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6:2 fluorotelomer carboxylic acid (6:2 FTCA) exposure induces developmental toxicity and inhibits the formation of erythrocytes during zebrafish embryogenesis

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ABSTRACT

Saturated fluorotelomer carboxylic acids (FTCAs) are intermediates in the degradation of fluorotelomer alcohols (FTOHs) to perfluorinated carboxylic acids (PFCAs). Recent studies have detected FTCAs in precipitation, surface waters, and wildlife, but few studies have focused on their toxicity. In this study, zebrafish embryos were exposed to different concentrations of 6:2 FTCA (0, 4, 8, and 12 mg/L) from 6 to 120 h post-fertilization (hpf) to investigate its developmental toxicity. Results showed that 6:2 FTCA exposure decreased the hatching and survival percentages, reduced the heart rate, and increased the malformation of zebrafish embryos. The median lethal concentration of 6:2 FTCA was 7.33 mg/L at 120 hpf, which was lower than that of perfluorooctanoic acid (PFOA), thus indicating higher toxicity for zebrafish. The most common developmental malformation was pericardial edema, which appeared in the 8 and 12 mg/L 6:2 FTCA-exposed embryos from 60 hpf. Using o-dianisidine staining, we found that the hemoglobin content in embryos was reduced in a concentration-dependent manner after 6:2 FTCA exposure at 72 hpf. Based on quantitative real-time polymerase chain reaction (q-RT-PCR) and whole-mount *in situ* hybridization, the transcriptional levels of hemoglobin markers (*hbae1*, *hbbe1*, and *hbae3*) were down-regulated at 48 and 72 hpf, even though no observed malformation appeared in zebrafish at 48 hpf. Moreover, 6:2 FTCA exposure decreased the protein level of *gata1*, a principal early erythrocytic marker, in Tg (*gata1*:DsRed) transgenic zebrafish at 72 hpf. We analyzed the transcriptional level of other erythrocyte-related genes using q-RT-PCR assay. For heme formation, the transcription of *alas2*, which encodes the key enzyme for heme biosynthesis, was down-regulated after 6:2 FTCA exposure, whereas the transcription of *ho-1*, which is related to heme degradation, was up-regulated at 48 and 72 hpf. The transcriptional patterns of *gata1* and *gata2*, which are related to erythroid differentiation, differed. At 48 hpf, the mRNA level of *gata2* was significantly increased, whereas that of *gata1* exhibited no significant changes in any treatment group. At 72 hpf, the expressions of both were down-regulated in a concentration-dependent manner. Taken together, 6:2 FTCA exposure decreased the erythrocyte number and disrupted erythroid differentiation during zebrafish embryonic development. Our results suggest that 6:2 FTCA can cause developmental toxicity in zebrafish embryos, and that FTCAs exhibit greater toxicity than that of PFCAs.

1. Introduction

Perfluorinated carboxylic acids (PFCAs) are persistent global contaminants, with their main source in the environment coming from direct spills and indirect degradation of their precursors (Cousins et al., 2011; Prevedouros et al., 2006). Fluorotelomer alcohols (FTOHs) are fluorinated precursors that can result in the formation of PFCAs through serial abiotic and aerobic biodegradation in the environment (Dinglasan et al., 2004; Ellis et al., 2004; Hurley et al., 2004; Wang et al., 2005a, 2005b). As industrial raw material, FTOHs are widely produced and used as intermediates for the synthesis of FTOH-based polymeric and surfactant products (Kissa, 2001; Prevedouros et al., 2006). Degradation of FTOH-based products and fugitive emissions during manufacturing can result in the occurrence of FTOHs in the environment (Dinglasan et al., 2004; Ellis et al., 2003; Dinglasan-Panlilio and Mabury, 2006). These volatile chemicals are primarily distributed in the ambient atmosphere, with their concentration in the North American troposphere reported to range from 11 to 171 pg/m^3 (Stock et al., 2004). Precipitation, discharge from wastewater treatment plants and degradation of FTOH-based products can also result in the

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occurrence of FTOHs in the aquatic environment (Dinglasan et al., 2004). For example, FTOHs have been detected recently in wastewater treatment plant effluent (23.2 ng/L) and surface water (10.8 ng/L) in urban Japan (Mahmoud et al., 2009).

In the atmosphere, FTOHs are oxidized to form saturated fluorotelomer carboxylic acids (FTCAs), which are then deposited in surface water (Ellis et al., 2004; Loewen et al., 2005). For example, 8:2 FTCA and 10:2 FTCA have been detected in North American rainwater (< 10 ng/L) and Arctic freshwater lakes (Loewen et al., 2005; Scott et al., 2006; Butt et al., 2010a, 2010b). In addition to oxidation, previous studies have also found that FTOHs can undergo a series of photolysis or biodegradation processes in the aquatic environment, forming FTCA intermediates and finally transforming into PFCAs (Lange, 2002; Dinglasan et al., 2004; Gauthier and Mabury, 2005; Wang et al., 2005a, 2005b; Zhao et al., 2013). Based on the worldwide occurrence of PFCAs and their precursor FTOHs, it is reasonable to speculate on the presence of FTCAs in the environment. So far, FTCAs (including 6:2, 8:2, and 10:2) have been detected in effluent waters from wastewater treatment plants (< 8.62 μg/L, in Germany), surface waters, precipitation (< 10 ng/L, in North American), and even aquatic mammals (1.5–9.6 ng/g) (Loewen et al., 2005; Houde et al., 2005; Taniyasu et al., 2005; Scott et al., 2006; Sinclair and Kannan, 2006; Butt et al., 2007; Gremmel et al., 2017).

Historically, 8:2 FTOH has been used as a major raw material since the 1970s (Prevedouros et al., 2006). As 8:2 FTOH is a potential precursor of perfluorooctanoic acid (PFOA) (Wang et al., 2005a, 2005b, 2009), short chain 6:2 FTOH has been approved by regulators to replace 8:2 FTOH as a key raw material in the manufacture of FTOH-based products (Ritter, 2010; OECD, 2012). Similar to 8:2 FTOH, manufacturing emissions and residual concentrations in products might also result in the environmental release of 6:2 FTOH (Buck et al., 2011). Several metabolic studies have suggested that 6:2 FTCA is a major transient intermediate during 6:2 FTOH biotransformation in various environments, including mammalian systems (Liu et al., 2010a, 2010b; Zhang et al., 2013; Zhao et al., 2013; Russell et al., 2015). With the wide application of 6:2 FTOH, the increase in the concentration of 6:2 FTCA in the environment is highly probable. A recent study demonstrated that 6:2 FTOH in wastewater treatment plant influent can result in an increase in the concentration of FTOH transformation products in corresponding effluent, with concentrations of 6:2 FTCA as high as 8.62 μg/L detected (Gremmel et al., 2017). In addition, 6:2 FTCA has been utilized as an alternative processing aid to PFOA in China (Wang et al., 2015; Xu et al., 2011). Thus, assessment of the potential risk of 6:2 FTCA in the environment is critical.

6:2 FTCA is structurally analogous to its precursor 6:2 FTOH and legacy PFOA (Fig. 1). Although structurally similar chemicals can result in similar effects on human and environmental health, current evidence on the potential risk of FTCAs remains insufficient and only limited reports are available on their toxicity to aquatic organisms (Phillips et al., 2007, 2010; Mitchell et al., 2011; Hoke et al., 2012). Acute aquatic toxicities have been assessed on *Chironomus dilutes*, *Daphnia magna*, *Lemna gibba*, *Pseudokirchneriella subcapitata*, *Oncorhynchus mykiss*, and *Pimephales promelas* (Phillips et al., 2007; Mitchell et al., 2011; Hoke et al., 2012). Based on 50% lethal concentrations (LC_{50}) and 50% effective concentrations (EC_{50}), the toxicity of FTCAs has been found to increase with increasing fluorocarbon (FC) chain length, and precursors of PFCAs exhibit greater toxicity than PFCAs with equivalent carbon chain lengths (Phillips et al., 2007, 2010; Mitchell et al., 2011; Hoke et al., 2012). Although evidence on the toxicity of FTCAs in aquatic environments has grown, studies assessing the potential effects of FTCAs on embryo development and the underlying mechanism have not yet been conducted.

Due to their high fecundity, rapid embryonic development, and optical transparency, zebrafish (*Danio rerio*) embryos are widely used for investigating the developmental toxicity of compounds (Embry et al., 2010; Scholz et al., 2008). To assess the developmental toxicity of

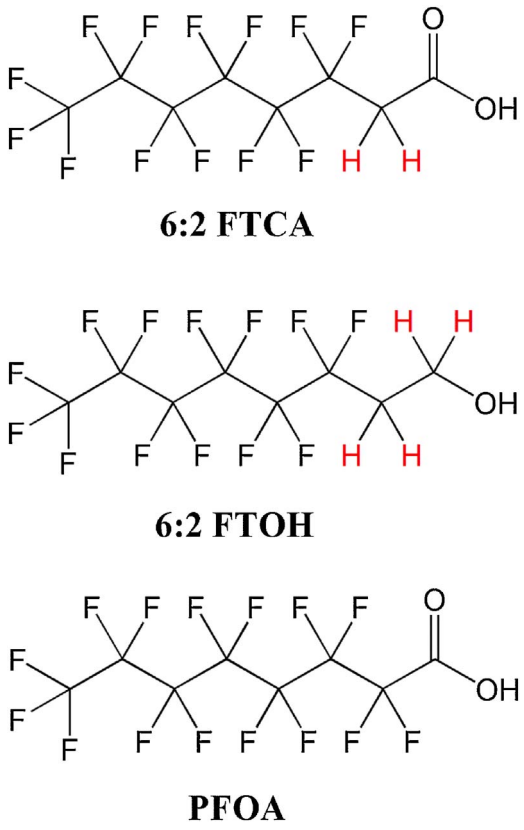

Fig. 1. Molecular structure of 6:2 FTCA, 6:2 FTOH, and PFOA. Red indicates the different atoms among them. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

6:2 FTCA, zebrafish embryos were exposed to various concentrations of 6:2 FTCA (0, 4, 8, and 12 mg/L) from 6 to 120 h post-fertilization (hpf). Different toxicity endpoints, including malformation assessment, hatching percentage, survival percentage, and heart rate, were determined. The o-dianisidine staining results showed that erythrocyte numbers were significantly reduced in 72 hpf 6:2 FTCA-exposed embryos compared with control embryos. *Gata1* is a principal early erythrocytic marker (Heicklen-Klein et al., 2005). Thus, Tg (*gata1*:DsRed) transgenic zebrafish embryos, which express DsRed in erythrocytes, were used to analyze the impact of 6:2 FTCA on erythrocytes at the protein level. To further explore the underlying molecular mechanisms of 6:2 FTCA exposure-induced toxicity, the expression of several erythrocyte-related genes was analyzed. Our study is the first to focus on the developmental toxicity and underlying molecular mechanism of FTCAs, and should be helpful for clarifying the ecological risks of FTCAs on fish.

2. Materials and methods

2.1. 6:2 FTCA stock solutions and exposure protocols

The 6:2 fluorotelomer carboxylic acid (6:2 FTCA $C_6F_{13}CH_2COOH$, CAS No. 53826-12-3, purity > 96%) was provided by Dr. Guo Yong (Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences), and was dissolved in 100% dimethyl sulfoxide (DMSO). To our knowledge, FTCA toxicity testing using zebrafish has not been conducted previously, thus preliminary experiments were performed based on the LogKow (4.636) and predicted LC_{50} values for fish (13.36 mg/L) at 96 h to establish suitable toxicity ranges and determine the LC_{50} value. Four doses of 6:2 FTCA (0, 4, 8, and 12 mg/L), which induced obvious malformation during early zebrafish embryo development, were chosen in our study. In general, relatively higher