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# Chronic PFOS Exposure Disrupts Thyroid Structure and Function in Zebrafish

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## Abstract

Perfluorooctane sulfonic acid (PFOS), as a potential endocrine disrupting chemical, is widely detected in the environment, wildlife and human. Currently few studies have documented the effects of chronic PFOS exposure on thyroid in aquatic organisms and the underlying mechanisms are largely unknown. The present study assessed the effect of chronic PFOS exposure on thyroid structure and function using zebrafish model. Zebrafish at 8 h post fertilization (hpf) were exposed to PFOS (250 μg/l) until 120 d post fertilization (dpf). Thyroid hormone ($T_3$ and $T_4$) level, thyroid morphology and thyroid function related gene expression were evaluated in zebrafish at 120 dpf. Our findings demonstrated that chronic PFOS exposure altered thyroid hormone level, thyroid follicular cell structure and thyroid hormone related gene expression, suggesting the validity of zebrafish as an alternative model for PFOS chronic toxicity screening.

**Keywords** Zebrafish · PFOS · Thyroid hormone · Thyroid morphology · Gene expression

Perfluorooctane sulfonic acid (PFOS), one of the two primary perfluoroalkyl acids, is environmentally and biologically stable and used widely in industrial and household applications (Lau et al. 2009). As a consequence, PFOS has been detected in the environment, wildlife and human tissues and it has recently emerged as a group of persistent organic pollutants (Wang et al. 2011b).

PFOS has been shown to cause developmental toxicity, reproductive toxicity, hepatotoxicity, immunotoxicity, endocrine disruption and neurotoxicity in mammalian and aquatic species (Austin et al. 2003; Hagenaars et al. 2008; Wang et al. 2011a). It has also been linked to a number of human diseases such as metabolic disorders, neurological disorders and reproductive diseases (Chang et al. 2009; Knox et al. 2011; Shi et al. 2009). The thyroid, as an endocrine gland, is one of the potential targets for PFOS. As the major cell type of thyroid gland, the thyroid follicular cells are mainly responsible for thyroid hormone production (Porazzi et al. 2009), which plays an important role in metabolism regulation in adult organisms and is also required throughout organogenesis. Previous studies using rat model revealed that short-term PFOS exposure led to a transient increase of thyroid hormones in tissues and an ultimate decrease in serum (Chang et al. 2009), proliferated thyroid follicular cells (Butenhoff et al. 2009), hypothyroxinemia in developing pups when exposure occurs during either prenatal or postnatal period (Yu et al. 2009). Besides rodent models, zebrafish (*Danio rerio*) has also been utilized as a model system for investigating PFOS toxicity in several studies (Cheng et al. 2016; Cui et al. 2017; Wang et al. 2011a). For example, it has been reported that acute waterborne exposure to PFOS (0-400 μg/l) during 1–15 d post fertilization (dpf) causes disruption of the hypothalamic -pituitary-thyroid (HPT) axis in zebrafish larvae by altering gene expression in the HPT axis (Shi et al. 2009). Although it has been demonstrated that acute exposure to PFOS disrupts thyroid function, adverse effect of chronic exposure at the relative low level of PFOS has not been well studied, especially the effects of PFOS on the morphological and molecular changes of thyroid have

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not yet been reported. The objective of this study was to characterize and further understand the thyroid toxicity of chronic PFOS exposure in zebrafish model.

## Materials and Methods

The zebrafish (*Danio rerio*) of wild type were raised at standard conditions in a recirculation system. Fish husbandry was maintained as previously described (Wang et al. 2011a). The use of zebrafish was approved by the Institutional Animal Care and Use Committee at Wenzhou Medical University. Embryos of high quality at 8 h post fertilization (hpf) were exposed to dimethyl sulfoxide (DMSO, 0.01% v/v) or PFOS (CAS: 1763-23-1, purity > 96%, 250 µg/l) till 120 dpf using the similar exposure paradigm and breeding protocol (Cheng et al. 2016). The PFOS concentration was based on our previous chronic exposure study (Wang et al. 2011a). Since PFOS exposure resulted in female biased population in zebrafish (Wang et al. 2011a), the exposed females were used for the following tests. A subset of fish was collected at 150 dpf and PFOS in whole body tissues of adults was quantified with Waters ACQUITY ultra performance liquid chromatography combined with mass spectrometer (Waters Corp, MA, USA) as previously described (Wang et al. 2011a). The quality assurance (QA) and quality control (QC) procedures for PFOS measurement, including recovery and limit of detection, and the detailed operating parameters of analytical instrumentation were conducted as our previously published methods (Huang et al. 2010; Zhang et al. 2011).

Thyroid hormones in the tissue were extracted using previous method (Crane et al. 2004). Total $T_3$ and $T_4$ levels were determined by radioimmunoassay (RIAs). The efficiency of the thyroid hormone extraction was determined by adding 100 µl of $^{125}I$ radio-labeled $T_3$ and $T_4$ to each sample before extraction, which was 59.9% and 57.3% for $T_3$ and $T_4$ in the present study, respectively. Three biological replicates were used for each group and each replicate was pooled by three individual fish with similar size. For each biological replicate, two technical repeats were used to reduce sampling error.

For morphological study, image of thyroid follicles were captured from paraffin sections under light microscope and nuclear size of thyroid follicular cells was quantitated as described (Patiño et al. 2003). Five nuclei per fish (4 fish per group) were measured with ten individual trails for each nuclear measurement to reduce sampling error. Routine transmission electron microscope (TEM) was used to observe the ultrastructural changes.

To investigate the transcriptional changes in genes related to thyroid function, total RNA was isolated from liver/brain samples at 120 dpf (3 replicates) using TRIzol Reagent (Invitrogen, CA, USA). The quantity and quality of RNA were determined using spectrophotometer and gel electrophoresis. Quantitative polymerase chain reaction (qPCR) was performed using previous protocol (Cheng et al. 2016). The mRNA level was calculated and normalized against housekeeping gene *β-actin* using the equation: fold change $= 2^{-\Delta\Delta CT}$ (Schmittgen and Livak 2008). The expression of *β-actin* gene was stable following PFOS treatments, which was used in our previous PFOS studies (Chen et al. 2014; Cheng et al. 2016). All oligonucleotide primers (Table 1) were synthesized by Sunny Biotechnology (Shanghai, China http://www.sunnybio.cn).

Nonparametric t test of Kolmogorvo-Smirnov was used to analyze the group difference. All statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL, USA) and $P < 0.05$ was considered as significant difference. The data were reported as mean ± standard error.

## Results and Discussion

Consistent with our previous study (Wang et al. 2011a), this exposure paradigm generated an internal PFOS concentration ranging from 8.4 to 12.4 µg/g (wet weight) in the whole body tissues of zebrafish (three replicates used for measurement), which are comparable to those commonly detected from various environmental fish samples (Hoff et al. 2005, 2003).

**Table 1** Primer sequences of qPCR used in this study

| Gene | Forward primer (5′–3′) | Reversed primer (5′–3′) |
|---|---|---|
| *β-actin* | AAGCAGGAGTACGATGAGTC | TGGAGTCCTCAGATGCATTG |
| *dio2* | TCAGCGCGAAATGGGCTTGCT | AGCAGCTCAGGAGTGACACCACA |
| *thrα* | GGCTCGGAGTGGTTTCTGA | GCGGTGGTTGATGTAGTGC |
| *thrβ* | GGCTCTGGCTCTTATGACATGGT | TTGTTGTCCACAGACTCGCTGA |
| *brd8* | AAGCAGGAAGAAGAGGAGGCGGA | TTGGCTGTGATGCGTCTGGCT |
| *ttr* | GTATCGGGTGGAGTTTGACA | CTCAGAAGGAGAGCCAGTGT |
| *ugt2a1* | CAAGCCATTACCCAAGGA | AGTGCTGAGGCGATTCTATT |
| *ugt1a5* | GGAGGAGTTTGTGAACGGCTCTGG | GCCTCAAAGAACTCTCTGGCTTTGG |

Thyroid gland plays an important role in the metabolism regulation for adult organisms and during organogenesis. As the major cell type of thyroid gland, thyroid follicular cells are mainly responsible for thyroid hormone production. As an endocrine disrupting chemical, PFOS exposure has been shown to increase $T_3$ levels following a 15 days treatment in a dose range of 100–400 µg/l and disrupted HPT axis by altering gene expression in zebrafish (Shi et al. 2009). Interestingly, the present study demonstrated a significantly decreased $T_4$ and increase of the $T_3$: $T_4$ ratio levels, and downward trend for $T_3$ level following chronic PFOS exposure at 250 µg/l (Fig. 1). It is likely that this discrepancy may just reflect the different effects of PFOS on thyroid hormones under different exposure paradigm (e.g. long-term vs. short-term exposure).

Consistent with the thyroid hormone assay, our morphological study further showed significant changes in the ultrastructure of thyroid gland. At the level of light microscopy, no significant changes of morphology were noticeable following chronic PFOS exposure. The thyroid of the fish from both groups had oval follicles of various size filled with colloid, and the follicles were lined with squamous or cuboidal follicle cells (Fig. 2a–d). However, the nuclear area of follicular epithelial cells in the thyroid of PFOS-treated fish was significantly lower when compared with the nuclear area of follicular epithelial cells from the controls (Fig. 2e). Further morphological analysis with TEM revealed significant alterations in the mitochondria and endoplasmic reticulum of zebrafish chronically exposed to PFOS. In controls, thyroid follicles are lined with a layer of squamous epithelium cells that connected by tight junctions (Fig. 3a). The mitochondria of follicular cells showed clearly crest without vacuoles and the rough endoplasmic reticulum (rER) cisterna displayed squamous shape and attached with many ribosomes (Fig. 3b). The lumen of follicular cells had lank, short and small microvilli (Fig. 3b). The nucleus of follicular cells is in the shape of ellipse (Fig. 3a). However, in PFOS-treated fish, the porosity was observed in some parts of the epithelium cells junction (Fig. 3d) and extensive vacuole formation observed in mitochondria (Fig. 3e), and the cisterna of rER exhibited scrotiform showing focal degranulation (Fig. 3e). Compared with the controls (Fig. 3c), PFOS exposure also caused edema of the interstitial tissue (Fig. 3f). These structural changes of thyroid follicular cells may affect the function of thyroid gland and account for the declined thyroid hormone level in the whole body.

In addition to the biochemical assay on thyroid hormone and the histological observations on thyroid gland morphology, we further investigated the expression of some genes associated with thyroid function at the molecular level. In

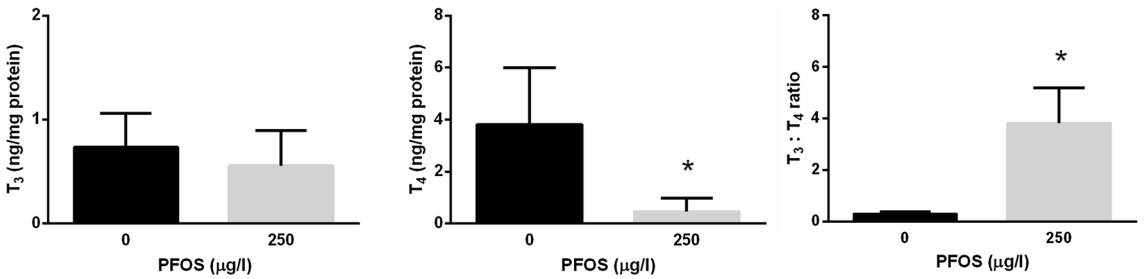


**Fig. 1** The whole-body content of triiodothyronine ($T_3$), thyroxine ($T_4$), and $T_3$: $T_4$ in female zebrafish exposed to 0 and 250 µg/l of PFOS at 120 dpf. Asterisks indicate significant difference in compared to control ($P < 0.05$)

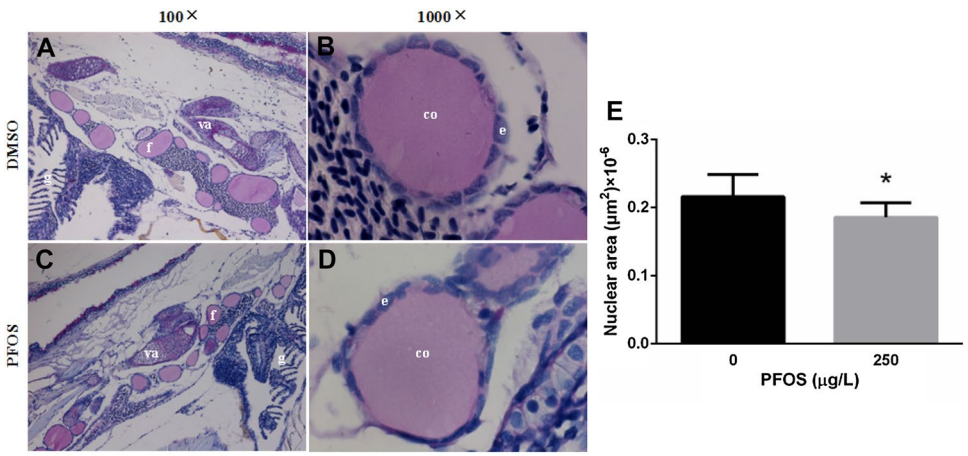


**Fig. 2** Representative micrographs of the cross section of fish head area showing the structure of thyroid follicles in controls (**a**–**b**) and 250 µg/l PFOS-exposed zebrafish (**c**–**d**). The measurement of nuclear area of the thyroid follicles were summarized in graph (**e**). *va* ventral aorta, *f* thyroid follicle, *co* colloid, *g* gill, and *e* thyroid follicle epithelial cell. Asterisks indicate significant difference when compared to control ($P < 0.05$)

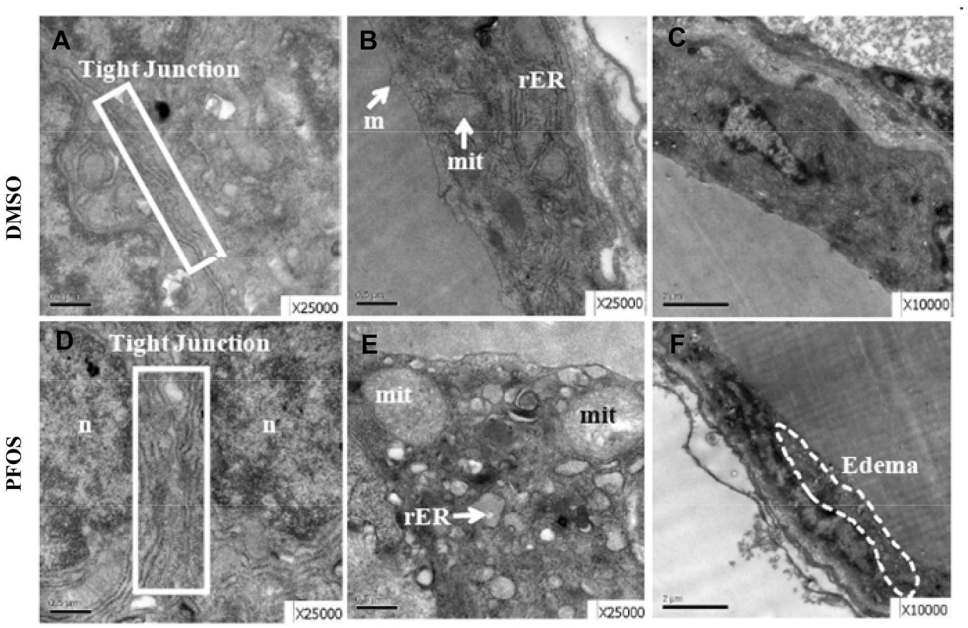


**Fig. 3** Representative micrographs from TEM of thyroid follicle cells of control (**a**–**c**) and 250 µg/l PFOS-treated fish (**d**–**f**) at 120 dpf. *n* nucleus, *mit* mitochondria, *rER* rough endoplasmic reticulum, *m* microvilli

the brains, all selected genes showed a decreased trend following chronic PFOS exposure, including *dio2, thrb, brd8, ttr, ugt2a1* and *ugt1a5* (Fig. 4). In the livers, all these genes except *ttr* were significantly decreased when compared with the controls (Fig. 4). The type 2 iodothyroninedeiodinase (encoded by gene *dio2)* catalyzes the conversion of $T_4$ into $T_3$, which subsequently binds to nuclear thyroid hormone receptors (Kotrschal et al. 1997) with a much higher affinity and is by far the predominant form secreted by the thyroid follicles (Walpita et al. 2009). Bromodomain-containing protein 8 (*brd8*) interacts with thyroid hormone receptor in a ligand-dependent manner and enhances thyroid hormone dependent activation from thyroid response elements. Serum $T_4$ and $T_3$ are transported to target tissues by the carrier proteins such as transthyretin (encoded by *ttr)*, thyroxine-binding globulin and albumin. The actions of thyroid hormones in target tissues are mediated by the binding of the thyroid hormone receptors (*thrα* and *thrβ*). Uridine diphosphate glucuronyl transferases (UGT), as an important phase II metabolic enzyme, is encoded by *ugt* and catalyze thyroid hormone glucuronidation in the liver (Builee and Hatherill 2004). In previous study some of these selected genes such as *ttr, thrα* and *thrβ* have been shown to be altered in juvenile zebrafish by a short-term (15d) PFOS exposure (Shi et al. 2009). In the present study, we demonstrated that chronic PFOS exposure down-regulated these genes relate to thyroid hormone production (*dio2*), transportation (*ttr*), binding (*thra, thrb, brd8*) and metabolism (*ugt2a1, ugt1a5*) in the brain and liver, except *ttr* was significantly up-regulated in the liver. We do not have a solid explanation for the up-regulated expression of *ttr* in the liver due to the limitation of our study. The over-expression of *ttr* in liver may just reflect the increased hormone transportation in response to decreased thyroid hormone level in the body. A detailed study is needed in future to address this hypothesis.

Although we demonstrated the significant effects of chronic PFOS exposure in zebrafish, there are several limitations associated with the present study. First, although the concentration of PFOS we applied in this study is relevant to some environment scenarios and the fish internal PFOS

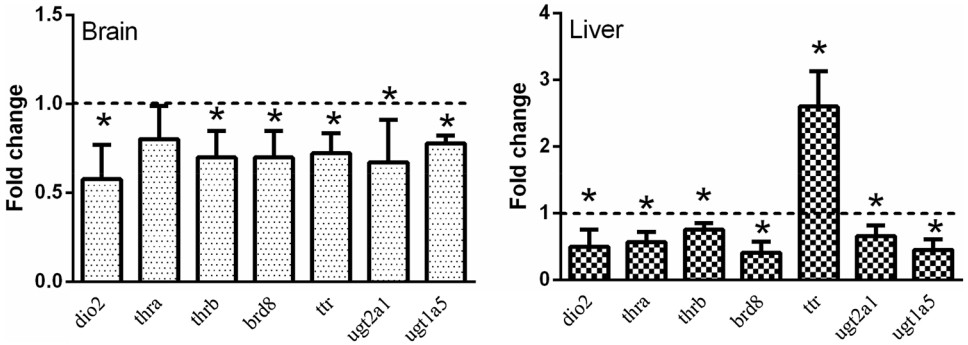


**Fig. 4** Fold changes of gene expression in the brain and liver tissues from adult zebrafish collected at 120 dpf following 250 µg/l PFOS exposure. The values represent relative mRNA levels as compared to the control group (n = 3). Asterisks indicate significant difference at $P < 0.05$

concentration is comparable to those commonly detected from various environmental fish samples, it is still slightly higher than the overall concentrations generally found in surface water. Future studies using even lower concentrations would be beneficial for better environmental risk assessment. Second, we only examined the exposed females. The sex specific effect of PFOS on the thyroid function is worth to be addressed in future study. Third, the present study is one-time point examination by design. Since the control of thyroid hormones is a dynamic process that depends on the balance among its synthesis, binding, transport, metabolism and the feedback regulation of HPT axis. A well-designed time-course study with multi endpoint points including blood thyroid hormone, thyroid-stimulating hormone (TSH) in future will help address the underlying mechanisms of PFOS induced thyroid metabolism disturbance. Taken together, our study demonstrated that chronic PFOS exposure at the environmental relevant level (250 µg/l) resulted in the decrease of thyroid hormone, ultrastructural alterations of thyroid follicular cells, and the changes of thyroid related gene expression in the brain and liver tissues of zebrafish, suggesting the validity of using zebrafish as an alternative animal model for PFOS chronic toxicity screening.

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