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Alteration in the expression of antioxidant and detoxification genes in *Chironomus riparius* exposed to zinc oxide nanoparticles

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ABSTRACT

Zinc oxide nanoparticles (ZnONPs) are widely used in several commercial products due to their unique physico-chemical properties. However, their release into the aquatic environments through various anthropogenic activities will lead to toxic effect in aquatic organisms. Although several investigations have been reported on the effect of ZnONPs in aquatic organisms using traditional end points such as survival, growth, and reproduction, the molecular level end points are faster and sensitive. In this study, the expression of different genes involved in oxidative stress response, detoxification, and cellular defense was studied in an ecotoxicologically important bio-monitoring organism *Chironomus riparius* in order to understand the subcellular effects of ZnONPs. The fourth instar larvae were exposed to 0, 0.2, 2, 10, and 20 mg/L of ZnONPs and Zn ions (in the form of $ZnSO_4.7H_2O$) for 24 and 48 h period. The expression of CuZn superoxide dismutase, manganese superoxide dismutase, catalase, phospholipid hydroperoxide glutathione peroxidase, thioredoxin reductase 1 and delta-3, sigma-4 and epsilon-1 classes of glutathione *S*-transferases, cytochrome p4509AT2, and heat shock protein 70 were studied using real-time polymerase chain reaction method. Gene expression results showed that the expression of genes related to oxidative stress response was more pronounced as a result of ZnONPs exposure as compared to Zn ions. The mRNA expression of genes involved in detoxification and cellular protection was also modulated. Significantly higher expression levels of oxidative stress-related genes shows that oxidative stress is an important mechanism of toxicity as a result of ZnONPs exposure in *C. riparius*.

1. Introduction

The increasing production and utilization of engineered nanoparticles (ENPs) in various consumer products and also in industrial applications has raised concerns over their release and subsequent exposure to various aquatic organisms (Handy et al., 2008). Among the different ENPs, zinc oxide nanoparticles (ZnONPs) are widely used in several industrial and consumer products viz. in cosmetics, sunscreens, as a food additive, tooth pastes, hair care products, packaging industries, paint formulations, and as a photocatalyst for pollution control (Sharma et al., 2012). Since the production, utilization, and subsequent release of ZnONPs to the environment are increasing, it has become important to study the potential environmental and health impacts of such nanoparticles (Lopes et al., 2014). Previous studies has shown that exposure to ZnONPs could cause toxic effects in various marine and fresh water organisms (Zhu et al., 2008; Bai et al., 2010; Wong et al., 2010; Fabrega et al., 2011; Xiong et al., 2011).

Once released into the aquatic environments, ZnONPs may settle down to the sediments, and therefore, organisms living in benthic sediments are most likely to be exposed to them (Klaine et al., 2008). In the aquatic environment, benthic organisms represent an important link in the aquatic food web and can accumulate pollutants from aqueous and solid sources (Lucan Bouché et al., 2000). The larvae of the aquatic midge *Chironomus riparius* have been widely used as a test organism to study the toxic effects of various environmental pollutants due to their association with benthic sediments (Lucan Bouché et al., 2000; OECD, 2001). Traditionally, the toxic effects of various environmental pollutants in *C. riparius* are studied using life cycle level end points (Pascoe et al., 1989; Postma and Davis, 1995; De Bisthoven et al., 2001; Martinez et al., 2003). However, due to their sensitivity, gene expression studies has been used to investigate the effect of environmental pollutants in several aquatic organisms (Snell et al., 2003; Vandegehuchte et al. 2010; Marinković et al., 2012). Suitability of gene expression studies to understand the effects of various environmental pollutants including ENPs has also been reported from *C. riparius* (Marinković et al., 2012; Nair et al., 2013a).

Recently, the toxic effects of ZnONPs and Zn ions has been reported from *C. riparius* using developmental level end points (Tomilina et al., 2014). As far as we are aware, no studies have been undertaken to determine the molecular levels effects ZnONPs and Zn ions in *C. riparius*. Therefore, in this study, the effect of different concentrations of ZnONPs and Zn ions (in the form of $ZnSO_4$) was assessed in the fourth instar

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larvae of *C. riparius* using stress-responsive gene expression. The transcriptional modulation of different antioxidant, detoxification, and stress-responsive genes such as CuZn superoxide dismutase (*CuZnSOD*), Mn superoxide dismutase (*MnSOD*), catalase (*CAT*), phospholipid hydroperoxide glutathione peroxidase (*PHGPx*), thioredoxin reductase 1 (*TrxR1*), three different classes of glutathione *S*-transferases (*GSTs*) (GSTDelta-3, GSTSigma-4, and GSTEpsilon-1), cytochrome p450 (*CYP9AT2*), and heat shock protein 70 (*HSP70*) was studied using real-time polymerase chain reaction (RT-PCR) method.

2. Materials and methods

2.1. Exposure conditions

The ZnONPs (size <100 nm) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Since dechlorinated water was used for the studies, the ZnONPs stock suspension was prepared in dechlorinated water, stirred well, and sonicated for 20 min. The characterization of ZnONPs prior to and at different time points of (24 and 48 h) was done using transmission electron microscopy (TEM; LIBRA 120, Carl Zeiss, Oberkochen, Baden-Wrttemberg, Germany) at 80–120 kV. For the preparation of sample for TEM analysis, the ZnONPs suspensions were drop coated on to 400 mesh carbon-coated copper grids (Tedpella Inc., Reading, USA) and dried over night. The size distribution was evaluated using a photal dynamic light scattering (DLS) spectrometer (DLS-7000; Otsuka Electronics Co., Inc., Osaka, Japan). Samples collected from prior to exposure and after 24 and 48 h from 0.2 and 20 mg/L of ZnONPs concentrations for the DLS analysis. For the preparation of different concentrations of Zn ions (0, 0.2, 2, 10, and 20 mg/L), $ZnSO_4.7H_2O$ (Sigma-Aldrich, St. Louis, MO) was used.

In order to determine the dissolution of ZnONPs, 10 mL of water was taken from three samples of 0.2, 2, 10, and 20 mg/L exposure sets at different time periods (24 and 48 h). The collected suspensions were centrifuged at 13000 rpm (Model VS-GooocFi, Vision Scientific Co. Ltd, Daejeon, Korea) for 20 min and then filtered through 0.2 μm filters (Chromedisc®, Seoul, South Korea) (Lopes et al., 2014). The samples were analyzed using Inductively Coupled Plasma-Mass Spectrometer (ICP-MS, Elan 6100 (2002), Perkin-Elmer Sciex, USA).

2.2. Exposure conditions

Different concentrations of ZnONPs and Zn ions viz. 0, 0.2, 2, 10, and 20 mg/L were added immediately after the preparation of the stock solutions and mixed well. The exposure concentrations were selected based on the previous studies by Timmermans et al. (1992) and Tomilina et al. (2014). The fourth instar larvae were exposed to ZnONPs and Zn ions for 24 and 48 h period at 21 ± 1 °C with a photoperiodic condition of 16 h light and 8 h dark periods. Three biological replications consisting of 15 fourth instar larvae in each exposure set were kept in beakers containing 100 mL of dechlorinated tap water. The controls and exposed larvae were not fed during exposure. The experiment was repeated two times. After the exposure period, the larvae were collected, frozen in liquid nitrogen, and stored at −80 °C.

2.3. Isolation of total RNA and cDNA synthesis

For the isolation of total RNA, the larvae collected from the control and treatments were pooled in 2 mL Eppendorf tubes and frozen using liquid nitrogen. The larvae were ground using a plastic pestle after adding 0.75 mL of Trizol™ (Invitrogen, USA) reagent. The homogenate was used for the isolation of RNA as per the manufacturer's (Invitrogen, USA) instructions. After isolation, the final RNA pellet was dissolved in 50 μL of deionized autoclaved water. The quality and quantity of RNA preparation was verified by agarose gel electrophoresis and absorbance spectrophotometry (A260/A280 > 1.8). For cDNA synthesis, 1 μg of total RNA was reverse-transcribed in a 20 μL reaction volume using QuantiTect® reverse transcription kit (Qiagen, USA) integrated with genomic DNA contamination removal step as per the manufacturer's instructions.

2.4. Quantitative real-time PCR

The *CuZnSOD*, *MnSOD*, *CAT*, *PHGPx*, *TrxR1*, *GSTd3*, *GSTs4*, *GSTe1*, *CYP9AT2*, *HSP70*, and *ACT* primers were selected based on previous reports (Lee et al., 2006; Nair and Choi, 2011; Nair et al., 2013a) (Supplementary Table 1). The expression levels of different genes were studied by running RT-PCR using samples collected from three biological replications, and the experiment was repeated two times. The reaction mixture included 1 μL of template cDNA, 0.2 μM of corresponding forward and reverse primers, and 10 μL of 2× IQ SYBR Green Super Mix (Bio-Rad, USA) in a final reaction volume of 20 μL. The RT-PCR reactions were run with an initial denaturing at 95 °C for 7 min followed by 44 cycles of 95 °C for 15 s, 55 °C for 1 min, and 72 °C for 0.15 s. A Melting curve analysis was done from 65 °C to 95 °C with a 0.2 °C increase per cycle. The amplification and the detection of various genes were performed using the CFX Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) and accompanying software (CFX Manager Software). Cycle threshold (CT) values were converted to relative gene expression levels by $2^{-\Delta\Delta CT}$ method using the gene expression analysis software in CFX PCR machine (Bio-Rad, USA).

2.5. Data analysis

The results were analyzed using one-way ANOVA with the SPSS 12.0 KO (SPSS Inc., Chicago, IL, USA). Dunnett's post hoc test was done to determine the effect of different treatments on gene expressions, and probability levels of $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered as significant.

3. Results

3.1. Characterization of ZnONPs

The characterization of ZnONPs using TEM prior to the exposure showed shapes and sizes similar to the data provided by the manufacturer (Suppl. Fig. 1A–D). The aggregation of ZnONPs was observed after 24 h (Suppl. Fig. 1E–H) and 48 h (Suppl. Fig. 1I–L) exposure periods. The size distribution analysis prior to exposure showed that the ZnONPs were mainly distributed in the range 20–50 nm (Suppl. Fig. 2. A). Size distribution of ZnONPs at the lowest (0.2 mg/L) and highest concentrations after 24 h showed 30–50 nm (Suppl. Fig. 2B, C). The size distribution after 48 h for 0.2 mg/L of ZnONPs was in the range of 30–60 nm and for 20 mg/L was in the range of 40–70 nm (Suppl. Fig. 2D, E). Dissolution analysis showed an increase in Zn ions release viz. 1.09 ± 0.08, 5.43 ± 0.28, 9.23 ± 1.02, and 14.72 ± 1.28 mg/L from 0.2, 2, 10, and 20 mg/L of ZnONPs after 24 h period. However, the release of Zn ions showed a decrease, i.e., 0.93 ± 0.09, 0.79 ± 0.08, 0.65 ± 0.12, and 0.31 ± 0.08 mg/L in 0.2, 2, 10, and 20 mg/L of ZnONPs exposure sets after 48 h period.

3.2. Expression of SOD and CAT genes

The relative mRNA expression of *CuZnSOD* gene was significantly increased after exposure to 0.2, 2, 10, and 20 mg/L of ZnONPs for 24 h period with the highest expression being observed after 24 h exposure to 10 mg/L of ZnONPs (Fig. 1A). A significant up-regulation of *CuZnSOD* gene was also observed after 48 h exposure to 0.2 and 2 mg/L of ZnONPs for 48 h period. However, the expression of *CuZnSOD* gene did not change significantly after exposure to 10 and 20 mg/L of ZnONPs for 48 h period (Fig. 1A). Exposure to 0.2, 2, 10, and 20 mg/L of Zn ions for 24 h period has not resulted in any significant change in the expression of *CuZnSOD* gene. Similarly, no significant change in the expression levels of *CuZnSOD* gene was observed upon exposure to 0.2 and 2 mg/L