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Research Paper

Combined toxicity of triclosan, 2,4-dichlorophenol and 2,4,6-trichlorophenol to zebrafish (*Danio rerio*)

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ABSTRACT

Triclosan (TCS), 2,4,6-trichlorophenol (2,4,6-TCP) and 2,4-dichlorophenol (2,4-DCP) are the most prevalent chlorinated phenolic pollutants in aquatic environments. Our results showed LC_{50} and EC_{50} values of 0.51, 1.11, 2.45 mg/L, and 0.36, 0.74, 1.53 mg/L for TCS, 2,4,6-TCP and 2,4-DCP, respectively, to 120 hpf zebrafish. The highest TCSD (the mixture of TCS, 2,4,6-TCP and 2,4-DCP) toxicity was observed at a TCS:2,4,6-TCP:2,4-DCP concentration ratio of 1:2:4. LC_{50} and EC_{50} values of TCSD mixtures for 120-hpf zebrafish were 2.28 and 1.16 mg/L, respectively. Two toxicity assessment methods (Toxic Unit and Mixture Toxicity Index) indicated that TCSD interactions produced partly additive toxicity. TCSD exposure decreased zebrafish hatching rate and led to a series of malformations. Following alkaline phosphatase staining, a large area of vascular ablation was observed with almost complete disappearance of vascular branches and a smaller coverage range. Prominent reddening of the yolk sac and visceral mass after oil red O staining implied that TCSD exposure severely affected fat metabolism. Following acridine orange staining, cell death occurred in eyes while high TCSD concentrations (0.84 mg/L) induced cardiovascular circulation dysfunction. Alcian blue staining increased the α angle between Meckel's cartilages and β angle between two ceratobranchial. Basihyal and palatoquadrate became shorter and developmental abnormality or defects occurred in the fifth ceratobranchial. Overall, these results provide a theoretical basis for systematically evaluating the combined toxicity of the prevalent chlorinated phenolic pollutants in real-world aquatic environments.

1. Introduction

Triclosan (5-chloro-2-(2,4-dichlorophenoxy) phenol; abbreviated as TCS) is widely used in personal care products such as shampoo, skin cream, soap and toothpaste due to its antimicrobial activity (MacIsaac et al., 2014). Due to its extensive use, TCS is detected in a wide variety of environmental matrices such as wastewaters, surface waters, sediments and even human breast milk and urine (Sanchez-Prado et al., 2006; Dayan, 2007; Moss et al., 2000). Although TCS is a stable and lipophilic compound, it can undergo a series of transformation reactions to produce more toxic and bio-accumulative compounds or derivatives once in the aquatic environment (Morales et al., 2005). For example, 2,8-dichlorodibenzo-*p*-dioxin is known as one of the most carcinogenic chemicals in the world (Wang et al., 2016), as well as 2,4-dichlorophenol (2,4-DCP) and 2,4,6-trichlorophenol (2,4,6-TCP). 2,4-DCP, an abundant member of chlorinated phenolic pollutants, is widely used to synthesize herbicides, insecticides and pharmaceutical intermediates, and thus poses great concern to human and ecological health (Zhang et al., 2014). 2,4,6-TCP is a common chemical intermediate and a by-product of water chlorination and combustion processes, and is considered a priority pollutant of aquatic environments in many countries (Jin et al., 2012). 2,4,6-TCP and 2,4-DCP can be formed through TCS photolysis or chlorinated reaction, and also they are widely used in agriculture and industry such as pesticides, wood preservatives and personal care products. Because of being detected frequently in ground and underground waters, industrial sewage, and drinking water, they are listed as the priority pollutants by China, USA and European Union, and also as carcinogens by International Agency for Research on Cancer (Chen et al., 2012; Gao et al., 2008; Xing et al., 2012; Olaniran and Igbinosa, 2011; USEPA, 1991).

Exposure to TCS in the environment causes several biological concerns, such as genotoxicity, hepatotoxicity, immunotoxicity, neurotoxicity, cardiotoxicity and carcinogenesis effects (Yueh and Tukey, 2016). TCS exposure impaired lipid metabolism (Ho et al., 2016), and

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produced DNA damage in unicellular alga *Closterium ehrenbergii* at 0.25 mg/L in the comet assay (Ciniglia et al., 2005). Moreover, the carcinogenicity of TCS in mice-model has attracted wide attention (Yueh and Tukey, 2016; Yueh et al., 2014; Olaniyan et al., 2016). Yin and coworkers reported that 2,4,6-TCP exposure resulted in elevated point mutations of the *p53* gene in the liver genome, which involved carcinogenesis by inducing point mutations in the somatic genome in zebrafish (Yin et al., 2009). A series of toxicological responses have been demonstrated, such as leukemias lymphomas and liver tumors in mice (Huff, 2012). Furthermore, low 2,4-DCP concentrations resulted in developmental disorders in zebrafish embryos (Sawle et al., 2010), decreasing body weight and food consumption in rats, and increased uterine weights of descendant female weanlings, suggesting that 2,4-DCP has a weak reproductive toxicity (Aoyama et al., 2005).

In recent years, zebrafish (*Danio rerio*) have been widely used as an alternative model in toxicity research due to several inherent advantages, such as their comparable genetic and physiologic make-up with mammals. As its embryos or larvae can absorb small molecules from the surrounding environment through skin and gills, drug-stress experiments are easily conducted (Hill et al., 2005; Kanungo et al., 2014). By 120 hpf, zebrafish develop transparent organs and tissues, which are similar to their mammalian counterparts at the anatomical, physiological and molecular levels. These unique features make zebrafish a promising animal model for developmental toxicity research (Knudsen et al., 2013). For example, zebrafish were successfully used to assess cardiovascular toxicity (Zhu et al., 2014), liver toxicity (He et al., 2013), ototoxicity and neurotoxicity (Ou et al., 2012; Fan et al., 2010). Most drug toxicity and safety evaluations using the zebrafish model obtained accuracy standards of “good” (75%–85%) or “excellent” (> 85%) from ECVAM (European Center for the Validation of Alternative Methods) (Kanungo et al., 2014). Thus, zebrafish are extensively used for screening large numbers of compounds, such as in drug discovery and toxicological studies (Eimon and Rubinstein, 2009).

Previous studies have mainly focused on the toxicity of a single TCS compound to explore its relevant toxicological mechanisms. However, TCS can produce a series of chlorinated derivatives from direct photolysis (Yueh and Tukey, 2016; Wang et al., 2015), which results in the co-existence of TCS and several chlorinated derivatives in aquatic environments and leads to complex and combined toxicity actions. Although single-compound TCS toxicological effects have been reported (Yueh and Tukey, 2016; Ho et al., 2016; Ciniglia et al., 2005), there are no data assessing the toxicity of mixtures of TCS and its derivatives on zebrafish. Therefore, mixtures of TCS, 2,4-DCP and 2,4,6-TCP were chosen to test their combined toxicity on zebrafish. The results of this study provide a theoretical basis for evaluating the ecological risks of exposure to TCS, 2,4-DCP and 2,4,6-TCP in real-world aquatic environments.

2. Materials and methods

2.1. Ethics statement

The Institutional Animal Care and Use Committee (IACUC) at Wenzhou Medical University approved our study plan for proper use of zebrafish. All studies were carried out in strict accordance with the guidelines of the IACUC. All dissection was performed on ice, and all efforts were made to minimize suffering.

2.2. Chemicals, fish husbandry and embryo collection

Triclosan (TCS, purity ≥99.9%), 2,4,6-trichlorophenol (2,4,6-TCP, ≥98%) and 2,4-dichlorophenol (2,4-DCP, ≥99%) were purchased from Sigma-Aldrich (St. Louis, USA); chemical structures are shown in Fig. 1. Acridine orange (Solarbio, Beijing, China), oil red O (Sigma-Aldrich, St. Louis, USA), nitroblue tetrazolium (Roche, Basel, Switzerland), 5-bromo-4-chloro-3-indolylphosphate (Roche, Basel, Switzerland) and alcian blue (Sigma-Aldrich, St. Louis, USA) were obtained from Shanghai Kejian Biology Science and Technology Co., LTD (Shanghai, China). Stock solutions of TCS, 2,4,6-TCP and 2,4-DCP were prepared in acetone and stored at −20 °C. Dosing solutions were made by diluting stock solutions in dechlorinated tap water (pH 6.5–7.5). A series of exposure concentrations were chosen according to LC_{50} values for zebrafish, environmentally relevant concentrations, and preliminary experiments.

An established line of AB strain and Tg (flk1:mCherry) of zebrafish (*Danio rerio*), acquired from China Zebrafish Resource Center (CZRC), was used in this study. They were raised using a light/dark, 14 h/10 h cycle in a circulation system with dechlorinated tap water (pH 6.5–7.5) at a constant temperature (27 ± 0.5 °C) according to standard protocols (Westerfield, 1995). Instant ocean salt was added to the water to raise the conductivity to 450–1000 μS/cm. Zebrafish embryos, used for chemical exposure, were collected from spawning adults in each tank overnight with a sex ratio of 2:2. Eggs were collected after turning on light for 30 min. The fertilized and normal embryos were inspected and staged for subsequent experiments under a stereomicroscope (Kimmel et al., 1995). At 6 hpf, these embryos were distributed into 96-well plates (one embryos per well) for exposure to TCS-2,4,6-TCP-2,4-DCP mixtures as described below. Each treatment included three biological replicates.

2.3. Toxicity assessment for single chemical species

According to preliminary exposure experiments, a series of gradient concentrations of TCS, 2,4,6-TCP or 2,4-DCP were set on the basis of mortality rates in the range of 10–95%. Embryos (n = 96) at 6 hpf were selected under a stereomicroscope. LC_{50} values for zebrafish exposed to TCS, 2,4,6-TCP or 2,4-DCP from 6 to 120 hpf: control (0 mg/L of TCS), 0.21, 0.26, 0.32, 0.40, 0.50 and 0.63 mg/L of TCS; control (0 mg/L of 2,4,6-TCP), 0.84, 1.05, 1.31, 1.64, 2.05, 2.56 mg/L of 2,4,6-TCP; and control (0 mg/L of 2,4-DCP), 0.88, 1.09, 1.37, 1.71, 2.13 and 2.67 mg/L of 2,4-DCP. EC_{50} values for zebrafish exposed to TCS, 2,4,6-TCP or 2,4-DCP from 6 to 120 hpf: control (0 mg/L of TCS), 0.14, 0.17, 0.21, 0.27, 0.33 and 0.42 mg/L of TCS; control (0 mg/L of 2,4,6-TCP), 0.56, 0.70, 0.87, 1.09, 1.37 and 1.71 mg/L of 2,4,6-TCP; and control (0 mg/L of 2,4-DCP), 1.31, 1.64, 2.05, 2.56, 3.20 and 4.00 mg/L of 2,4-DCP. The EC_{50} (median effective concentration) and LC_{50} (median lethal concentration) values were computed by the Boltzmann equation (Eq. (1)):

$$Y = A2 + (A1–A2)/(1 + \exp((X–X0)/dX)) \quad (1)$$

where X is the concentration of a mixture or a given contaminant, and Y is the malformation effect or lethal rate that a mixture or a given contaminant has on the test organism (Zhang et al., 2014). Embryos were kept in the sterilized 96-well plates with one embryo per well containing 200 μL circulating water. The observational indexes included 72-hpf hatching rate (percentage of hatch producing live zebrafish), 120-hpf malformation rate (percentage of malformations in living zebrafish; phenotypic endpoint including any kinds of malformation (pericardial cyst, yolk sac, bent spine, swim sac, sinus venosus)) and 120-hpf mortality rate (percentage of mortality in all zebrafish).

2.4. The compound ratio of TCS-2,4,6-TCP-2,4-DCP (TCSD) and combined toxicity

In order to determine the maximum toxicity concentration ratio of TCS, 2,4,6-TCP and 2,4-DCP, an orthogonal test with five factors and three levels was carried and evaluated using Latin 3.1 software (Sharetop, Shenzhen, China). The average mortality rate of TCSD (n = 3) and concentrations of single chemicals were entered into an orthogonal table, and the optimal ratio among TCSDs was computed. Subsequently, LC_{50} and EC_{50} values were determined on the basis of the