively. Utilizing three databases, 2523 potential target genes were predicted, and they were assigned to 19 high-abundance KEGG pathways and 20 functional categories by COG analysis. Among 11 significantly differential expression and high-abundance miRNAs, the expression levels for 7 miRNAs (miR-144, -124, -499, -125b, -430b, -430c and -152) assessed by qRT-PCR were consistent with those determined by sRNA-seq. A potential network was plotted between 11 miRNAs and their target genes based on differential expression and binding effectiveness. The high degree of connectivity between miRNA-gene pairs suggests that these miRNAs play critical roles in zebrafish development. The expression of miR-124 and miR-499 in whole-mount *in situ* hybridization was in general agreement with those from qRT-PCR and miRNA-seq and were DKA concentration-dependent. DKA exposure induced severe histopathological changes and damage in F0-zebrafish ovary tissue, as reflected by an increased number of early developmental oocytes, irregular cell distribution, decreased yolk granules, cytoplasmic shrinkage, cell lysis in mature oocytes, and dissolution of internal corona radiata. Chronic DKA exposure affected reproduction of F0-zebrafish and development of F1-zebrafish. These observations demonstrate the toxic effect transfer relation across parent and their offspring, and enhance our understanding of drug-induced diseases.

1. Introduction

The β-diketone antibiotic compounds (DKAs), including fluoroquinolones (FQs) and tetracyclines (TCs), are prominent constituents in pharmaceuticals and personal care products. They are widely used in humans and veterinary practice to prevent and treat a large variety of infectious diseases (Alavi et al., 2015). The

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widespread and frequent application of DKAs leads to "pseudo-persistent" in the environment, even though the half-lives for most DKA species are relatively short (~8 h) (Yoon et al., 2010). As a result, DKAs maintain background concentrations in the environment at ng/L to mg/L levels. DKAs originate in the environment from sources such as hospital sewage where large temporal variations in concentrations are observed: 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 (Lindberg et al., 2004). What is noteworthy is the relatively high concentration (up to 0.355 mg/L) of ofloxacin found in hospital and residential effluent in New Mexicol (Brown et al., 2006). Golet et al. (2001) determined concentrations of ciprofloxacin and norfloxacin in real-world environmental waters in the concentration range of 249–405 ng/L and 45–120 ng/L, respectively. Consequently, DKAs pose a potential threat to aquatic organisms and human health due to their prevalence and mixed exposure in the environment.

Long-term exposure to low-doses of DKA mixtures can induce behavioral, biomarker and histopathological changes resulting in the origin of human diseases (Wang et al., 2016a,b). Therefore, the toxicological effects of DKAs have raised great concern in the fields of environmental, ecological and health sciences (Hagenbuch and Pinckney, 2012). With respect to detoxification metabolism, studies found that DKAs affected the activities of cytochrome P450, acetylcholinesterase (AChE) and superoxide dismutase (SOD) (Wang et al., 2014). Some DKAs, such as TCs, were found to be more toxic to aquatic organisms than FQs based on their inherent chemical properties (Ambili et al., 2012). Previous investigations by our group demonstrated that DKAs induced deleterious effects on the zebrafish nervous system and led to abnormal behavior and neurotoxicity (Wang et al., 2016a,b), as well as changes in heart development and skeletal muscle formation (Zhang et al., 2016). Wang et al. (2014) concluded that DKA exposure to F0-zebrafish embryos resulted in a series of developmental toxicity to F1-zebrafish, such as pericardial edema, uninflated swim bladder and yolk sac edema. FQ exposure was also reported to reduce the fertility and cloacal gland area in male Japanese quail (Mohan et al., 2004). Administration of tetracycline caused a reduction in the epididymal sperm motility, percentage of live spermatozoa, sperm count, and an increase in abnormal sperm morphology, as well as induction of adverse histopathological changes in the testes of African catfish (*Clarias gariepinus*) (Farombi et al., 2008). Tetracycline treatment of preovipositional *Otiorhynchus sulcatus* females specifically inhibited egg hatching and influenced reproduction of *Wolbachia*-infected parthenogenetic *O. sulcatus* females. However in *vivo* experiments, changes in metabolic transformation and distribution of DKAs make reproductive toxicity research more complicated (Melvin et al., 2014). Therefore, it is challenging to explore chronic toxicological effects of DKA exposure in studies evaluating reproductive risk.

In recent years, omics technology provides a novel approach for assessing pollutant toxicology in model organisms. MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression by inducing cleavage of their target mRNA or by inhibiting their translation (Krol et al., 2010). Bhattacharya et al. (2016) found that miRNAs act as regulators in most biological processes, and as modulatory factors in developmental processes of zebrafish. Rapamycin exposure to zebrafish embryo resulted in significant suppression of melanocyte development and senescence-associated beta-galactosidase (SA-β-gal) activity, and perturbed the development of intersegmental vessels (ISVs) due to expression changes in zTOR-associated miRNAs (Khor et al., 2016). However, few studies have examined the reproductive toxicological effects from mixed DKA exposure, especially using next generation miRNA-sequencing techniques. Therefore, in this study we screened the significantly differential expression and high-abundance miRNAs based on miRNA-sequencing analyses, which are hidden in the genomic information and regulate target genes at the transcriptional level due to DKA exposure to zebrafish.

Zebrafish (*Danio rerio*) are a preferred model organism in human health risk and environmental toxicology research due to their tiny size, transparent embryo and rapid external embryonic development and high genetic and physiologic homology with humans (Sipes et al., 2011). Therefore, we chose zebrafish as a model organism to investigate the effects of DKA exposure on reproduction of F0-zebrafish and development of F1-zebrafish. This study aimed to screen and identify the significantly differential expression and high-abundance miRNAs from 7-dpf F1-zebrafish by miRNA sequencing after chronic DKA exposure to F0-zebrafish. Next, we systematically analyzed the functions of the screened miRNAs and their regulatory target genes. The functions of genes targeted by 193 mature miRNAs, which were differentially expressed (p-value ≤ 0.05, $\log_2$(fold-change) ≥ 1 or ≤ -1) between control and treatment groups, were elucidated by GO annotation, KEGG pathway analysis and COG protein classification. The expression was verified using qRT-PCR for 11 significantly differential expression and high-abundance miRNAs ($100 \leq$ FPKM ≤ 110000), and their potential target genes and regulatory network were predicted using DIANA miRPath software (v.2.0) (Vlachos et al., 2012). Seven miRNAs were consistent between qRT-PCR and sRNA-seq. Finally, *in situ* hybridization (ISH) was conducted to characterize the spatial expression pattern of miRNAs in F0-zebrafish ovary and whole-mount *in situ* hybridizations (W-ISH) in 7-dpf F1-zebrafish. Histopathological observation on F0-zebrafish ovary was performed to investigate structural changes due to DKA exposure. This study systemically evaluates the reproductive toxicity to zebrafish under the joint exposure of FQ and TC mixtures, and also enhances our understanding of drug-induced diseases.

2. Materials and methods

2.1. Ethics statement

All experimental protocols involving zebrafish followed the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Wenzhou Medical University, Wenzhou, China. All zebrafish surgery was performed on ice to minimize suffering.

2.2. Chemical reagents

Certified DKA reference standards were sourced from Amresco (Solon, OH, USA) and used as received: ofluoxacin (CAS No. 82419-36-1, purity of 99%), ciprofloxacin (85721-33-1, 99%), enrofloxacin (93106-60-6, 99%), doxycycline (24390-14-5, 99%), chlortetracycline (64-72-2, 95%) and oxytetracycline (79-57-2, 99%). The chemical structures and molecular weights of the DKAs are shown in Fig. S1.

2.3. Zebrafish maintenance, embryonic collection and exposure experiment

Adult wild type zebrafish (AB strain) were purchased from a local supplier and adapted to the laboratory with a light/dark, 14 h/10 h cycle in a circulation system with dechlorinated tap water (pH 7.0–7.5) at a constant temperature (28 ± 0.5 °C). Zebrafish were fed twice daily with live *Artemia* (Jiahong Feed Co., Tianjin, China) and dry flake diet (Zeigler, Aquatic Habitats, Apopka FL, USA). Before spawning, male and female zebrafish (1:1) were paired in spawning boxes overnight. Once the light was turned on the following