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# Tris (2-butoxyethyl) phosphate affects motor behavior and axonal growth in zebrafish (*Danio rerio*) larvae

Fan Jiang[a,1], Jue Liu[b,1], Xinyue Zeng[a], Liqin Yu[a], Chunsheng Liu[a], Jianghua Wang[a,*]

[a] College of Fisheries, Huazhong Agricultural University, Wuhan 430070, China
[b] Department of Pharmacy, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430014, China

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ABSTRACT

Tris (2-butoxyethyl) phosphate (TBOEP) is an environmental contaminant that poses serious risks to aquatic organisms and their associated ecosystem. Recently, the reproductive and developmental toxicology of TBOEP has been reported. However, fewer studies have assessed the neurotoxic effects in zebrafish (*Danio rerio*) larvae. In this study, zebrafish embryos were subjected to waterborne exposure of TBOEP at 0, 50, 500, 1500 and 2500 μg/L from 2 to 144-h post-fertilization (hpf). Behavioral measurements showed that TBOEP exposure reduced embryonic spontaneous movement and decreased swimming speed of larvae in response to dark stimulation. In accordance with these motor effects, TBOEP treatment reduced neuron-specific GFP expression in transgenic *Tg* (*HuC*-GFP) zebrafish larvae and inhibited the growth of secondary motoneurons, as well as decreased expression of marker genes related to central nervous system development in TBOEP treated group. Furthermore, increased concentrations of reactive oxygen species (ROS) and malondialdehyde (MDA), as well as reduction of SOD activity were detected in TBOEP exposure group. The present results showed that the alteration in motor neuron and oxidative stress could together lead to the motor behavior alterations induced by TBOEP.

## 1. Introduction

Tris (2-butoxyethyl) phosphate (TBOEP) has been used as an organophosphate flame retardant (OPFR) during the past decades in various products, with an estimated global production ranging from 5000 to 6000 tons per year (van der Veen and de Boer, 2012; WHO, 1998). These additives are not covalently bound to the end-product and can easily diffuse into the surrounding environment by volatilization, leaching and/or abrasion (Sundkvist et al., 2010). TBOEP has been found to be the most abundant OPFR measured in effluent, surface and ground water as well as drinking water samples due to its water solubility (1.2 g/L) and low removal rate from wastewaters treated with conventional methods (Marklund et al., 2005; Andresen et al., 2004; Cristale et al., 2013; Loos et al., 2013; Martínezcarballo et al., 2007). Moreover, the presence of TBOEP in mothers' milk and placenta along with its high octanol-water coefficient ($\log K_{ow}$ = 3.75) indicating lipophilicity demonstrates its potential for bioaccumulation (van der Veen and de Boer, 2012). It has been reported that concentrations of TBOEP ranged 0.07–3.50 ng/g wet mass (wm) in Lake Trout (*Salvelinus namaycush*) (McGoldrick et al., 2014), and 8.10–13.40 ng/g wm in herring gull (*Larus argentatus*) (Greaves and Letcher, 2014).

The growing ubiquity of TBOEP in water ecosystems increases the urgency to continually address the knowledge about its risk to the aquatic organisms. It has been reported that exposure of zebrafish (*Danio rerio)* embryos to different concentrations of TBOEP, ranging from 0.8 to 100 mg/L produced a concentration- and time-dependent decrease in embryonic survival and percent hatching (Han et al., 2014). And the toxicity of TBOEP in the developing zebrafish may result from inhibiting the degradation and utilization of nutrients from the mother and inducing apoptosis. Furthermore, TBOEP has shown to affect development of zebrafish embryos by inducing malformations, ultimately resulting in death (Ma et al., 2016). These developmental effects were also observed concurrently with alterations to endocrine functions, by potentially causing perturbation of GH/IGF and HPT axis (Liu et al., 2017), altering cortisol homeostasis, and sex-hormone homeostasis (Ma et al., 2016). In addition, our previous study showed that TBOEP could alter serum 17β-estradiol and testosterone levels which led to a decrease in egg production, hatching success, and survival rates while also retarding oocyte maturation and spermiation in zebrafish (Xu et al., 2017).

Exposure of many environmental contaminants during the early developmental stages has been associated with adverse health effects

* Corresponding author.
*E-mail address:* whtjwjh@163.com (J. Wang).
[1] These authors contributed equally to this work.

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throughout the life span (Heindel and Vandenberg, 2015; Linares et al., 2015). The developing brain is especially sensitive to perturbation, and even very low levels of chemical exposure may result in persistent impairments in cognitive functions and other types of behavior (Grandjean and Landrigan, 2006, 2014). Behavioral anomalies can also happen without any obvious morphological deformations or decrease in survival rates, thus quantifiable behavioral changes have proved to be a sensitive supplemental endpoint for traditional toxicological studies (Little and Finger, 1990). Exposure to the tested TBOEP for 96 h resulted in hypoactivity for zebrafish larvae (Sun et al., 2016b) in both the free-swimming and the dark-to-light photoperiod stimulation test. In Japanese medaka (*Oryzias latipes*), TBOEP has been confirmed to interfere with neurobehavioral development (Sun et al., 2016a). Changes of acetylcholinesterase activity and transcriptional responses of genes related to the nervous system likely provide a reasonable explanation for the neurobehavioral disruption. However, the specific alterations in neurodevelopmental morphology in the brain and mechanisms underlying these alterations are not yet completely understood.

Zebrafish has been considered to be a reliable, sensitive and economic model for assessments of the developmental neurobehavioral toxicity of various chemicals (Bailey et al., 2013). Transient spontaneous movement and the ability to swim were determined to be two major endpoints for quantitative behavioral study (Saint-Amant and Drapeau, 1998). In order to confirm previous studies (Sun et al., 2016a, 2016b), and serve as a basis of study for investigating the underlying mechanisms, we also evaluated the effects of TBOEP on neuronal development, axonal growth of motor neurons as well as expressions of genes related to axonal growth, and oxidative stress, which may help to elucidate the neurobehavioral alterations.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Tris(2-butoxyethyl) phosphate (TBOEP, purity: 94%, CAS Number: 78-51-3), TBOEP was purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solution of TBOEP was prepared in dimethyl sulfoxide (DMSO, Nanjing Chemical Reagent Co., Ltd, Nanjing, China), stored at −20 °C and diluted with embryo rearing water (60 mg $L^{-1}$ instant ocean salts in aerated distilled water) to final concentrations immediately before use. The final concentration of solvent (DMSO) in test solutions did not exceed 0.1%. Early study has demonstrated that 0.1% DMSO exposure, just like blank control, couldn't cause any development defect to zebrafish embryos (Han et al., 2014; Hallare et al., 2006). The TRIzol reagent, PrimeScript Reverse Transcription (RT) Reagent kits and SYBR Green kits were obtained from TaKaRa (TaKaRa, Dalian, China). All the other reagents used in our research were of analytical grade.

### 2.2. Maintenance of zebrafish and embryo toxicity test

Adult zebrafish (*Danio rerio*; AB strain) maintenance and embryo exposure were performed according to our previous study (Liu et al., 2017). Based on the maximum no observed effect concentration (NOEC) with larvae (50 μg/L) and the reported 96-h LC50 of TBOEP (3489 μg/) (Liu et al., 2017), a gradient nominal concentrations were chosen (50, 500, 1500 and 2500 μg/L, which are equivalent to 0.13, 1.3, 3.9, and 6.5 μM, respectively). Briefly, normally developed zebrafish embryos that reached blastula stage (2 h post fertilization; hpf) were selected and randomly distributed among 200 mL glass beakers (150 embryos per beaker) containing different nominal concentrations of TBOEP. Both control and treated groups received 0.1% (v/v) DMSO. Each concentration included four replicates (beakers). During semi-static exposure, solutions were replaced for every 24 h with fresh carbon filtered water containing corresponding concentrations of TBOEP. A total of four replicates were used for each treatment, and the embryos were raised at 28 °C ± 1 °C in a 14-h light/10-h dark cycle. After behavior analysis at 144 hpf after exposure, larvae were sampled, frozen with liquid nitrogen immediately, and stored at −80 °C until subsequent analysis. Acute endpoints, including mortality, hatching, and malformation were recorded. The body length was determined by measuring from the anteriormost part of the head until the tip of the tail along the body axis (10 larvae/replicate) with Image Pro Plus software (Media Cybernetics).

### 2.3. Locomotor behavior measurement of zebrafish larvae

The spontaneous movement test was videotaped via a CCD camera mounted to a dissection microscope (Leica, Germany), as depicted in our previous study (Chen et al., 2017). Embryos were exposed to TBOEP in 6-well plate beginning at 2 hpf. Starting from 20 hpf, alternating tail coiling or bending was recorded with the videotape as spontaneous movement for 1 min every two hours until 28 hpf. All recordings on the recording station started after 5 min adaptation. For each treatment group, a total of 30 embryos from three replicates were used for analysis.

Larval locomotor activity at 144 hpf was quantified according to the previous study (Wu et al., 2016) using a Video-Track system (ViewPoint Life Sciences, Inc., Montreal, Canada). Larvae that were dead or showed deformities were excluded from the assay. The swimming speed in response to dark and light transition (5 min dark and 5 min light) was monitored. Data on distance traveled, movement frequency, and movement duration were recorded every 30 s, and each assay was repeated 4 times. Data were analyzed with Videotrack V3 software (ViewPoint Life Sciences, Inc., Montreal, Canada). The net speed changes in response to transitions between light and dark time periods were recorded as the differences between the average swimming speeds at the end of one light state (for instance, the light period) and the beginning of the following light states (for instance, the dark period).

### 2.4. Whole-mount immunohistochemistry

At 144 hpf, the larvae were sampled and anesthetized in 0.03% tricaine methanesulfonate (MS-222), and then were fixed in 4% paraformaldehyde. Well characterized antibodies were used to perform the assay of whole-mount immunohistochemistry in both control and TBOEP-exposed larvae to visualize subsets of neurons as well as their axons based on previously described methods (Chen et al., 2012a,b). Secondary motor neurons (SMNs) were immunolabeled with zn-5 (1:200, Zebrafish International Resource Center, University of Oregon). Samples were imaged with an inverted fluorescence microscope (Leica DMI 6000B, Leica Microsystems, Germany). Axon length was quantified with image J analysis software (V. 1.44p, NIH, USA) for the larvae (10, n = 3 replicates), and the average value of axon length of each larva was then normalized to the body width. The body width was determined by drawing a straight line across the trunk region to the point just above of the termination of the yolk extension, according to a previously described method (Yang et al., 2011).

### 2.5. Transgenic zebrafish larvae assay

Adult transgenic (*HuC*-GFP) zebrafish (Danio rerio, AB strain) were purchased from the China Zebrafish Resource Center at the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The protocol of adult fish maintenance and embryo exposure was the same as described above in Section 2.2. After144 hpf exposure of TBOEP (0, 50, 500, 1500 and 2500 μg/L), images of the *Tg* (*HuC*-GFP) embryos were acquired using a fluorescence microscope (Leica M205 FA, Leica Microsystems, Germany). GFP fluorescence for *Tg* (*HuC*-GFP) larvae was measured with Image J software based on 2D images.