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Acute ZnO nanoparticles exposure induces developmental toxicity, oxidative stress and DNA damage in embryo-larval zebrafish

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ABSTRACT

Nano-scale zinc oxide (nano-ZnO) is widely used in various industrial and commercial applications. However, the available toxicological information was inadequate to assess the potential ecological risk of nano-ZnO to aquatic organisms and the publics. In this study, the developmental toxicity, oxidative stress and DNA damage of nano-ZnO embryos were investigated in the embryo-larval zebrafish, the toxicity of Zn^{2+} releasing from nano-ZnO were also investigated to ascertain the relationship between the nano-ZnO and corresponding Zn^{2+}. Zebrafish embryos were exposed to 1, 5, 10, 20, 50, and 100 mg/L nano-ZnO and 0.59, 2.15, 3.63, 4.07, 5.31, and 6.04 mg/L Zn^{2+} for 144 h post-fertilisation (hpf), respectively. Up to 144 hpf, activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and malondialdehyde (MDA) contents, the genes related to oxidative damage, reactive oxygen species (ROS) generation and DNA damage in zebrafish embryos were measured. The nano-ZnO was found to exert a dose-dependent toxicity to zebrafish embryos and larvae, reducing the hatching rate and inducing malformation and the acute toxicity to zebrafish embryos was greater than that of the Zn^{2+} solution. The generation of ROS was significantly increased at 50 and 100 mg/L nano-ZnO. DNA damage of zebrafish embryo was evaluated by single-cell gel electrophoresis and was enhanced with increasing nano-ZnO concentration. Moreover, the transcriptional expression of mitochondrial inner membrane genes related to ROS production, such as Bcl-2, in response to oxidative damage, such as Nqo1, and related to antioxidant response element such as Gstp2 were significantly down-regulated in the nano-ZnO treatment groups. However, the nano-ZnO up-regulated the transcriptional expression of Ucp2-related to ROS production. In conclusion, nano-ZnO induces developmental toxicity, oxidative stress and DNA damage on zebrafish embryos and the dissolved Zn^{2+} only partially contributed to the toxicity of nano-ZnO. The adverse effects of nano-ZnO may be the important mechanisms of its toxicity to zebrafish embryos.

1. Introduction

With the development of nanoparticles (NPs), nanotechnology is moving toward larger scale production and increasing applications, hence, it is inevitable that NPs and respective byproducts will be released directly and indirectly into the aquatic environment. The unique properties of small size (one or more dimensions of the order of 100 nm or less), chemical composition, agglomeration, high persistence, worldwide distribution and biocompatibility of NPs have received great concern over their toxicity. Several studies have focused on the toxicity of various NPs in the early life stages of fish. For example, silver NPs induce developmental retardation and abnormalities in the early life stages of Japanese medaka (*Oryzias latipes*) (Wu et al., 2010). Silver NPs at 0.25 mM is more toxic than gold NPs at the same concentration in zebrafish embryos (Bar-Ilan et al., 2009). Zebrafish specimens loaded with functionalized multi-walled carbon nanotubes (MWCNTs) were found to have normal primordial germ cells at early stages and damaged reproduction potential (Cheng et al., 2009). Copper NPs at ≥0.1 mg/L induces gill injury to adult zebrafish (Griffitt et al., 2007). Exposure to TiO_2 NPs causes several gill pathologies, including edema and thickening of the lamellae, and significantly increases the total glutathione levels in rainbow trout (*Oncorhynchus mykiss*) (Federici et al., 2007).

Nano-scale zinc oxide (nano-ZnO), due to their unique properties and ability to form diverse nanostructures, has been widely applied in optoelectronics, cosmetics, catalysts, ceramics, and pigments, etc. The increased use of nano-ZnO has inevitably led to elevated human and environmental exposures and induced the toxicological effects environmental organisms. On the other hand, nano-ZnO particles are easily bio-accumulated by aquatic organisms, wherein they elicit toxic effects. The limited information currently available on the toxic effects of nano-ZnO in fish revealed that nano-ZnO induced the development (Yu et al., 2011) and hatch inhibition (Xia et al., 2011) in zebrafish embryo. The

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possible toxic mechanisms of nano-ZnO in fish are quite complex. Zhu et al. (2009) suggested that nano-ZnO and Zn^{2+} elicited embryonic toxicity by increasing the reactive oxidative species (ROS) and/or compromising the cellular oxidative stress response. Moreover, nano-ZnO suspensions have other toxic mechanisms, such as mechanical damage to gill cells by direct contact with the particles (Yu et al., 2011).

Recent studies have investigated the relationship between the toxicity of nano-ZnO and the release of Zn^{2+} ions (Franklin et al., 2007; Bai et al., 2010; Xia et al., 2011; Fukui et al., 2012; Gilbert et al., 2012). However, some disputes emerged on the role of dissolved Zn^{2+} in the toxic mechanisms of nano-ZnO. For example, some studies suggested that the toxicity of nano-ZnO in freshwater microalga (*Pseudokirchneriella subcapitata*) and marine organisms was significantly influenced by the release of Zn^{2+} (Franklin et al., 2007; Wong et al., 2010). By contrast, other studies seldom ascribed the toxicity of nano-ZnO to dissolved Zn^{2+} (Bai et al., 2010; Xia et al., 2011). To date, the role of soluble Zn^{2+} in the toxicity of nano-ZnO in aquatic organisms and the mechanism of the interaction between them have not been fully elucidated (Bai et al., 2010; Wong et al., 2010; Xia et al., 2011).

It has been reported that reactive oxygen species (ROS) can be generated in living organisms exposed to environmental contaminants. The production of ROS has long been regarded as a possible mechanism of nanoparticles-induced toxicity as evidenced by triggered oxidative stress in recent study (Xiong et al., 2011). Moreover, the induction of the ROS results in oxidative damage to macromolecules such as proteins, DNA and lipids, finally leading to the damage of different cellular organelles (Sabatini et al., 2009). Additionally, DNA damage is mainly caused by the hydroxyl radical and superoxide anion radical and this damage is of particular concern because it can cause heritable effects and disease. Under normal conditions, in living organisms damaging effects of oxidative stress are counteracted by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Malondialdehyde (MDA), an indicator of lipid peroxidation contents, has been considered as one of the molecular mechanisms involved in nanoparticles-induced toxicity (Ma et al., 2010) and its predictive importance as a biomarker for oxidative stress is indicated in different investigation (Xu et al., 2011). Ma et al. had demonstrated that adult mice abdominal cavity injected nanoparticulate anatase (5 nm) exhibited reduced level of SOD, CAT, APX and GSH-Px the activities of brain. Additionally, a recent study suggested that acute exposure 5 mg/L nano-ZnO to adult zebrafish induced oxidative stress in the gills and elevated MDA level the liver (Xiong et al., 2011). Moreover, silver NPs (1 and 25 μg/L) caused cellular and DNA damages as well as oxidative stress in medaka, consequently activating the genes related to metal detoxification/metabolism regulation and radical scavenging action (Chae et al., 2009). Furthermore, exposure of zebrafish to silver NPs at 30 mg/L to 120 mg/L increased the mRNA expression of metallothionein-2 (MT2) in the liver and decreased the activities of the oxyradical-scavenging enzymes catalase (Cat) and glutathione peroxidase 1a (Gpx1a) (Choi et al., 2010). Although the previous studies showed that the nanoparticles could induce oxidative stress and DNA damage, the researches about genes related to oxidative damage were so inadequate that they could not supply the comprehensive information at the molecular level to indicate how the regulation of antioxidant enzyme activities and other negative effects in vertebrates is affected by nanoparticles stress.

The zebrafish embryo is a useful research model because it is small in size and transparent, easy to maintain and rapid embryogenesis and continuous reproduction. In addition, the zebrafish genome has been sequenced and genetic information is rapidly accumulating, 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. Previous studies have demonstrated that embryonic and larval zebrafish were useful for investigating chemical toxicity by using survivorship and development, as well as gene expression as endpoints. Based on the fact that zebrafish embryo represents a good model to assess the toxicity of nanoparticles (Lee et al., 2007), this study has utilized zebrafish embryo as a model to evaluate the developmental toxicity of nano-ZnO. Furthermore, to better understand the oxidative stress process induced by nano-ZnO and at the molecular level to indicate how the regulation of antioxidant enzyme activities in vertebrates is affected by nano-ZnO stress, the present study determined MDA contents, and the activities of SOD, CAT, and GPx, as well as the mRNA expression levels of genes encoding antioxidant proteins. Moreover, employing single cell gel electrophoresis (SCGE) to detect zebrafish embryo DNA damage after nano-ZnO treated. Finally, to determine whether the toxicity of nano-ZnO was attributed to the released Zn^{2+} from the nano-ZnO, we analyzed the level of Zn^{2+} ions released from nano-ZnO, and investigated any potential negative effects. The results reveal new insights into the mechanisms underlying the oxidative damages caused by nano-ZnO on zebrafish embryos.

2. Materials and methods

2.1. Nanoparticle characterization

Nano-ZnO dispersion with a published particle size of less than 100 nm was purchased from Sigma (St. Louis, MO, USA). A series of exposure suspensions (1, 5, 10, 20, 50, and 100 mg/L) were prepared by stepwise dilution with zebrafish culture medium (consisting of 64.75 mg/L, $NaHCO_3$, 5.75 mg/L, KCl, 123.25 mg/L, $MgSO_4{\cdot}7H_2O$, and 294 mg/L $CaCl_2{\cdot}2H_2O$). And then the suspending solutions containing nano-ZnO particles dispersed by an Ultrasonic processor JY92-IID 900W (Scientz, China) (frequency 25 kHz, amplitude 250 μm, continuous/pulse 30 s, tip diameter 6 mm) for 30 min. The morphology abnormalities of the nano-ZnO aggregates were observed using a scanning electron microscope (SEM, Philips XL30, FEI Company) operated at 15 kV. Samples for transmission electron microscopy (TEM) analysis were prepared by 50 mg/L solution of nano-ZnO on carbon-coated copper grids. TEM measurements were performed on a TecnaiTM G2 Spirit (FEI, The Netherlands) instrument operated at an accelerating voltage at 80 kV. The characteristics of nano-ZnO aggregates in medium were determined using a dynamic light scattering device (DLS, Brookhaven Instrument Corporation, Holtsville, NY, USA).

After the series of exposure suspensions (1, 5, 10, 20, 50, and 100 mg/L) freshly diluted and dispersed, each dose of nano-ZnO suspension was sampled at 24 h for analysis of dissolved Zn^{2+}. Sampled nano-ZnO suspensions were centrifuged at 10,000 × *g* for 30 min, and the supernatant was taken for determination of dissolved Zn^{2+} by inductively coupled plasma-mass spectrometer (ICP-MS, Thermo Elemental X7, Thermo Electron Co., USA). According to the results of ICP-MS, the corresponding soluble Zn^{2+} released from the series of exposure suspensions (1, 5, 10, 20, 50, and 100 mg/L) were 0.59, 2.15, 3.63, 4.07, 5.31 and 6.04 mg/L, respectively. The series of Zn^{2+} solutions were prepared by adding specific quantities of $ZnSO_4{\cdot}7H_2O$ to medium. The Zn^{2+} solutions prepared were for the exposure test following.

2.2. Zebrafish culture and embryo selection

Adult AB strain zebrafish (*Daniorerio*) were purchased from State Key Laboratory of Freshwater Ecology and Biotechnology, Chinese Academy of Sciences (Wuhan, China). Fish were fed with