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Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS

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Abstract

Perfluorooctanesulfonate (PFOS) is a persistent organic pollutant, the potential toxicity of which is causing great concern. In the present study, we employed zebrafish embryos to investigate the developmental toxicity of this compound. Four-hour post-fertilization (hpf) zebrafish embryos were exposed to 0.1, 0.5, 1, 3 and 5 mg/L PFOS. Hatching was delayed and hatching rates as well as larval survivorship were significantly reduced after the embryos were exposed to 1, 3 and 5 mg/L PFOS until 132 hpf. The fry displayed gross developmental malformations, including epiboly deformities, hypopigmentation, yolk sac edema, tail and heart malformations and spinal curvature upon exposure to PFOS concentrations of 1 mg/L or greater. Growth (body length) was significantly reduced in the 3 and 5 mg/L PFOS-treated groups. To test whether developmental malformation was mediated via apoptosis, flow cytometry analysis of DNA content, acridine orange staining and TUNEL assay was used. These techniques indicated that more apoptotic cells were present in the PFOS-treated embryos than in the control embryos. Certain genes related to cell apoptosis, *p53* and *Bax*, were both significantly up-regulated upon exposure to all the concentrations tested. In addition, we investigated the effects of PFOS on marker genes related to early thyroid development (*hhex* and *pax8*) and genes regulating the balance of androgens and estrogens (*cyp19a* and *cyp19b*). For thyroid development, the expression of *hhex* was significantly up-regulated at all concentrations tested, whereas *pax8* expression was significantly up-regulated only upon exposure to lower concentrations of PFOS (0.1, 0.5, 1 mg/L). The expression of *cyp19a* and of *cyp19b* was significantly down-regulated at all exposure concentrations. The overall results indicated that zebrafish embryos constitute a reliable model for testing the developmental toxicity of PFOS, and the gene expression patterns in the embryos were able to reveal some potential mechanisms of developmental toxicity.

Keywords: PFOS; Developmental toxicity; Gene expression; Apoptosis; Embryo; Zebrafish

Introduction

Perfluorinated chemicals (PFCs) have been used in a variety of commercial and industrial applications for over 50 years, including in protective coatings for food packaging, textiles, and carpets and in insecticides. The widespread use of these compounds has resulted in their global distribution and detection in the environment, wildlife and humans (e.g., Giesy and Kannan, 2001; Kannan et al., 2002; So et al., 2004, 2006; Dai et al., 2006; Yeung et al., 2006; Kärrman et al., 2007; Zhao et al., 2007; for reviews, see Houde et al., 2006; Lau et al., 2007). Among PFCs, perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) have been routinely measured and found to be the predominant compounds in environmental matrices, wildlife and human populations, and thus have been extensively studied (Giesy and Kannan, 2001; Hansen et al., 2001; So et al., 2006; Yeung et al., 2006). PFOS is an end product of the metabolic breakdown or environmental degradation of many other PFCs and is not itself subject to environmental degradation or metabolic converion. The environmental persistence, bioaccumulative tendency and potential toxicity of PFOS have generated great concern.

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In the aquatic environment, PFOS concentrations are generally low. However, environmental monitoring studies showed that PFOS can bioaccumulate through the food chain (Kannan et al., 2005). As a consequence, higher concentrations of PFOS have been detected in a variety of fish species. For example, PFOS concentrations in the liver of smallmouth bass (*Micropterus dolomieu*) and largemouth bass (*Micropterus salmoides*) from New York State in the United States ranged from 9 to 315 ng/g wet weight, and the average concentrations of PFOS in the fish were 8850-fold greater than those in surface water (Sinclair et al., 2005). Likewise, higher concentrations of PFOS have been detected in the liver (7760 ng g^{-1} wet weight) of plaice (*Pleuronectes platessa*) (Hoff et al., 2003) and feral gibel carp (*Carassius auratus gibelio*) (up to 9031 ng g^{-1} wet weight) in Belgium (Hoff et al., 2005). It is worth noting that high concentrations of PFOS were also detected in fish eggs (145–381 ng/g) in lake whitefish (*Coregonus clupeaformis*) from Michigan waters in the United States (Kannan et al., 2005), as well as in the eggs of fathead minnows (*Pimephales promelas*) exposed to PFOS in the laboratory (Ankley et al., 2005), which suggests oviparous transfer of this compound.

Fish, and in particular small freshwater fish species such as the zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*) and fathead minnow (*P. promelas*), have been used for acute and chronic tests to investigate the toxic properties of chemicals and for ecotoxicological regulatory purposes. Zebrafish are also proving increasingly useful for investigating the developmental toxicity of chemical exposure during early life stages, using survivorship and development as well as gene expression as endpoints. Fish embryo assays are considered pain-free *in vivo* tests and are therefore accepted as a replacement for other types of animal experiments (Voelker et al., 2007). Acute toxicity endpoints, such as morphological alternations, hatching and survivorship, do not provide information on the mechanism of action leading to toxic effects; however, using gene expression patterns as endpoints may result in a greater mechanistic understanding of toxicity, as examining effects on the molecular level is likely to provide highly sensitive, mechanism-based and potentially predictive molecular biomarkers (Voelker et al., 2007).

The zebrafish embryo is a useful research model because it is small in size and transparent, and easy to maintain and handle, and the species has high fecundity, rapid embryogenesis and continuous reproduction. In addition, the zebrafish genome has been sequenced and genetic information is rapidly accumulating (http://zfin.org), which places this freshwater fish in a privileged position for toxicological studies (Berry et al., 2007). Therefore, it is feasible to select toxicological endpoints to find the genes that may be involved in toxicant exposure. Recently, zebrafish embryos have been employed for rapid and high-throughout screening of compounds for developmental toxicity and mechanisms of toxicant exposure, including β-naphthoflavone (Voelker et al., 2007), polybrominated diphenyl ethers (Lema et al., 2007), algal toxins (Berry et al., 2007), dioxins (Bello et al., 2004), polycyclic aromatic hydrocarbons (Incardona et al., 2005), pesticides (Stehr et al., 2006), heavy metals (Chan and Cheng, 2003; Linbo et al., 2006) and carbon nanotubes (Cheng et al., 2007).

Despite extensive studies on the widespread distribution of PFOS in the environment, little is known about its potential developmental toxicity in fish. In the present study, zebrafish embryos were employed to investigate the developmental toxicity induced by PFOS exposure. We selected different endpoints, such as morphology, hatching rates, survivorship of the larvae and apoptosis, as well as certain gene expressions. However, the endpoints employed in this study (e.g., hatching rates, malformation, larvae survival) may provide low sensitivity compared to effects detected during chronic exposure, and these endpoints generally do not provide any information on the mode of action leading to toxic effects. Alternatively, gene expression approaches are likely to identify highly sensitive, mechanism-based markers that may also have the potential to indicate long-term detrimental effects (Corvi, 2002; Voelker et al., 2007). Therefore, genes that are involved in apoptosis (i.e., *p53* and *Bax*) and genes that play critical roles in zebrafish early thyroid development (i.e., *hhex* and *pax8*) were investigated. To evaluate whether the expression of genes involved in steroid homoeostasis was altered during early development by PFOS exposure, we also studied *cyp19*. The *cyp19* gene encodes aromatase and plays an important role in steroid homoeostasis by converting androgens to estrogens.

Materials and methods

Chemicals

PFOS of >99% purity was obtained from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan) and a stock solution was prepared by dissolving it in dimethyl sulfoxide (DMSO). Ethidium bromide (EB), propidium iodide (PI) and MS-222 were obtained from Sigma (St. Louis, MO, USA). Acridine orange (AO) was purchased from Amresco (Solon, OH, USA). All other chemicals used in this study were analytical grade.

Zebrafish maintenance and embryo toxicity test

Wild-type (AB strain) zebrafish were maintained at 28±0.5 °C in a 14:10 h light:dark cycle in a closed flow-through system in charcoal-filtered tap water. The fish were fed with *Artemia nauplii* twice daily. Zebrafish embryos were obtained from spawning adults in groups of about 20 males and 10 females in tanks overnight. Spawning was induced in the morning when the light was turned on. At 4–5 h post-fertilization (hpf), embryos were examined under a dissecting microscope, and those embryos that had developed normally and reached the blastula stage were selected for subsequent experiments. Briefly, 30 normal embryos were randomly distributed into beakers containing 50 ml of exposure solution (0, 0.1, 0.5, 1, 3 and 5 mg/L PFOS), containing 0.2 mM Ca $(NO_3)_2$, 0.13 mM $MgSO_4$, 19.3 mM NaCl, 0.23 mM KCl and 1.67 mM HEPES (Westerfield, 1995). The range of concentrations was selected based on our previously ascertained range-finding studies which identified a concentration to induce clear phenotypic effects in short time exposure period, and information from the available literatures (Ankley et al., 2005; Kannan et al., 2005). The control and exposure embryos received 0.005% DMSO. Three replicates for each concentration were used and each replicate consisted of a glass beaker containing 50 ml of the respective treatment solutions and 30 viable embryos. Mortality was identified by coagulation of the embryos, missing heartbeat, failure to develop somites and a non-detached tail. Sublethal endpoints included embryo malformation, heart rate, hatching success and mortality. During the 132 h exposure, embryos and larvae were examined under a stereomicroscope to screen for morphological abnormalities, and survival rates were recorded within each treatment. Mortality was recorded at 12, 24, 36, 48, 60, 72, 84, 96, 108, 120 and 132 hpf. Likewise, additional experiments were carried out where embryos were exposed for heart rates, length measurement, apoptosis and gene