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Effects of environmentally relevant concentrations of tris (2-butoxyethyl) phosphate on growth and transcription of genes involved in the GH/IGF and HPT axes in zebrafish (*Danio rerio*)

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HIGHLIGHTS

- Growth of zebrafish was inhibited by environmentally relevant levels of TBOEP.
- TBOEP had no persistent effect on concentrations of THs.
- Bioaccumulation of TBOEP contributed to the growth inhibition.
- Disruption of GH/IGF axis was induced by environmentally relevant levels of TBOEP.

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ABSTRACT

Tris (2-butoxyethyl) phosphate (TBOEP), as one of the most widely used organophosphate flame retardants (OPFRs), is applied in nearly all manufactured items and materials. It has been reported that TBOEP could cause developmental impairments and disrupt the endocrine regulation of fish growth during acute toxic experiments. However, concentrations to which fish were exposed in these studies were greater than environmentally relevant concentrations ever reported. This study examined effects on growth associated with exposure of zebrafish to 0, 0.1, 1 and 10 μg/L TBOEP during 20–90 days post fertilization (dpf). The changes in growth indicators and bioaccumulation of TBOEP were examined along with the transcription of related genes in the growth hormone/insulin-like growth factor (GH/IGF) axis and the hypothalamic-pituitary-thyroid (HPT) axis. The average body contents of TBOEP were higher in females than in males in all the exposure groups. Exposure to environmentally relevant concentrations of TBOEP significantly decreased body length and body mass and down-regulated expression of several genes involved in the GH/IGF and HPT axes. Exposure to TBOEP decreased plasma thyroxine (T4) content accompanied by decreased mRNA level of thyrotropin β-subunit (*tshβ*) in females at 60 dpf, but no effects were observed at 90 dpf. These results suggested that bioaccumulation of TBOEP and down-regulation of genes involved in the GH/IGF axis might be responsible for the observed growth inhibition in zebrafish exposed to TBOEP.

1. Introduction

Owing to phasing out of main commercial polybrominated diphenyl ethers (PBDEs) mixtures, the worldwide consumption of organophosphate flame retardants (OPFRs) has been increasing over the past ten years (Reemtsma et al., 2008; Stapleton et al., 2009). Tris (2-butoxyethyl) phosphate (TBOEP) is one of the most common OPFRs, with an estimated global output ranging from 5000 to 6000 tons per year (van der Veen and de Boer, 2012). Lots of TBOEP can enter surrounding environment, because TBOEP is not

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covalently bound to polymeric substrates, (Sundkvist et al., 2010). Furthermore, TBOEP is a semi-volatile compound and possesses high water solubility (1.20×10^3 mg/L) (Wei et al., 2015). It has been frequently detected in aquatic environments in many countries, including the United States (Benotti et al., 2009), Italy (Bacaloni et al., 2008), Spain (Gorga et al., 2015), and China (Zeng et al., 2014; Wang et al., 2015). In water samples from Iberian rivers in Spain, TBEOP concentrations ranged from 5.3 to 659 ng/L (Gorga et al., 2015). TBOEP was one of the predominant OPFRs detected in surface water samples collected in Pearl River Delta of China, with a mean concentration of 200 ng/L (Zhang et al., 2018). TBOEP was detected even in drinking water from Nanjing of China, with a mean concentration of 70.1 ng/L (range: 24.1–151.0 ng/L) (Li et al., 2014). In addition, TBOEP has a relatively large low Kow (3.75), which could be transferred via food web from low-to high-trophic level organisms, as evidenced by its frequent detection in various aquatic species of fish (Chen et al., 2012; Campone et al., 2010). Levels of TBOEP ranged from 86.0 to 98.4 ng/g dry weight in mullet fish (Álvarez-Muñoz et al., 2015), and from 0.07 to 3.50 ng/g wet weight in Lake trout (McGoldrick et al., 2014). Therefore, a comprehensive understanding of the toxic effects of TBOEP on aquatic organism is required.

Previous toxicological information suggested that exposure to TBOEP has the potential to disrupt the endocrine system, and impair developmental, reproductive, and neurological functions (Jiang et al., 2018; Xu et al., 2017; Ma et al., 2016). For example, a high concentration of TBOEP was shown to be related to malformation, growth delay, decreased heart rate in zebrafish larvae (Liu et al., 2017). Exposure of zebrafish to relatively small concentrations (2.1, 11, and 118 μg/L) for 21 d significantly affected the reproduction of zebrafish and development of progeny generations (Kwon et al., 2016). However, concentrations to which fish were exposed in these studies were greater than environmentally relevant concentrations ever reported, and thus could not provide reliable thresholds for effects that could be used in assessments or risk. Therefore, in-depth studies are needed to assess the potential toxicological effects of long-term exposure to environmentally relevant concentrations of TBOEP.

In teleost fish, growth is a multi-factorial characteristic coming out of complex genetic and molecular interactions (de Azevedo Figueiredo et al., 2007). The growth hormone/insulin-like growth factor (GH/IGF) axis is known to play a predominate role in the endocrine regulation of fish growth (Reinecke et al., 2005; Reinecke, 2010; Castell et al., 2013). Besides GH, thyroid hormones (THs) including tri-iodothyronine (T3) and thyroxine (T4) also have important effects on regulation of growth in vertebrates (Crane et al., 2004). Hypothalamus-pituitary-thyroid (HPT) axis is essential for homeostasis of THs because it regulates hormone synthesis, secretion, transport and metabolism (Porazzi et al., 2009). Results of our previous study have demonstrated that exposures to relatively high concentrations of TBOEP change expression of genes involved in the GH/IGF and HPT axes in zebrafish (Liu et al., 2017). However, it was unknown whether changes in expression of genes involved in GH/IGF and HPT axes due to exposure to TBOEP are also responsible for effect on growth after exposure to environmentally relevant concentrations. Meanwhile, previous studies have focused on a single time point, such as 120 post-fertilization (hpf) (Ma et al., 2016) and 144 hpf (Liu et al., 2017), ignoring the time-varying characteristic of toxicity. This could lead to a bias in evaluation of hazard or risk of TBOEP to aquatic organisms.

In the present study, time-varying effects on growth were examined after long-term, water-borne exposures of zebrafish to environmentally relevant concentrations of TBOEP. Specifically, transcript levels of genes involved in GH/IGF and HPT axes were examined.

2. Material and methods

2.1. Chemicals and reagents

TBOEP (purity: 94%, CAS Number: 78-51-3) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solution of TBOEP was prepared in dimethyl sulfoxide (DMSO, Nanjing Chemical Reagent Co., Nanjing, China), stored at −20 °C 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 0.1%. TRIzol reagent and reverse transcription and SYBR Green kits were obtained from Takara (Dalian, Liaoning, China). Thyroid hormone detection kits were purchased from Cloud-Clone Company (Houston, TX, USA). Other reagents used in this study were of analytical grade.

2.2. Zebrafish maintenance and treatment protocol

Zebrafish maintenance and embryo collection were performed according to the protocol described by Zhu et al. (2017). Briefly, zebrafish embryos were collected and cultured in 10-cm glass Petri dishes until larvae could swim (about 4–5 days post fertilization (dpf)). 100 embryos were put into each dish contained 30 mL culture media. The survival rates and hatching rates in each dish were greater than 90% in our experiment, which could confirm that the density of the embryo in per dish not affects embryonic development. After that, larvae were transformed into 25-L glass tanks, where each tank contained 50 fish and were fed twice a day with 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. After 15 dpf, *Artemia nauplii* was the only dietary source. During all the culture stages, zebrafish were cultured in a closed flow-through system with charcoal-filtered tap water at 28 ± 1 °C in a 14-h light/10-h dark cycle.

Ten-day old zebrafish were acclimated in 25-L glass tanks for ten days and then exposed to 0, 0.1, 1 and 10 μg/L TBOEP (equivalent to 0, 0.26, 2.6 and 26 nM TBOEP), respectively. Given to the detected concentrations of TBOEP in water samples ranged from 5.3 to 659 ng/L (Gorga et al., 2015), the exposure concentrations used in this study were environmental relevant concentrations. Fifteen fish were exposed in each of 6 replicate tanks for each concentration. Exposure solutions were replaced every 2 day with fresh carbon-filtered water containing corresponding concentrations of TBOEP. During the exposure period, fish sampling was performed at 30, 60 and 90 dpf. At 30 dpf, fish were anesthetized with a 0.02% tricaine methanesulfonate (MS-222), thirty fish were randomly selected from 6 replicate tanks (five fish per tank), and body mass (g) and body length (mm) were recorded. Then, six fish were randomly selected from 6 replicate tanks (one fish per tank), and the whole body of fish was collected for use in real-time PCR reactions. At 60 and 90 dpf, fish were anesthetized, thirty fish of each sex were randomly selected from 6 replicate tanks, and body mass (g) and body length (mm) were recorded. Then one 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. Quantification of TBOEP in zebrafish

Eighteen zebrafish (3 per replicate) per treatment were sampled at 90 dpf for analysis of TBOEP. TBOEP concentrations in biotic samples were determined using a Waters ACQUITY UPLC system