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Multiple bioanalytical method to reveal developmental biological responses in zebrafish embryos exposed to triclocarban

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HIGHLIGHTS

- Triclocarban (TCC) exposure decreased heart rate, delayed yolk absorption and swim bladder development in zebrafish larvae.
- TCC exposure disrupted thyroid system in zebrafish larvae.
- Low concentration of TCC induced protein dysregulation in zebrafish larvae.

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ABSTRACT

Triclocarban (TCC) is a well-known antibacterial agent that is frequently detected in environmental, wildlife and human samples. The potential toxicological effects and action mechanism of TCC on vertebrate development has remained unclear. In the present study, we analyzed phenotypic alterations, thyroid hormone levels, thyroid hormone responsive genes, and proteomic profiles of zebrafish embryos after exposure to a series of concentrations of TCC from 6 h post-fertilization (hpf) to 120 hpf. The most nonlethal concentration (MNLC), lethal concentration 10% (LC_{10}) and lethal concentration 50% (LC_{50}) of TCC for exposures of 96 h were 133.3 μg/L, 147.5 μg/L and 215.8 μg/L, respectively. Our results showed that exposure to TCC decreased heart rate, delayed yolk absorption and swim bladder development at MNLC and LC_{10}. Exposure to MNLC of TCC inhibited thyroid hormone and altered expression of thyroid hormone responsive genes. Furthermore, exposure to 1/20 MNLC of TCC altered expression of proteins related to binding and metabolism, skeletal muscle development and function, as well as proteins involved in nervous system development and immune response, indicating TCC has potential health risks in wildlife and humans at low concentration level.

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1. Introduction

Triclocarban [TCC; 3-(4-chlorophenyl)-1-(3,4-dichlorophenyl) urea] is an antibacterial active ingredient common in personal care products such as antibacterial soaps and body washes (Halden, 2014). Although TCC was revealed to have potential health risks to human beings and was banned for use in over-the-counter wash products by the U.S. Food and Drug Administration in 2016, it is still used for other consumer products such as toothpaste, fabrics and plastic goods (Halden, 2014; Smith, 2013).

Due to incomplete removal in wastewater treatment plants or direct discharge of wastewater without treatment, TCC has been ubiquitously diffused in aquatic environment via discharge of these personal care products into domestic sewage (Halden and Paull, 2005; Xia et al., 2010). A large number of studies have reported TCC contamination in wastewaters and surface waters at ng/L to even μg/L or mg/kg levels. For example, in the United States, the water streams of intense urbanization and municipal wastewater treatment sites have a higher TCC level of 6.7 μg/L (Halden and Paull, 2005). In China, TCC has been found to be widely distributed in aquatic environments. In the Pearl River system, the detected concentrations ranged from 6.0 to 338 ng/L for TCC (Zhao et al., 2010), whereas, TCC in the Xiaoqing River was estimated to be at a concentration between 32 ng/L and 382 ng/L (Wang et al., 2014). Furthermore, TCC has been detected in pregnancy, suggesting potential health risks **to** expecting mothers and their unborn children (Pycke et al., 2014). Therefore, it is of particular importance to evaluate the effects of TCC pollution in the aquatic environment

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and its threat to aquatic organisms and human health.

Previous studies have demonstrated that TCC is persistent in the environment and bioaccumulates in aquatic animals (Halden, 2014; Halden et al., 2017). This will cause detrimental effects on aquatic organisms. For example, TCC affects reproduction function in fathead minnow (Villeneuve et al., 2017) and causes genotoxicity in fresh water (Gao et al., 2015) at environmentally relevant concentrations. TCC can also interfere with the action of thyroid hormones in cultured frog tadpole tail fin at low concentrations (Hinther et al., 2011). In mammals, TCC has been shown to disrupt androgen systems (Chen et al., 2008; Duleba et al., 2011). *In vitro* study has also shown a weak estrogenic effect of TCC in a reporter gene assay (Ahn et al., 2008; Huang et al., 2014; Tarnow et al., 2013). In addition, a human study has suggested that prenatal TCC exposure is associated with decreased gestational age at birth (Geer et al., 2017).

The embryonic or fetus stage is the critical period of development and is a time of great susceptibility to hazardous effects of chemicals. The developmental origins of health and disease (DOHaD) paradigm indicates exposure to environmental factors during early life could play a role in a latent disease outcome (Gore et al., 2015; Schug et al., 2011). From this perspective, exposure to environmental chemicals in early-life stage can increase proportion of disease burden later in life in the absence of apparent alterations in the normal developmental program. In mammal studies, early life exposure to TCC reduced survival of neonate rats (Kennedy et al., 2015). However, the mechanism of these correlations is still largely unknown. In addition, TCC has been detected in umbilical cord blood at birth and breast milk, raising health risks in developing fetus by prenatal and postpartum exposure (Halden et al., 2017). Therefore, the detrimental effects of early life exposure to TCC urgently need documentation.

In the present study, we use zebrafish embryos as an animal model to evaluate the toxicity of TCC at early-life stage. We aimed to examine the developmental toxicity of TCC by phenotypic changes in exposed zebrafish embryos, to reveal thyroid-disrupting effects of TCC in zebrafish larvae, and to identify protein changes targeted by environmentally relevant concentrations of TCC during development of embryonic zebrafish.

2. Materials and methods

2.1. Chemicals

TCC (CAS number: 101-20-2, 99%) was purchased from Sigma-Aldrich. TCC was dissolved in dimethyl sulfoxide (DMSO) as a stock solution and stored at 4 °C. All other chemicals used were of analytical grade.

2.2. Animals

The wild-type zebrafish (AB strain) stock used in this study originated from the China Zebrafish Resource Center (Wuhan, China). Adult zebrafish were cultured at 28 ± 0.5 °C under a 14 light: 10 h dark cycle in a flow-through system in dechlorinated tap water and fed with brine shrimp (*Artemia nauplii*) twice daily in our laboratory. At 6 h post-fertilization (hpf), embryos were examined under a stereomicroscope and those embryos that had developed normally and reached the blastula stage were selected for subsequent experimentation.

2.3. Experimental exposure I

Based on a preliminary acute toxicity test experiment (data not shown), 30 zebrafish embryos were randomly distributed into a dish as a group and exposed to 100, 125, 150, 175, 200 and 250 μg/L TCC until 120 hpf in triplicate at each treatment condition. The solvent control group received 0.005% DMSO (v/v). During the exposure period, hatching and survival were recorded daily. All tested embryos were cultured at 28 ± 0.5 °C under a 14 light: 10 h dark cycle and fresh solutions were renewed every 24 h. After 96 h exposure, we calculated the most nonlethal concentration (MNLC), lethal concentration 10% (LC_{10}) and lethal concentration 50% (LC_{50}) of TCC.

Then, 30 new zebrafish embryos were randomly distributed into a dish as a group and exposed to 1/10 MNLC, 1/3 MNLC, MNLC and LC_{10} of TCC until 120 hpf in triplicate at each treatment condition. At the exposure endpoint, developmental parameters such as embryo malformation, heat rate, hatching success and mortality were examined under a stereomicroscope.

2.4. Experimental exposure II

Based on the results of Experimental exposure I, 150 zebrafish embryos were randomly distributed into a dish as a group and exposed to 1/20 MNLC, 1/10 MNLC, 1/5 MNLC, 1/3MNLC and MNLC of TCC until 96 hpf in triplicate at each treatment condition. After exposure at 96 hpf, zebrafish larvae were collected, rinsed and frozen in liquid nitrogen. The samples from each dish were divided into two parts: one part for thyroid hormone (TH) analysis while the other for analysis of thyroid system gene expression.

2.5. Experimental exposure III

Based on the results of Experimental exposures I and II, and the high TCC level of 6.7 μg/L in water resource (Halden and Paull, 2005), 200 zebrafish embryos were randomly distributed into a dish as a group and exposed to 1/20 MNLC of TCC until 96 hpf in triplicate at each treatment condition. After exposure at 96 hpf, zebrafish larvae were collected, rinsed and frozen in liquid nitrogen. The samples from each dish were divided into two parts: one part for proteomic analysis and another for gene expression analysis.

2.6. Thyroid hormone (TH) analysis

Whole body TH contents including triiodothyronine (T3) and thyroxine (T4) from zebrafish larvae were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (Uscnlife, China) by following the manufacturer's instructions. Briefly, 200 larvae from each dish were homogenized in 0.3 mL ELISA buffer from the kits. Next, the homogenate was sonicated intermittently on ice for 5 min and then centrifuged at 5000 × g for 10 min at 4 °C. The supernatant was collected and stored at −80 °C for TH assays. The TH levels were measured using a microplate reader (Infinite® M1000 PRO, Tecan, Switzerland).

2.7. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from 30 homogenized zebrafish larvae from each dish (n = 3 per treatment) using TRIzol reagent following the manufacturer's instructions (Sangon, China). The quality and quantity of RNA were evaluated by UV spectrophotometry and by 1.2% agarose gel electrophoresis. First-strand cDNA synthesis was performed using the AMV First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Sangon, China). Real-time PCR with SYBR green detection was performed on the LightCycler® 480 System (Roche, USA) according to protocols established by the manufacturer (LightCycler® 480 SYBR Green I Master, Roche). Melt curve analyses were performed to validate the specificity of the PCR amplicons. The primer sequences were designed

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