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Exposure of zebrafish embryos/larvae to TDCPP alters concentrations of thyroid hormones and transcriptions of genes involved in the hypothalamic–pituitary–thyroid axis

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ABSTRACT

Tris(1,3-dichloro-2-propyl) phosphate (TDCPP) has been frequently detected in the environment and in various biota, including fish, and has been implicated in disruption of the thyroid endocrine system. In the present study, zebrafish (*Danio rerio*) embryos were exposed to different concentrations of TDCPP (10, 50, 100, 300 and 600 μg/L) from 2 h post-fertilization (hpf) to 144 hpf. Developmental endpoints, and whole-body concentrations of thyroid hormones and transcriptional profiles of genes involved in the hypothalamic–pituitary–thyroid (HPT) axis were examined. Exposure to TDCPP caused a dose-dependent developmental toxicity, including decreased body weight, reduced hatching, survival and heartbeat rates, and increased malformation (spinal curvature). Treatment with the positive control chemical 3,3′,5-triiodo-L-thyronine (T3) significantly decreased whole-body thyroxin (T4) concentrations, increased whole-body T3 concentrations, and upregulated mRNA expression involved in the HPT axis as a compensatory mechanism. These results suggested that the HPT axis in 144-hpf zebrafish larvae was responsive to chemical exposure and could be used to evaluate the effects of chemicals on the thyroid endocrine system. TDCPP exposure significantly decreased whole-body T4 concentrations and increased whole-body T3 concentrations, indicating thyroid endocrine disruption. The upregulation of genes related to thyroid hormone metabolism (*dio1* and *ugt1ab*) might be responsible for decreased T4 concentrations. Treatment with TDCPP also significantly increased transcription of genes involved in thyroid hormone synthesis (*tshβ*, *slc5a5* and *tg*) and thyroid development (*hhex*, *nkx2.1* and *pax8*) as a compensatory mechanism for decreased T4 concentrations. Taken together, these results suggest that TDCPP alters the transcription of genes involved in the HPT axis and changes whole-body concentrations of thyroid hormones in zebrafish embryos/larvae, thus causing an endocrine disruption of the thyroid system.

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1. Introduction

Since the ban on some brominated flame retardants (BFRs), the use of organophosphorus flame retardants (OPFRs) has been gradually increasing (Van der Veen and de Boer, 2012). Recently, Tris(1,3-dichloro-2-propyl) phosphate (TDCPP) was identified as one of the primary OPFRs found in polyurethane foam used in furniture (Stapleton et al., 2009). The annual production in the United States was estimated to be between 4500 and 22,700 tons in 1998, 2002 and 2006 (Van der Veen and de Boer, 2012).

Environmental monitoring has frequently detected TDCPP in indoor air, dust, surface water, drinking water, influents, effluents, sediments and biota (Van der Veen and de Boer, 2012). In surface water for example, the highest level of TDCPP reported was 50 ng/L (Andresen et al., 2004). Particularly high levels of TDCPP were found in the effluent of sewage treatment plants in Germany and Norway, with concentrations ranging from 20 ng/L to 740 ng/L (Andresen et al., 2004; Green et al., 2008; Van der Veen and de Boer, 2012). In biota, TDCPP was detected in freshwater perch at 36–140 μg/kg lipid weight (Sundkvist et al., 2010). In China, TDCPP

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were also detected in water from the Songhua River and sediments from Taihu Lake, with the concentrations of 2.5–40 ng/L and 0.62–5.54 μg/kg, respectively (Cao et al., 2012; Wang et al., 2011)

Limited information is currently available about the toxic effects and mechanisms of TDCPP. Results of acute toxicity demonstrated that TDCPP is relatively toxic compared with other OPFRs. The median lethal concentrations were 1.1 mg/L and 7.0 mg/L in rainbow trout and in zebrafish embryos/larvae after 96 h and 116 h exposure, respectively (Liu et al., submitted for publication; WHO, 1998). Toxicological studies demonstrated that TDCPP could cause neuro-developmental toxicity and endocrine disruption of the reproductive system (Dishaw et al., 2011; Liu et al., 2012). Exposure to TDCPP significantly inhibited DNA synthesis and promoted neuro-differentiation in the absence of nerve growth factor in PC12 cells (Dishaw et al., 2011). Liu et al. (2012) demonstrated that treatment with TDCPP upregulated mRNA expression associated with steroidogenesis, downregulated mRNA expression of two sulfotransferase genes, and increased testosterone (T) and 17β-estradiol (E2) productions in H295R cells and in zebrafish. Furthermore, results of epidemiological and controlled laboratory studies suggested that TDCPP affects the thyroid endocrine system and alters development-related mRNA expression (Crump et al., 2012; Liu et al., submitted for publication; Meeker and Stapleton, 2010). For example, Meeker and Stapleton (2010) reported that increased concentrations of TDCPP in house dust were associated with decreased concentrations of free thyroxin (T4) in the serum of adult men. Using zebrafish embryos and larvae, a study demonstrated that TDCPP could significantly upregulate mRNA expression of thyroid hormone receptors and associated genes in a dose-dependent manner (Liu et al., submitted for publication).

In fish, the thyroid endocrine system is controlled primarily by the hypothalamic–pituitary–thyroid (HPT) axis, which is responsible for maintaining homeostasis of thyroid hormones by regulating their synthesis, secretion, transport and metabolism. In the present study, we evaluated the effects of TDCPP on mRNA expression involved in the HPT axis using zebrafish embryos/larvae by using a HPT-PCR array. Furthermore, an enzyme-linked immunosorbent assay (ELISA) was employed to measure whole-body thyroid hormone levels after chemical exposure.

2. Materials and methods

2.1. Materials and reagents

Tris(1,3-dichloro-2-propyl) phosphate (TDCPP) was purchased from TCI Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). T3 (3,3′,5-triiodo-L-thyronine) and 3-amino-benzoic acid ethyl ester, methanesulfonate salt (MS-222) were purchased from Sigma (St. Louis, MO, USA). TDCPP and T3 were dissolved in dimethyl sulfoxide (DMSO) as stock solutions and stored at −20 °C. MS-222 was dissolved directly in water and was used immediately. TDCPP standard was obtained from Dr. Ehrenstorfer, GmbH (Germany). Tributyl-d27-phosphate (TnBP-d27) was purchased from Cambridge Isotope Laboratories (UK) and was used as internal standard. Trizol Reagent and ELISA kits for detection of thyroid hormones were obtained from Invitrogen (Carlsbad, CA, USA) and Uscn Life (Wuhan, China), respectively. PrimeScript® RT reagent kits were purchased from TaKaRa (Dalian, China). SYBR® Real-time PCR Master Mix -Plus-kits were obtained from Toyobo (Osaka, Japan).

2.2. Animals and chemical exposure protocol

Adult zebrafish (AB strain, aged 5 months) maintenance and embryo exposure were performed according to published protocols (Shi et al., 2008; Yu et al., 2010). 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 of TDCPP exposure solutions at different concentrations (0, 10, 50, 100, 300 and 600 μg/L) or the positive control chemical T3 (30 μg/L). The embryos were exposed until 144 hpf, by which time they had developed into free-swimming larvae and most organs had completed development, such as liver, heart, brain, and thyroid (Amsterdam et al., 2004; Chan et al., 2009; Elsalini et al., 2003). The exposure solutions were renewed daily, and each exposure concentration was replicated in three separate glass beakers. Control groups received 0.01% DMSO (v/v). Exposure concentrations of TDCPP were selected based on a range-finding study to determine concentrations that slightly decreased survival. For T3, the exposure concentration (30 μg/L) was selected based on previous studies (Duarte-Guterman et al., 2010; Liu et al., submitted for publication) in which the expression of genes involved in the thyroid endocrine system was significantly altered. During the exposure period, heartbeat (48 and 96 hpf), hatching (144 hpf), survival (144 hpf) and malformation (144 hpf) were recorded. In the present study, hatching rate was the percentage of successful hatching larvae, which included alive individuals at 144 hpf and dead individuals after hatching. Survival rate was the percentage of alive individuals at 144 hpf, which included hatched and not hatched individuals. Malformation was the percentage of malformed individuals, including alive and dead malformed embryos/larvae. After exposure, zebrafish larvae were anesthetized with MS-222 and sampled for body weight determination, mRNA expression analysis and thyroid hormones measurement.

2.3. Quantification of TDCPP in exposure solutions

Since exposure solutions were renewed daily, TDCPP concentrations in exposure solutions were determined only at 120 hpf (after renewing of exposure solutions) and 144 hpf, by which time the embryos had developed into free-swimming larvae and had relative strong metabolizing ability of chemical compared to early developmental stages. The measurement of TDCPP was performed according to a previously published method (Wang et al., 2011). Briefly, exposure solutions were filtered through 0.22 μm cellulose acetate membrane filters (Sartorius, Germany) and immediately used for LC–MS/MS analysis. The quantification of TDCPP was performed by using a TDCPP standard and a TnBP-d27 internal standard.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Isolation of total RNA, synthesis of first-strand cDNA and qRT-PCR were carried out as previously described by Yu et al. (2010). Briefly, 20 larvae per sample were homogenized and used for total RNA isolation using Trizol reagent following the manufacturer's instructions. Genomic DNA contamination was removed by using RNase-free DNase I (Promega, Madison, WI, USA). Total RNA content was measured at 260 nm using a spectrophotometer (M2; Molecular Devices, CA, USA), and the purity and quality were determined by measuring 260/280 nm ratios and by 1% agarose–formaldehyde gel electrophoresis with ethidium bromide staining. Synthesis of first-strand cDNA was performed by using a PrimeScript® RT Reagent Kit (TaKaRa, Dalian, China) following the manufacturer's instructions. The qRT-PCR was carried out using SYBR® Real-time PCR Master-Mix-Plus kits (Toyobo, Osaka, Japan) and analyzed on an ABI 7300 System (PerkinElmer Applied Biosystems, Foster City, CA, USA). The primer sequences of genes were obtained as previously described (Liu et al., 2011; Shi et al., 2008; Yu et al., 2010) and are shown in Table 1. The amplification protocol was as follows: initial denaturation for 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s, and a final

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