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Research Paper

Thyroid disrupting effects of halogenated and next generation chemicals on the swim bladder development of zebrafish

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ABSTRACT

Endocrine disrupting chemicals (EDCs) can alter thyroid function and adversely affect growth and development. Halogenated compounds, such as perfluorinated chemicals commonly used in food packaging, and brominated flame retardants used in a broad range of products from clothing to electronics, can act as thyroid disruptors. Due to the adverse effects of these compounds, there is a need for the development of safer next generation chemicals. The objective of this study was to test the thyroid disruption potential of old use and next generation halogenated chemicals. Zebrafish embryos were exposed to three old use compounds, perfluorooctanoic acid (PFOA), tetrabromobisphenol A (TBBPA) and tris (1,3-dichloro-2-propyl) phosphate (TDCPP) and two next generation chemicals, 9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxdie (DOPO) and perfluorobutyric acid (PFBA). Sub-chronic (0–6 days post fertilization (dpf)) and chronic (0–28 dpf) exposures were conducted at 1% of the concentration known to kill 50% (LC_{50}) of the population. Changes in the surface area of the swim bladder as well as in expression levels of genes involved in the thyroid control of swim bladder inflation were measured. At 6 dpf, zebrafish exposed to all halogenated chemicals, both old use and next generation, had smaller posterior swim bladder and increased expression in the gene encoding thyroid peroxidase, *tpo* and the genes encoding two swim bladder surfactant proteins, *sp-a* and *sp-c*. These results mirrored the effects of thyroid hormone-exposed positive controls. Fish exposed to a TPO inhibitor (methimazole, MMI) had a decrease in *tpo* expression levels at 28 dpf. Effects on the anterior swim bladder at 28 dpf, after exposure to MMI as well as both old and new halogenated chemicals, were the same, i.e., absence of SB in ~50% of fish, which were also of smaller body size. Overall, our results suggest thyroid disruption by the halogenated compounds tested via the swim bladder surfactant system. However, with the exception of TBBPA and TDCPP, the concentrations tested (~5–137 ppm) are not likely to be found in the environment.

1. Introduction

Halogenated chemicals, such as per/polyfluorinated and brominated chemicals are commonly used in everyday items such as electronics, furniture and baby products. These chemicals can persist in the environment and bioaccumulate in organisms, thus increasing probability of exposure (Segev et al., 2009; Stahl et al., 2011). Some members of this group can cause detrimental effects to the nervous, reproductive and endocrine systems (Stahl et al., 2011). More specifically, laboratory and epidemiological studies have linked exposure to certain halogenated chemicals with thyroid toxicity, which is of special concern for developing organisms as it could lead to significant developmental delays and abnormalities (Hartoft-Nielsen et al., 2011). Importantly, exposure to some halogenated chemicals like perfluorooctanoic acid (PFOA) can occur prenatally as they are commonly detected in cord plasma and breast milk (de Cock et al., 2014).

The exact mechanism(s) behind the thyroid toxicity of these halogenated chemicals is not well understood. Studies have shown they can bind to thyroid receptors and/or outcompete for binding to the carrier protein transthyretin (TTR) due to structural similarities to thyroxine (T_4) (Kar et al., 2017; Meerts et al., 2000; Ren et al., 2015; Weiss et al., 2009). For instance, tetrabromobisphenol A (TBBPA) is a thyroid hormone disruptor as it outcompetes for TTR binding causing disruption of thyroid homeostasis (Shaw et al., 2010). In amphibians, TBBPA suppresses triiodothyronine (T_3) driven tail shortening (Kitamura et al., 2005). A chlorinated flame retardant, tris (1,3-dichloro-2-propyl)

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phosphate (TDCPP) dysregulates the hypothalamic-pituitary-thyroid (HPT) axis in zebrafish, decreasing body weight and increasing malformations (Wang et al., 2013). Other toxic halogenated chemicals, such as polychlorinated biphenyls (PCBs), have also been shown to cause thyroid disruption in fish at environmentally relevant concentrations (Dong et al., 2014).

Swim bladder development in fishes is under thyroid control. In zebrafish, two distinct swim bladder chambers develop under thyroid hormone control: the posterior swim bladder is developed by 5 days post fertilization (dpf) and the anterior swim bladder by 21 dpf (Chang et al., 2012). The posterior swim bladder is responsible for buoyancy and the anterior swim bladder is thought to play a role in the auditory system as well as buoyancy (Nelson et al., 2016). Zebrafish (Stinckens et al., 2016) and fathead minnows (Nelson et al., 2016) treated with a thyroid peroxidase (TPO) inhibitor that prevents oxidation of iodide for addition onto tyrosine residues on thyroglobulin for the production of T_4 and T_3, resulted in impaired swim bladder inflation. Deflation occurs because a decline in thyroid hormones results in a decline in the production of surfactant proteins which lower the surface tension of the swim bladder to prevent it from collapsing (Zheng et al., 2011). The putative thyroid disruptor perfluorooctane sulphonate (PFOS) causes deflation of the posterior swim bladder in zebrafish larvae (Hagenaars et al., 2014; Shi et al., 2008) but it is unclear whether this deflation is associated with thyroid disruption and surfactant protein levels. A similar surfactant protein system under thyroid control occurs in mammalian lungs (Daniels et al., 2004). Children with thyroid transcription factor 1 (*ttf-1*) deficiency have pulmonary dysfunction and have been shown to have reduced and/or dysregulated surfactant proteins, which contribute to their obstructed airways and overall respiratory problems (Galambos et al., 2010).

There is a need for the development of next generation, safer, alternative chemicals because of the high environmental persistence and potential toxicity of halogenated chemicals. DOPO, 9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxdie, is an alternative for TBBPA: it replaces the bromine with a phosphate. Heptafluorobutanoic acid (PFBA) is a potentially safer alternative to PFOA because it has a shorter carbon chain which has been shown to decrease toxicity and bioaccumulation potential (Hagenaars et al., 2011). Currently, there is no safer alternative to TDCPP. Although less overtly toxic, it remains unknown whether these next generation chemicals can also act as thyroid disrupting chemicals.

The objective of this study was to assess the thyroid disrupting potential of old and next generation halogenated chemicals via effects on fish swim bladder development. We used swim bladder inflation as a key event in our studies since it is known to be under direct thyroid control in fishes. We hypothesized that old use halogenated chemicals (PFOA, TBBPA and TDCPP) would act as thyroid disruptors causing a delay/absence in swim bladder inflation and a decrease in expression of surfactant proteins. Next generation alternative chemicals PFBA and DOPO were not expected to elicit this response. To our knowledge, this is the first study that has assessed the thyroid disrupting potential of these halogenated chemicals via the swim bladder development in fishes.

2. Materials and methods

2.1. Exposure conditions

We tested the thyroid disrupting activity of the following chemicals: TBBPA (CAS No. 79-94-7, Sigma-Aldrich, St. Louis, MO), TDCPP (CAS No. 13674-87-8, TCI America, Portland, OR), PFOA (CAS No. 335-67-1, Sigma-Aldrich), DOPO (CAS No. 35948-25-5, ChemReagents, Watertown, MA) and PFBA (CAS No. 375-22-4, Sigma-Aldrich). Methimazole (MMI, CAS No. 60-56-0) was used as a negative control as it inhibits TPO-catalyzed thyroid hormone synthesis (Reider and Connaughton 2014). Thyroid hormones T_3 (CAS No. 6893-02-3) and T_4 (CAS No. 51-48-9) were also used as controls (both from Sigma-Aldrich). T_4 is converted to T_3, the biologically active form, via the removal of an iodine atom. **Table S1** summarizes the chemical structures of the different compounds tested. A stock solution was prepared for each chemical by dissolving it in 1 L of Reverse Osmosis (RO) water containing 12.5 mL Replenish (Seachem Laboratories Inc., Madison, GA) and pH was adjusted to neutral (7-7.5). Stock solutions were then diluted to 1% of their respective lethal concentration 50% (LC_{50}) based on data from a previous study (TBBPA 1.3 ppm; TDCPP 1.9 ppm; PFOA 473 ppm; DOPO 989 ppm; and PFBA 13,795 ppm) (Godfrey et al., 2017). Tested exposure concentrations were as follows: TBBPA (0.013 ppm), TDCPP (0.019 ppm), PFOA (4.7 ppm), DOPO (9.8 ppm) and PFBA (137 ppm). Concentrations for T_3, T_4 and MMI were based on values derived from Liu and Chan (2002) who observed swim bladder deflation at 30 nM of T_4 and 5 nM of T_3 and used 0.3 mM MMI for recovery experiments. Preliminary tests indicated no differences in swim bladder development between 10 nM and 30 nM for thyroid hormones and between 30 nM and 300 μM for MMI; therefore, we decided to use the lower dose for all follow-up experiments.

Adult AB wild type zebrafish were maintained at the Purdue University Aquatic Laboratory. We kept genders separate overnight at a ratio of 2 M:1F and removed the dividers the next morning. Embryos were collected (gastrula stage, 4.5 h post fertilization (hpf)) and randomly placed into petri dishes containing 25 mL of test solution which was renewed daily throughout the duration of the exposure. Each petri dish contained 20 embryos and each test consisted of a minimum of three replicates per dose with experiments repeated three times. In order to cover the complete period of swim bladder development (see more on this below under 2.2), zebrafish embryos were exposed starting immediately after fertilization either subchronically for 6 days (0–6 dpf) or chronically for 28 days (0–28 dpf). For the subchronic exposures, embryos were maintained in petri dishes for 6 days, after which they were imaged and flash frozen for qPCR analysis. For the chronic exposures, larvae were moved after 6 days to a 500 mL glass mason jar containing 200 mL solution. Fish were not fed during the subchronic exposures since they rely on their yolk sac until swim-up. From 6–14 dpf larvae were fed ad libitum paramecia once a day, and from 15 to 28 dpf larvae were fed *Artemia* nauplii in the morning and Tetramin® in the afternoon. Embryos and larvae were maintained in an environmental chamber at a temperature of 28 °C ± 1 °C and a photoperiod of 14L:10D. Zebrafish were exposed to all of the chemicals for 6 dpf, however, we did not pursue the 28 dpf zebrafish exposure with TBBPA or DOPO.

2.2. Body size and swim bladder surface area

A timeline of exposure and sampling in relation to the development of the swim bladder is depicted in **Fig. S1**. In zebrafish, the posterior swim bladder starts developing at 3 dpf and completes development by 5 dpf, while the anterior swim bladder completes development by 21 dpf (see **Fig. S1**, Winata et al., 2009). Zebrafish are able to sustain their own T_4 levels by 4 dpf; prior to that their hormones are maternally-derived, which are depleted by 5 dpf (Porazzi et al., 2009). Therefore, we finished our subchronic experiments at 6 dpf to reduce the influence of potential maternally derived thyroid effects on the development of the posterior swim bladder, and our chronic experiments at 28 dpf to ensure the anterior swim bladder was fully developed. A representative number of fish (12–50 individuals/dose/chemical/time point) were imaged using an Olympus stereo microscope (Tokyo, Japan) for later measurements using CellSens imaging software. Prior to imaging, fish were anesthetized with tricaine MS-222 (40–75 mg/L). Standard lengths, as well as the anterior and posterior swim bladder surface areas of larvae were measured. After imaging, fish were transferred to clean water to ensure proper functioning and swimming before being flash frozen in liquid nitrogen. Only fish that recovered from the anesthesia and were observed swimming were then