OXIDATIVE STRESS AND APOTOSIS TO ZEBRAFISH (DANIO RERIO) EMBRYOS EXPOSED TO PERFLUOROOCTANE SULFONATE (PFOS) AND ZNO NANOPARTICLES

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Abstract

Objectives: Perfluorooctane sulfonate (PFOS) and zinc oxide nanoparticles (ZnO-NPs) are frequently detected in the environment but few studies have assessed the joint toxicity of them. Oxidative stress and apoptosis to zebrafish (Danio rerio) embryos induced by the PFOS and ZnO-NPs were investigated in this study. Material and Methods: The embryos were exposed to the PFOS (0, 0.4, 0.8 and 1.6 mg/l), ZnO-NPs (12.5, 25, 50 mg/l) and PFOS plus ZnO-NPs (0.4+12.5, 0.8+25 and 1.6+50 mg/l) mixture solutions until 96 h post-fertilization. Activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (Gpx) and malondialdehyde (MDA) content were measured in zebrafish embryos after exposure lasting for 96 h. At the same time, the genes expression related to reactive oxygen species (ROS) generation, oxidative damage and apoptosis were also measured. Results: A significant induction of the ROS accompanied by the increase in the activity of the Gpx and MDA contents were found in co-treatment groups. Furthermore, several apoptosis pathway related genes such as Bax, p53, caspase-3 and caspase-9 were significantly up-regulated in the PFOS plus ZnO-NPs exposure groups, while anti-apoptotic gene Bcl-2 was significantly down-regulated in the PFOS plus ZnO-NPs exposure groups. In addition, some oxidative stress-related genes such as Cat, GSH peroxidase 1 (Gpx1a) and superoxide dismutase 1 (Sod1) were also significantly down-regulated after the PFOS plus ZnO-NPs co-treatments. Conclusions: The results demonstrated that the PFOS plus ZnO-NPs co-exposure could cause more serious oxidative stress and apoptosis than the PFOS and ZnO-NPs exposure alone at the exposure concentrations above. The synergistic effects of the PFOS and ZnO-NPs may be the important mechanisms of their toxicity to zebrafish embryos. Int J Occup Med Environ Health 2017;30(2):213–229

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INTRODUCTION
Zinc oxide (ZnO) nanomaterials have been widely used in the rubber industry, coating, magnetic substance, ceramic industry and many other processed products [1]. The increased use of the ZnO nanomaterials in industrial material has inevitably increased human and environmental exposure. Zinc oxide nanoparticles (ZnO-NPs) have accumulated in the lake and ocean in Europe [2]. Zinc oxide nanoparticles have biological toxicity in the nano-sized properties [3]. Zinc oxide nanoparticles induced inflammation response in vascular endothelial cells [4]. Zinc oxide nanoparticles also induced mitochondrial toxicity and cytotoxicity in human lung epithelial cells [5]. Zinc oxide nanoparticles induced oxidative deoxyribonucleic acid (DNA) damage and apoptosis in HepG2 cell [6].

The perfluorooctane sulfonate (PFOS) has been widely used in processed products, such as textiles, carpets, paper, propellants and insecticides for many years [7–9]. Though the PFOS has been banned in many countries in recent years, this chemical substance may still be found all over the world [10]. The concentration of the PFOS is 100 ng/l in the Tennessee River [11]. The largest concentration of the PFOS was 4385 ng/l in the Ruhr River [12]. Jin et al. (2009) found out that median concentrations (largest concentration) of the PFOS in the water were 0.4 (2.4) ng/l for the downtown (N = 13), and 4.0 (14.1) ng/l – for the city (N = 22) in China, respectively [13]. The toxicity of the PFOS has also claimed much attention in the aquatic environment. Du et al. (2009) found that the PFOS exposure to maternal induced in malformation offspring [14]. The perfluorooctane sulfonate caused DNA damage in common carp (Cyprinus carpio) [15]. In addition, the PFOS led to bioaccumulation and histological alterations in liver, thyroid and gonads of zebrafish (Danio rerio) [16].

The biology of particle-induced oxidative stress is an important mechanistic paradigm, on the basis of which a predictive model for studying engineered nanomaterials toxicity has been developed [17]. The imbalance between reactive oxygen species (ROS) and antioxidant defense induced the oxidative stress [18]. Deoxyribonucleic acid damage and cellular apoptosis induced the ROS generation [6]. The antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx)) reduced the damage of the excessive ROS in organism [19]. Zinc oxide nanoparticles through the oxidative stress induced oxidative damage [20]. Zinc oxide nanoparticles exposure to zebrafish embryo induced the cellular oxidative stress response and the ROS generation [21]. The perfluorooctane sulfonate could induce oxidative stress and apoptosis in the lung of rat offspring [22]. Apoptosis is the process of programmed cell death (PCD) that occurs in multicellular organisms. The caspase family plays an important role in the induction, transduction and amplification of intracellular apoptotic signals [23]. The p53 protein regulates the cell cycle and apoptosis induction in multicellular organisms [24]. When p53 was activated, it induced the expression of Bax gene and activated the effector caspase-3 [25]. Bel-2 was an apparent antioxidant to prevent apoptosis [26]. The perfluorooctane sulfonate triggered the transactivation of Bax and activate caspase-3 and caspase-9 in zebrafish embryos [27]. Zinc oxide nanoparticles were found to elicit caspase-3 activation and induce Bax gene expression in cultured primary astrocytes [28]. Ahamed et al. (2011) found that ZnO-NPs induced apoptotic cell death by oxidative stress [20].

Aquatic organisms are exposed to mixtures of chemicals for a long time. Many studies have evaluated the biological toxicity of the PFOS and ZnO-NPs by investigating exposure to individual toxicant [16,29]. Some studies have focused on the combination of organic contaminants or inorganic contaminants to study the joint toxicity [30]. Combined toxicity assessment should adopt co-exposure models [31]. Sarigianannis and Hansen (2012) have provided that combined toxicity assessment should consider the cumulative effects [32]. There is inadequate data used for assessing the joint effect of the PFOS and ZnO-NPs exposure on oxidative stress and
apoptosis. In addition, the molecular mechanism of their joint toxicity remains largely unclear.

In this research, zebrafish was used for evaluating the joint effects of the PFOS and ZnO-NPs on oxidative stress and apoptosis pathways. Indicators such as the SOD activity, malondialdehyde (MDA) content, CAT activity, Gpx activity, caspase-9 activity, caspase-3 activity and the level of ROS were examined. Gene expression patterns related to the apoptosis pathway (pro-apoptotic p53, and Bax), apoptosis protease-activating factor (caspase-9 and caspase-3), anti-apoptotic (Bcl-2) and oxidative stress (Cat, Gpx1a and Sod1) were also examined to elucidate the potential molecular mechanism underlying the induction of oxidative stress and apoptosis by the PFOS and ZnO-NPs.

MATERIAL AND METHODS

Chemicals and preparation of stock solutions
Heptadecafluorooctanesulfonic acid (PFOS) potassium salt (Chemical Abstracts Service (CAS) No. 2795-39-3; ≥ 98% purity), heptadecafluorooctanesulfonic acid potassium salt (PFOS; CAS No. 2795-39-3; analytical standard (Fluka), dimethyl sulfoxide (DMSO, ≥ 99.9%), ZnO-NPs (CAS No. 1314-13-2, St. Louis, MO, USA). Other chemicals and solvents were of analytical grade.

Characterization of ZnO-NPs
Zinc oxide nanoparticles solutions were dispersed by the Ultrasonic processor for 20 min. A series of the ZnO-NPs solutions were prepared by means of stepwise dilution with zebrafish culture medium. The morphology of the ZnO-NPs aggregates were observed using a scanning electron microscope (Photo 1a) and a transmission electron microscopy (TEM) system (Photo 1b). The images revealed that the average size of ZnO-NPs was 40 nm. The particle size distribution of the ZnO-NPs was obtained by a dynamic light scattering (DLS) device, and the results showed polydispersed aggregates characterized by single particles of 30–100 nm in size (Photo 1b).

Photo 1. a) Zinc oxide nanoparticles (ZnO-NPs) suspension of 50 mg/l (scanning electron microscopy), b) ZnO-NPs suspension of 50 mg/l (transmission electron microscopy) and particle size distribution (dynamic light scattering – DLS)
Chemical analysis
Zinc oxide nanoparticles solutions were centrifuged at 10 000 g for 30 min, and dissolved zinc (Zn\textsuperscript{2+}) was measured by the inductively coupled plasma-mass spectrometer (ICP-MS) (Thermo Elemental X7, Thermo Electron Co., USA). The perfluorooctane sulfonate concentrations were analyzed by means of the solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [12].

Zebrafish maintenance
Adult AB strain zebrafishes were fed twice daily with frozen blood worms, and kept at approximate 28°C under 14 h light: 10 h dark in the beaker. Zebrafishes were paired in spawning boxes overnight. Spawning was accomplished at 7 a.m., and then the fertilized eggs were collected and transferred to culture water to rinse.

Samples exposure
The normal embryos were exposed to the ZnO-NPs solution (1, 5, 10, 20, 50, 100, 200 mg/l) and PFOS solution (1, 2, 4, 8, 16 mg/l). It was concluded that the 96 h lethal concentration (LC\textsubscript{50}) of ZnO-NPs and PFOS were 60 mg/l and 3.502 mg/l, respectively (Table 1). The mortality of zebrafish was the same after having been exposed to the PFOS (1.6 mg/l) and ZnO-NPs (50 mg/l) (Table 2). The results showed that the PFOS (1.6 mg/l) and ZnO-NPs (50 mg/l) exposure caused the obvious symptom as compared with the control. The exposure concentrations of the ZnO-NPs were 0, 12.5, 25, 50 mg/l and PFOS were 0, 0.4, 0.8, 1.6 mg/l, respectively. The exposure concentrations of the PFOS were higher than those measured in the water of China in this research [13]. However, it was an appropriate method to use the relatively higher concentration of toxicants to elicit the possible toxicity mechanisms in the laboratory short-term exposures [29,33,34]. Four hundred normal embryos were put into every beaker containing 500 ml of the ZnO-NPs plus PFOS (12.5+0.4, 25+0.8, 50+1.6 mg/l),

| Study group exposure | Zebrafish embryo 96-h mortality (M±SD) |
|-----------------------|----------------------------------------|
| particle concentration [mg/l] | ZnO-NPs | 1.0 | 15.00±2.10 |
|                         | 5.0 | 22.25±4.67 |
|                         | 10.0 | 27.67±3.58 |
|                         | 20.0 | 23.00±7.89 |
|                         | 50.0 | 25.33±4.43 |
|                         | 100.0 | 31.00±3.41 |
| PFOS                  | 0.3 | 10.00±4.80 |
|                         | 0.5 | 14.36±6.34 |
|                         | 0.8 | 11.71±2.60 |
|                         | 1.2 | 15.00±3.95 |
|                         | 1.6 | 25.10±5.32 |
|                         | 2.0 | 30.00±2.34 |
| Control                | 0.0 | 9.66±0.25 |

M – mean; SD – standard deviation.

PFOS single (0, 0.4, 0.8, 1.6 mg/l) and ZnO-NPs single (0, 12.5, 25 and 50), respectively. Reactive oxygen species measurement was performed after 96 h exposure to larvae. The tested samples were stored at ~80°C.

Antioxidant enzymes activity analysis
Three replicates of samples were homogenized in buffer solution (0.01 M Tris hydrochloride (Tris-HCl), 0.0001 M ethylenediaminetetraacetic acid 2Na (EDTA-2Na), 0.01 M

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**Table 1.** The 96-h lethal concentration (LC\textsubscript{50}) of zinc oxide nanoparticles (ZnO-NPs) and perfluorooctane sulfonate (PFOS) to zebrafish embryo

| Particle     | 96-h LC\textsubscript{50} | 95% CI       |
|--------------|-----------------------------|---------------|
| ZnO-NPs      | 60.000                      | 83.35–44.16   |
| PFOS         | 3.502                       | 2.369–5.051   |

CI – confidence interval.

**Table 2.** The 96-h mortality of zebrafish embryo after exposure to zinc oxide nanoparticles (ZnO-NPs) and perfluorooctane sulfonate (PFOS)
The 96-well plate was kept at 37°C dark incubator for 30 min. The fluorescence intensity was determined by means of the micro plate reader with excitation and emission at 485 nm and 530 nm. The total protein was determined using protein assay kits (Jiancheng Biochemistry Co., Nanjing, China). The concentrations of the ROS were determined in arbitrary units (dichlorofluorescein milligram per protein).

Caspase activity assay

The caspase activity was determined by means of the colorimetric assay according to the extent to acetyl-Asp-Glu-Val-Asp-nitroanilide and acetyl-Leu-Glu-His-Asp-p-nitroanilide, respectively. The chemicals converted into yellow formazan products. In a typical procedure, after 96 h, 80 hatched larvae were rinsed with cold buffer solution and homogenised for 300 s. The solution was then centrifuged at 12,000 g for 15 min at 4°C, and the supernatant was collected. The caspase activities were expressed in terms of the percentage enzyme activity compared with the control.

Quantitative real-time PCR assay

Ribonucleic acid was extracted using Trizol Reagent after having been exposed for 96 h. Then the RNA was digested...
Concentrations of the PFOS from the series of exposure suspensions (0.4, 0.8 and 1.6 mg/l) were 0.33, 0.64 and 1.23 mg/l, respectively. The series of the PFOS and ZnO-NPs exposure suspensions (0.4+12.5, 0.8+25 and 1.6+50 mg/l) were 0.31, 0.54 and 1.19 mg/l, respectively. After having been verified analytically, the exposure concentrations used in this study in terms of the results proved that renewed solutions daily was appropriate for the PFOS and ZnO-NPs to ensure that exposures concentrations were at the intended level.

Activity of antioxidant enzymes

The superoxide dismutase activity was significantly increased in a dose-dependent manner in all groups (except for 0.4 and 0.8 mg/l PFOS single-treatment) compared with that in the control (p < 0.05) (Figure 1a). The superoxide dismutase activity was significantly increased in 0.4, 0.8 and 1.6 mg/l of the PFOS joint exposure groups relative to 0.4, 0.8 and 1.6 mg/l PFOS single exposure groups, respectively.

The catalase activity was significantly decreased in 1.6 mg/l of the PFOS joint exposure groups compared to 1.6 mg/l of the PFOS single exposure group (p < 0.05) (Figure 1b). The catalase activity was significantly decreased in a dose-dependent manner in all the ZnO-NPs single-treatment, 12.5 mg/l of the ZnO-NPs co-treatment and 50 mg/l of the ZnO-NPs joint exposure groups compared to the control (p < 0.05). In addition, the CAT activities were not significantly decreased in the PFOS single-treatment groups as compared to the control.

Glutathione peroxidase activities in zebrafish embryo were found to be increased significantly in all groups (except for 0.4 and 0.8 mg/l PFOS single exposure groups) as compared to the control (Figure 1c). Significant increases were similarly observed in 0.4, 0.8 and 1.6 mg/l of the PFOS joint exposure groups compared to 0.4, 0.8 and 1.6 mg/l of the PFOS single exposure group (p < 0.05). Glutathione peroxidase activities were also significantly increased in a dose-dependent manner in all groups (except for 0.4 and 0.8 mg/l PFOS single-treatment) compared with that in the control (p < 0.05) (Figure 1a).
**Fig. 1.** a) Superoxide dismutase (SOD), b) catalase (CAT), and c) glutathione peroxidase (GPx) activity and d) malondialdehyde (MDA) content in zebrafish embryos after exposure to perfluorooctane sulfonate (PFOS) and zinc oxide nanoparticles (ZnO-NPs) at 96 h post-fertilisation.

* Statistically significant difference from PFOS and ZnO-NPs co-exposure at p < 0.05 level.
# Statistically significant difference from control at p < 0.05 level.
Values represent the mean ± standard error of 3 replicates (p < 0.05).
increased as compared with that in the control (p < 0.05) (Figure 2). The levels of the ROS in 0.8 and 1.6 mg/l of the PFOS joint exposure groups were significantly increased as compared with that in 0.8 and 1.6 mg/l of the PFOS single exposure groups (p < 0.05). Moreover, the level of the ROS in 1.6 mg/l of the ZnO-NPs co-treatment group was significantly increased to 50 mg/l of the ZnO-NPs single-treatment group.

Effects of ZnO-NPs on the expression of gene related to the antioxidant defence system

The mRNA expression levels of the PFOS and ZnO-NPs exposure were measured by RT-PCR (Figure 3). The expression level of the Cat gene was significantly down-regulated in 1.6 mg/l of the PFOS single exposure group, 0.8 and 1.6 mg/l of the PFOS joint exposure groups (0.81, 0.77 and 0.75-fold; p < 0.05) (Figure 3a). The transcription of the Cat gene in 0.8 mg/l of the PFOS and 25 mg/l of the ZnO-NPs joint exposure group was significantly increased as compared with that in 0.8 mg/l of the PFOS and 25 mg/l of the ZnO-NPs single-treatment groups. The mRNA levels of Gpx1a in zebrafish larvae were significantly decreased by 0.72, 0.72 and 0.38-fold after treatment with 1.6 mg/l of the PFOS single-treatment, 0.8 mg/l of the PFOS co-treatment and 1.6 mg/l of the PFOS joint exposure groups compared with that in the control (Figure 3b). In addition, the expression levels of Gpx1a in zebrafish larvae were significantly decreased in 1.6 mg/l of the PFOS and 50 mg/l of the ZnO-NPs co-treatment groups compared to 1.6 mg/l of the PFOS single-treatment and 50 mg/l of the ZnO-NPs single-treatment groups. Sod1 mRNA expression was significantly affected by 1.6 mg/l of the PFOS single and joint exposure groups compared to the control (Figure 3c). The expression of Sod1 mRNA in 1.6 mg/l of the PFOS and 50 mg/l of the ZnO-NPs co-treatment group was significantly decreased to 1.6 mg/l of the PFOS and 50 mg/l of the ZnO-NPs single-treatment groups.
Effects of PFOS and ZnO-NPs on Caspase-3 and Caspase-9 activities

In order to determine whether the PFOS and ZnO-NPs induced apoptosis through the caspase pathway, the Caspase-3 and Caspase-9 activities were examined. After the PFOS and ZnO-NPs joint exposure, the activities were significantly increased in a concentration-dependent manner (Figure 4). As shown in the Figure 4a, the Caspase 3 activities were significantly increased in 1.6 mg/l of the PFOS single-treatment, 0.8 mg/l of the PFOS and 1.6 mg/l of the PFOS joint exposure groups compared with that in the control (p < 0.05). The level of the Caspase 3 activity in 0.8 mg/l of the PFOS joint exposure group was significantly increased to 0.8 mg/l of the PFOS single exposure group (p < 0.05). In addition, the Caspase 3 activities in the ZnO-NPs co-treatment groups were significantly increased as compared with that in the ZnO-NPs single-treatment groups. The Caspase 9 activities were significantly increased in 1.6 mg/l of the PFOS single exposure group, 0.8 mg/l of the PFOS co-treatment and 1.6 mg/l of the PFOS co-treatment groups as compared to the control (Figure 4b).
addition, the level of \( Bax \) expression in 1.6 mg/l of the PFOS joint exposure group was significantly increased to 1.6 mg/l of the PFOS single treatment and 50 mg/l of the ZnO-NPs single treatment groups. The expression of the anti-apoptotic gene \( Bcl-2 \) was significantly down-regulated by 0.31-fold in 1.6 mg/l of the PFOS joint exposure group as compared with that in the control (Figure 5c). Moreover, the expression of \( Bcl-2 \) was significantly down-regulated by 0.76 and 0.70-fold in 1.6 mg/l of the PFOS single- and 50 mg/l of the ZnO-NPs single-treatment groups \((p < 0.05)\). The transcription of \( caspase-3 \) in the groups of 25 mg/l of the ZnO-NPs single- and co-treatment were significantly up-regulated by 2.05 and 2.1-fold as compared to the control, respectively (Figure 5d). Moreover, the expression of \( caspase-3 \) gene in the groups of 50 mg/l of the ZnO-NPs single- and co-treatment were significantly up-regulated by 2.46 and 2.96-fold in relation to the control. The levels of \( caspase-3 \)
expression in the PFOS co-treatment groups were significantly increased as compared with that in the PFOS single-treatment groups ($p < 0.05$). The expression of the apoptosis gene caspase-9 were significantly up-regulated by 3.66, 3.46, 3.56 and 4.96-fold in 1.6 mg/l of the PFOS single, 50 mg/l of the ZnO-NPs single, 25 mg/l of

**Fig. 5.** a) p53, b) Bax, c) Bcl-2, d) caspase-3, and e) caspase-9 genes expression levels in zebrafish embryos after exposure to perfluorooctane sulfonate (PFOS) and zinc oxide nanoparticles (ZnO-NPs) at 96 h post-fertilization.
Synergistic effects play a more important role during the PFOS and ZnO-NPs joint exposure. In our study, the PFOS and ZnO-NPs were found to generate intracellular ROS when analysed using cell permeable dye DCFH-DA. The concentration-dependent increase of the ROS in the organism exposure to the PFOS and ZnO-NPs indicated that the embryos sustained more serious oxidative damage after joint exposure to the PFOS and ZnO-NPs. Qian et al. (2010) reported that the PFOS induced ROS production in human microvascular endothelial cells [41]. Zinc oxide nanoparticles exposure enhanced the ROS production in primary human T cells [42]. Malondialdehyde is a by-product of cellular lipid peroxidation. The results indicated that the PFOS and ZnO-NPs single and co-treatment induced oxiradicals (Figure 1d). Glutathione peroxidase has a complementary role in hydrogen peroxide (H$_2$O$_2$) detoxification and may reduce the tissue injury by removing H$_2$O$_2$. The results indicated that the PFOS and ZnO-NPs single- and co-treatment groups (Figure 1c). The CAT activity was significantly decreased in 1.6 mg/l of the PFOS joint exposure group as compared to 1.6 mg/l of the PFOS single exposure group (p < 0.05) (Figure 1b). The superoxide dismutase plays an important role in the anti-oxidant system and the CAT is a member of important antioxidases and both of them may eliminate the ROS in vivo. It was observed that the level of the SOD activity, Gpx activity and the MDA content were significantly increased in co-treatment groups as compared with those in single-treatment groups (Figure 1). Zinc oxide nanoparticles exposure induced developmental toxicity, oxidative stress and DNA damage in embryo-larval zebrafish [29]. Zinc oxide nanoparticles exposure induced oxidative stress in the gills, live and gut of zebrafish [43]. The perfluorooctane sulfonate exposure induced oxidative stress in the lung of rat off-spring [22]. The results showed the similar trend as the description of Kim and they found that

**DISCUSSION**

This study has presented some evidence for the first time that the PFOS and ZnO-NPs co-exposure induce oxidative stress, apoptosis, as well as alterations in the expression of oxidant- and apoptosis-related genes in the early stages of zebrafish development. It has turned out that the
pre-exposure to the PFOS affected antioxidant defence mechanisms and potentially increased of cadmium toxicity [44]. Alteration of these parameters indicated that oxidative stress-related processes were involved in the induced PFOS and ZnO-NPs in vivo toxicity.

The expression levels of gene encoding for antioxidant proteins Sod1, Cat and Gpx1a were also analysed in the PFOS and ZnO-NPs treated zebrafish embryos. The expression level of Gpx1a and Cat gene were down-regulation after having been exposed to toxicants compared with that in the control (Figure 3a and 3b). In addition, the expression levels of Sod1 were not significantly affected by the PFOS and ZnO-NPs (Figure 3c). It had been previously demonstrated that expression levels of Sod1 were not affected after exposure to the ZnO-NPs and heavy metals (Zn and Cd) [45,46]. Zebrafish treated with silver nanoparticles also displayed down-regulated Cat and Gpx translation in liver [47], which provided further support to our results. In addition, it was demonstrated that the combination of the PFOS and ZnO-NPs had synergistic effects on the repression of antioxidant gene expression as well as the generation of oxidative stress in developmental zebrafish embryos.

To further investigate apoptosis caused by the PFOS and ZnO-NPs in zebrafish embryos, the caspase activity was measured using specific chromogenic substrates. From the Figure 4, it could be found that the PFOS and ZnO-NPs induced the enhancement of the caspase-3 and -9 activities in the zebrafish larvae. Wang et al. (2014) also reported that the ZnO-NPs induced the caspase-3 activity in cultured primary astrocytes [28]. Chen et al. (2012) proved that the PFOS induced activity of the caspase-3 and caspase-9 up-regulation in the lung of rat [22]. The Figure 5 showed that the PFOS and ZnO-NPs induced apoptosis by up-regulation of p53 and Bax gene expression in a dose-dependent manner. The p53-related pro-apoptotic Bcl-2 family genes were induced after the exposure to the PFOS and ZnO-NPs. The decrease was found in Bcl-2 ratio along with increased caspase-3 and caspase-9 expression (Figure 5). Liu et al. (2007) also reported that the PFOS triggered the transactivation of Bax and activated caspase-3 and caspase-9 in zebrafish embryos [27]. Wang et al. (2014) reported that the ZnO-NPs induced Bax gene expression in cultured primary astrocytes [28]. Some researchers also found out that the ZnO-NPs induced apoptotic cell death via p53, Bax/Bcl-2, and caspase pathways mediated by oxidative stress [20].

Nanoparticles interacted with other chemicals and induced joint toxicity effects on biology or environment. Wang et al. (2014) found out that some surfactants with different ion types could alter the properties of nanoparticles (particle size or surface charge) and present complex joint effects on nanoparticles toxicity [48]. Mixtures of different nanoparticles and surfactants exhibited antagonistic, synergistic, and additive effects. In recent decades, some models have been developed to predict the joint effect of chemical mixtures on organisms and processes in the environment [30]. From the result of the ICP-MS, there was more soluble Zn$^{2+}$ released from the ZnO-NPs co-exposure suspensions than that from the ZnO-NPs single-exposure suspensions. Zinc oxide nanoparticles released Zn$^{2+}$ [46] and the PFOS released anionic n-alkyl surfactants into solution [49]. The joint toxicity effect of the PFOS and ZnO-NPs exposure could be explained in terms of interactions between the Zn$^{2+}$ and the surfactants. Based on the structural characteristics of the PFOS and ZnO-NPs, the potential reasons and mechanism hypotheses are as follows: the surfactants may damage the membranes by perturbing the lipid bilayers [50,51]. Therefore, nanoparticles penetrated through cell membranes easier and caused more serious damage to zebrafish. A previous study proved that the mitochondrial membrane potential mediated the pathway involved in the ZnO-NPs induced apoptosis [52]. In this research, the joint effect of the PFOS and ZnO-NPs was presumed to be synergistic. The perfluorooctane sulfonate and some nanoparticles impaired the multi-xenobiotic resistance (MXR) mechanism, which resulted in the increased intracellular
levels of other compounds, thereby potentiating their toxicity [53]. This study suggested that the PFOS increased the intracellular levels of the ZnO-NPs and induced more serious damage to organism. It suggests that the impact of other coexist compounds should be considered when the ecological risk assessments of the ZnO-NPs are made. Further work is required to find different mechanisms behind synergistic interactions, such as chemical uptake or chemical interactions outside the organism.

CONCLUSIONS

This is the first time to investigate the joint toxicity induced theFOS and ZnO-NPs on zebrafish embryos. This study has demonstrated the occurrence of oxidative stress and apoptosis in the embryo-larval stages of zebrafish exposed to the PFOS and ZnO-NPs. It has been proven that oxidative stress and apoptosis induced by the PFOS at different concentrations are enhanced in the presence of the ZnO-NPs after 96 h exposure. The acute PFOS and ZnO-NPs exposure induce oxidative stress and apoptosis through the excessive ROS, change the activities of the defence enzymes (SOD, CAT and Gpx), apoptosis enzymes (Caspase-3 and Caspase-9), and increase the MDA concentration in zebrafish larvae. In addition, It has also been revealed that the mRNA levels of genes, which encode in response to the oxidative damage (such as Cat and Gpx1a), induction genes in response to apoptosis (such as p53, Bax, Bcl-2, caspase-3 and caspase-9), are also significantly altered in larvae zebrafish after the PFOS and ZnO-NPs joint exposure. In conclusion, the combination of the PFOS and ZnO-NPs has synergistic effects on induction of oxidative stress and apoptosis. The observed synergism suggests that the inclusion of mixture considerations in the risk assessment of the PFOS and ZnO-NPs is needed.

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