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Developmental neurotoxicity of triphenyl phosphate in zebrafish larvae

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ABSTRACT

Triphenyl phosphate (TPhP), a typical organophosphate ester, is frequently detected in the environment and biota samples. It has been implicated as a neurotoxin as its structure is similar to neurotoxic organophosphate pesticides. The purpose of the present study was to investigate its potential developmental neurotoxicity in fish by using zebrafish larvae as a model. Zebrafish (*Danio rerio*) embryos were exposed to 0.8, 4, 20 and 100 μg/L of TPhP from 2 until 144 h post-fertilization. TPhP was found to have high bioconcentrations in zebrafish larvae after exposure. Further, it significantly reduced locomotor activity as well as the heart rate at the 100 μg/L concentration. TPhP exposure significantly altered the content of the neurotransmitters γ-aminobutyric and histamine. Downregulation of the genes related to central nervous system development (e.g., *α1-tubulin*, *mbp*, *syn2a*, *shha*, and *elavl3*) as well as the corresponding proteins (e.g., α1-tubulin, mbp, and syn2a) was observed, but the gap-43 protein was found to upregulated. Finally, marked inhibition of total acetylcholinesterase activity, which is considered as a biomarker of neurotoxicant exposure, was also observed in the larvae. Our results indicate that exposure to environmentally relevant concentrations of TPhP can affect different parameters related to center nervous system development, and thus contribute to developmental neurotoxicity in early developing zebrafish larvae.

1. Introduction

Triphenyl phosphate (TPhP) is used as a high production volume organophosphate ester (OPE) in polyurethane foam and as a plasticizer in unsaturated polyester resins, polyvinyl chloride polymers, printed circuit boards, photographic films, hydraulic fluids, glues, foam, electronic equipment and casting resins (van der Veen and de Boer, 2012; Wei et al., 2015). Recently, the use of TPhP has increased because the use of other flame retardants such as polybrominated diphenyl ethers has been phased out on account of their persistent property. It is estimated that the production and use of TPhP within Western Europe was 20,000–30,000 tons in 2000, while the production and use of TPhP within the United States alone was 4500–22,700 tons (van der Veen and de Boer, 2012). Although there are no clear data on the production of TPhP in China, more than 100,000 tons of OPEs were produced in 2011 and the usage of OPEs has increased rapidly since then (Hou et al., 2016).

As an additive flame retardant, TPhP easily leaches into the environment (van der Veen and de Boer, 2012): for example, TPhP enters the environment mainly through volatilization of plastics, manufacturing processes, and hydraulic fluid leakages as well as leaching (Lassen and Lokke, 1999). As a consequence, TPhP is one of the most frequently detected OPEs in the environment and biota (Wei et al., 2015). In general, its concentration in surface water is in the range of several to hundreds of nanograms per liter (Andresen et al., 2004; Li et al., 2014). However, concentrations of up to 7.9 μg/L have been reported in river water (Lassen and Lokke, 1999). Moreover, TPhP is hydrophobic (log *Kow* = 4.59) (Van der Veen and de Boer, 2012), and it has relatively high bioconcentration factors (110–300) in fish (Hou et al., 2016). Hence, relatively high concentrations of TPhP have been

Abbreviations: AChE, acetylcholinesterase; BCF, bioconcentration factor; BCF_{lw}, bioconcentration factor on the basis of the lipid weight; BCF_{ww}, bioconcentration factor on the basis of the wet weight; d.w, dry weight; DA, dopamine; Ep, epinephrine; GABA, γ-aminobutyric; Glu, glutamic acid; l.w, lipid weight; OPE, organophosphate ester; TDCIPP, 1,3-dichloro 2-propyl phosphate; TPhP, triphenyl phosphate; w.w, wet weight; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid

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found in fish samples. For example, 21–180 μg/kg lipid weight (l.w) TPhP has been detected in perch (*Perca fluviatilis*) (Sundkvist et al., 2010), while concentrations of up to 350 ng/g l.w have been detected in the coral grouper (Epinephelus corallicola) (Kim et al., 2011) and concentrations of 280 μg/kg l.w have been detected in the lake trout (*Salvelinus namaycush*) (Guo et al., 2017). In China, up to 45.7 μg/kg l.w of TPhP has been reported in muscle samples of catfish (*Claris fuscus*) and carp (*Ctenopharyngodon idellus*) from the Pearl River Delta region of South China (Ma et al., 2013). Further, a recent study has shown that the concentration of TPhP in fish tissue samples was 16.3–85.0 ng/g l.w in several freshwater fish species from the same region (Liu et al., 2018).

A few studies have reported that TPhP may have the potential to disrupt thyroid function in cultured cells and in zebrafish larvae (Kim et al., 2015; Liu et al., 2013a). Disruption of steroidogenesis and impairment of reproductive performance have also been reported in cultured TM3 Leydig cells and in adult zebrafish (Chen et al., 2015; Ding et al., 2017; Liu et al., 2013b). In addition, several studies have shown that exposure to TPhP can induce cardiotoxicity in zebrafish (Du et al., 2015; McGee et al., 2013; Mitchell et al., 2018). Another important concern is its potential neurotoxic effect, as several studies have shown that exposure of zebrafish embryos/larvae to TPhP could result in hypoactive locomotor responses (Jarema et al., 2015; Noyes et al., 2015). Moreover, high concentrations of TPhP have been detected in the brain of zebrafish exposed to it (Wang et al., 2016a,b). This implies that TPhP can cross the blood—brain barrier, and that the brain may be a key target organ for TPhP bioconcentration. With this background information, in the present study, it is hypothesized that exposure to TPhP causes developmental neurotoxicity.

The objectives of the present study were to investigate the developmental neurotoxicity of TPhP by using zebrafish embryos/larvae as a model, as zebrafish in the early developmental stages are typically more sensitive to toxicant stress, particularly the developing brain (Rice and Barone, 2000). Several key genes related to central nervous system (CNS) development and neurotransmitters were measured, as these parameters are regarded as biomarkers of developmental neurotoxicity as well as neurochemical and behavioral changes (Rico et al., 2011). Furthermore, we measured acetylcholinesterase (AChE) activity, since AChE has been widely used as a biomarker of neurotoxicants (e.g., organophosphorus pesticides) in aquatic organisms (Payne et al., 1996).

2. Materials and methods

2.1. Chemicals

Triphenyl phosphate (CAS#115-86-6; > 99% purity), dimethyl sulfoxide (DMSO; CAS 67–68-5; ≥99.5% purity) and methanesulfonate (MS-222) were purchased from Sigma-Aldrich (St. Louis, MO, USA); Triphenyl phosphate-d_{15} (TPhP-d_{15}, 99%) was obtained from Toronto Research Chemicals, Inc. (Toronto, ON, Canada); dopamine (> 98.5% purity), histamine (> 97.0% purity), γ-aminobutyric acid (GABA) (> 99.0% purity), serotonin (> 98.0% purity), glutamic acid (> 99% purity), epinephrine hydrochloride (≥98% purity) and 5-hydroxyindol acetic acid (≥98% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trizol reagent and PrimeScript® reverse-transcription reagent kits were purchased from Takara (Dalian, China). SYBR® Real-time PCR Master Mix-Plus-kits was obtained from Toyobo (Osaka, Japan). All other chemicals used in the present study were analytical or high-performance liquid chromatography grade.

2.2. Zebrafish maintenance and embryos exposure

The culture of adult zebrafish (Wild type, AB strain, four-month-old) and embryos exposure were performed as previously described (Shi et al., 2008). Briefly, 500 embryos that had developed normally and reached the blastula stage (2 h post fertilization, hpf) were randomly distributed into glass beakers containing 500 mL exposure solutions of 0.8 μg/L (2.5 nM), 4 μg/L (12.3 nM), 20 μg/L (61.4 nM) and 100 μg/L (306.7 nM) TPhP. The exposure concentrations were based on environmentally relevant concentrations in surface waters (Lassen and Lokke, 1999). The embryos were exposed until 144 hpf, because most organs have well developed this time. Both the control and exposure groups received 0.01% (v/v) DMSO. There were 4 replicates for each exposure concentration and the exposure solutions were totally renewed daily. The embryos were observed two times daily, and the dead embryos/larvae were removed. At the end of treatment, a subset of the larvae was used for assessing locomotor activity, the remaining were anesthetized with 0.03% MS-222 (300 mg/L) and immediately frozen in liquid nitrogen, and stored at -80°**C** for later analyses. The hatching, heartbeat, malformation, and survival were recorded at 144 hpf. The heartbeat (beats/min) of 10 larvae selected randomly from each exposure group (n = 4) was counted under a stereomicroscope, and morphological deformities were examined. All studies were conducted in accordance with the guidelines for the care and use of laboratory animals of the National Institute for Food and Drug Control of China.

2.3. Quantification of TPhP in exposure solutions and zebrafish larvae

The concentrations in exposure solutions were measured just after (T_0) and before (T_{24}) renewing of exposure solutions. TPhP was extracted and analyzed according to a previous method (Wang et al., 2011). Briefly, the exposure solutions (3 mL) from each replicate (n = 4 replicates) were filtered through 0.7 μm glass-fiber filters (Whatman). TPhP was extracted using a preconditioned solid-phase extraction cartridge (Oasis HLB, 3 mL, 60 mg, Waters, USA) and then, the cartridges were washed with 3 mL water, dried with air for 10 min, and were eluted three times with 3 mL acetonitrile (ACN). The extracts were dried and subsequently diluted to 1 mL with water/ACN (60/40) for instrumental analysis. The TPhP concentrations were analyzed using an ultra-performance liquid chromatography system (UPLC, Waters, USA) equipped with a Waters BEH C8 column (2.1 mm × 50 mm, 1.7 μm). The limits of detection and quantitation for TPhP were 0.3 ng/L and 1 ng/L, respectively, based on 50 mL water samples. The recovery of water samples ranged from 100%–110% (n = 4). As a quality control for the analytical techniques used in this study, 20 ng of TPhP-d_{15} was added to each sample as the internal standard. The calibration was conducted using an internal standard method with 0.1–200 μg/L of TPhP standard prepared in water/ACN (60/40), with 20 μg/L of TPhP-d_{15} internal standard.

Bioconcentration of TPhP in zebrafish larvae was measured according to previous study (Wang et al., 2016a,b). Briefly, 100 zebrafish larvae of each replicate (n = 4 replicates) were weighed after freeze-drying, and spiked with 20 ng of TPhP-d_{15} as internal standard, and then homogenized with 1 mL of ethyl acetate/hexane (1:1 v/v) by homogenizer. The homogenates were ultrasonicated (15 min, 20 °C) and centrifuged at 12,000 × *g* for 10 min, and then the supernatants were collected. This procedure was repeated two additional times for each sample. The total supernatants were transferred to a preconditioned solid-phase extraction cartridge (CNWBOND NH_2, 500 mg, 3 mL), and eluted three times with 3 mL ethyl acetate. Finally, the extracts were evaporated to dryness under the nitrogen stream and redissolved in 1 mL of water/ACN (60/40) for instrumental analysis by UPLC-MS/MS (Waters, USA). The method limits of detection and quantitation for TPhP were 2.7 ng/g and 8 ng/g, respectively, based on 40 mg zebrafish larvae samples. The recovery of zebrafish larvae samples ranged from 87%–100%.

2.4. Locomotor behavior assay

The larvae swimming activity was monitored under 15 min continuous light and response to 30 min dark-to-light transition under the photoperiod stimulation test (cycle of 5 min light followed by 5 min