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Time-dependent inhibitory effects of Tris(1, 3-dichloro-2-propyl) phosphate on growth and transcription of genes involved in the GH/IGF axis, but not the HPT axis, in female zebrafish☆

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ABSTRACT

Growth curves were used to determine sensitive exposure windows for evaluation of developmental toxicity of chemicals to zebrafish. Dose- and time-dependent effects on body mass, body length and expression of genes involved in the growth hormone/insulin-like growth factor (GH/IGF) axis and the hypothalamic-pituitary-thyroid (HPT) axis were examined after exposure to environmentally relevant concentrations of tris(1,3-dichloro-2-propyl) phosphate (TDCIPP). Based on growth curves, zebrafish grew most rapidly between 60 and 90 days post fertilization (dpf). Exposure to environmentally relevant concentrations of TDCIPP significantly decreased body mass and body length and down-regulated expression of several genes involved in the GH/IGF axis of female zebrafish, but no such effects were observed in male zebrafish. Exposure to TDCIPP did not change concentrations of thyroid hormones or expression of genes along the HPT axis in female and male zebrafish. These results suggest that growth stages of zebrafish between 60 and 90 dpf might be most appropriate for evaluation of developmental toxicity of chemicals, and down-regulation of genes involved in the GH/IGF axis, but not the HPT axis, might be responsible for the observed growth inhibition in females exposed to TDCIPP.

1. Introduction

Because of persistence, potential for bioaccumulation and biomagnification and toxic potency, some brominated flame retardants (BFRs), such as polybrominated diphenyl ethers (PBDEs), are restricted or are being phased out of use (van der Veen and de Boer, 2012). As a result, production and usage of alternative flame retardants (FRs) are increasing. Organophosphate esters used as flame retardants (OPFRs) are one class of alternative FRs that have been produced in large volumes since the 1970s, and are added to foams, plastics, textiles, varnishes, waxes, floor polishes and electronic equipment (Reemtsma et al., 2008). Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) is one major and environmentally relevant OPFR. According to USEPA's 2012 Chemical Data Reporting, in 2010 and 2011, 4500–22,700 tons/year of TDCIPP were manufactured or imported into the USA (Schreder and La Guardia, 2014). Because it is not chemically bonded into materials, TDCIPP can be easily released into aquatic environments (Kai, 2007). TDCIPP is routinely detected in natural waters (Shi et al., 2015), snow and rainfall (Regnery and Püttmann, 2009) as well as influents and

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effluents from wastewater treatment plants (Meyer and Bester, 2004; Rodil et al., 2005). For example, TDCIPP was observed at a mean concentration of 46.3 (<LOD–855) ng/L in urban surface water in Beijing, China (Shi et al., 2015). Concentrations of 24–377 ng TDCIPP/L were measured in seawaters near the cities of Qingdao and Xiamen, China (Hu et al., 2014). Concentrations as great as 3250 ng TDCIPP/L have been observed in effluents from a wastewater treatment plant in the State of Washington, USA (Schreder and La Guardia, 2014). Furthermore, TDCIPP has been detected in various aquatic wildlife (Hallanger et al., 2015; Mcgoldrick et al., 2014). For example, concentrations of TDCIPP as great as 251 ng/g lipid mass (lm) were measured in grass carp (*Cyprinus idellus*) and catfish (*Clarius fuscus*) from the Pearl River, China (Ma et al., 2013). These data suggest that TDCIPP might pose hazards or risks to exposed wildlife.

Although multiple toxic effects have been reported in various organisms exposed to relatively high concentrations of TDCIPP (Dishaw et al., 2014; Farhat et al., 2013, 2014; Fu et al., 2013; Kojima et al., 2013; Liu et al., 2013, 2016; Volz et al., 2016; Wang et al., 2015a), results of recent studies suggest that inhibition of growth is a primary apical response of both zebrafish and the protozoan *Tetrahymena thermophila* (Li et al., 2015, 2016; Yu et al., 2017; Zhu et al., 2015). For example, exposure of zebrafish to relatively small concentrations of TDCIPP (4, 20, or 100 μg/L) for 6 months resulted in significantly smaller and shorter bodies (Wang et al., 2015a). Exposure of zebrafish embryos/larvae to various concentrations of TDCIPP (50, 100, 300 or 600 μg/L) resulted in lesser body mass (Wang et al., 2013). Long-term exposure to environmentally relevant concentrations of TDCIPP resulted in significantly lesser growth of female zebrafish, relative to unexposed individuals (Yu et al., 2017; Zhu et al., 2015).

In vertebrates, growth is a multi-factorial characteristic resulting from complex genetic and molecular interactions in which growth hormone (GH) plays a major role. In teleost fish, as in other vertebrates, growth is regulated by the growth hormone/insulin-like growth factor (GH/IGF) axis (de Azevedo Figueiredo et al., 2007). Besides GH, thyroid hormones (THs), triiodothyronine (T3) and thyroxine (T4) also play major roles in regulation of growth of vertebrates (Crane et al., 2004). In fish, the thyroid endocrine system is controlled primarily by the hypothalamic–pituitary–thyroid (HPT) axis, which is responsible for maintaining thyroid hormone homeostasis (Blanton and Specker, 2007). Results of previous studies have demonstrated that exposures to TDCIPP inhibit growth and change expression of genes involved in the GH/IGF axis in zebrafish exposed to environmentally relevant concentrations (Yu et al., 2017; Zhu et al., 2015), and expression of genes involved in the HPT axis of zebrafish exposed to relatively high concentrations (Wang et al., 2013). However, it was unknown whether changes in expression of genes involved in the HPT axis due to exposure to TDCIPP are also responsible for inhibition of growth after exposure to environmentally relevant concentrations. Meanwhile, previous studies have focused on a single time point, such as 116 h (Liu et al., 2013), 142 h (Wang et al., 2013), 3 months (Wang et al., 2015a), 4 months (Zhu et al., 2015) or 8 months (Yu et al., 2017), ignoring the fact that toxicity is a time-varying process. This could have led to a bias in assessments of hazard or risk of TDCIPP to aquatic organisms (Baas et al., 2009). Therefore, the objectives of this study were to: (1) establish growth curves of zebrafish and then determine the most appropriate exposure stage and (2) evaluate time-dependent effects of environmentally relevant concentrations of TDCIPP on growth and expression of genes involved in the GH/IGF and HPT axes of zebrafish, and (3) determine which axis is the more likely primary target of TDCIPP that results in inhibition of growth.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and reagents were purchased from the following sources: TDCIPP from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan); TRIzol reagent and reverse transcription and SYBR Green kits from Takara (Dalian, Liaoning, China); Thyroid hormone detection kits from Cloud-Clone Company (Houston, TX, USA); MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt) from Sigma-Aldrich (St. Louis, MO, USA). Other reagents used in this study were of analytical grade.

2.2. Zebrafish maintenance, growth curves and TDCIPP exposure protocols

Zebrafish were maintained in flow-through tanks according to a previously published method (Zhu et al., 2015). Growth curves of zebrafish were established to determine the most appropriate exposure stage. Briefly, zebrafish embryos were collected as described previously (Zhu et al., 2015), and then were cultured in 10-cm glass Petri dishes until larvae could swim (almost 4–5 days post fertilization (dpf)). After that, larvae were transformed into 25-L glass tanks, where each tank contained 50 fish and were fed twice a day with the egg yolk of milled fresh hens. From 10 to 15 dpf, zebrafish larvae were co-fed with *Artemia* nauplii (Tianjin Fengnian Aquaculture Co., Ltd. Tianjin, China) and the egg yolk of milled fresh hens, and unconsumed food in tanks was cleaned every day. From 15 to 180 dpf, *Artemia* nauplii was the only dietary source. During all the culture stages, zebrafish were maintained at 28 °C under a light regime of 12 h light/12 h dark. Body mass and body length of 11 time points (3, 7, 15, 30, 45, 60, 90, 105, 120, 150 and 180 dpf) were recorded in order to establish zebrafish growth curves.

Stock solutions of TDCIPP were prepared in dimethyl sulfoxide (DMSO). Seven-day old zebrafish were acclimated in 25-L glass tanks for 1 week and then exposed to 0, 50, 500 or 5000 ng TDCIPP/L until 120 dpf. Fifty fish were exposed in each of 3 replicate tanks for each concentration. Exposure solutions were replaced daily with fresh carbon-filtered water containing corresponding concentrations of TDCIPP. Both control and treated groups received 0.005% DMSO. This experiment was ran twice in order to guarantee sufficient samples. During the exposure period, fish sampling was performed at 30, 60, 90 and 120 dpf according to the obtained growth curves of zebrafish. At 30 dpf, fish were euthanized with MS-222, thirty fish were randomly selected from 3 replicate tanks (ten fish per tank), and body mass (g) and body length (mm) were recorded. Then, six fish were randomly selected from 3 replicate tanks (two fish per tank), and the whole body of fish was collected for use in real-time PCR reactions. At 60, 90 and 120 dpf, fish were euthanized, thirty fish of each sex were randomly selected from 3 replicate tanks (ten fish each tank), and body mass (g) and body length (mm) were recorded. Then, two fish of each sex per tank were randomly selected, and a total of six fish were dissected. Brain and liver samples were collected for real-time PCR reactions.

2.3. Quantity assurance & quality control (QA&QC) on concentrations of TDCIPP in the exposure system

The QA & QC for quantification of TDCIPP during exposure to zebrafish was performed according to previously reported methods. TDCIPP was quantified in both exposure solutions and tissues of fish by use of a method based on analysis using a Waters ACQUITY UPLC® I-Class system (UHPLC) coupled to Waters® Xevo™ TQ-S mass spectrometer (TQ-S/MS) (Milford, MA, USA) using electrospray ionization (ESI(+)) and operated in multiple reaction