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Reproductive toxicity of β-diketone antibiotic mixtures to zebrafish (*Danio rerio*)

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ABSTRACT

So far, few data are available on the reproductive toxicological assessment of β-diketone antibiotics (DKAs), a class of ubiquitous pseudo-persistent pollutant, in zebrafish (*Danio rerio*). Herein, we reported the reproductive effects of DKAs by means of transcriptome analysis (F1-zebrafish), changes in a series of reproductive indices (F0-zebrafish) and histopathological observations. A total of 1170, 983 and 1399 genes were found to be differentially expressed when compared control *vs.* 6.25 mg/L, control *vs.* 12.5 mg/L and 6.25 *vs.* 12.5 mg/L DKA-exposure treatments, respectively. Among three comparison groups, 670, 569 and 821 genes were respectively assigned for GO analyses based on matches with sequences of known functions. In 149 KEGG-noted metabolic pathways, the preferential one was the MAPK (mitogen-activated protein kinase) signaling pathway, followed by oxidative phosphorylation, neuroactive ligand-receptor interaction and so on. By qPCR verification, 6 genes (*c6ast4, igfbp1b, mrpl42, tnnc2, emc4 and ddit4*) showed consistent gene expression with those identified by transcriptome sequencing. Due to DKA-exposure, the concentrations of plasma estradiol and testosterone, and the gonado-somatic index were significantly dose-dependently declined. Also, DKA-exposure led to declining in zebrafish reproductive capacity, reflecting in fertilization, hatchability and egg production. Histopathological observations demonstrated that zebrafish ovary and testis suffered serious damage after DKA-exposure. The 4-oxo-TEMP signals increased obviously with increasing DKA-exposed concentrations, implying disruption of balance between generation and clearance of $^1O_2$. In summary, DKAs not only produce reproductive toxicological effects on F0-zebrafish, but also result in adverse consequences for growth and development of F1-zebrafish.

1. Introduction

Fluoroquinolones and tetracyclines are widely used antibiotics in humans and veterinary practice, and they are known as β-diketone antibiotics (DKAs) due to incorporation of a diketone group into their molecular structure (Qu et al., 2010). DKAs can not be fully absorbed by humans and animals resulting in excretion of DKAs and/or their metabolites with feces and urine into waters and other environments (Hirsch et al., 1999). In recent years, DKA residues in concentration levels of μg/L-mg/L were widely detected in aquaculture, poultry farms and sewage treatment facilities (Bueno et al., 2007). Their detected levels varied greatly according to the different matrices, such as in the range of 0.2–101.0 mg/L for ciprofloxacin, ofloxacin, and doxycycline in hospital sewage waters (Lindberg et al., 2004). Several researches, conducted in Beijing and Tianjin suburbs of China, reported that tetracycline concentration even reached as high as 119–307 mg/kg in some agricultural soils irrigated with domestic wastewater and pig feces (Xie et al., 2011). The pseudo-persistence of DKAs and their metabolites at ng/L-mg/L levels in natural waters may induce resistance genes (Baquero et al., 2008), resulting in potentially toxic effects to aquatic organisms and humans.

Long-term low-dose DKA exposure can induce behavioral abnormality, biomarker and histopathological changes, and finally cause a series of human diseases (F.H. Li et al., 2016; J.Y. Li et al., 2016; Zhang et al., 2016). Some DKAs interfere with the endocrine system, such as estrogen activity, immune toxicity, feminization, reproductive disorder, abortion, and cancer of the reproductive organs (Li et al., 2010). Especially, DKAs have strong genetic toxicity, including DNA damage, cross-linking and adduct formation, DNA local hypermethylation of kidney cells in *Carassius auratus*, and teratogenic effects on *Cyprinus carpio* (Khadra et al., 2012). However, most of the previous researches on DKA toxicology focused on acute toxicity of a single species to

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examine effects on biological behavior, morphological development, hatching and survival, angiogenesis, etc (H.L. Wang et al., 2016; X.D. Wang et al., 2016; Hegedus et al., 2009; Alsop et al., 2008). In aquatic environments, the different composition and different concentrations of DKA mixtures may lead to wholly different toxicological action on target organisms (Zhang et al., 2016). Also, the different metabolic transformation and distribution for the different DKA species make the toxicological research more complicated (Melvin et al., 2013). Therefore, the joint toxicological assessment of DKAs is a complex and challenging issue. Our group investigated the joint toxicity of DKAs, including three fluoroquinolones and three tetracyclines to zebrafish, and found that it was comparable with or slightly less than those of tretracyclines, suggesting that tetracyclines played a major toxicological role in the combined DKAs and that the interaction between FQs and TCs was antagonistic effect (Zhang et al., 2016). The EC_{50} values of fluoroquinolones, tetracyclines and DKAs were 481.3, 16.4 and 135.1 mg/L, respectively, based on malformation rates of zebrafish (Zhang et al., 2016). Because ciprofloxacin, ofluoxacin and enrofloxacin, and oxytetracycline, chlortetracycline and doxycycline were frequently detected in aquatic environments, they were chosen as the three representative fluoroquinolones and three representative tetracyclines (equal quality proportion) to conduct the combined DKA reproductive toxicological assessment in this study.

So far few data are available on the reproductive toxicological effects of mixed DKA exposure, especially using modern omics technologies. The advanced omics technologies can acquire the reliable data for systems toxicology researches (Heijne et al., 2005). Zebrafish (*Danio rerio*) is a kind of popular model organisms because of many advantages such as rapid life cycle, transparent development, high fecundity, and their embryos being amenable to genetic manipulation using transgenic approaches and morpholino gene knockdowns (Su et al., 2009). The homology of zebrafish genes with humans reaches as high as 87% (Zou et al., 2009). The complete genome sequence is available for zebrafish providing a valuable resource for high-throughput analyses. Transcriptomics is important for interpreting the functional elements of the genome and for revealing the molecular constituents of cells and tissues. Based on our previous researches, biomarkers, morphological development and biological behavior of zebrafish under mixed DKA exposure were all affected (Zhang et al., 2016). Using next generation RNA-sequencing to analyze the transcriptome in DKA-exposed zebrafish, we found some significant differentially expressed genes functioning in immune and reproductive systems, and oxidoreductase activity (Wang et al., 2014). However, deep insights into the effects of DKAs on zebrafish reproduction were not assessed in our previous studies.

Reactive oxygen species (ROS) in biology are closely related to reproduction processes (Shah et al., 2004), and for example singlet oxygen ($^1O_2$) can affect reproduction because it is closely linked with cell division, body aging and Gonado-somatic index (GSI) (Oikawal et al., 2003). Singh et al. (2014) found that prenatal VEN-exposed young-adult rat offspring showed increased anxiety-like and stereotypic responses. The prenatal VEN-exposure enhanced ROS generation, which affected proliferation, migration and differentiation of cells by regulating release of proapoptotic factors from mitochondria. According to the above-mentioned data, we aimed to explore whether or not chronic DKA-exposure is related to ROS changes and reproductive abnormality, and to further analyze the reproductive toxicological mechanisms.

Based on the above considerations, the purpose of this study was to sequence F1-zebrafish for screening the differentially expressed genes, and analyzing gene annotation and signaling pathways. We used the next generation sequencing platform for high throughput analysis of F1-zebrafish transcriptome after DKA exposure to F0-zebrafish. Parts of the differentially expressed genes, which possessed high abundance, high differential fold and co-difference in three comparison groups, were screened and validated by qRT-PCR. Also, whether or not DKA exposure to zebrafish affected their reproductive capacity was rigorously investigated by a series of reproductive indices such as GSI, plasma sex steroid hormones, fertilization, hatchability, egg production, level of ROS and histopathological observations in the tested F0-zebrafish.

2. Materials and methods

2.1. Ethics statement

The use of zebrafish was approved by the Institutional Animal Care and Use Committee (IACUC) at Wenzhou Medical University, Wenzhou, China. We performed the experiments on the basis of the IACUC guidelines. All dissection was conducted on ice or specimens anesthetized with 0.03% tricaine (buffered MS-222) to decrease suffering.

2.2. Chemicals

Six representative DKAs were used in this study, and their origin and purities were as follows: ciprofloxacin (Amresco, CAS No. 85721-33-1, 99%), ofloxacin (82419-36-1, 99%), oxytetracycline (79-57-2, 99%), chlortetracycline (64–72-2, 95%), enrofloxacin (93106-60-6, 99%), and doxycycline (24390-14-5, 99%).

2.3. Embryo collection and zebrafish maintenance

Wild-type (AB strain) adult zebrafish from Oregon State University (USA) were raised in a circulation system with dechlorinated tap water (pH 7.0–7.5) at a constant temperature (28 ± 0.5 °C). The light photoperiod was 14 h light:10 h dark. Fish were fed twice a day with live *Artemia nauplii*. Before spawning, females were separately housed to optimize egg production. Male and female were paired with a sex ratio of 1:1 in spawning boxes on the afternoon before the spawning date. Spawning was triggered once the light was turned on the following morning. The fertilized and normal embryos were incubated in Petri dishes at 28 ± 0.5 °C until DKA-exposure.

2.4. Exposure experiment

The DKA-exposed concentrations were set at 0 (control), 6.25, 12.5 and 25 mg/L, and the total exposure concentration for the six DKAs was mixed with equal weight concentrations and equal volumes of each DKA species. The selected dosage was chosen according to dosage responses in our preliminary experiments (data not shown). Embryos were collected at 6 h post fertilization (hpf) with embryo medium (EM) for control and DKAs-exposed treatment groups. The embryos were cultivated and exposed until 144 hpf without malformation. Then, the F0-larvae were transferred to 2 L tanks from 6 to 30 days. Subsequently, they were raised in 12 L tanks until the end of the experiment (90 dpf). In case of DKA degradation in the exposure process, all of the DKA-exposed solutions were renewed each day to ensure constant concentration during the experimental period. In order to ensure accuracy and reproducibility, a minimum survival rate should be greater than 95% for the control group.

2.5. Basic information on adult zebrafish and preparation of biological samples

At 90 dpf, the sexually mature zebrafish in control and DKA-exposed groups were randomly selected. Each biological replicate included 9 zebrafish, and thus 27 zebrafish were anesthetized with 0.03% tricaine (buffered MS-222). An electronic balance (AUW320, Shimadzu, Japan) was used to measure zebrafish weight, and subsequently body mass index (BMI) of males and females were calculated in each group. Subsequently, 27 zebrafish were used for RNA-seq (F1-larvae at 7 dpf), qRT-PCR (F1-larvae at 7 dpf), sex index analysis (F0-zebrafish at 90 dpf), hispathological observation (F0-zebrafish at 90 dpf) and ROS detection (F0-zebrafish at 90 dpf). Each index value was the average of