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6:2 fluorotelomer sulfonamide alkylbetaine (6:2 FTAB), a novel perfluorooctane sulfonate alternative, induced developmental toxicity in zebrafish embryos

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ABSTRACT

6:2 fluorotelomer sulfonamide alkylbetaine (6:2 FTAB) is a major component of Forafac®1157, a novel perfluorooctane sulfonate (PFOS) alternative used globally in aqueous film forming foams (AFFFs). Although 6:2 FTAB has been recently detected in the aquatic environment, its toxic effects on aquatic organisms remain unclear. Here, zebrafish embryos were exposed to various concentrations of 6:2 FTAB (0, 5, 10, 20, 40, 60, 80, and 100 mg/L) from 6 to 120 h post-fertilization (hpf) to investigate its developmental toxicity and possible mechanism of action. Results showed that exposure to 40 mg/L or higher concentrations of 6:2 FTAB significantly decreased the survival percentage and increased the malformation percentage. The median lethal concentration (LC_{50}) at 120 hpf was 43.73 ± 3.24 mg/L, and the corresponding benchmark dose lower limit (BMDL) of lethal effect was 33.79 mg/L. These values were both higher than those for PFOS, supporting the notion that 6:2 FTAB is less toxic than PFOS to zebrafish embryos. The most common developmental defect in 6:2 FTAB-treated embryos was rough-edged skin/fins. TUNEL assay showed that 6:2 FTAB exposure induced cell apoptosis in the tail region compared with that of the control, which might explain the rough-edged skin/fins. The increased transcriptional levels of *p53*, *bax*, and *apaf1* and the increased activities of caspase-3, -8, and -9 provided further evidence of 6:2 FTAB-induced apoptosis. We also analyzed the effects of 6:2 FTAB on oxidative stress and the immune system. Results showed that reactive oxygen species and malondialdehyde accumulated in concentration-dependent manners after exposure to 6:2 FTAB, and antioxidant enzyme activities (catalase and glutathione peroxidase) also changed. Exposure to 6:2 FTAB also altered the transcriptional levels of *ccl1*, *il-1β*, *il-8*, *tnfα*, *ifn*, and *cxcl-c1c*, which play important roles in the innate immune system. Collectively, our data suggest that 6:2 FTAB exposure can induce cell apoptosis, oxidative stress, and immunotoxicity, thus highlighting the developmental toxicity of 6:2 FTAB in zebrafish embryos.

1. Introduction

Aqueous film forming foams (AFFFs) are hydrocarbon and fluorochemical surfactant blends, which are routinely used to extinguish hydrocarbon fuel fires (Ranjbar and Shahraki, 2013; Sontake and Wagh, 2014; Seow, 2013). Historically, perfluorooctane sulfonate (PFOS), as well as its derivatives, has been the most utilized fluoroalkyl surfactant used in AFFF formulations. However, due to its persistence, bioaccumulation, and toxicity, its production was phased out by the 3 M Company in 2002 (OECD, 2002), with PFOS and its salts also added to Annex B of the Stockholm Convention on Persistent Organic Pollutants in 2009 (UNEP, 2009). Since then, considerable effort has been made to develop effective and environmentally-friendly compounds to replace PFOS used in AFFFs (Cortina and Korzeniowski, 2008). Forafac®1157, as a novel PFOS alternative, was developed by DuPont and has been used globally in AFFF production (Hagenaars et al., 2011a). Forafac®1157 is a mixture, with 6:2 fluorotelomer sulfonamide alkylbetaine (6:2 FTAB) as its major component (Moe et al., 2012). 6:2 FTAB contains a 6:2 fluorotelomer sulfonamide (6:2 FTSA) moiety, i.e. $[F_3C(CF_2)_5CH_2CH_2SO_2NH\text{-}]$, and a betaine $[\text{-}N(R,R')^+CH_2COO^-]$ moiety, separated by an alkyl spacer (Fig. 1) (Pabon and Corpart, 2002; Jensen et al., 2008; Moe et al., 2012). In an investigation of 10 fluorinated AFFFs used in Ontario, Canada, 6:2 FTAB was detected in four of them and appeared to be the second most commonly utilized

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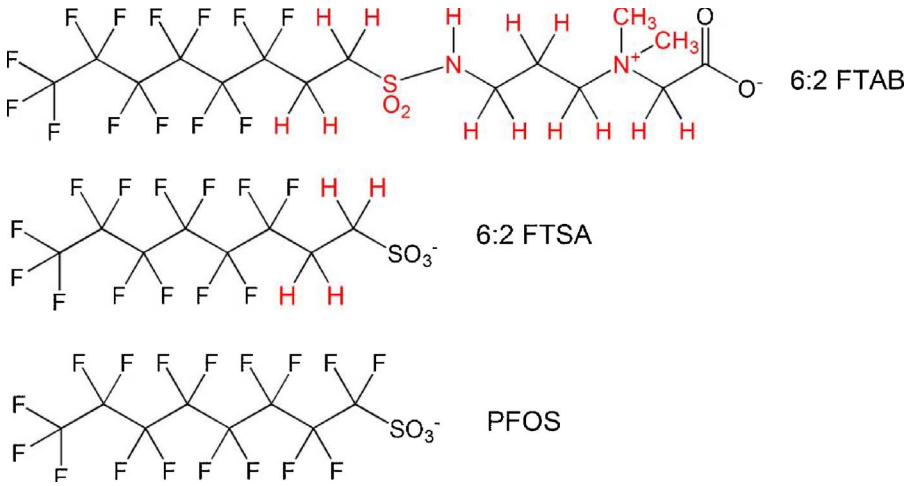

Fig. 1. Molecular structures of 6:2 FTAB, 6:2 FTSA and PFOS, red color indicates different atoms between them. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fluorotelomer AFFF component (D'Agostino and Mabury, 2014). In addition, 6:2 FTAB has also been detected in AFFFs from U.S. military bases (Backe et al., 2013).

The release of AFFFs is a known source of perfluoroalkyl and polyfluoroalkyl substance (PFASs) contamination in surface water (de Solla et al., 2012), groundwater (Schultz et al., 2004), soil (Houtz et al., 2013), and biota (Moody et al., 2002; de Solla et al., 2012). Recently, 6:2 FTAB has been detected in various environmental matrices where AFFFs have been produced or used (Gomez-Ruiz et al., 2017a, 2017b; Dauchy et al., 2017a, 2017b; Mejia-Avendaño et al., 2017; Boiteux et al., 2017). For example, 6:2 FTAB was found to be the predominant PFAS with concentration ranging from 4 to 45.5 mg/L in raw effluent from a wastewater treatment plant (WWTP), which is located in the north of France and receives the wastewater from four plants and one of them produces fluorotelomer-based products (including 6:2 FTAB and 6:2 FTSA for fire-fighting foams) (Dauchy et al., 2017b). Effluent discharged into a major French river resulted in 6:2 FTAB-contaminated surface water (estimated highest mass flow: 3830 g/day) and drinking water (up to 84 ng/L) (Boiteux et al., 2017). 6:2 FTAB has also been detected in soil from Norwegian airports and the Lac-Mégantic Railway; both of them previously used AFFF products (Moe et al., 2012; Mejia-Avendaño et al., 2017). A study on 6:2 FTAB degradation found that it could transform into 6:2 fluorotelomer alcohol, 6:2 saturated and unsaturated fluorotelomer carboxylic acid, and ultimately short-chain perfluoroalkyl carboxylates in aerobic WWTP sludge (D'Agostino and Mabury, 2017). However, perfluoroalkyl carboxylic acids, as the degradation products of 6:2 FTAB, were detected in the aqueous phase after one month, and even 6:2 FTAB was still retained in substantial quantities of at least 20% of the spike at the end of the 109-day study. These results suggested that 6:2 FTAB biodegrades quite slowly and may be relatively persistent under aerobic biodegradation conditions (D'Agostino and Mabury, 2017). Another study focused on the biological transformation process of 6:2 FTAB in blue mussel and turbot and found its major metabolite was a deacetylated betaine species and the 6:2 fluorotelomer sulfonamide moiety was retained in most of the identified metabolites (Moe et al., 2012).

Although evidence for the presence of 6:2 FTAB in the environment has increased, research assessing the potential effects of this compound has been limited to a single study on Forafac® 1157 (Hagenaars et al., 2011a). It demonstrated that 6:2 FTAB can accumulate in the liver of turbot (*Scophthalmus maximus*) after 14 days of exposure with concentrations ranging from 0.5 to 2.5 μg/g (w.w), inconsistent with results from 6:2 FTSA which found that the accumulation of 6:2 FTSA could be negligible in aquatic organisms (Hoke et al., 2015). The accumulation of 6:2 FTAB in the liver of turbot indicated that it might be harmful to aquatic organisms. Meanwhile, the study also found that Forafac® 1157 exposure influenced the immune system, but did not alter food intake, energy reserves, or growth of turbot, which differs from the effects of PFOS, supporting the notion that Forafac® 1157 was less toxic than PFOS at the organismal level (Hagenaars et al., 2011a). However, the evidence is not adequate to determine whether Forafac® 1157 is an environmentally-friendly PFOS alternative, and further study is necessary to fully evaluate the ecological risks of this fluorosurfactant used in AFFFs.

In recent years, studies have shown that PFOS was harmful to fish, including their reproduction, development, endocrine, and immune systems (Ankley et al., 2005; Huang et al., 2010; Shi et al., 2008; Shi et al., 2009; Zheng et al., 2012). Although 6:2 FTAB contains a betaine moiety, it still retains six perfluorinated carbons in backbone. It has two perfluorinated carbons less than PFOS with eight perfluorinated carbons (Fig. 1). Structurally similar chemicals often have similar effects on biota, and thus it is reasonable to hypothesize that 6:2 FTAB might induce similar developmental toxicity as PFOS. Zebrafish (*Danio rerio*) is a well-established model used for investigating the developmental toxicity of compounds (Embry et al., 2010; Scholz et al., 2008). Thus, to testify this hypothesis, zebrafish embryos were placed into 6-well plates with various concentrations of 6:2 FTAB (0, 5, 10, 20, 40, 60, 80, and 100 mg/L) from 6 to 120 h post-fertilization (hpf) to explore the developmental toxicity of the compound. Various toxicity endpoints, including hatching, survival, malformation, and heart rate, were examined at specific times. For further analysis of the molecular response underlying 6:2 FTAB-induced malformation, TUNEL assay was performed first to visually observe the region of cell apoptosis, and the enzyme activities of caspase 3, 8 and 9 and mRNA expression pattern of *p53*, *bcl2*, *bax*, *apaf1* and *mdm2* were examined. In addition, reactive oxygen species (ROS) and malondialdehyde (MDA) concentrations, anti-oxidative enzyme activities, as well as transcriptional profiles of genes (*il-1β*, *tnfa*, *il-8*, *cxcl-clc*, *ccl1* and *ifn*) that are involved in immune response were investigated to evaluate whether 6:2 FTAB exposure induced oxidative stress and immune toxicity during embryo development. The present study is the first to focus on the effects of 6:2 FTAB on an aquatic organism, and will thus provide insight into the environmental risks of 6:2 FTAB.

2. Materials and methods

2.1. Chemicals and reagents

The 6:2 FTAB ($C_6F_{13}C_2H_4S(O)_2N(H)C_3H_6N(CH_3)_2CH_2CO_2H$; CAS # 34455-29-3, purity > 96%) was provided by Dr. Guo Yong from the Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences. As per Hagenaars et al. (2011a) and Moe et al. (2012), the solid 6:2 FTAB was directly dissolved fish water (3.5 g/L NaCl, 0.05 g/L KCl, 0.1 g/L $CaCl_2$, and 0.025 g/L $NaHCO_3$, pH: 7.2–8.0) and the stock

solution was 100 mg/L (Liu et al., 2015; Westerfield, 1995). Since the compound is well soluble in water, the working solution was obtained by serial dilution with fish water.

2.2. Zebrafish maintenance and embryo collection

Adult wild-type zebrafish (Tuebingen strain) were housed in automatic flow-through feeding aquariums (ESEN, EnvironScience, China). The pH and conductivity of the water were maintained at 7.2–8.0 and 500–580 μS, respectively. All fish were kept at approximately 28 °C with a 14/10 h light/dark photoperiod, following standard zebrafish breeding protocols (Westerfield, 1995). Fishes were fed with live brine shrimp twice a day. A total of ten male and ten female fishes were used in our study. Embryos were obtained by natural spawning of adult fish at a sex ratio of 1:1 in breeding tanks. Embryos were collected from different spawning boxes and washed with fish water. The embryos were observed under an optical microscope (LEICA DFC290, Germany), and their stage was defined according to previous study (Kimmel et al., 1995). Healthy fertilized embryos were selected for subsequent experiments. The embryos were maintained at 28 °C and their ages were defined as hours-post-fertilization (hpf).

2.3. Phenotypic observation

Based on previous studies (Hagenaars et al., 2011a,b) and our primary concentration range studies, zebrafish embryos were exposed to 0, 5, 10, 20, 30, 40, 60, 80, and 100 mg/L of 6:2 FTAB from 6 hpf to determine the median lethal concentration (LC_{50}), bench mark dose (BMD), and BMD lower limit (BMDL) of lethal effect at 96, and 120 hpf, respectively. The LC_{50} values were calculated using Origin 8.5.0 software with a nonlinear curve fit. The values of BMD and BMDL were calculated using benchmark dose software (BMDS) version 2.6 from the USEPA (USEPA, 2012, 2016), with BMDL corresponding to the lower 95% confidence limit of BMD. Five concentrations of 6:2 FTAB (0, 5, 10, 20, and 40 mg/L), which induced obvious malformation during early zebrafish embryo development, were chosen for further study.

The fertilized and healthy embryos were randomly distributed into each well of 6-well plates, and each well contained thirty embryos and 5 mL of test solution. Six wells were used for each treatment group. All exposure experiments were started from 6 hpf and the solution was renewed daily until 120 hpf. Dead embryos were removed in a timely manner. Hatching percentages were recorded from 48 to 72 hpf. Heart rates for 10 s of 30 embryos per group were recorded at 72 hpf (five embryos per replicate and six replicates per treatment). The embryos were identified as dead when coagulation of embryos, failure to develop somites, lack of heartbeat, or non-detachment of the tail from the yolk sac were observed. Abnormal morphological structures, including heart, head, eye, muscle, tail, and swim bladder, were recognized as malformations. The percentage of survival and malformation were recorded and calculated at 12, 24, 36, 48, 60, 72, 96, 108 and 120 hpf. At 96 hpf, embryos were collected from each group to further analyze the molecular mechanism of 6:2 FTAB-induced developmental toxicity.

2.4. TUNEL assay

Cell apoptosis was detected using an *in-situ* cell death detection kit, POD (Roche Diagnostics GmbH, Mannheim, Germany) following Shi et al. (2008). Briefly, embryos were fixed in 4% paraformaldehyde at 4 °C overnight. After washing with phosphate-buffered saline (PBS), 3% hydrogen peroxide was added to block endogenous peroxidases. After incubation for 15 min at room temperature, the zebrafish embryos were rinsed using PBS and then incubated with TUNEL reaction mixture at 37 °C for 60 min without light. Subsequently, the embryos were rinsed with PBS and transferred to converter POD solution. After incubation for 30 min at 37 °C, the embryos were again washed with PBS. The embryos were then placed in diaminobenzidine (DAB) solution for 30–90 min to stain the apoptotic regions brown. The results were photographed using an optical microscope (LEICA DFC290, Germany).

2.5. Determination of caspase-3, -8, and -9 activities

The activities of caspase-3, -8, and -9 were measured using corresponding caspase assay kits (Beyotime Institute of Biotechnology, Nantong, China). Briefly, the zebrafish embryos were homogenized on ice in a lysis buffer provided in the kit. After centrifugation at 12000*g* at 4 °C for 15 min, 50 μL of supernatant was placed in 96-well plates, with 40 μL of reaction buffer and 10 μL of caspase substrate then added. The mixture was incubated at 37 °C for 3 h without light. Caspase-3, -8, and -9 can change the substrate to *p*-nitroaniline (*p*NA). The fluorescence intensity of *p*NA was detected using a microplate reader (Gen 5, BioTek Instruments, Inc., Winooski, VT, USA) at 405 nm. The enzyme activity was calculated based on the fluorescence intensity of *p*NA. Protein concentrations were determined using a BCA protein assay kit (Tiangen Company, China). Each group included six biological replicates.

2.6. ROS, antioxidant enzyme assays, and lipid peroxidation assessment

The content of ROS in embryos exposed to 6:2 FTAB (0, 5, 10, 20, and 40 mg/L) was analyzed at 96 hpf using dichlorofluorescein-diacetate (DCF-DA), as described in Zeng et al. (2014). The embryos were washed using cold PBS, and then homogenized in cold buffer. After centrifugation at 15000*g* at 4 °C for 20 min, 20 μL of supernatant was added to one well of a 96-well plate, after which 100 μL of PBS and 8.3 μL of DCF-DA solution (10 mg/mL) were added to the corresponding well. The mixture was incubated at 37 °C for 2 h without light. The fluorescence intensity was measured using a microplate reader (Gen 5, BioTek Instruments, Inc., Winooski, VT, USA) with excitation and emission at 485 and 530 nm, respectively. Protein concentrations were determined as described above. The ROS level was given as a percentage of the control. Each group included six biological replicates.

At 96 hpf, zebrafish embryos were collected from each group and rinsed with cold PBS. Each group included six biological replicates. The samples were homogenized in cold buffer. After centrifugation at 2500 × rpm for 10 min, the supernatant was collected for further analysis of biochemical parameters. Lipid peroxidation was assessed by measuring the amount of MDA using a trace MDA assay kit (Jiancheng, Nanjing, China). The activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were determined using assay kits (Jiancheng, Nanjing, China). Assays were performed following the manufacturer's recommendations. Protein concentrations were analyzed as described above.

2.7. Gene expression

Embryos were collected at 96 hpf and washed three times with PBS. Total RNA of 30 homogenized zebrafish embryos was extracted using TRIzol reagent (Ambion, Life Technologies, USA) following the manufacturer's instructions. The RNA concentration of each sample was detected using a UV1240 spectrophotometer (Shimadzu, Japan). The cDNA was synthesized via reverse transcription (RT) using an oligo-$(dT)_{15}$ primer and M-MLV reverse transcriptase (Promega, Madison, USA) per the manufacturer's instructions. Real-time PCR was performed with the Stratagene Mx3000P q-PCR system (Stratagene, USA). The SYBR Green Real Master Mix (Tiangen, China) was used for quantification of gene expression.

The transcriptional level of *β-actin* was first analyzed, and its mRNA expressions were not changed after 6:2 FTAB exposure. Thus, *β-actin* was used as an internal control in our study. In order to evaluate the effect of 6:2 FTAB on cell apoptosis, immune systems, the transcriptional level of genes (*p53*, *apaf1*, *bcl2*, *bax*, *mdm2*, *il-1β*, *tnfα*, *il-8*, *ccl1*, *cxcl-clc*, and *ifn*) were analyzed, and the primer sequences were obtained from Deng et al. (2009) and Jiang et al. (2014). Each group

included six biological replicates. The fold change of target genes was analyzed using the $2^{-D\Delta Ct}$ method (Pfaffl, 2001).

2.8. Statistical analysis

All results obtained were statistically analyzed using SPSS for Windows 17.0 software (SPSS Inc., Chicago, IL, USA). Normality and homogeneity of data were examined using the Kolmogorov-Smirnow and Levene's tests, respectively. One-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) tests were used as post hoc tests for determining differences between groups. All experimental data were represented as means with standard errors (mean ± SE). *$p < 0.05$ indicates significant difference between the control and exposure groups.

3. Results

3.1. Developmental toxicity

Embryos were exposed to various concentrations of 6:2 FTAB from 6 to 120 hpf. Toxicity endpoints, including hatching, survival, malformation, and heart rate of zebrafish embryos, were recorded and calculated at specific time points (Fig. 2). As shown in Fig. 2A, exposure to 5, 10, and 20 mg/L of 6:2 FTAB did not induce significant embryo death from 6 to 120 hpf. The survival percentage was reduced in a concentration-dependent manner in the 40 and 100 mg/L 6:2 FTAB-treated groups after 24 hpf, with 100% embryo mortality at 72 hpf following 80 and 100 mg/L 6:2 FTAB exposure, and 100% mortality at 120 hpf following 60 mg/L exposure. At 72, 96 and 120 hpf, the LC_{50} values were 67.12 ± 3.82, 64.39 ± 4.23 and 43.73 ± 3.24 mg/L, respectively, the BMD lethal effect values were 56.32, 53.90, and 34.99 mg/L, respectively, and the BMDL values were 55.87, 53.33, and 33.79 mg/L, respectively (Table 1). Because lethal toxicity was high, the higher exposure concentrations (60, 80, and 100 mg/L) were not used for further analyses.

In the control groups, over 90% of embryos hatched from the chorion at 72 hpf (Fig. 2B). In the 6:2 FTAB-treated groups, hatching started from 48 hpf, and the hatching percentage decreased in a concentration-dependent manner at 54 and 60 hpf (Fig. 2B). However, 6:2 FTAB exposure had no effect on hatching percentage, and live embryos hatched in all groups at 72 hpf (Fig. 2B).

The embryos developed normally in the control group. Malformations first appeared after 72 hpf in the 40 mg/L 6:2 FTAB-treated group (Fig. 2C). Following 6:2 FTAB exposure, the malformation percentage increased in the higher concentration groups (20 and 40 mg/L) (Fig. 2C). The main morphological defect occurred in the tail region, with the formation of rough-edged skin/fins in the 20 and 40 mg/L 6:2 FTAB-treated groups (Fig. 2D). In addition, about 10% and 23.3% of embryos exhibited the malformation type of uninflated swim bladders in 20 and 40 mg/L 6:2 FTAB treated groups at 120 hpf, respectively (Fig. 2D). The heartbeat of embryos was not changed significantly after 6:2 FTAB exposure at 72 hpf, even in 40 mg/L treated groups (Fig. 2E).

3.2. Effect of 6:2 FTAB on cell apoptosis

3.2.1. TUNEL assay

TUNEL assay was used to detected whether 6:2 FTAB exposure induced cell apoptosis during zebrafish embryogenesis. At 96 hpf, no obvious apoptotic cells were detected in the control group (Fig. 3). In the 40 mg/L 6:2 FTAB-treated embryos, however, a large number of apoptotic cells were observed in the tail area (Fig. 3).

3.2.2. Expression of genes related to apoptosis

To further analyze the possible molecular response involved in 6:2 FTAB-induced apoptosis, the transcriptional levels of genes that were related to cell apoptosis were detected in 96 hpf zebrafish embryos (Fig. 4). The transcriptional levels of *p53* and *apaf1* were significantly up-regulated (1.3- and 1.22-fold, respectively) in the 40 mg/L 6:2 FTAB-treated group at 96 hpf relative to the control groups (Fig. 4). Similarly, the transcriptional level of *bax* was up-regulated in the 20 and 40 mg/L-treated groups (1.37- and 1.54-fold, respectively), whereas *bcl2* expression showed no significant change in any treatment group (Fig. 4). The transcriptional level of *mdm2* increased 1.5-, 1.3-, and 1.48-fold in the 10, 20, and 40 mg/L 6:2 FTAB-treated groups, respectively (Fig. 4).

3.2.3. Caspase activity

The activities of caspase-3, -8, and -9 were detected in the zebrafish embryos at 96 hpf. As shown in Fig. 5, there were no significant differences in caspase activities between the 5 mg/L 6:2 FTAB-treated group and the control, but caspase enzyme activities increased with 10, 20 and 40 mg/L 6:2 FTAB exposure. In the 40 mg/L 6:2 FTAB-treated group, the activities of caspase-3, -8, and -9 increased by 143.1%, 138.2%, and 146.4%, respectively, compared with the control.

3.3. Effect of 6:2 FTAB on oxidative stress

As shown in Fig. 6, 6:2 FTAB exposure induced a concentration-dependent increase in ROS and MDA levels in zebrafish embryos at 96 hpf. Compared with the control, ROS and MDA levels were significantly higher in the 20 and 40 mg/L exposure groups, with a slight increase also appearing in the lower exposure group. 6:2 FTAB exposure exhibited different effects on the antioxidant enzyme activities (SOD, CAT, and GPx) (Fig. 6). SOD activity in embryos was unchanged following 6:2 FTAB exposure. Compared with the control, CAT activity increased 2.2-, 2.5-, 2.7-, and 3.0-fold in the 5, 10, 20, and 40 mg/L 6:2 FTAB-treated groups, respectively. A slight but significant decrease in GPx activity was observed the 5, 20, and 40 mg/L 6:2 FTAB-treated embryos.

3.4. Effect of 6:2 FTAB on the immune system

At 96 hpf, the transcriptional levels of genes involved in the immune system, including *ccl1*, *il-1β*, *il-8*, *cxcl-c1c*, *tnfα*, and *ifn*, were detected in zebrafish embryos (Fig. 7). The transcriptional level of *ccl1* was down-regulated in a concentration-dependent manner and was 0.76-, 0.72-, 0.62-, and 0.47-fold of the control in the 6:2 FTAB-treated groups, respectively. The transcriptional levels of *il-1β*, *il-8*, and *tnfα* were significantly up-regulated after exposure to 10, 20, and 40 mg/L of 6:2 FTAB. Compared with the control, significant increases in *cxcl-c1c* and *ifn* mRNA expression (3.36- and 1.7-fold, respectively) were observed after exposure to 40 mg/L of 6:2 FTAB.

4. Discussion

6:2 FTAB is the main component of Forafac®1157, which is used as a PFOS alternative and fluorosurfactant in AFFFs worldwide. Since the 3 M Company phased out PFOS production, the application of 6:2 FTAB has increased, with its subsequent detection in the environment and predominance in final effluent from WWTPs. Effluent from WWTPs is a major route of PFASs, including 6:2 FTAB, into aquatic environments. To date, however, only one study has focused on the effect of 6:2 FTAB on marine fish (Hagenaars et al., 2011a).

We attempted to ascertain whether 6:2 FTAB is toxic in the early developmental stages of zebrafish, and to elucidate its potential mechanism. The 72 hpf LC_{50} for 6:2 FTAB was 67.12 mg/L. This value was similar to PFOS (68 mg/L provided by Zheng et al., 2012). The 96- and 120-hpf LC_{50} values for 6:2 FTAB were 64.39 and 43.73 mg/L, respectively, similar to those of PFOS (58.47 mg/L at 96 hpf and 28.21 mg/L at 120 hpf provided by Hagenaars et al., 2011b; and 71 mg/L at 96 hpf provided by Ye et al., 2009), but higher than the 120 h LC_{50}

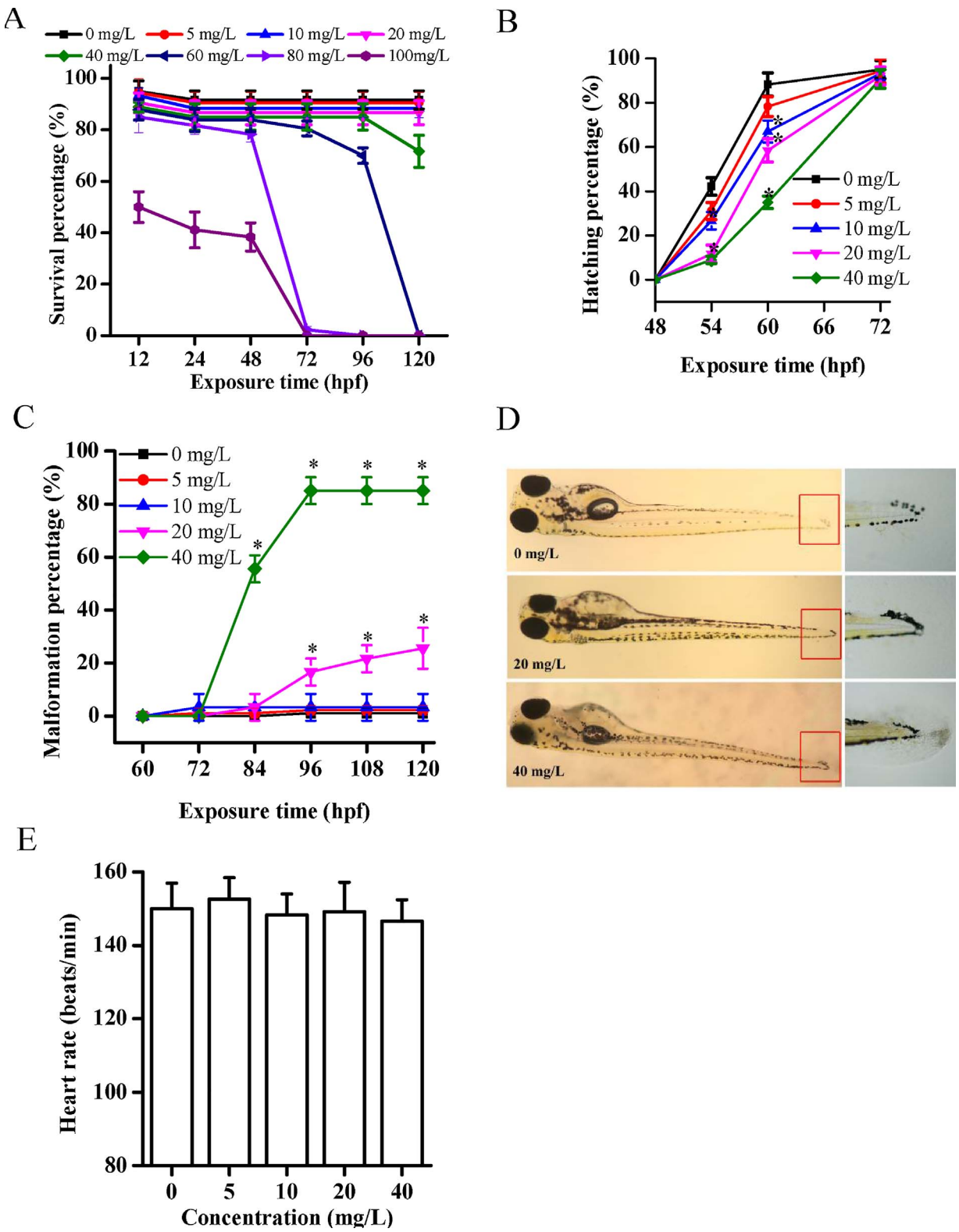

Fig. 2. Developmental endpoints of zebrafish embryos following exposed to 6:2 FTAB from 6 to 120 hpf. (A) Survival percentage from 12 to 120 hpf. (B) Hatching percentage from 48 to 72 hpf, (C) Malformation percentage from 60 to 120 hpf. (D) Microscope photographs of zebrafish larvae at 120 hpf. (E) Heart rate at 72 hpf. Values are given as means ± SE (n = 6), and the asterisk represents a statistically significant difference compared with the control (one-way ANOVA, followed by a post hoc test: LSD: *p < 0.05).

value of PFOS provided by Huang et al. (2010) (2.2 mg/L at 120 hpf). The BMDL levels were 53.33 and 33.79 mg/L at 96 hpf and 120 hpf, respectively, which were higher than those determined for PFOS (10 and 0.1 mg/L, respectively; Hagenaars et al., 2011b). Furthermore, the predominant morphological defect observed in the 6:2 FTAB-treated embryos was rough-edged skin/fins, with only a few embryos also exhibiting uninflated swim bladders. These malformations have appeared in zebrafish embryos exposed to all tested PFASs (Hagenaars et al., 2011b; Ankley et al., 2005; Shi et al., 2008; Huang et al., 2010; Liu et al., 2015). However, different from PFOS exposure, pericardial and

Table 1
The values of LC_{50}, BMD and BMDL of lethal effect in 6:2 FTAB-treated embryos at 72, 96 and 120 hpf. The embryos were exposed started from 6 hpf, and the test solution was refreshed daily.

	LC_{50} (mg/L)	BMD (mg/L)	BMDL (mg/L)
72 hpf	67.12 ± 3.82	56.32	55.87
96 hpf	64.39 ± 4.23	53.90	53.33
120 hpf	43.73 ± 3.24	34.99	33.79

yolk sac edemas were not detected after exposure to 6:2 FTAB, and heart rates were not significantly changed, even at relatively high concentrations. These results support that 6:2 FTAB toxicity is lower than that of PFOS to zebrafish embryos, consistent with previous research (Huang et al., 2010).

Effects on tails and swim bladders appear to be a general outcome in zebrafish embryos after PFAS exposure, and the possible molecular mechanisms that result in tail malformations have been explored in zebrafish embryos after PFOS exposure (Hagenaars et al., 2011b; Shi et al., 2008). Previous studies have demonstrated that cell apoptosis is a plausible explanation for tail malformations caused by PFOS (Shi et al., 2008). Therefore, we used TUNEL assay to analyze cell apoptosis in 6:2 FTAB-treated embryos. Results showed that 40 mg/L of 6:2 FTAB exposure induced obvious apoptosis in zebrafish embryos and apoptotic cells mainly appeared in the tail area. Stress-induced cell apoptosis is a major factor accounting for abnormal development during embryonic growth in zebrafish (Cohen, 1997; Yamashita, 2003). Thus, cell apoptosis might explain the observed tail malformations of embryos after 6:2 FTAB exposure. To further analyze the underlying molecular response, the transcriptional levels of several genes related to cell apoptosis, including *p53*, *apaf1*, *bax*, and *bcl2*, were analyzed in zebrafish embryos after 6:2 FTAB exposure at 96 hpf. The transcriptional levels of *p53* and *apaf1* were up-regulated, whereas the *Bcl2/Bax* expression ratio decreased in the 40 mg/L 6:2 FTAB-treated group. DNA damage or cells under stress can trigger the expression of tumor suppressor gene *p53* (Langheinrich et al., 2002). When P53 is translocated to the nucleus, the expression of pro-apoptotic gene *Bax* is activated, whereas that of anti-apoptotic gene *Bcl2* is suppressed (Langheinrich et al., 2002). A

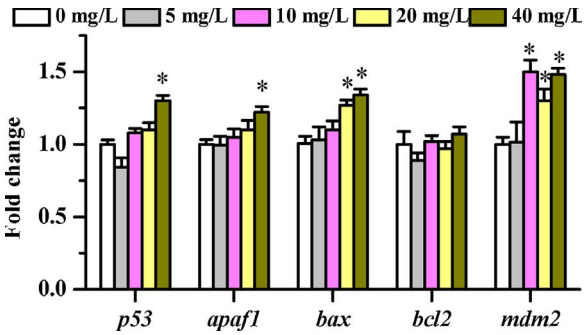

Fig. 4. Transcriptional levels of *p53*, *apaf1*, *bax*, *bcl2*, and *mdm2* in zebrafish larvae exposed to different 6:2 FTAB concentrations (0, 5, 10, 20, and 40 mg/L) until 96 hpf. Values are given as means ± SE (n = 6), and the asterisk represents a statistically significant difference compared with the control (one-way ANOVA, followed by a post hoc test: LSD: *$p < 0.05$).

decrease in the *Bcl2/Bax* expression ratio can result in the release of cytochrome c from mitochondria into cytosol, which triggers oligomerization of *apaf1* and caspase-9 and catalysis of downstream caspase-3 (Yamashita, 2003; Gross et al., 1999; Desaghera, 2000). Caspase-3 carries out one molecular step of programmed cell death (Yamashita, 2003). In our study, the activities of caspase-9 and −3 were increased after 6:2 FTAB exposure. We also analyzed the effects of 6:2 FTAB on another apoptotic pathway, the extrinsic death receptor pathway, involving the TNF superfamily, TNFR, Fas, and caspase-8 (Fulda and Debatin, 2006). The transcriptional level of *tnfα* and the activation of caspase-8 increased in 10, 20, and 40 mg/L 6:2 FTAB-treated groups. Taken together, these results show that 6:2 FTAB-induced cell apoptosis was likely through *p53* and *tnfα* activation, leading to caspase-dependent cell apoptosis, and thus embryonic malformation in the tail region.

ROS-induced oxidative stress can induce cell apoptosis during zebrafish embryogenesis (Yamashita, 2003). Exposure to PFASs (e.g., PFOS, perfluorooctanoic acid, perfluorododecanoic acid and perfluorononanoic acid) can trigger the generation of ROS in mammalian liver cells (Hu and Hu, 2009), chicken embryos (Zhao et al., 2017), zebrafish livers (Liu et al., 2015), and zebrafish embryos (Liu et al.,

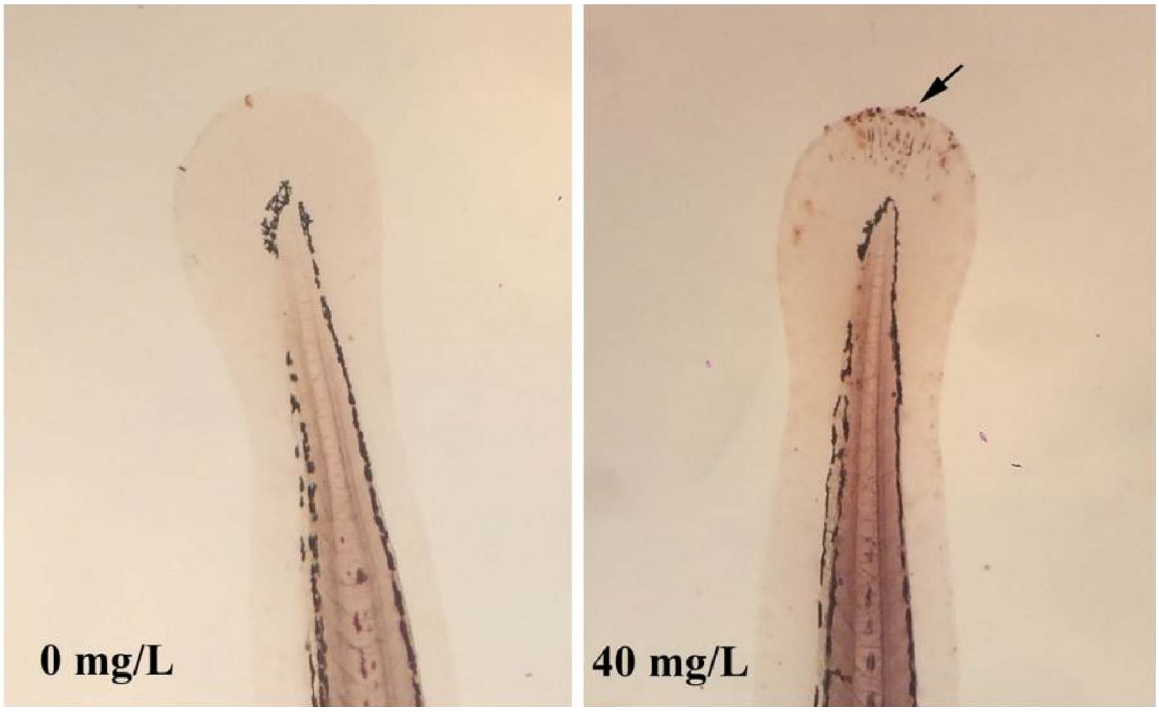

Fig. 3. Detection of apoptosis in zebrafish embryos at 96 hpf using TUNEL assay. Apoptotic cells were found in the embryos, with the highest numbers in the tail region (arrow).

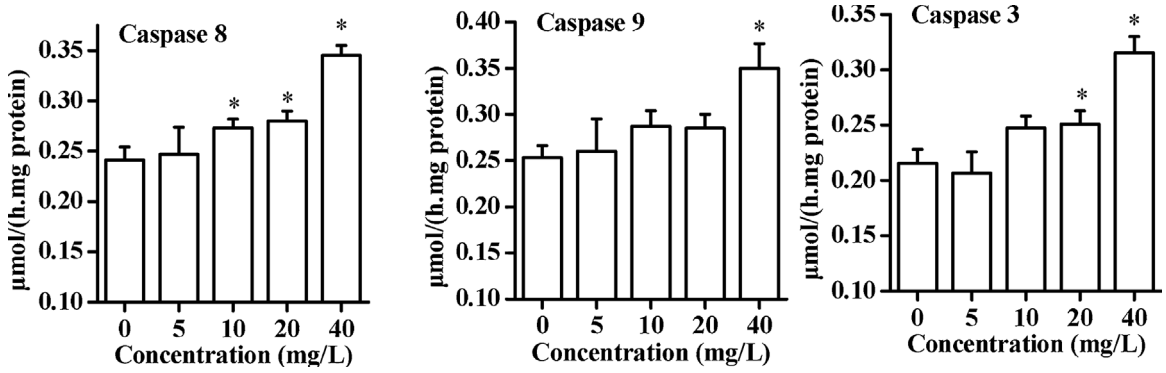

Fig. 5. Activities of caspase-3, -8, and -9 in zebrafish embryos after exposure to various concentrations of 6:2 FTAB for 96 hpf. Values are given as means ± SE (n = 6), and the asterisk represents a statistically significant difference compared with the control (one-way ANOVA, followed by a post hoc test: LSD: *p < 0.05).

2015; Shi and Zhou, 2010). We found that 6:2 FTAB exposure increased ROS levels in the zebrafish, indicating that the embryos suffered from serious oxidative stress. MDA is the product of the reaction between free radicals and unsaturated fatty acids in cellular membranes, and was recognized as an important parameter in evaluating the level of oxidative stress in organisms (Zhao et al., 2013). The concentration-dependent increase in MDA in zebrafish embryos exposed to 6:2 FTAB in our study also indicated that the embryos sustained serious oxidative stress. Antioxidative enzyme activity plays an important role in protecting cells from oxidative stress. SOD can convert superoxide radical O_2^- to H_2O_2, thus preventing lipid peroxidation (Pi et al., 2010). Furthermore, CAT and GPx can transform H_2O_2 into oxygen and water (Pi et al., 2010). In a previous study, PFOS exposure increased the activities of SOD, CAT, and GPx to protect zebrafish embryos from oxidative stress (Shi and Zhou, 2010). In our study, a slight but non-

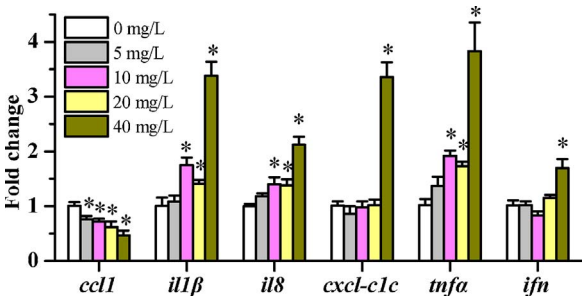

Fig. 7. Transcription of genes related to innate immunity in zebrafish after exposure to various concentrations of 6:2 FTAB until 96 hpf. Values are given as means ± SE (n = 6), and the asterisk represents a statistically significant difference compared with the control (one-way ANOVA, followed by a post hoc test: LSD: *p < 0.05).

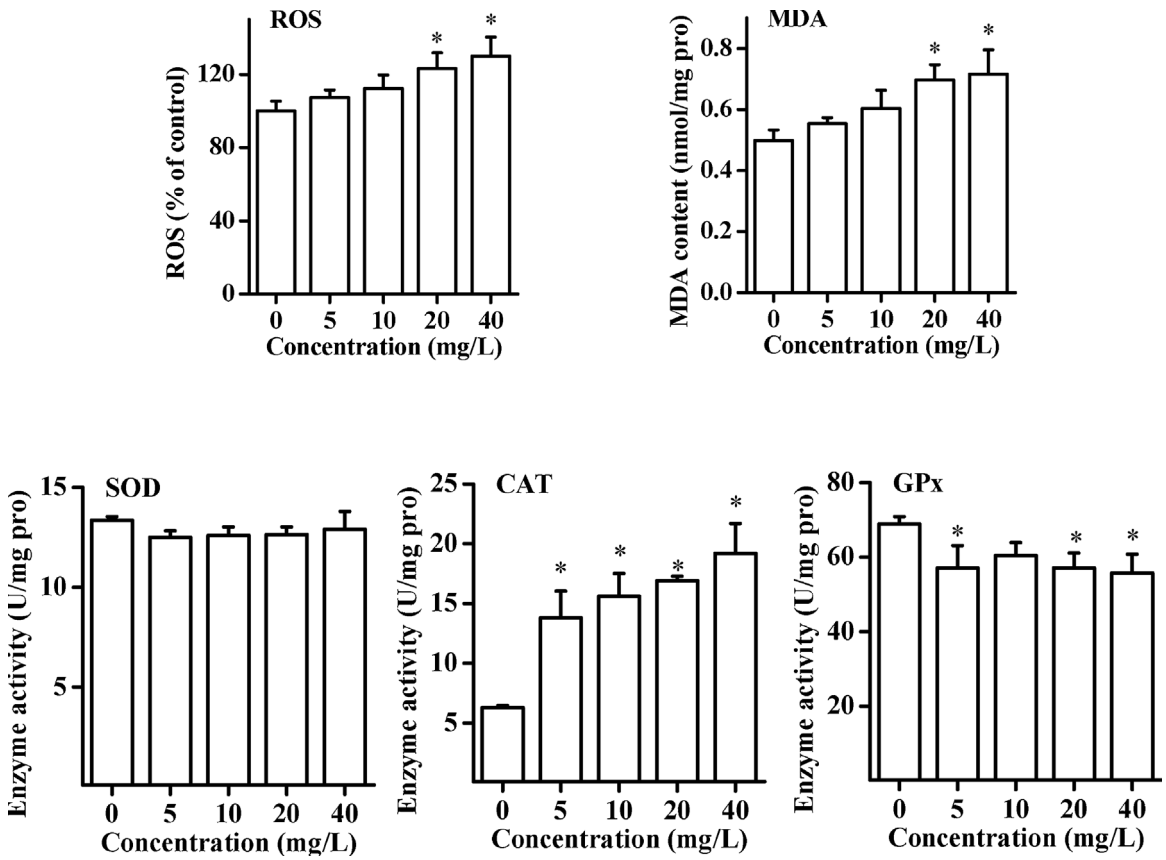

Fig. 6. Oxidative stress in zebrafish embryos exposed to different concentrations of 6:2 FTAB at 96 hpf. Values are given as means ± SE (n = 6), and the asterisk represents a statistically significant difference compared with the control (one-way ANOVA, followed by a post hoc test: LSD: *p < 0.05).

significant decrease in SOD activity was observed in zebrafish embryos exposed to 6:2 FTAB, which probably resulted in the excessive production of free radicals or increased the probability of ROS scavenging by other antioxidant enzymes (Azooz et al., 2012). We also determined that CAT activity was increased in all 6:2 FTAB-treated groups, whereas GPx activity decreased. Based on the above results, 6:2 FTAB exposure enhanced ROS generation and changed antioxidative defense, but the antioxidative capacity was insufficient to clean excessive production of ROS, further resulting in lipid peroxidation and oxidative damage.

Earlier research found that Forafac®1157 exposure altered the transcriptional levels of genes related to immune responses and altered leukocyte profiles (Hagenaars et al., 2011a). Our study also analyzed the transcriptional levels of immune-related genes to explore the possible response for 6:2 FTAB-induced immunotoxicity. The cytokines and chemokines secreted by immune cells play important roles in regulating immune responses (Sieger et al., 2009; Trede et al., 2004). For example, IL-1β is critical for activating neutrophils and macrophages at the site of injury (Dinarello, 1996); TNFα plays an important role in the normal function of T cells, natural killer cells, macrophages, and dendritic cells (So et al., 2006); IL-8, CXCL-C1C, and CCL1 mediate immune and inflammatory responses (Baggiolini et al., 1994). The transcriptional level of immune-related genes can be induced or suppressed in organism after environmental chemical exposure (Corsini et al., 2011; Eder et al., 2008; Jin et al., 2011; Mollenhauer et al., 2010; Pressley et al., 2005). In the present study, the transcriptional levels of *il-1β*, *tnfα*, *il-8*, *cxcl-clc*, and *ifn* were increased in the higher 6:2 FTAB concentration groups, indicated that 6:2 FTAB might induce immune response during zebrafish embryonic development. It has been demonstrated that cell apoptosis could destroy immune cells and decrease the defensive ability of immune system (Battaglia et al., 2010; Roh et al., 2011). It was possible that the zebrafish embryos might increase the transcriptional level of immune-related genes to deal with the 6:2 FTAB-induced cell apoptosis, but the specific relationship between cell apoptosis and immune response need further study.

In conclusion, this study is the first to assess the developmental toxicity of 6:2 FTAB using zebrafish embryos. Results showed that 6:2 FTAB exposure caused an increase in malformation percentage and decrease in survival percentage during 6–120 hpf. Based on the LC_{50}, BMD, and BMDL values at 96 and 120 hpf, 6:2 FTAB appeared to be less toxic than PFOS. The possible molecular responses underlying 6:2 FTAB exposed zebrafish embryos was explored at 96 hpf. Results showed that 6:2 FTAB exposure up-regulated the transcriptional levels of certain genes involved in the apoptosis signaling pathway, and then stimulated the activities of caspase-3, −8, and −9. Accumulation of ROS and MDA and changes in antioxidant enzyme (CAT and GPx) activity were observed in the 6:2 FTAB-treated groups. Moreover, the transcriptional levels of several genes involved in the immune system increased following 6:2 FTAB exposure. Taken together, our data demonstrated that 6:2 FTAB had the potential to induce oxidative stress, apoptosis, and immunotoxicity and increased the malformation and death of embryos during zebrafish embryonic development. Although the exposure concentrations in our study were similar to those observed in WWTP effluent (up to 45.5 mg/L), they were much higher than the concentrations of PFASs in surface water, which generally range from pg/L to μg/L (Boiteux et al., 2017). Thus, the long-term effects of 6:2 FTAB at environmentally relevant concentrations need to be explored in future studies.

Competing financial interests

The authors declare no conflicts of interest.

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