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Reproduction impairment and endocrine disruption in adult zebrafish (*Danio rerio*) after waterborne exposure to TBOEP

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ABSTRACT

Tris (2-butoxyethyl) phosphate (TBOEP) is widely used as a substitute of polybrominated diphenyl ethers (PBDEs). It has been frequently measured at concentrations of micrograms per liter (μg/L) in surface waters and waste water. However, limited information is available about the reproduction toxicology of TBOEP. In this study, adult zebrafish pairs were exposed to TBOEP at concentrations of 0, 5, 50, and 500 μg/L for 21 days. The effects on reproduction, hormone concentration, transcription of genes along the hypothalamic-pituitary-gonadal (HPG) axis, and gonadal development were investigated. After exposure to TBOEP, plasma concentrations of 17β-estradiol were significantly increased in both sexes of fish, while increase of testosterone was observed only in male fish. Transcription of genes along the HPG axis was significantly influenced by exposure to TBOEP in both male and female fish. Moreover, TBOEP decreases the average number of eggs production, as well as hatching success and survival rates in offspring. Histological examination shows inhibition of oocyte maturation in females and retardation spermiation in males, respectively. The results demonstrate that TBOEP could disturb the sex hormone balance by altering regulatory circuits of the HPG axis, affect gonadal development, eventually leading to disruption of reproductive performance and the development of progeny.

1. Introduction

Due to phased-out of main commercial polybrominated diphenyl ethers (PBDEs) mixtures, such as PentaBDE, the production and use of alternative flame retardants such as organophosphate flame retardants (OPFRs) have increased (Reemtsma et al., 2008). Among OPFRs, tris (2-butoxyethyl) phosphate (TBOEP) has been increasingly used in a number of applications and products as a substitute for PBDEs (McGee et al., 2012). Furthermore, TBOEP is used as a plasticizer in various products such as textiles, floor polish, varnish, plastics, furniture, foams, and electronic equipment (Marklund et al., 2003). It is an additive OPFR and is not chemically bind into final products. Thus, TBOEP can be discharged into the surrounding environment (Rodriguez et al., 2006). Previous studies have been demonstrated that TBOEP is frequently found in waste water, effluent, surface water, ground water, soil, drinking water and even human milk (Fries and Puttmann, 2003; Cequier et al., 2014; Marklund et al., 2005; Andresen, 2006; Bacaloni et al., 2007; Stapleton et al., 2009; Sundkvist et al., 2010). In Sweden, the concentrations of TBOEP in influents and effluents of municipal waste water treatment plants were 35 μg/L and 30 μg/L, respectively, which demonstrated its resistance to waste water treatment processes (Marklund et al., 2005). In China, concentration of TBOEP in sediment has been reported to range from 1.00 to 5.00 mg/kg dm (dry mass) and it was the most prominent chemical in Tai Lake (Ch:Taihu) (Cao et al., 2012). In addition, a recent study reported that concentrations of TBOEP ranged from 0.07 to 3.50 ng/g wet weights in Lake trout (McGoldrick et al., 2014) and from 86.0 to 98.4 ng/g dry weights in mullet fish (Alvarez-Muñoz et al., 2015).

Although neurotoxicity, developmental toxicity, reproductive toxicity, and systemic effects of OPFRs have been reported (Veen and Boer, 2012), limited information is currently available on reproduction impairment and the underlying mechanisms of TBOEP. A recent report demonstrated that TBOEP increased concentrations of 17β-estradiol (E2) and testosterone (T) by altering transcriptions of major steroidogenic genes in H295R cells (Liu et al., 2012). In

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contrast to this, after TBOEP treatment, the expression of genes relating to T synthesis and the concentration of T declined strongly in TM3 Leydig cells (Jin et al., 2015). Another study also reported that TBOEP induced pregnane X receptor agonistic activity in cell based on transactivation assays (Kojima et al., 2013).

In fish, reproduction is regulated mainly by the hypothalamic-pituitary-gonadal (HPG) axis (Ma et al., 2012). Theoretically, disrupting any point in the HPG axis can adversely affect the function of the reproductive endocrine system. It is well-known that OPFR families have endocrine disruption effect in fish. Tris (1, 3-dichloro-2-propyl) phosphate (TDCPP) and triphenyl phosphate (TPP) exposure resulted in a decline in fecundity, which was associated with significant increases of plasma E2 level and several transcriptions of steroidogenic genes (Liu et al., 2013). After TDCPP exposure, remarkable increases of plasma E2 and T levels in females, reduced egg quality, and increased malformation rates in the F1 generation have been observed (Wang et al., 2015). A recent study about TBOEP reported that exposure to TBOEP caused developmental malformations and changed the transcription of genes involved in the endocrine axis in zebrafish larva (Ma et al., 2016). Nevertheless, whether exposure to TBOEP can affect fish reproduction remains unknown.

The present study was conducted to evaluate the impact of TBOEP on reproduction function in zebrafish. The influences on the sex steroid hormone level, mRNA transcription of vital genes in HPG axis, reproductive performance and gonadal histology in zebrafish were examined after 21 days exposure. This study will contribute to a better understanding of TBOEP reproductive toxicology in adult zebrafish and the relevant endocrine disruption potentials following exposure to TBOEP.

2. Material and methods

2.1. Zebrafish maintenance

Wild-type adult male and female zebrafish (18-week old, AB wild-type) were obtained from zebrafish breeding center in Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China) and were acclimated for approximately 7 days in a temperature controlled room (28 °C ± 1 °C). Male and female fish were cultured separately in glass tanks filled with dichlorinated tap water (pH 7.0–7.4). The culture water was renewed every 24 h. Fish were maintained under a photoperiod of 14:10 h light: dark and fed with fairy shrimp (Tianjin Red Sun Aquaculture Co., Ltd., Tianjin, China) three times a day. Water quality parameters, such as pH, conductivity, temperature, and dissolved oxygen, were measured weekly. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Huazhong Agriculture University for laboratory animal use, Wuhan of China. Culturing and breeding of fish was performed according to the common OECD protocol for fishes.

2.2. Chemicals reagents

TBOEP (CAS NO.: 78-51-3; purity: 94%, product of Japan) was purchased from Sigma-Aldrich. A stock solution of TBOEP was prepared in dimethyl sulfoxide (DMSO), and diluted with DMSO to final concentrations immediately before use. The final concentration of solvent (DMSO) in test solutions did not exceed 0.01%. Test medium was prepared in fish culture water which is filtered and aerated (>24 h) tap water. TRIzol reagent and reverse transcription and SYBR Green kits were from Takara (Dalian, Liaoning, China), hormone detection kits were from Cayman Chemical Company (Ann Arbor, MI). All the reagents used in this study were of analytical grade.

2.3. TBOEP exposure protocols

Based on environmentally relevant concentrations of TBOEP (surface water 127 ng/L and wastewater up to 35 μg/L, respectively) (Cao et al., 2012), a gradient nominal concentrations were chosen (5, 50, and 500 μg/L, which are equivalent to 0.013, 0.13, and 1.3 μM, respectively). During exposure experiment, 8 male and 8 female mating fish were randomly selected and placed in each of three replicate tanks for each concentration of TBOEP for 21 d. Both control and treated groups received 0.01% (v/v) DMSO, which has no significant effects on development and reproduction in zebrafish (Han et al., 2014). During semi-static exposure, solutions were replaced for every 24 h with fresh carbon-filtered water containing corresponding concentrations of TBOEP. We have changed our description of exposure method in the revised version.

2.4. Evaluation of TBOEP on reproduction

To examine the effects of TBOEP on reproduction, fish were spawned in different groups throughout the last 14 days of exposure. Spawned eggs were collected 2 h after the light turned on every morning, because the embryos developed normally and reached the blastula stage at 2 h post fertilization (hpf). The number of eggs per spawning event was recorded daily. Fecundity was reported as cumulative eggs per female during the last 14 days of exposure.

Up to 100 fertilized eggs were randomly collected from each tank and separately cultured in glass dishes containing fresh water without TBOEP on the final day of spawning, and were observed for hatching rate and survival success at 5 dpf. Another fifteen randomly selected eggs from each tank were used to determine the egg diameter. The egg diameter was evaluated on a Leica M205FA microscope with a digital camera and software system, and the digital image was analyzed using LAS V4.5 software.

2.5. Evaluation of adult zebrafish exposure endpoint parameters

After the 21 d of exposure, all fish in the mating tank were anesthetized on ice, and body weights and snout-vent lengths were measured. In each group, eight fish (4 male and 4 female) were selected randomly from each tank and the gonads were removed and weighed to determine the GSI. Then the gonads were prepared for sex hormones determination. Following Vitale et al. (2006), gonadosomatic index (GSI = 100 × [gonad weight (g)/body weight (g)]), hepatosomatic index (HSI = 100 × [hepar weight (g)/body weight (g)]), brain index (BSI = 100 × [brain weight (g)/body weight(g)]), and condition factor (K = 100 × [body weight (g)/total length^3 (cm)]) values were calculated.

2.6. Sex hormones measurements

After exposure, blood samples were collected as described by Liu et al. (2009). Briefly, samples of 4–10 μL of blood were collected from the caudal vein of each fish, and blood samples from 4 fish of the same sex were pooled as one replicate. The blood samples were centrifuged at 3500g for 10 min at 4 °C, and the supernatant (females 8 μL; males 6 μL) was collected and stored at −80 °C until analysis. Before ELISA could be performed, free steroids were extracted from the samples by using a previously reported method (Yu et al., 2014). Briefly, each blood sample was diluted to 490 μL with Milli-Q water in clean glass tubes, 2 mL diethyl ether was added to each glass and vortexed. The supernatants were removed and transferred to a clean tube. Repeat the extraction process three times. The collected supernatants were evaporated to dryness by heating to 30 °C under a gentle stream of nitrogen and then stored at −80 °C. The dried extracts were resuspended in 0.5 mL ELISA buffer.