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Evaluation of the effect of silver nanoparticles and silver ions using stress responsive gene expression in *Chironomus riparius*

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HIGHLIGHTS

- Toxicogenomic response of AgNPs and Ag^+ ions is studied in *Chironomus riparius*.
- AgNPs and Ag^+ ions showed different toxicogenomic patterns.
- AgNPs promoted pronounced induction of antioxidant genes.

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ABSTRACT

Silver nanoparticles (AgNPs) are extensively used in many commercial products because of their antimicrobial properties and they are therefore released into the environment from various products. A number of genes, especially those representing antioxidant and detoxification pathways, have potential application for studying mechanism of action of environmental pollutants at molecular level. In the present study, the stress responsive transcription of antioxidant and detoxification genes in response to AgNPs and Ag^+ ions exposure is studied in the ecotoxicologically important model species *Chironomus riparius*. The selected genes were superoxide dismutases (CuZnSOD and MnSOD), catalase (CAT), phospholipid hydroperoxide glutathione peroxidase 1 (PHGPx1), thioredoxin reductase 1 (TrxR1), and delta-3, sigma-4 and epsilon-1 classes of glutathione S-transferases (GSTs). The mRNA expression levels of each gene were determined after exposure of animals for 24 h to three different AgNP and Ag^+ ion concentrations using Real-Time PCR method. Significant up-regulation of CuZnSOD and MnSOD was found after exposure to Ag^+ ions and AgNPs, respectively. The transcript levels of CAT, PHGPx1 and TrxR1 were significantly up-regulated only after exposure to AgNPs and no significant change was observed after exposure to Ag^+ ions. The expression levels of all the GSTs were more pronounced after exposure to AgNPs as compared to Ag^+ ions. The overall results suggest that AgNPs led to pronounced induction of genes related to oxidative stress and detoxification than Ag^+ ions.

1. Introduction

The application of nanomaterials in various commercial products has increased with the fast growth of nanotechnology (http://www.nanotechproject.org). Among the various nanomaterials, silver nanoparticles (AgNPs) are extensively used in many commercial products due to their antibacterial properties (Sondi and Salopek-Sondi, 2004; Maynard et al., 2006; Cohen et al., 2007; Perelshtein et al., 2008; Chen and Schluesener, 2008; Johnston et al., 2010; Ananth et al., 2011). AgNPs are used on various textiles, toys, deodorants, shampoos, toothbrushes, toothpaste, washing machines, refrigerators, detergents, and also in several medical applications (Samuel and Guggenbichler, 2004; Vigneshwaran et al., 2007; Benn and Westerhoff, 2008; Kim et al., 2010a; Nowack et al., 2011; Park et al., 2011). The AgNPs released from these products might ultimately reach the aquatic environment (Benn and Westerhoff, 2008; Farkas et al., 2011) and cause adverse effects at various levels in aquatic organisms (Asharani et al., 2008; Griffitt et al., 2009; Nair et al., 2011a). The chances of human exposure are also high with the release of AgNPs into the environment (Wijnhoven et al., 2009; Johnston et al., 2010).

In the aquatic environment, the benthic fauna is of great importance because it represents an important link in the aquatic food web and benthic organisms can accumulate metals from aqueous and solid sources (Lucan Bouché et al., 2000). The larvae of the aquatic midge *Chironomus riparius* is a well-established aquatic

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biomonitoring species, and is widely used as a test organism in aquatic toxicology because of its association with sediments (OECD, 2001). The organism level effects of AgNPs were studied in *C. riparius*, and significantly affected the growth, pupation, emergence and egg production (Nair et al., 2011a).

The application of biochemical measurements and molecular biomarkers based on the expression of genes involved in the detoxification of xenobiotics, metal responsive genes and oxidative stress regulation has been used in many aquatic environmental bio-monitoring studies (Snell et al., 2003). For example, several studies have been conducted to check environmental pollution using the gene expression and biochemical activity of acetylcholine esterase, glutathione s-transferase (GST), metallothioneins, vitellogenin and cytochrome P450's (Hyne and Maher, 2003; Snell et al., 2003; Lee and Choi, 2006; Rhee et al., 2007; Viarengo et al., 2007). Previous studies in our lab and elsewhere have demonstrated that environmental stressors may induce changes in activity of enzymes as well as alter the expression of genes associated with short term and/or long term stress responses in *C. riparius* (Matthew and David, 1998; Choi et al., 2000, 2002; Lee et al., 2006; Park et al., 2012). Earlier studies suggest that AgNPs induce changes in gene expression, especially in oxidative stress-related genes in nematode *Caenorhabditis elegans* and in animal cells (Roh et al., 2009; Bouwmeester et al., 2011).

The antioxidant enzymatic system of organisms protects them from the toxic effects of activated oxygen species and helps to maintain cellular homeostasis by removing reactive oxygen species (ROS) (Mackay and Bewley, 1989). Superoxide dismutases (SODs) are metalloenzymes, which catalyze the dismutation of superoxide radicals to hydrogen peroxide and oxygen. Eukaryotes possess two major kinds of SOD: CuZnSOD, which is present mostly in the cytosol and nucleus, and MnSOD, which is present in mitochondria (Kroll et al., 1995). Catalase (CAT), is a tetrameric heme-containing enzyme, and is one of the key antioxidant enzymes present in all aerobic organisms, catalyzing the breakdown of hydrogen peroxide to water and molecular oxygen to protect cells against the toxic effects of hydrogen peroxide (Chance et al., 1979). Phospholipid glutathione peroxidase (PHGPx) prevents lipid peroxidation and protects biomembranes against oxidative stress (Ursini et al., 1982). The role of PHGPx in signal transduction, inflammation and apoptosis has also been reported (Hermesz and Ferencz, 2009). The insect PHGPxs are stress-inducible antioxidant enzymes that act on phospholipid hydroperoxide and H_2O_2 (Li et al., 2003; Hu et al., 2010). The thioredoxin reductases (TrxRs) are homodimeric proteins, belonging to the flavoprotein family of pyridine nucleotide-disulfide oxidoreductases, with each monomer containing a FAD prosthetic group, a NADPH-binding site and an active site that contains a redox-active disulfide (Holmgren, 1985; Tamura and Stadtman, 1996; Arnér and Holmgren, 2000; Mustacich and Powis, 2000). TrxR is also an antioxidant enzyme induced under stress conditions that plays an important role in the cellular defense to scavenge ROS (Mustacich and Powis, 2000; Sakurai et al., 2005; Nair and Choi, 2012). GSTs are multifunctional phase II detoxification enzymes and are members of a multi gene family found in almost all living organisms. Their isozymes have great structural diversity, which provides the capability of binding to different compounds and products of oxidative stress (Leaver et al., 1992; Barata et al., 2005; Hayes et al., 2005).

In our previous study, we observed the effect of AgNPs on *C. riparius* (Nair et al., 2011a) and it may be possible that some of the adverse effect might be caused by the dissolved metal ions. On the other hand, Kim et al. (2009) reported the toxicity of AgNPs is mainly due to oxidative stress and is not dependent on the release of Ag^+ ions. According to some other studies (Navarro et al., 2008; Kawata et al., 2009) both Ag^+ ions and AgNPs contribute to the toxicity. Therefore, it might be possible that some part of the toxicity of AgNPs might be contributed through the release of Ag^+ ions from them. However, despite the widespread use of products with AgNPs, no studies have been undertaken to determine the sub cellular effects of AgNPs and Ag^+ ion exposure in ecotoxicologically important model test organism *C. riparius*. In this study, therefore, the adverse effects of different concentrations of AgNPs and Ag^+ ion on *C. riparius* were assessed using stress-response gene expression. The expression of different antioxidant and detoxification genes such as CuZnSOD, MnSOD, CAT, PHGPx1, TrxR1, and three different classes of GSTs (GSTDelta-3, GSTSigma-4 and GSTEpsilon-1) were studied using Real-Time PCR method.

2. Materials and methods

2.1. Animal maintenance

C. riparius (Insecta: Chironomidae) larvae were originally obtained from Korea Institute of Toxicology (Daejeon, South Korea). They were grown in the lab in 2L glass tanks containing dechlorinated tap water and acid washed sand with aeration. The animals were fed with finely ground fish flake (Tetramin, Tetrawerke, Melle, Germany) once in alternate days. The temperature was adjusted at 21 ± 1 °C and photoperiod was 16 h light and 8 h dark.

2.2. Experimental setup

The experimental setup consisted of three experimental groups: (1) controls (2) AgNPs exposed larvae and (3) Ag^+ ion (in the form of $AgNO_3$) exposed larvae. Aqueous suspensions of AgNPs (size <100 nm, Sigma–Aldrich Chemical, St. Louis, MO) were prepared in deionized water by sonication for 13 h (Branson-5210 sonicator, Branson Inc., Danbury, CT), stirring for 7 d, and filtered through a cellulose membrane (pore size 100 nm, Advantec, Toyo Toshi Kaisha, Japan) to remove nanoparticle aggregations. The particles size was determined using a LIBRA 120 TEM (Carl Zeiss, Oberkochen, Baden-Wrttemberg, Germany) at 80−120 kV and size distribution was evaluated using a Photal dynamic light scattering (DLS) spectrometer, DLS-7000 (Otsuka Electronics Co., Inc., Osaka, Japan) as described in our previous report (Nair et al., 2011a). Fourth instar larvae of *C. riparius* were exposed to sub-lethal concentrations of AgNPs (0, 0.2, 0.5 and 1 mg L^{-1}; Sigma–Aldrich Chemical, St. Louis, MO, USA) and Ag^+ ions in the form of $AgNO_3$ (0, 0.2, 0.5 and 1 mg L^{-1}) for 24 h. The exposure conditions were selected based on our previous studies in which we observed that the dissolutions of Ag^+ ions from AgNPs was highest after 24 h exposure (Nair et al., 2011a). All the exposure and controls were done in triplicates consisting 15 fourth instar larvae in each exposure set in beakers containing 100 mL dechlorinated tap water. The controls and exposed larvae were not fed during exposure. After the exposure the larvae were collected, frozen in liquid nitrogen and stored at −80 °C.

2.3. RNA isolation, cDNA synthesis and quantitative Real-Time RT-PCR

Total RNA from chemical exposed and control larvae was isolated using Trizol™ (Invitrogen, USA) as per manufacturer's instructions, cleaned using Nucleospin RNA-Clean up kit (Macherey-Nagel, Germany). The quality of RNA preparation was verified by agarose gel electrophoresis and absorbance spectrophotometry (A260/A280 > 1.8). For cDNA synthesis, 1 μg total RNA was reverse transcribed in 20 μL reaction volume with oligo dT_{20} primer using iScript™ select cDNA synthesis kit (Bio-Rad, USA) as per the manufacturer's instructions. The primers for all the genes and *C. riparius* GAPDH (GenBank accession no. EU999991) were designed using online Primer3 program (http://frodo.wi.mit.edu/primer3/) (Table 1). The substrate specificity of the primers was tested on a