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Toxic effects of diclofenac on life history parameters and the expression of detoxification-related genes in *Daphnia magna*

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ABSTRACT

Diclofenac (DCF), as a widely used drug, has been detected in various environmental media such as municipal wastewater effluent. However, there is little information on the effects of DCF on freshwater invertebrates potentially exposing to its residues in surface water. In the present study, we investigated the toxic effects of DCF on the physiological parameters (e.g., survival, growth rate, and reproduction) of a crustacean, *Daphnia magna*, via a 21-d chronic toxicity test, and we also evaluated the effects of DCF on the expression of the genes related to the detoxification metabolism, growth, development and reproduction (e.g., *HR96*, *P-gp*, *CYP360A8*, *CYP314*, *GST*, *EcR* and *Vtg*) in acute exposure (up to 96 h) with RT-PCR. Significant toxic effects of DCF to *D. magna* were observed at 50 $\mu g\,L^{-1}$, the expression of these selected genes was inhibited with 24 h of exposure, and induced after 48 h to some extents. The expression of *Vtg* was induced at high concentrations of DCF (500 $\mu g\,L^{-1}$ and 5000 $\mu g\,L^{-1}$) after 24 h and 48 h of exposure, but also significantly induced at low concentration (50 $\mu g\,L^{-1}$) after 96 h of exposure. Dose- and time-dependent relationships were observed for gene expression of the seven selected genes. In the 21-d chronic toxicity test, the days to the first brood and the days to the first egg production were both significantly delayed at 50 $\mu g\,L^{-1}$. However, there were no significant differences observed among the molting frequency, number of eggs produced in the first brood, total number of eggs per individual, total number of broods per individual, body length and intrinsic growth rate. Our results suggested that the reproduction parameters are more sensitive endpoints than the survival and growth for evaluating the toxicity of DCF to aquatic invertebrates.

1. Introduction

Presence of pharmaceuticals and personal care products (PPCPs) in the aquatic environment is considered to be one of the biggest environmental concerns in recent years (Jones et al., 2001) attributed to their potential harmful effects to both aquatic organisms and humans via direct and/or indirect exposure (Stackelberg et al., 2004). Many studies found that these contaminants in surface and groundwater have shown a remarkable increase worldwide (Montforts et al., 2007; Spindler et al., 2007). Although extensive studies have been done on the toxic effects of PPCPs on aquatic organisms, the relationship between toxic mechanisms and eco-physiological responses to PPCPs still remains a great challenge in ecotoxicology (Roos et al., 2012).

Diclofenac (DCF) is a classic non-steroidal, anti-inflammatory drug (NSAID) which is widely used in humans and animals. Therefore they were found in different aquatic media. Ternes et al. (1999) reported its presence at concentrations above 1.0 $\mu g\,L^{-1}$ in wastewater treatment plant effluent and lower concentrations in surface water (Ternes et al., 1999). Other studies have reported DCF concentrations ranging from 10 to 2200 $ng\,L^{-1}$ in effluent from wastewater treatment plants in many European countries (Letzel et al., 2009; Stülten et al., 2008). In China, it was reported that the median concentration of DCF was 7.0 $ng\,L^{-1}$ (the Yellow River), 14.5 $ng\,L^{-1}$ (Hai River), 13.4 $ng\,L^{-1}$ (Liao River) and 17.6 $ng\,L^{-1}$ (the Pearl River), respectively (Wang et al., 2010; Zhao et al., 2010). Studies indicated that DCF has the moderate toxic effects on algae, zooplankton and fish. Oaks et al. (2004) demonstrated that DCF was the main cause of decrease of vultures population in Pakistan (Oaks et al., 2004).

Abbreviations: PPCPs, pharmaceuticals and personal care products; NSAID, anti inflammatory drug; DCF, diclofenac; *GST*, glutathione S-transferase; *P-gp*, P-glycoprotein; MFO, mixed-function oxidase; *Vtg*, vitellogenin; *EcR*, ecdysone receptor; ROS, reactive oxygen species.

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Daphnia magna, a freshwater crustacean, has been widely used in toxicity evaluation of chemicals because of its high sensitivity to a wide range of chemicals, a short life cycle, and ease of manipulation in laboratory. In addition, it is ubiquitously distributed among diverse freshwater lakes and ponds, and plays a key role in transfer of energy and nutrients to upper food webs, so it is also an important ecological sentinel species (Baird and Barata, 1998; Soetaert et al., 2006). It was reported that 48-h LC_{50} of DCF for a freshwater crustaceans, *Daphnia magna*, was 67 mg L^{-1} (Quinn et al., 2011). Studies have shown that 25 mg L^{-1} of DCF could inhibit the breeding of *D. magna* under a long-term exposure (Lee et al., 2011; Milan et al., 2013; Schuetz et al., 1996). These studies showed that DCF exposure may have detrimental effects on the growth and reproduction of *D. magna*. However, many questions still remain unresolved, such as the underlying toxic mechanisms of DCF to *D. magna*.

Many environmental pollutants can change the expression of the detoxification- and antioxidant-related genes (Huggett et al., 1992), such as P450, glutathione S-transferase (*GST*), P-glycoprotein (*P-gp*), etc. and subsequently cause physiological changes of the exposed organisms. *HR96*, a putative toxicant receptor in the *NR1J* group of nuclear receptors in invertebrates, is a homologous gene of *NR1I (VDR/CAR/PXR)* group of nuclear receptors in mammals (Bertrand et al., 2004), which can regulate the expression of phase I, II and III detoxification genes (Hernandez et al., 2009; Qatanani and Moore, 2005; Swales and Negishi, 2004). Karimullina et al. observed repression of *HR96* expression induced by chemicals such as triclosan, rostanol and fluoxetine (Karimullina et al., 2012). P450-dependent mixed-function oxidase (MFO) and antioxidant enzyme system play a pivotal role in the detoxification process of xenobiotics. It is reported that *CYP3A*, an important member in P450 family, is involved in various oxidative metabolism of drugs *in vivo* (Yamano et al., 1990). Therefore, the changes in *CYP 3A* subfamily are often used as a biomarker of exposure to xenobiotics. *CYP360*A8, homologous to *CYP3A* subfamily in invertebrate, is expected to respond to the exposure of DCF. *CYP314* is involved in the biosynthesis of ecdysone, a crucial hormone related to the molt of crustaceans. Ecdysone combined with *EcR* can regulate targeted genes involved in development and reproduction of invertebrates (Rewitz and Gilbert, 2008; Rewitz et al., 2007). P-glycoprotein (*P-gp*) is mainly involved in the transport of extra- and endogenous substances across cellular membrane and plays an important role in the process of detoxification of exogenous substances. Antioxidant such as *GST, SOD* and CAT serve as scavengers to protect cells against ROS (reactive oxygen species) insult. There was reported antioxidant enzyme responses including *CAT, SOD, GST* and oxidative tissue damage to UV radiation and varying oxygen concentrations in *Daphnia* spp. (Borgeraas and Hessen, 2000; Borgeraas and Hessen, 2002a,b; Vega and Pizarro, 2000). Vitellogenin (*Vtg*), a precursor of yolk protein, is a major lipoprotein in many oviparous animals. Because the production of vitellogenin is controlled by some estrogen hormones so it is often used as a biomarker for exposure to estrogenic compounds (Jones et al., 2000; Matozzo et al., 2008). Thus, the physiological responses of organisms (e.g., survival, growth rate, and reproduction) may be closely related to the changes in gene expression at molecular level. It is possible that the changes of expression of these detoxification-related genes may provide an early warning before any irreversible damages in physiology occur.

In the present study, *D. magna* was employed as our experimental organism and we evaluated the toxic effects of DCF on the expression of the seven genes (i.e. *HR96*, *P-gp*, *CYP360*A8, *CYP314*, *GST*, *EcR* and *Vtg*) of *D. magna* via an acute toxic test (96 h). Meanwhile, we investigated the physiological changes (e.g., survival, growth rate and reproduction) of *D. magna* through a 21-d chronic toxicity test. The objective of this study is aimed to provide insights into the relationship between changes of growth and reproduction in *D. magna* and its responses of detoxification-related genes under the DCF exposure. The results of this study should improve our understanding of the underlying mechanisms of DCF toxicity on aquatic invertebrates and provide a scientific basis for development of early diagnosis and bio-indicators for PPCPs such as DCF.

2. Materials and methods

2.1. Chemicals and reagents

Diclofenac sodium (purity ≥96%, Beijing), and rifampicin (RIF, Beijing) was obtained from J&K Scientific Ltd. Trizol Reagent (Invitrogen, USA) was obtained from Ruishu, Guangzhou, China. All other reagents were analytical grade and obtained from commercial sources.

2.2. Daphnia magna

Our culture of *D. magna* was originally obtained from Division of Life Science, Hong Kong University of Science and Technology (Kowloon, Hong Kong, China) and maintained under a constant 18-h light/6-h dark cycle and a temperature of 23 ± 1 °C at the Institute of Hydrobiology of Jinan University (Guangzhou, China) for over 6 years. They were cultured with M4 medium (OECD, 2004) and fed with *Scenedesmus obliquus* daily (0.5×10^{-6} cells mL^{-1}) and the medium was renewed twice a week.

2.3. Sample collection and chemical exposure system

Healthy and reproductive *D. magna* were selected to produce neonates (<24 h) for experiments, about 6 h before the toxicity experiments. We chose third or fourth brood neonates (<24 h) because they can be reproduced exclusively by parthenogenesis under this condition.

In the acute toxicity test, exposure was carried out in a 250 mL glass beaker containing 200 mL of DCF solution. Test concentrations were prepared by dissolving diclofenac sodium salt in culture media with 10-fold serial dilution (control, 5, 50, 500 and 5000 μg L^{-1}) for 24 h, 48 h and 96 h. One hundred and eighty individuals of *D. magna* were used per each concentration and each test was performed in triplicate. The range of test concentrations in this study was based on the preliminary experiment and the environmentally relevant concentration of DCF reported previously (Letzel et al., 2009; Stülten et al., 2008).

In the chronic toxicity test, *D. magna* was exposed to five different concentrations of DCF: control, 5 μg L^{-1}, 50 μg L^{-1}, 500 μg L^{-1} and 5000 μg L^{-1} for 21 days. Exposure was carried out in a 50 mL glass beaker containing 20 mL of diclofenac solution, one neonate was placed in each beaker, and eight replicates were set up for each concentration. *D. magna* were fed daily (0.5×10^6 cells mL^{-1} of *S. obliquus*) and the test solution was refreshed daily.

2.4. Total RNA isolation and reverse transcription for quantitative real-time

In each treatment, sixty DCF-treated neonates were transferred into a 2 mL centrifuge tube, rinsed with distilled water and kept on ice immediately. Total RNA was isolated from neonates with Trizol reagent (Invitrogen, USA), following the instructions of the manufacturer. After isolation, the concentrations and purity of the total RNA in the samples were measured using a Scientific NanoDrop2000 (NanoDrop Technologies Wilmington, USA). We then diluted the RNA samples with RNase free H_2O to maintain consistent concentrations. Reverse transcription was performed by using