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Research Paper

Acute exposure to tris (2-butoxyethyl) phosphate (TBOEP) affects growth and development of embryo-larval zebrafish

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ABSTRACT

Tris (2-butoxyethyl) phosphate (TBOEP), is used as a flame retardant worldwide. It is an additive in materials and can be easily discharged into the surrounding environment. There is evidence linking TBOEP exposure to abnormal development and growth in zebrafish embryos/larvae. Here, using zebrafish embryo as a model, we investigated toxicological effects on developing zebrafish (*Danio rerio*) caused by TBOEP at concentrations of 0, 20, 200, 1000, 2000 μg/L starting from 2 h post-fertilization (hpf). Our findings revealed that TBOEP exposure caused developmental toxicity, such as malformation, growth delay and decreased heart rate in zebrafish larvae. Correlation analysis indicated that inhibition of growth was possibly due to down-regulation of expression of genes related to the growth hormone/insulin-like growth factor (GH/IGF) axis. Furthermore, exposure to TBOEP significantly increased thyroxine (T4) and 3,5,3′-triiodothyronine (T3) in whole larvae. In addition, changed expression of genes involved in the hypothalamic-pituitary-thyroid (HPT) axis was observed, indicating that perturbation of HPT axis might be responsible for the developmental damage and growth delay induced by TBOEP. The present study provides a new set of evidence that exposure of embryo-larval zebrafish to TBOEP can cause perturbation of GH/IGF axis and HPT axis, which could result in developmental impairment and growth inhibition.

1. Introduction

Tris (2-butoxyethyl) phosphate (TBOEP) belongs to the family of organophosphate flame retardants (OPFRs), is widely used in plastics, varnish, textiles, furniture and electronic equipment (Marklund et al., 2003). The current worldwide production of TBOEP ranges between 5000 and 6000 tons per years, consequently, the concentration of TBOEP has been increasing during the last decade (Jin et al., 2016). It is an additive in materials and is not chemically bound into final products, thus it can be easily discharged into the surrounding environment (Rodriguez et al., 2006). Studies have demonstrated that TBOEP can be detected everywhere, such as in the air (Quintana et al., 2007; Bergh et al., 2011), natural water (Cristale et al., 2013), soil (Cristale et al., 2013) and sediments (García-López et al., 2009). Monitoring study in Taihu lake has reported that TBOEP was an ubiquitous contaminant of aquatic environment, and concentration of TBOEP in sediment ranged from 1.00 to 5.00 mg/kg dm (dry mass) (Cao et al., 2012). TBOEP, which has a relatively large log Kow (3.75), has potential to be transferred via the food web from low- to high-trophic level organisms (Chen et al., 2012; Campone et al., 2010), therefore, threatening the entire ecosystem and human health.

Although TBOEP is frequently detected in the environment, limited information is available on the thresholds and mechanisms of TBOEP toxicity. A recent study reported that exposure to TBOEP (2–5000 μg/L) from 3 h post-fertilization (hpf) to 120 hpf induced developmental malformations in zebrafish, and the predicted no observed effect concentration (PNOEC) was 2.4 μg/L (Ma et al., 2016). Concentrations of TBOEP observed in aquatic systems are comparable or greater than values of PNOEC. The TBOEP concentration ranged from 5.2 μg/L to 35 μg/L in the influent, and from 3.1 μg/L to 30 μg/L in the effluent of municipal wastewater treatment plants in Sweden (Marklund et al., 2005). It is likely that TBOEP at the current level of occurrence may cause potential adverse effects to aquatic ecosystem and to human health. Recently, cardiotoxicity during the period of zebrafish embryogenesis of TBOEP was reported, and the 96 h median lethal concentration (LC50) value in the fertilized eggs was 3.34 mg/L (Du et al., 2015). Furthermore, TBOEP has been reported to cause disruption of the endocrine system, leading to reproduction impairment and developmental toxicity (Xu et al., 2017; Ma et al., 2016). However, the definite correlation between disruption of endocrine system and

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developmental toxicity induced by TBOEP in embryo-larval zebrafish is lacking.

The growth hormone/insulin-like growth factor (GH/IGF) axis plays a predominate role in the endocrine regulation of fish growth (Reinecke et al., 2005; Reinecke, 2010; Castell et al., 2013). Almost all major physiological processes in fish including the regulation of osmotic balance (Sakamoto et al., 1993), energy homeostasis, immune function (Pérez-Sánchez, 2000), reproduction (Le Gac et al., 1993) as well as primarily growth (Peng and Peter, 1997) are regulated by GH. In addition, thyroid hormones (THs) including tri-iodothyronine (T3) and thyroxine (T4), play an important role in the control of osmoregulation, metabolism, somatic growth, skin pigmentation, development, reproduction, post-hatching metamorphosis and behavior (Scott and Sloman, 2004; Blanton and Specker, 2007; Brar et al., 2010; Schnitzler et al., 2011; Yu et al., 2010). Abnormal function of THs causes malformation, developmental impairments, and cancer in vertebrates (Goldey et al., 1995; Soto and Sonnenschein, 2010; Gilbert et al., 2012). The hypothalamus-pituitary-thyroid (HPT) axis is responsible for maintaining homeostasis of THs by regulating hormone synthesis, secretion, transport and metabolism (Carr and Patiño, 2011; Porazzi et al., 2009). However, there are few studies discussed interference effects of TBOEP on the development in embryo-larval zebrafish from the perspective of the GH/IGF and HPT system.

In this study, we employed zebrafish embryos as a model and they were exposed to various concentrations of TBOEP for 144 h. The adverse effects of TBOEP on embryo-larval stage of zebrafish by monitoring *in vivo* endpoints of survival rate, hatching rate, malformation rate, heart rate and body length was elucidated. Specifically, transcript levels of genes involved in GH/IGF axis, expression levels of genes related to HPT axis, as well as thyroid hormone levels were examined.

2. Material and methods

2.1. Chemicals

TBOEP (CAS NO.: 78-51-3; purity: 94%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of TBOEP was prepared in dimethyl sulfoxide (DMSO, Nanjing Chemical Reagent Co. Ltd, Nanjing, China), and diluted with culture media (60 mg/L instant ocean salts in aerated distilled water) to final concentrations immediately before use. The final concentration of DMSO in test solutions did not exceed 0.01%. TRIzol reagent and reverse transcription and SYBR Green kits were from Takara (Dalian, Liaoning, China). All the reagents used in this study were of analytical grade.

2.2. Maintenance of zebrafish and developmental toxicity test

Zebrafish maintenance and embryo exposure were carried out according to published protocol (Qi et al., 2016). Wild-type adult male and female zebrafish were obtained from zebrafish breeding center in Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China) and were raised at 28 °C in a 14-h light/10-h dark cycle in a closed flow-through system. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Huazhong Agricultural University for laboratory animal use. Fertilized eggs from natural crosses were collected and examined under the stereomicroscope at 2 hpf. Embryos developing normally at blastula stage were selected for the subsequent experiments. The embryos (approximately 400) were randomly distributed into 1000 ml glass beakers containing 500 ml TBOEP exposure solution (0, 20, 200, 1000, or 2000 μg/L, which are equivalent to 0, 0.6, 6, 30 and 60% of the 96 h EC50 value). Each concentration included six replicates (beakers). During semi-static exposure, solutions were replaced for every 24 h with fresh carbon-filtered water containing corresponding concentrations of TBOEP.

After 96 h lasting exposure, the mortalities of embryos were recorded for the 96 h LC50 calculation. Malformations like coagulation of embryo, lack of somite formation, tail detachment and heart beat are considered lethal (OECD, 2013). The hatching rate of embryos and heart rate of larvae at 96 hpf were calculated, and the larvae developmental parameters for body length, survival rate and malformation rate at 144 hpf were recorded. Ten zebrafish larvae were randomly selected from each beaker as one replicate for body length measurement. Each treatment group has six biological replicates. The length of each larva along the body axis from the anterior-most part of the head to the tip of the tail was measured with digital images produced using the Image Pro Plus software (Leica, Germany).

2.3. Quantitative real-time polymerase chain reaction assay

Thirty larvae from each beaker were pooled as one replicate at termination of the experiment and preserved in Trizol reagent at −80 °C. Total RNA was isolated using Trizol reagent and digested with RNase-free DNaseI (Promega) following the manufacturer's instructions. Concentrations of total RNA were estimated by spectrophotometric analysis at 260 nm. The purity of the RNA in each sample was verified by determining the A260/A280 ratio and by confirming the purity of 1.0 μg RNA using 1% agarose-formaldehyde gel electrophoresis with ethidium bromide staining. The purified RNA was used immediately for reverse transcription (RT) or stored at −80 °C until analysis. Synthesis of first-strand cDNA was performed using a PrimeScript® RT Reagent Kit (TaKaRa) following the manufacturer's instructions. Quantitative real-time PCR (q-RT-PCR) was performed on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The 20 μl reaction mixture contained 10 μl iQ™ SYBR® Green Supermix (Bio-Rad Laboratories), 1 μl of each of the specific primers at a concentration of 0.25 μmol/L, 7.8 μl of Rnase-free water, and 0.2 μl of cDNA template. The primer sequences of the selected genes were obtained using the online Primer 3 program (http://frodo.wi.mit.edu/) and are listed in Table S1. The thermal cycle was set at 95 °C for 2 min; this was followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 1 min and a final cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The *rpl8* gene was used as internal control, because mRNA expression of this gene was not affected by TBOEP exposure in the present study (data not shown). The amplification efficiencies of the target genes and *rpl8* were approximately equal, ranged from 95.6% to 105.3% (Table S1). The mRNA expression level of each target gene was normalized to the content of the reference gene, and changes of the relevant genes were analyzed by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Six biological replicates and two technical replicates were used in the q-RT-PCR analysis.

2.4. Extraction and analysis of thyroid hormone

The method for extraction of whole-body thyroid hormone was modified from previous study (Yu et al., 2010). The samples of 200 zebrafish larvae from the same beaker were pooled as one replicate, and each treatment group has six biological replicates and two technical replicates. Briefly, the larvae were homogenized in 0.3 ml ELISA buffer using an Ultra-Turrax T8 basic homogenizer (IKA, Staufen, Germany). Then samples were disrupted by intermittent sonic oscillation for 15 min on ice. Next, the samples were centrifuged at 5000 × *g* for 10 min at 4 °C. The supernatants were collected and stored at −80 °C for T3 and T4 assay following the manufacturer's instruction by using commercial kit, which was purchased from Cayman Chemical Company (Ann Arbor, MI). The detection limits of T4 and T3 were 3 and 0.12 ng/mL, respectively.

2.5. Statistical analysis

All statistical analyses were performed using SPSS 19.0 (SPSS, Chicago, IL). The normality of the data and the homogeneity of variances were analyzed with the Kolmogorov-Smirnov test and Levene's