Effects of ZnO nanoparticles on perfluorooctane sulfonate induced thyroid-disrupting on zebrafish larvae

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ABSTRACT

Perfluorooctane sulfonate (PFOS) and ZnO nanoparticles (nano-ZnO) are widely distributed in the environment. However, the potential toxicity of co-exposure to PFOS and nano-ZnO remains to be fully elucidated. The test investigated the effects of co-exposure to PFOS and nano-ZnO on the hypothalamic–pituitary–thyroid (HPT) axis in zebrafish. Zebrafish embryos were exposed to a combination of PFOS (0.2, 0.4, 0.8 mg/L) and nano-ZnO (50 mg/L) from their early stages of life (0–14 days). The whole-body content of TH and the expression of genes and proteins related to the HPT axis were analyzed. The co-exposure decreased the body length and increased the malformation rates compared with exposure to PFOS alone. Co-exposure also increased the triiodothyronine (T3) levels, whereas the thyroxine (T4) content remained unchanged. Compared with the exposure to PFOS alone, exposure to both PFOS (0.8 mg/L) and nano-ZnO (50 mg/L) significantly up-regulated the expression of corticotropin-releasing factor, sodium/iodidesymporter, iodothyronine deiodinases and thyroid receptors and significantly down-regulated the expression of thyroid-stimulating hormone, thyroglobulin (TG), transthyretin (TTR) and thyroid receptors. The protein expression levels of TG and TTR were also significantly down-regulated in the co-exposure groups. In addition, the expression of the thyroid peroxidase gene was unchanged in all groups. The results demonstrated that PFOS and nano-ZnO co-exposure could cause more serious thyroid-disrupting effects in zebrafish than exposure to PFOS alone. Our results also provide insight into the mechanism of disruption of the thyroid status by PFOS and nano-ZnO.

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Introduction

Perfluorinated chemicals (PFCs) have received much attention due to their extensive use in surfactants, agrochemicals, lubricants, adhesives, fire retardants, propellants and medicines (Key et al., 1997; Renner, 2001). The widespread use of PFCs has resulted in their global distribution and detection in the environment (Giesy and Kannan, 2001; Kannan et al., 2002). Perfluorooctane sulfonate (PFOS) is an environmental degradation product of many PFCs. PFOS has been widely detected in wildlife and humans (Giesy and Kannan, 2002; Lien et al., 2008), and its contamination can be found globally distributed in the environment (So et al., 2004). Because PFOS can be detected in human tissues, there are increasing concerns about its potential...
detrimental effects on human health (Apelberg et al., 2007; Inoue et al., 2004; Karmann et al., 2007; Midsch et al., 2007). It has been previously shown that PFOS exposure induced oxidative stress and apoptosis in the lungs of rat offspring (Chen et al., 2012). Keiter et al. (2012) reported that PFOS led to bioaccumulation and histological alterations in the liver, thyroid and gonads of zebrafish (Danio rerio). Kim et al. (2010) reported that PFOS caused DNA damage in common carp (Cyprinus carpio).

Nanotechnology has developed rapidly over the past decade and exhibits great promise for numerous applications (Chen et al., 2004). ZnO nanomaterials (nano-ZnO) are one of five metal oxide nanoparticles (NPs) and have been employed in many applications, such as optoelectronics and cosmetics, and as catalysts and ceramic pigments (Lanone and Boczkowski, 2006). The hazards caused by nano-ZnO exposure include damage to DNA (Sharma et al., 2012), induction of the inflammation response (Gojova et al., 2007), developmental toxicity (Zhao et al., 2013b) and the production of reactive oxygen species (ROS) (Sharma et al., 2012).

Thyroid hormones (THs) are important regulators of vertebrate growth and development and are under the control of the hypothalamic–pituitary–thyroid (HPT) axis (Erik et al., 2008). THs play significant roles in embryonic development and the metamorphosis of species (Kawakami et al., 2008; Marchand et al., 2004; Walpita et al., 2007). The thyroid function is dependent upon iodine uptake, TH synthesis, TH transport, tissue-specific TH deiodination and TH binding to thyroid hormone nuclear receptors (TRs). Thyroxin (T4) and triiodothyronine (T3) are the two major forms of THs. T4 is the primary thyroid hormone synthesized, and T3 is major biological thyroid hormone. When serum T3 and T4 levels are too low, thyrotropin releasing hormone (TRH) is secreted by the hypothalamus and thyroid stimulating hormone (TSH) is released by the anterior pituitary to promote thyroidial iodide uptake and thyroid hormone synthesis. The sodium iodide symporter (NIS) is a component of the thyroid hormone production pathway and a membrane protein that translocates iodide into thyroid follicular cells (Dohan et al., 2003; Eskandari et al., 1997). Thyroglobulin peroxidase (TPO) is an enzyme that oxidizes iodide (I\(^{-}\)) after iodide (I\(^{-} \)) is transported into the thyroid follicular cells. De Groef et al. (2006) found that corticotrophin-releasing hormone (CRH) can stimulate TSH secretion and the regulation of TH synthesis within the HPT axis. PFOS can cause developmental toxicity in rodents, fathead minnows (Pimephales promelas) and zebrafish embryos (Lau et al., 2003; Ankley et al., 2005; Shi et al., 2008). Zhao et al. (2013b) reported that nano-ZnO also causes developmental toxicity in zebrafish embryos. Du et al. (2009) reported that PFOS induced an increase in the triiodothyronine (T\(_3\)) levels in zebrafish embryos after chronic exposure. In addition, Shi et al. (2009) reported that PFOS exposure could alter the expression of genes in the HPT axis in zebrafish larvae. Varghese et al. (2001) reported that disruption of thyroid homeostasis might affect the responses of organisms to oxidative stress, and it was found that both PFOS and nano-ZnO single-exposure could induce oxidative stress (Chen et al., 2012; Wang et al., 2014b).

The retinoid X receptor (RXR) is a type of nuclear receptor that is activated by 9-cis retinoic acid, which has never been convincingly proven to be of physiological relevance and to be present endogenously, as well as 9-cis-13, 14-dihydro-retinoic acid. A lot of gene families participated in fish embryo development and morphogenesis. Early morphogenesis and differentiation in the process of fish development are rapid and complex. Vitamin A is essential nutrients in fish development, deficiency of vitamin A can lead to growth slowly, and other the symptom such as bleeding eyes, fins and skin (Moren et al., 2004; Hemre et al., 2004). The main metabolite of Vitamin A is retinoic acid in the fish body, and retinoic acid signaling pathway is mainly composed of two nuclear receptor family such as retinoic acid receptor (RAR) and retinol X receptor (RXR) mediated. RAR and RXR passways maybe another mechanisms to affect the development of fish. Other mechanisms also need to be discussed. But in this article we mainly discussed the mechanism of thyroid-disrupting in regulation of development and deformities of fish.

Toxicity studies of co-exposures to different substances are becoming increasingly common (Zhao et al., 2013a). This is because people have realized that studies of the toxicity of single substances cannot fully evaluate the risks associated with exposure because organisms are commonly exposed to mixtures of contaminants in the environment. Therefore, it is essential to understand the mechanisms underlying the combined toxicity and to correctly evaluate the risks to the environment, humans and animals. Altenburger et al. (2012) suggested that mixture toxicity assessments should adopt mixture toxicity models. The cumulative effects of the individual substances can be used to evaluate the risk of some mixtures (Sarigiannis and Hansen, 2012). There have been only limited studies of the toxicity of co-exposure to PFOS and nano-ZnO in animals, and inadequate data exist to assess the molecular mechanism(s) underlying their joint effects leading to disruption of the thyroid status.

In the present study, zebrafish embryos were exposed to a combination of PFOS and nano-ZnO to investigate the mechanisms underlying the responses of the HPT axis to co-exposure and to identify the potential mechanisms by which these agents disrupt the THs in zebrafish. Indicators such as developmental toxicity, the thyroid hormone contents, and the transcriptional profiles of genes and proteins related to the HPT axis were examined. This study will enhance the understanding of the actions of PFOS co-exposure and will facilitate environmental risk assessment.

### 1. Materials and methods

#### 1.1. Chemicals

Perfluorooctane sulfonate (PFOS; CAS no. 2795-39-3; ≥98% purity), perfluorooctane sulfonate (PFOS; CAS no. 2795-39-3; analytical standard (Fluka), dimethyl sulfoxide (DMSO, ≥99.9%) and a nano-sized ZnO particle dispersion (CAS no. 1314-13-2, less than 100 nm) were purchased from Sigma (St. Louis, MO, USA). The biological reagents used for experiments and phosphate-buffered saline (PBS, pH 7.4 biotechnology performance certified) were also purchased from Sigma. All other chemicals and solvents were of analytical grade.

#### 1.2. Nano-ZnO characterization

Stock solutions of nano-ZnO (50 mg/L) dispersed by an Ultrasonic processor JY92-IID 900W (Scientz, China)
(frequency 25 kHz, amplitude 250 μm, continuous/pulse 30 min, tip diameter 6 mm) for 30 min before use. PFOS and nano-ZnO were both delivered to the test vessels without use of a carrier solvent. A series of exposure suspensions (1, 5, 25, 50, 100 mg/L) were prepared by stepwise dilution with zebrafish culture medium (consisting of 64.75 mg/L NaHCO₃, 5.75 mg/L KCl, 123.25 mg/L MgSO₄·7H₂O, and 294 mg/L CaCl₂·2H₂O). The morphology of the ZnO-NP aggregates was observed using a scanning electron microscope (SEM, Philips XL30, FEI Company, USA) operated at 15 kV (Fig. 1a). A drop of nano-ZnO solution was placed onto a carbon-coated copper grid, air dried, and observed using a transmission electron microscopy (TEM) system (JEOL, Tokyo, Japan) (Fig. 1b). The SEM and TEM images of nano-ZnO revealed that nano-ZnO in the suspensions gathered into large aggregates of irregular shapes, with an average size of 40 nm (Fig. 1).

1.3. Chemical analysis

After the series of exposure suspensions freshly diluted and dispersed, each dose of nano-ZnO suspensions was sampled at 0 and 24 hr for size distribution analysis using a dynamic light scattering (DLS) device (Brookhaven Instrument Corporation, Holtsville, NY, USA). The particle size distribution of the nano-ZnO obtained by DLS showed polydispersed aggregates characterized by single particles with sizes of 30–100 nm (inset in Fig. 1b).

1.4. Zebrafish maintenance and embryo exposure

Adult AB strain zebrafish (D. rerio) were purchased from the State Key Laboratory of Freshwater Ecology and Biotechnology, Chinese Academy of Sciences (Wuhan, China). Fish were fed twice daily with frozen freshly hatched Artemia nauplii blood worms and were maintained at approximately 28°C under a 14 hr:10 hr light/dark cycle. Males and females were paired in spawning boxes overnight, and the culture medium (pH 7.2, DO > 6.3 mg/L) was kept at approximately 28°C. Spawning was completed at the beginning of the light cycle; then, the fertilized embryos were collected from spawn traps using plastic disposable pipettes and were transferred to clean water to rinse them.

Approximately 400 normal embryos were randomly distributed into every glass beaker containing 500 mL of nano-ZnO plus PFOS (P 0.2 + Z 50, P 0.4 + Z 50, P 0.8 + Z 50 mg/L), PFOS alone (0, 0.2, 0.4, 0.8 mg/L), nano-ZnO alone (50 mg/L) and 0.003% DMSO. Because PFOS has low water solubility, we used DMSO as a solvent to enhance its solubility. There were three replicates for each exposure concentration, and the exposure solution was renewed daily. The larvae were randomly sampled until day 14 and were immediately frozen in liquid nitrogen and stored at −80°C for further gene expression, protein expression and thyroid hormone assays. Ten individual larvae from each beaker (n = 3) were randomly sampled to measure the body length using a digital camera as described by Shi et al. (2008).

The survival and malformation rates of larvae were also recorded following observation under a stereo-microscope (Olympus, Japan) equipped with a digital camera. The concentrations of PFOS and nano-ZnO were selected based on preliminary experiments performed in our lab (Du et al., 2014). The embryos were co-exposed to PFOS (0.2, 0.4 or 0.8 mg/L) and nano-ZnO (50 mg/L). The exposure concentrations of PFOS in this study were higher than those measured in the surface waters in China (Jin et al., 2009), because researchers often use relatively higher concentrations of toxicants to study the possible mechanisms of toxicity during short-term laboratory exposures (Lema et al., 2007; Zhao et al., 2013b; Shi et al., 2009).

Fig. 1 – SEM images (a) of a nano-ZnO suspension of 50 mg/L; TEM images (b) of a nano-ZnO suspension of 50 mg/L; particle size distribution (inset in panel b). Linear graph of nano-ZnO obtained from DLS. SEM: scanning electron microscope; ZnO-NP: zinc oxide nanoparticle; TEM: transmission electron microscopy; DLS: dynamic light scattering.
1.5. RNA isolation and quantitative RT-PCR

Total RNA was extracted from 20 homogenized zebrafish larvae using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). To remove genomic DNA contamination, total RNA was digested by RNase-free DNase I (Promega Madison, WI, USA) and then purified. The total RNA recovered after the DNase I digestion was measured at 260 and 280 nm using a spectrophotometer (M2, Molecular Devices, Sunnyvale, CA, USA). Quantitative RT-PCR was carried out with an ABI 7000 instrument using the SYBR Green PCR kit (TaKaRa Biotechnology CO. LTD., Japan). The primer sequences used to amplify β-actin, CRH, TSH, NIS, TG, TPO, TTR, Deio1 and Deio2 are shown in Table 1. The thermal cycling conditions were as follows: initial denaturation for 10 min at 95°C followed by 40 cycles of 95°C for 30 sec, 60°C for 20 sec and 72°C for 1 min. For each selected gene, RT-PCR reactions were performed on three replicate samples. The expression level of each target gene was normalized to its β-actin mRNA content. The fold change of the tested genes was analyzed by the 2−ΔΔCt method (software automation) (Livak and Schmittgen, 2001).

1.6. Thyroid hormone extraction and radioimmunoassay

The method used for extracting whole body THs was based on that reported by Zhao et al. (2013b). A total of 200 zebrafish larvae from each group were homogenized in 0.5 mL of ice-cold methanol and centrifuged at 10,000 g for 15 min at 4°C. Then, the homogenates were disrupted by intermittent sonic oscillation for 5 min on ice and were vortexed vigorously for 10 min. After centrifugation at 5000 g for 15 min at 4°C, the supernatants were collected, and the pellets were re-extracted with 0.5 mL methanol and centrifuged. The freshly collected supernatant was mixed with the original extraction. The extracts were stored at −80°C until the radioimmunoassays (RIAs) were performed. The efficiency of the thyroid hormone extraction was determined by adding 100 μL of 125I-labeled T3 or T4 to each larval homogenate prior to extraction. The RIAs were performed using commercial kits (Beijing North Institute of Biotechnology, China), according to the manufacturer’s instructions. The minimum detectable levels of T3 and T4 in the radioimmunoassay were 0.5 and 20 ng/mL, respectively. The inter-assay coefficients of variance (CV) were <10% for both T3 and T4.

1.7. Protein extraction and Western blot analysis

Protein extraction was performed using the method reported by Shi et al. (2009) and Zhao et al. (2013a), with slight modification. Zebrafish larvae were homogenized in 500 μL of lysis buffer containing 7 mol/L urea, 2 mol/L thiouria, 4% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 1 wt.% dithiothreitol (DTT), 40 mmol/L Tris base, 1% protease inhibitor cocktail, 0.5 μL benzonase, and 20 μL/mL Bio-Lyte. The samples were then disrupted by intermittent sonic oscillation for 5 min and were incubated on a shaker for 30 min at 4°C. Insoluble particles were removed by centrifugation at 12,000 xg for 60 min at 4°C, after which the supernatants were collected. Four volumes of 100% ice-cold acetone were added to 1 volume of supernatant. The samples were precipitated at 20°C for 1 hr and then centrifuged at 12,000 xg for 15 min at 4°C. The supernatants were then discarded, and the protein pellets were dissolved in a protein solution buffer (7 mol/L urea, 2 mol/L thiouria, 4% CHAPS, 1 wt.% DTT, and 0.5% Immobilized pH Gradient (IPG) buffer). The protein concentration was determined using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ, USA). Approximately 40 μg of each protein sample was separated by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences, USA). The nitrocellulose membrane was blocked for 1 hr with 5% non-fat dried milk in Tris-buffered saline (TBS) and incubated with a primary antibody against TG (Sigma, St. Louis, MO, USA) or TTR (Abcam, USA) at 37°C for 2 hr. The blots were washed six times for 30 min with Tris-buffered saline Tween-20 (TBST) and then were incubated with an AP-conjugated secondary antibody at 37°C for 1 hr. Then, the blots were washed six times and developed using a BCIP/NBT kit (Amresco, USA). A rat GAPDH antibody (ProteinTech, USA) was also used to detect the zebrafish protein.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence of the primers (5′-3′)</th>
<th>Gene bank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>Forward: GGGTGGGAGTTGCACTT</td>
<td>Reverse: CTCCTGATGCTGGTGCCT</td>
</tr>
<tr>
<td>CRF</td>
<td>Forward: TCCGGGAAGTACCAACAGG</td>
<td>Reverse: CTGCACTTATGCGCCTTCC</td>
</tr>
<tr>
<td>TSHβ</td>
<td>Forward: GCAGCTCTCCACTCCATCC</td>
<td>Reverse: GACAGGTGTTGCAGATCTCA</td>
</tr>
<tr>
<td>NIS</td>
<td>Forward: GGTTGCGATAGGCTGTAAT</td>
<td>Reverse: GATACGGGATGCCATTAAGGA</td>
</tr>
<tr>
<td>TG</td>
<td>Forward: GCAGCGGAAAGGATAGTGG</td>
<td>Reverse: AGTCTGGCCGTGAAATAGGA</td>
</tr>
<tr>
<td>TPO</td>
<td>Forward: GGGATGCTGGACTT</td>
<td>Reverse: GCACGAGCCATGCACCATCA</td>
</tr>
<tr>
<td>TTR</td>
<td>Forward: GGGTGGGAGTTGCACTT</td>
<td>Reverse: GATACGGGATGCCATTAAGGA</td>
</tr>
<tr>
<td>Dio1</td>
<td>Forward: GCTCAAACAGGGTCTGGAAGGACT</td>
<td>Reverse: AGCAACACACCCACCCAAAT</td>
</tr>
<tr>
<td>Dio2</td>
<td>Forward: GCATAGGCGATCCTGCACTTT</td>
<td>Reverse: TGGTGGCTCTCTGTCACACC</td>
</tr>
<tr>
<td>Trα</td>
<td>Forward: CTAGAAGACACATTCGGCAAGA</td>
<td>Reverse: CACACACACACGGCATTAC</td>
</tr>
<tr>
<td>TRβ</td>
<td>Forward: TGGGAGATGATACTGGCTT</td>
<td>Reverse: ATAGGGCCATGCAATGTCG</td>
</tr>
</tbody>
</table>

TSHβ: beta-subunit of thyroid stimulating hormone; NIS: iodide symporter; TG: thyroglobulin; TPO: thyroglobulin peroxidase; TTR: transthyretin.
1.8. Statistical analysis

All data are presented as the means with standard errors (mean ± SEM), and homogeneity of variances was checked by Levene’s test. If the data failed to pass the test, a logarithmic transformation of the data was performed, and the data were checked again for the homogeneity of variances. If the assumptions of the homogeneity of variances were met, the differences were evaluated by a one-way analysis of variance (ANOVA) followed by Tukey’s test using the SPSS 18.0 software program (SPSS, Chicago, IL, USA). A p-value <0.05 was deemed statistically significant in all experiments.

2. Results

2.1. Chemical analysis

According to the results of ICP-MS, the corresponding soluble Zn²⁺ released from exposure suspension (50 mg/L) was 22.58 mg/L. In addition, the concentrations of soluble Zn²⁺ were 21.74, 20.25, and 23.15 mg/L in the series of PFOS and ZnO-NP exposure suspensions (0.2 + 50, 0.4 + 50 and 0.8 + 50), respectively. From the analysis results of LC-MS/MS, the linear equation is obtained: Y = 88,133.5 + 14,149.75X (R² = 0.996) and Y secondary subion (m/z 499.2 > 79.9). The results showed that PFOS linear relation is good in the concentration of 0.05 to 1.8 mg/L. The concentrations of PFOS from the series of exposure suspensions (0.2 + 50, 0.4 + 50 and 0.8 + 50) were 0.10, 0.36 and 0.64 mg/L, respectively. The series of PFOS and ZnO-NP exposure suspensions (0.2, 0.4 and 0.8 mg/L) were 0.12, 0.33 and 1.8 mg/L. The concentrations of PFOS from the series of exposure suspensions (0.2 + 50, 0.4 + 50 and 0.8 + 50), were 21.74, 20.25, and 23.15 mg/L in the series of PFOS and ZnO-NP exposure suspensions (0.2 + 50, 0.4 + 50 and 0.8 + 50), respectively. From the analysis results of LC-MS/MS, the linear equation is obtained: Y = 88,133.5 + 14,149.75X (R² = 0.996) and Y secondary subion (m/z 499.2 > 79.9). The results showed that PFOS linear relation is good in the concentration of 0.05 to 1.8 mg/L. The concentrations of PFOS from the series of exposure suspensions (0.2 + 50, 0.4 + 50 and 0.8 + 50) were 0.10, 0.36 and 0.63 mg/L, respectively. After verifying analytically the exposure concentrations used in this study, the results proved that renewing solutions daily was appropriate for PFOS and nano-ZnO to ensure that exposure concentrations were at the intended level.

2.2. Developmental toxicity

As shown in Table 2, the survival rates were decreased in a dose-dependent manner in all exposure groups (p < 0.05). The survival rates were significantly decreased in the PFOS 0.8 mg/L and nano-ZnO 50 mg/L single-exposure groups and in all of the co-exposure groups (p < 0.05). The malformation rates were significantly increased in the 0.8 mg/L PFOS single-treatment and (P 0.2 + Z 50) mg/L group compared to the control group (p < 0.05). Moreover, significant increases in the malformation rate were also observed in the (P 0.4 + Z 50) and (P 0.8 + Z 50) groups compared with the control group (p < 0.01). The measured body lengths (control: (5.20 ± 0.35 mm) were significantly reduced (exposure: (4.47 ± 0.24 mm and (4.17 ± 0.14) mg in the 0.4 and 0.8 mg/L PFOS single-exposure groups. However, there were no significant effects on the body weight after exposure to the PFOS and nano-ZnO relative to the control. Three developmental indices changed in a concentration-dependent manner in both the combination and single exposure groups.

Shorter body lengths, lower survival rates and higher malformation rates were observed in the co-exposure groups compared with single-exposure groups. The results showed that co-exposure to PFOS and nano-ZnO induced more severe developmental toxicity than exposure to either agent alone. Moreover, DMSO treatment group did not affect the development compared with the control. As shown in Fig. 2, the observed malformations following PFOS single-treatment and PFOS plus nano-ZnO treatment were the swimming bladder deformity (SBD), tail deformity (TD), spinal curvature (SC), yolk sac edema (YSE) and pericardial edema (PE).

2.3. T3 and T4 contents of the whole body

In the present study, the extraction efficiency for T3 and T4 were 50% and 85%, respectively. The reported T3 and T4 contents in the entire body were corrected based on the extraction efficiency. The measured T3 content was (0.75 ± 0.02) ng/g in the control, and there was no significant change in the 0.2 or 0.4 mg/L PFOS single-treatment or 0.2 mg/L PFOS co-treatment groups relative to the control. Significant increased to (1.27 ± 0.012) ng/g, (1.29 ± 0.01) ng/g and (1.30 ± 0.04) ng/g were observed in the 0.5 mg/L nano-ZnO single-treatment, 0.8 mg/L PFOS single-treatment and 0.4 mg/L PFOS co-exposure groups.

| Table 2 – Development index of zebrafish larvae after exposure to PFOS and nano-ZnO for 14 days. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Exposure groups                 | Survival rate (%)| Malformation rate (%) | Body length (mm) | Body weight (µg) |
| Control                         | 75 ± 2.64       | 4.03 ± 0.67      | 5.20 ± 0.35     | 486.66 ± 0.03   |
| DMSO                            | 72 ± 1.73       | 4.02 ± 0.73      | 5.12 ± 0.31     | 478.38 ± 0.01   |
| Z 50 mg/L                       | 50 ± 2.64*      | 17.64 ± 4.14*    | 4.73 ± 0.11     | 472.13 ± 0.01   |
| P 0.2 mg/L                      | 69 ± 1.52       | 11.02 ± 2.31     | 4.93 ± 0.11     | 479.67 ± 0.04   |
| P 0.4 mg/L                      | 68.33 ± 1.00    | 11.29 ± 1.17     | 4.47 ± 0.24*    | 456.33 ± 0.24   |
| P 0.8 mg/L                      | 60 ± 2.89*      | 16.48 ± 5.74*    | 4.17 ± 0.14     | 467.16 ± 0.02   |
| P 0.2 mg/L + Z 50 mg/L          | 46.67 ± 2.30*   | 15.78 ± 2.08*    | 4.99 ± 0.06     | 496.67 ± 0.01   |
| P 0.4 mg/L + Z 50 mg/L          | 45.67 ± 7.37**  | 22.07 ± 1.79**   | 4.15 ± 0.07*    | 485.62 ± 0.07   |
| P 0.8 mg/L + Z 50 mg/L          | 42 ± 4.58**     | 24.79 ± 3.29**   | 3.98 ± 0.15**   | 443.33 ± 0.02   |

P and Z represent PFOS and nano-ZnO. The values represent the mean ± SEM of three replicate groups. The change of surviving rate, malformation rate, length and weight exposed to single and joint PFOS and nano-ZnO for 14 days.

DMSO: dimethyl sulfoxide; Z 50 mg/L: nano-ZnO 50 mg/L; P 0.2 mg/L: PFOS 0.2 mg/L; P 0.4 mg/L: PFOS 0.4 mg/L; P 0.8 mg/L: PFOS 0.8 mg/L; P 0.2 mg/L + Z 50 mg/L: PFOS 0.2 mg/L + nano-ZnO 50 mg/L; P 0.4 mg/L + Z 50 mg/L: PFOS 0.4 mg/L + nano-ZnO 50 mg/L; P 0.8 mg/L + Z 50 mg/L: PFOS 0.8 mg/L + nano-ZnO 50 mg/L.

* p < 0.05 indicated significant difference between exposure groups and the corresponding control.

** p < 0.01 indicated significant difference between exposure groups and the corresponding control.
respectively (Fig. 3a). Notably, the T3 content in the highest co-exposure group (P 0.8 + Z 50 mg/L) was significantly increased compared with that in the PFOS (0.8 mg/L) single-treatment group (p < 0.05). However, the measured T4 content was (63.76 ± 0.34) ng/g in the control and no significant differences were observed in the T4 content in any of the PFOS single-exposure groups ((59.14 ± 1.75), (50.99 ± 0.20), (52.24 ± 0.34) for 0.2, 0.4 and 0.8 mg/L, respectively) relative to the control (Fig. 3b). The T4 content in the co-exposure groups was significantly decreased compared with that in the control group.

### 2.4. Gene expression profiles

The expression of genes in the HPT axis of the zebrafish larvae were analyzed after 14 days of exposure. The CRF gene transcription was significantly up-regulated by more than 3- and 4-fold relative to the control in the 0.8 mg/L PFOS single- and

![Fig. 2 – Morphology of zebrafish larvae exposed to PFOS and nano-ZnO for 96 hr. (a) The head of a control larva. (b) The tail of a control larva. (c) SBD: swimming bladder deformity. (d) TD: Tail deformity. (e) SC: spinal curvature; (f) YSE and PE: yolk sac edema and pericardial edema. PFOS: perfluorooctane sulfonate; nano-ZnO: ZnO nanoparticles.](image)

![Fig. 3 – The whole body content of T3 (a) and T4 (b) in larvae exposed to PFOS and Nano-ZnO alone or in combination. *p < 0.05 and **p < 0.01 indicate significant differences between the exposure groups and the corresponding control. *p < 0.05 indicates a significant difference between the single-exposure group and the corresponding co-exposure group. T3: triiodothyronine; T4: thyroxine; PFOS: perfluorooctane sulfonate; nano-ZnO: ZnO nanoparticles.](image)
co-exposure groups (Fig. 4a1). However, the beta-subunit of TSH (TSHβ) gene transcription was significantly down-regulated 4.6- and 4.1-fold in the 0.4 mg/L PFOS single- and co-exposure groups, respectively (Fig. 4a2). Notably, TSHβ gene transcription was significantly down-regulated in the highest co-exposure group (P 0.8 + Z 50) mg/L compared with the control (Fig. 4b2).

As shown in Fig. 4b1, the transcription of the NIS gene was significantly up-regulated in all of the co-exposure groups (p < 0.05), but no significant changes were observed in the single-exposure groups. For the combination of PFOS with nano-ZnO, significant up-regulation was observed in the 0.4 and 0.8 mg/L PFOS co-exposure groups compared with the 0.4 and 0.8 mg/L PFOS single-exposure groups (p < 0.05). The gene transcription of thyroglobulin (TG) after single-exposure to 0.2, 0.4 and 0.8 mg/L PFOS was depressed by 2.9, 3.9 and 2.1-fold compared to the control, respectively (Fig. 4b2). The combination resulted in a significant down-regulation relative to the control at concentrations of both 0.4 and 0.8 mg/L PFOS (p < 0.05) (Fig. 4b2). There was no significant difference in the gene transcription of TPO in zebrafish larvae exposed to any of the concentrations of the agents alone or in combination (Fig. 4b3).

Transthyretin (TTR) is a major TH carrier protein in fish and is known to be involved in TH transport. TTR transcription was significantly down-regulated in a concentration-dependent manner (5.2 and 5.6-fold) in the 0.4 and 0.8 mg/L PFOS single-exposure groups (Fig. 4c1). Co-exposure caused a significant reduction in transcription compared with the control (p < 0.05) (Fig. 4c1). Two isoforms of deiodinase (Deio1 and Deio2) showed a similar trend. PFOS exposure significantly up-regulated Deio1 gene transcription (1.7-fold) in the 0.8 mg/L PFOS single-exposure group compared with the control group (Fig. 4c2). It was also observed that the expression of Deio1 in the 0.4 and 0.8 mg/L PFOS combination groups was significantly increased (1 and 0.7-fold) compared with the levels in the PFOS single-exposure groups (p < 0.05) (Fig. 4c2). However, the expression levels of Deio2 in the 0.4 and 0.8 mg/L PFOS groups were not significantly different from those in the control group (Fig. 4c3). The expression levels of Deio2 in the 0.2, 0.4 and 0.8 mg/L PFOS combination groups were significantly increased compared with the groups treated with PFOS alone (p < 0.05).

The expression of two isoforms of TRα (TRα1 and TRα2) was also examined. The transcription of TRα1 was significantly down-regulated in the 0.2, 0.4 and 0.8 mg/L PFOS single-exposure groups by 0.28-, 0.28- and 0.18-fold, respectively. Co-exposure also caused a significant reduction in transcription compared with the control group (Fig. 4d1). In contrast, the TRα2 gene transcription was significantly up-regulated by 3.3- and 3.7-fold after exposure to 0.8 mg/L PFOS in the single and co-treatment groups (p < 0.01) (Fig. 4d2). The trend toward up-regulation in the co-treatment groups was higher than that in the single-treatment groups (Fig. 4d2).

The expression of proteins in the zebrafish larvae HPT axis was examined on day 14 (Fig. 5a). The levels of the TG and TTR proteins decreased in a concentration-dependent manner when zebrafish larvae were exposed to 0.2, 0.4 and 0.8 mg/L PFOS in both the single- and co-exposure groups compared to the control group (Fig. 5b, c). Co-exposure resulted in a significant down-regulation of the TG protein levels relative to the control level at concentrations of 0.4 and 0.8 mg/L (p < 0.05) (Fig. 5c). Regarding the expression of the TTR protein, co-exposure (0.8 mg/L) caused a significant reduction in transcription compared with the control (p < 0.01). In addition, the nano-ZnO single-exposure group also exhibited a tendency toward decreases in both the TG and TTR protein levels.

3. Discussion

The persistent presence of PFOS and nano-ZnO in aquatic environments and the potential disturbance of the endocrine system by these chemicals could influence the normal thyroid function in fish. In the present study, we evaluated the joint effects of PFOS and nano-ZnO on the thyroid hormone levels in zebrafish. The results demonstrated that co-exposure to PFOS and nano-ZnO significantly disturbed the TH levels and changed the transcription levels of genes in the HPT axis, as well as the corresponding protein levels in zebrafish.

THs play a key role in the early development of fish (Nelson and Habibi, 2009). Liu and Chan (2002) found that THs play an important role in this embryonic to larval transitory phase in zebrafish. In the present study, we found that there was significant inhibition of growth at the highest level of co-exposure compared with the control group. For example, there was a significant difference in the body lengths between the (P 0.8 + Z 50) mg/L co-exposure group and the control group (p < 0.05). There was a dose-dependent inhibitory effect on larval development observed in the PFOS single-exposure groups. These results agreed with the results reported by Shi et al. (2008). These authors found that a significant decrease in the growth of zebrafish larvae was detected following PFOS exposure (0.4 mg/L). In the nano-ZnO single-exposure group, there were significant changes in the survival and malformation rates, but the body length and weight were not significantly changed. Supporting our findings, Zhao et al. (2013a) reported that nano-ZnO could induce development toxicity in zebrafish larvae.

A significant increase in the T3 level was observed following the combined exposure relative to the changes induced by PFOS alone, which suggested that the combination of PFOS and nano-ZnO enhanced the effects of PFOS on the level of T3. The T4 level was unchanged in the PFOS and nano-ZnO single-exposure groups but was decreased in the co-exposure groups in this study. These results suggested that there was a significant negative effect on the THs in the co-exposure groups. Wang et al. (2011) found that co-exposure to PFOS and 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) affected the serum T3 and T4 levels differently in adult rats and offspring. In their study, Zhao et al. (2013b) suggested that the T3 level was not altered but that a significant decrease in the T4 levels was induced by exposure to the combination of PER and BDE-47. In an earlier study, Tomy et al. (2004) reported that the plasma thyroxine levels (T4) were lower in lake trout (Salvelinus namaycush) exposed to polybrominated diphenyl ethers (PBDEs) compared with control fish. The different responses in rats and zebrafish could be explained by differences in the species and in the exposure concentrations, as well as by tissue specificity.

The up-regulated expression of the CRH gene may be attributable to stimulation of the pituitary synthesis of adrenocorticotropic hormone (ACTH), a regulator of the HPT axis during larval development in fish (De Groef et al., 2006).
TSH is a pituitary hormone that stimulates the thyroid gland to produce thyroxine (T4). T4 is converted to triiodothyronine (T3), which is the active hormone that stimulates metabolism (Nishiyama, 2000). In the present study, there was a significant up-regulation of CRH expression in the group exposed to the highest combination relative to the group exposed to PFOS.
alone. These results are in agreement with those reported by Lema et al. (2009) who found that the up-regulation of the CRH gene was associated with a reduction of the T4 concentration in fathead minnows. The level of TSHβ expression was not affected by nano-ZnO exposure. However, there was a significant down-regulation of TSHβ expression in the PFOS co-exposure groups relative to the control group. TSH is a central regulator that can induce the generation and secretion of TH. Chiamolera et al. provided evidence for negative feedback inhibition of TSH expression by TH. In addition, another study showed that the level of TSHβ expression was down-regulated after the levels of T3 and T4 were changed (MacKenzie et al., 2009). These results suggest that PFOS and nano-ZnO co-exposure might disrupt the homeostasis of thyroid hormone and enhance the toxic effects of exposure.

The NIS-mediated uptake of iodide into follicular cells of the thyroid gland is the first step in the synthesis of thyroid hormone (Dohan et al., 2003). TG is a 660 kDa, dimeric protein produced by the follicular cells of the thyroid and is used entirely within the thyroid gland to produce T4 and T3 (Venturi et al., 2000). TPO is an enzyme expressed mainly in the thyroid, where it is secreted into the colloid. TPO is stimulated by TSH, which up-regulates gene expression (Ruf and Carayon, 2006). In the present study, the NIS gene transcription was significantly up-regulated, while the TG gene expression was down-regulated following co-exposure of PFOS at the highest concentration. Shi et al. (2009) showed that PFOS treatment increased the NIS gene expression in zebrafish larvae, which suggested that PFOS might induce thyroid gland development in the early developmental stages of zebrafish. Zhao et al. (2013a) reported that BDE-47 treatment also increased the NIS gene expression in zebrafish larvae. In our study, we found that exposure to both PFOS and nano-ZnO could change the transcription levels of the TG gene and protein in zebrafish. Manchado et al. (2008) also reported...
that THs mediate metamorphosis and down-regulate TG at the transcriptional level in Senegalese sole (Solea senegalensis Kaup). The TPO enzyme catalyzes the iodination of the tyrosyl residues in TG and their coupling to iodothyronines (Dunn and Dunn, 2001). Our study showed that after exposure to PFOS and nano-ZnO, the transcription of the TPO gene was unchanged. TPO may be less sensitive to PFOS and nano-ZnO stress. Similarly, Yu et al. (2009) found that the activity of TPO in the thyroid was unaffected by PFOS treatment in rats. Yu et al. (2010) also reported that TPO may not be a reliable endpoint for analyzing the adverse effects of PBDE exposure.

TTR functions in concert with two thyroid hormone-binding proteins in the serum. TTR can bind THs and transports them to target tissues. Some researchers have suggested that TTR is a potential target for some chemicals that induced TH disruption in rodents, birds and fish (Yu et al., 2010; Chen et al., 2012; Kawakami et al., 2006). Yamauchi et al. (2003) also reported that the TTR assay could be applied as a primary screen for possible thyroid-disrupting chemicals in environmental wastewater. In our study, co-exposure to PFOS and nano-ZnO resulted in the down-regulation of the transthyretin (TTR) gene and protein transcription. Our results were consistent with those of a report in which TTR gene expression was significantly down-regulated in BDE-209 and Pb co-exposed zebrafish (Zhu et al., 2014). Down-regulation of TTR mRNA expression after exposure to PFOS had been observed in fish and rats (Wang et al., 2011; Shi et al., 2009). The down-regulated TTR gene expression might result in less TTR protein synthesis and thus an increase in the TH levels induced by PFOS co-exposure. Deio1 and Deio2 are members of a subfamily of deiodinase enzymes. They are important in the activation and deactivation of THs in tissues. The activation of T4 occurs by conversion to the active T3 through the removal of an iodine atom on the outer ring (Orozco and Valverde, 2005). Deiodinase activity is critical for regulating the TH concentrations (Mol et al., 1999). The mRNA expression levels of Deio1 and Deio2 were significantly increased in both the 0.8 mg/L PFOS single- and co-exposure groups compared with the control group. There was a significant difference in the transcription of the TTR β genes in the single-exposure groups and the control group between all of the PFOS single-exposure groups and the control group. There was a significant difference in the expression of the TR α and TR β genes in the common frog (Rana temporaria) following exposure to DDE. In the present study, the TRα gene expression was significantly decreased in the 0.4 and 0.8 mg/L PFOS co-exposure groups compared with the control group (p < 0.01). TRβ gene expression was also significantly changed in the co-exposure groups compared with the control group. These observations following co-exposure to PFOS and nano-ZnO showed that such mixtures had more pronounced negative effects on TRα and TRβ mRNA expression than exposure to either agent alone. Liu et al. (2000) found that the expression of the TRα and TRβ genes differed because they perform different functions in specific organization. Arukwe and Jenssen (2005) also reported that the TRα and TRβ gene expression differed in the brain tissue of frogs exposed to p-chlorophenyl.

PFOS and nano-ZnO can interfere with homeostasis in the thyroid gland; consequently, exposure to a mixture of the two chemicals can cause more profound adverse effects on the THs than exposure to either agent alone. Nano-ZnO have a strong adsorption capacity for other trace pollutants due to their small size, large specific surface area and high surface energy. Sulfonic groups of PFOS might be adsorbed onto nano-ZnO and then enter into the zebrafish, with the accumulation of nano-ZnO, and the physical properties (i.e., small particle size and strong penetrability) of nano-ZnO could induce more cell membrane damage, resulting in the transmembrane transport of sulfonic groups of PFOS through the membrane and the enhancement of the cellular uptake of sulfonic groups. Nanoparticles can interact with other environmental chemicals and thereby exert joint effects on biological systems and the environment. Some surfactants with different ion types could alter the properties of NPs in different ways and lead to complex effects on the toxicities of NPs (Wang et al., 2014a). Our present study also suggested that the toxicity of mixed materials should be studied in greater depth to more comprehensively and scientifically evaluate the ecological risks.

4. Conclusions

The findings of this study suggest that exposure to PFOS and nano-ZnO might lead to endocrine disruption in developing zebrafish larvae by causing changes in the TH levels. There were some exposure-dependent alterations and enhance effects of PFOS and nano-ZnO co-exposure on the HPT-axis-mediated gene and protein expression in developing zebrafish larvae. The results also demonstrated that co-exposure to these two chemicals might result in an increase in toxicological effects in organisms. There are complex compositions of chemicals present in the environment. However, there are currently very limited data
available on the mechanisms of toxicity and interactions of mixtures of chemicals. Other mechanisms are not fully understood. It is important to understand the toxic effects of mixtures of different environmental pollutants. Therefore, further studies are required to determine the mechanisms by which the various agents exert their toxic effects, and studies focusing on the chemical uptake or chemical interactions in vivo and/or in vitro are needed.

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