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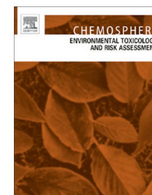

Evaluation of the effect of silver nanoparticles and silver ions using stress responsive gene expression in *Chironomus riparius*

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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.

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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

Table 1
PCR primers used in the study.

Gene name	Genbank accession number	Primer name and sequence (5′–3′)	Amplicon length (bp)
CuZnSOD	JQ342170	CuZnSOD-F-GTCGTGCTGTTGTCGTTCAT CuZnSOD-R-CAGCATTGCCAGTTTTGTGT	81
MnSOD	JQ342169	MnSOD-F-CTGATGCACTCCAAAAAGCA MnSOD-R-AACTCCAACAGCAGCGACTT	86
CAT	JL641904	CAT-F-CGTGATCTTCGTGGTTTTGCTG CAT-R-GGATTGGATCGCGGATGAAG	100
PHGPx	JQ762261	PHGPx1-F-AAGTGTGGTTACACAGCTAAGCATT PHGPx1-R-GATATCCAAATTGATTACACGGAAA	112
TrxR1	JN600620	TrxR1-F-GACATTTTCTCATTAGACCGTGAAC TrxR1-R-ACGAACCAAAATTGTTGACTCATAG	120
GSTd3	EZ966119	GSTd3-F AGCAGTTGTCGCCTGAGTTT GSTd3-R-TGTGCAATTGCTCTCGACTC	100
GSTs4	EZ966123	GSTs4-F-CCAGTCCTCGAAGTTGATGG GSTs4-R-GGCAGTTTGGATTTGCATTT	119
GSTe1	EZ966125	GSTe1-F-CCTAATGCGCACAAAAGTCA GSTe1-R-TCTTGAGTCGGTTCCTTCCT	117
GAPDH	EU999991	GAPDH-F-GTATTTCATTGAATGATCACTTTG GAPDH-R-TAATCCTTGGATTGCATGTACTTG	110

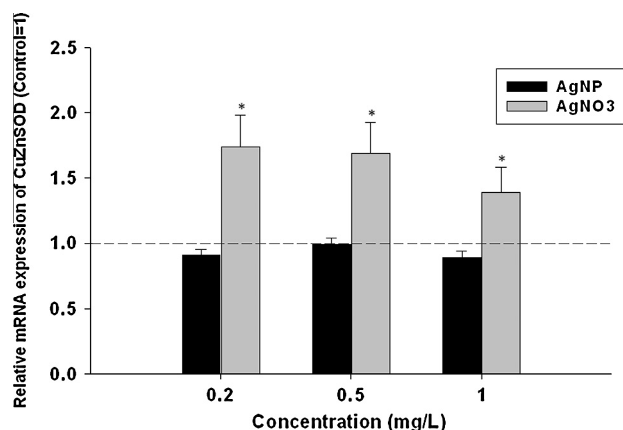

Fig. 1. Expression of *Chironomus riparius* CuZnSOD mRNA after exposure for 24 h to 0.2, 0.5 and 1 mg L^{-1} of silver nanoparticles and Ag^{+} ions (in the form of $AgNO_3$) exposure for 24 h. The mRNA expression of CuZnSOD was quantified using real time PCR and normalized using *C. riparius* GAPDH gene. Data are mean ± SE of three independent experiments. An asterisk indicates a statistically significant difference, $p < 0.05$ compared with the control (control = 1) group, analyzed using ANOVA test.

representative *C. riparius* cDNA preparation using the following reaction conditions with 94 °C for 4 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C or 30 s and 72 °C for 10 min using PTC 100 thermal cycler (MJ Research, Lincoln, MA, USA) with the PCR mix (Bioneer, South Korea) according to the manufacturers' manual. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide to verify the amplicon length and to assure that only one product was amplified.

The mRNA expression of different genes after exposure to different concentrations of AgNPs and Ag^{+} ions were studied using quantitative Real-Time RT-PCR using samples from three independent exposure and control sets. Each reaction included 1 μL of template cDNA, 0.2 μM of corresponding forward and reverse primers, 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 and a melting curve analysis was done. Amplification and detection were performed using a CFX Connect Real-Time PCR detection system (Bio-Rad, USA) and accompanying software (CFX Manager Software) according to the manufacturer's instructions. The expression level of different genes under different exposure conditions was calculated relative to the expression levels of the *C. riparius* GAPDH mRNA used as an internal standard to normalize the expression levels.

2.4. Data analysis

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 Connect PCR machine (Bio-Rad, USA). Effects of AgNPs or Ag^{+} on gene expressions were tested by one-way ANOVAs. Dunnett's post-doc tests were done to determine treatments that differ significantly from control. Statistics were done with SPSS 12.0 KO (SPSS Inc., Chicago, IL, USA). A probability level of $p < 0.05$ was considered significant.

3. Results

The relative expression levels of the CuZnSOD and MnSOD genes were significantly different between the tests with AgNPs and Ag^{+} ions. The relative expression level of CuZnSOD mRNA significantly increased after exposure to Ag^{+} ions at all tested concentrations (Fig. 1). However, no significant difference as compared to the control was observed with AgNP exposure (Fig. 1). In the case of MnSOD, exposure to different concentrations of AgNPs significantly down-regulated mRNA expression level. However, no significant change was observed upon exposure to Ag^{+} ions (Fig. 2).

The expression profile of CAT mRNA after exposure to different doses of AgNPs and Ag^{+} ions was also investigated after 24-h exposure. No significant modulation of CAT mRNA was noticed after exposure to 0.2, 0.5 and 1 mg L^{-1} of Ag^{+} ions (Fig. 3). However, significantly increased expression was observed after exposure to 0.5 and 1 mg L^{-1} of AgNPs compared to the control sets. Among the different AgNP concentrations tested, 0.5 mg L^{-1} was found to be the most effective in modulating CAT mRNA expression level (Fig. 3).

The mRNA expression of PHGPx1 was quantified after 24 h exposure to 0.2, 0.5 and 1 mg L^{-1} of AgNPs and Ag^{+} ions. Exposure concentration-dependant increase was observed in the mRNA expression of PHGPx1 by AgNP exposure (Fig. 4). However, in the case of Ag^{+} ion, significant up-regulation of PHGPx1 gene was observed only at 1 mg L^{-1} of Ag^{+} ions exposed *C. riparius* (Fig. 4).

The qRT-PCR results revealed significant modulation of TrxR1 mRNA after different concentrations of AgNP exposure. There was a steady increase in the expression of TrXR1 with highest expression being observed after exposure to 1 mg L^{-1} of AgNPs (Fig. 5). Even though not significant, down-regulation of TrxR1

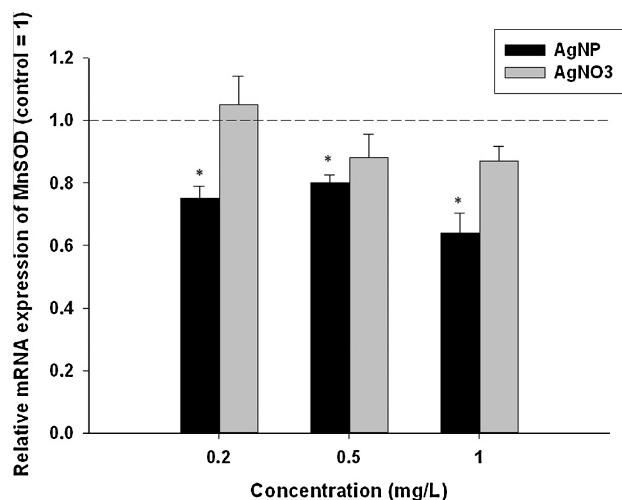

Fig. 2. Expression of *Chironomus riparius* MnSOD mRNA after exposure for 24 h to 0.2, 0.5 and 1 mg L^{-1} of silver nanoparticles and Ag^{+} ions (in the form of $AgNO_3$) exposure for 24 h. The mRNA expression of MnSOD was quantified using real time PCR and normalized using *C. riparius* GAPDH gene. Data are mean ± SE of three independent experiments. An asterisk indicates a statistically significant difference, $p < 0.05$ compared with the control (control = 1) group, analyzed using ANOVA test.

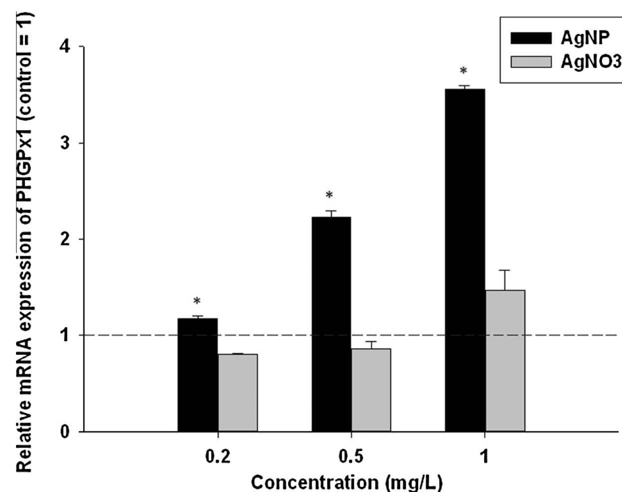

Fig. 4. Expression of *Chironomus riparius* PHGPx1 mRNA after exposure for 24 h to 0.2, 0.5 and 1 mg L^{-1} of silver nanoparticles and Ag^{+} ions (in the form of $AgNO_3$) exposure for 24 h. The mRNA expression of PHGPx1 was quantified using real time PCR and normalized using *C. riparius* GAPDH gene. Data are mean ± SE of three independent experiments. An asterisk indicates a statistically significant difference, $p < 0.05$ compared with the control (control = 1) group, analyzed using ANOVA test.

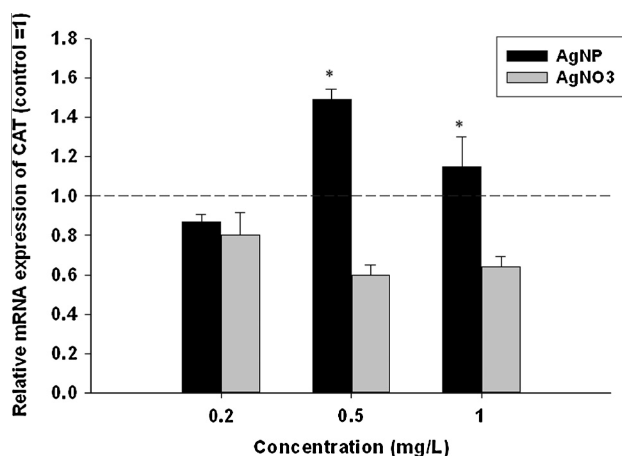

Fig. 3. Expression of *Chironomus riparius* CAT mRNA after exposure for 24 h to 0.2, 0.5 and 1 mg L^{-1} of silver nanoparticles and Ag^{+} ions (in the form of $AgNO_3$) exposure for 24 h. The mRNA expression of CAT was quantified using real time PCR and normalized using *C. riparius* GAPDH gene. Data are mean ± SE of three independent experiments. An asterisk indicates a statistically significant difference, $p < 0.05$ compared with the control (control = 1) group, analyzed using ANOVA test.

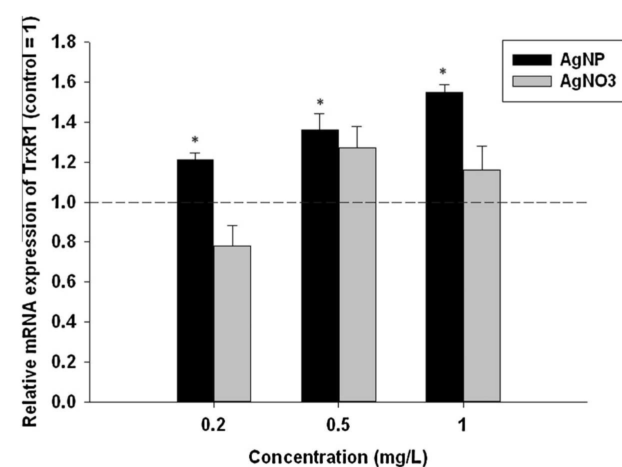

Fig. 5. Expression of *Chironomus riparius* TrxR1 mRNA after exposure for 24 h to 0.2, 0.5 and 1 mg L^{-1} of silver nanoparticles and Ag^{+} ions (in the form of $AgNO_3$) exposure for 24 h. The mRNA expression of TrxR1 was quantified using real time PCR and normalized using *C. riparius* GAPDH gene. Data are mean ± SE of three independent experiments. An asterisk indicates a statistically significant difference, $p < 0.05$ compared with the control (control = 1) group, analyzed using ANOVA test.

was observed after exposure to 0.2 mg L^{-1} of Ag^{+} ions. There was also a non-significant increase in the mRNA expression of TrxR1 after exposure to 0.5 and 1 mg L^{-1} of Ag^{+} ions (Fig. 5).

The relative expression of different classes of GST genes viz. GST delta 3 (GSTd3), GST Sigma 4 (GSTs4) and GST epsilon 1 (GSTe1) was studied using quantitative RT-PCR. The mRNA expression levels of GSTd3, GSTs4 and GSTe1 were significantly increased to varying levels upon exposure to all concentrations of AgNPs and Ag^{+} ions tested in this study (Fig. 6A–C). All three tested GSTs showed similar trend to AgNPs and Ag^{+} ion exposure: dramatic and exposure concentration-dependant increase with AgNPs, but, mild increase with Ag^{+} ions (Fig. 6A–C).

4. Discussion

AgNPs may undergo many transformations such as reactions with biomacromolecules, redox reactions, aggregation, and dissolution, in both environmental and biological systems (Lowry et al., 2012). The uptake of AgNPs in living organisms may also result in their interactions with sub cellular compartments of the cell (Costa et al., 2010). One of the main mechanisms through which AgNPs mediate their effects in organisms is the production of different types of ROS from different sub cellular compartments (Nel et al., 2006; Xia et al., 2006; Costa et al., 2010). In the present study, the effects of AgNPs and Ag^{+} ions were studied in the transcriptional modulation of antioxidant genes in the ecotoxicologically important biomonitoring species *C. riparius*.

The uptake and interactions of AgNPs with sub cellular structures has been reported from previous studies using mammalian and animal cells causing increased oxidative stress (Asharani et al., 2009; Costa et al., 2010). Almofti et al. (2003) reported that AgNPs perturb mitochondria through interactions with thiol groups of the mitochondrial inner membrane and cause oxidative stress. Previously it was reported that AgNPs reduce mitochondrial

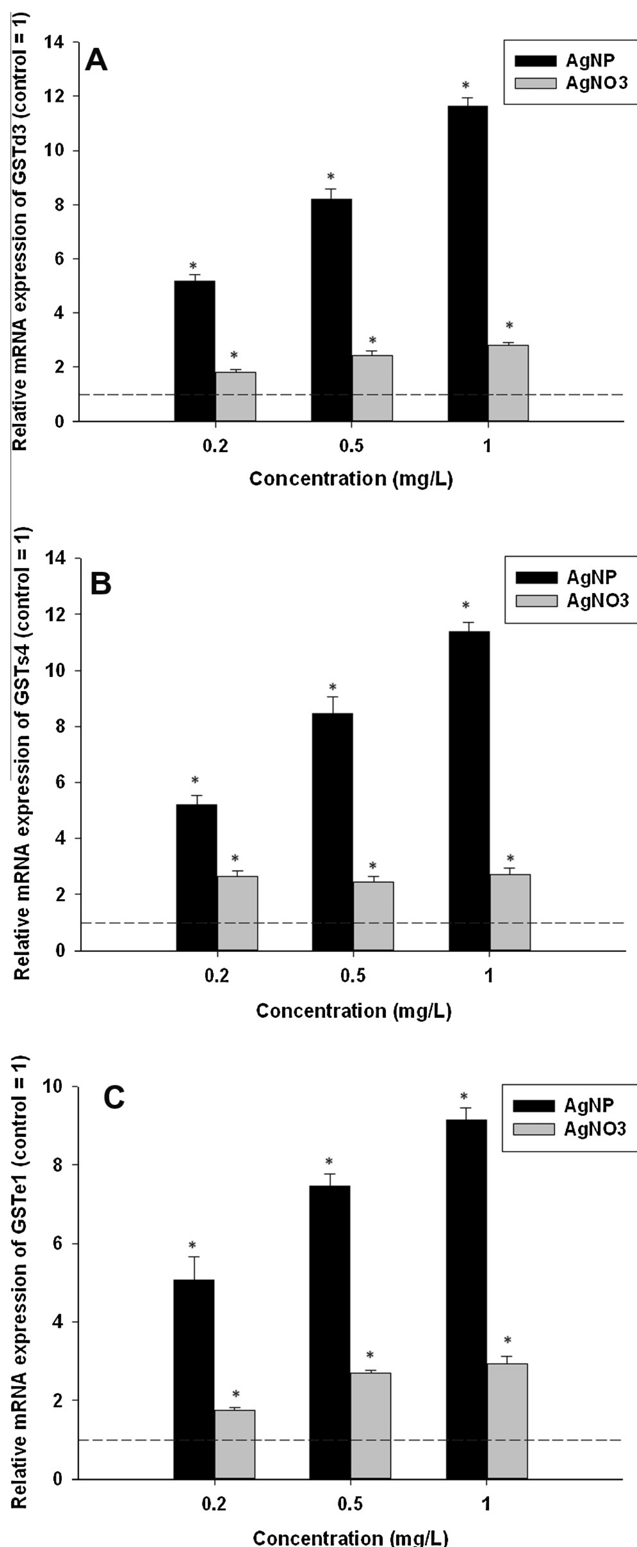

Fig. 6. Expression of *Chironomus riparius* (A) GSTdelta3 (GSTd3) (B) GSTsigma4 (GSTs4) and (C) GSTepsilon1 (GSTe1) mRNAs after exposure for 24 h to 0.2, 0.5 and 1 mg L^{-1} of silver nanoparticles and Ag^{+} ions (in the form of $AgNO_3$) exposure for 24 h. The mRNA expression of GSTd3, GSTs4 and GSTe1 was quantified using real time PCR and normalized using *C. riparius* GAPDH gene. Data are mean ± SE of three independent experiments. An asterisk indicates a statistically significant difference, $p < 0.05$ compared with the control (control = 1) group, analyzed using ANOVA test.

function in mammalian germline stem cells and increase ROS generation, deplete antioxidant GSH content, and reduce mitochondrial function in BRL 3A rat liver cells (Braydich-Stolle et al., 2005; Hussain et al., 2005). Mitochondria are the source of ROS production in the cell and the electron transport system is affected by AgNPs exposure producing ROS in human cells (Asharani et al., 2009). Paula et al. (2009) reported that AgNPs inhibit the in vitro activity of mitochondrial enzymes and Costa et al. (2010) demonstrated that AgNPs decrease the activity of mitochondrial respiratory chain complexes leading to the generation of ROS by impairing the mitochondrial respiratory chain.

It is well known that the Ag^{+} ion is a sulfhydryl group-reactive cytotoxin and Ag^{+} ions may interact and oxidize thiol groups of cell membrane proteins and cause changes in membrane permeability which disrupts mitochondrial functions leading to the production of excess ROS and hence to the depletion of antioxidant defense mechanisms (Passow et al., 1961; Gritzka and Trump, 1968; Rangachari and Matthews, 1985; Kone et al., 1988; Almofti et al., 2003). Choi et al. (2010) suggested that Ag-conjugated NPs release Ag^{+} in solution, and that the dissolved Ag^{+} ion can induce oxidative stress. Several previous reports also show that Ag^{+} affects the cellular oxidative status and production of ROS in various systems (Hultberg et al., 1997; Baldi et al., 1998; Yoshimaru et al., 2006).

In order to counteract with excess ROS, various antioxidant mechanisms are activated in organisms. The initial mechanisms that act to adjust antioxidant levels to protect the cells include changes in antioxidant gene expression (Cushman and Bohnert, 2000). Since ROS production occurs in different sub cellular compartments of the cells, activation of different types of antioxidant and detoxification genes occurs in different sub cellular locations of the cell.

In the present study, it was observed that after exposure to different concentrations of AgNPs, MnSOD mRNA levels were significantly down-regulated and no significant change was observed in the expression of CuZnSOD. Upon exposure to different concentrations of Ag^{+} ions, a non-significant change in the expression of MnSOD was observed. However, the expression of CuZnSOD mRNA was significantly increased after exposure to different concentrations of Ag^{+} ions. Based on previous reports (Almofti et al., 2003; Braydich-Stolle et al., 2005; Hussain et al., 2005; Asharani et al., 2009), it could be assumed that there might be interactions of AgNPs with sub cellular structures such as mitochondria, leading to excess oxidative stress, which reduces the mRNA expression of MnSOD in *C. riparius*. The expression levels of CuZnSOD also showed a slight decrease, though in a non-significant manner, after exposure to 1 mg L^{-1} of Ag^{+} ions, which might have also been due to the excess oxidative stress caused by AgNP exposure. The modulation of MnSOD according to the different Ag^{+} ion concentrations may have been due to the interactions of Ag^{+} ions with thiol groups of mitochondrial membranes causing oxidative stress in mitochondria, as described by earlier reports (Passow et al., 1961; Gritzka and Trump, 1968; Rangachari and Matthews, 1985; Kone et al., 1988). The up-regulation of CuZnSOD as observed in this study might have arisen in order to counteract the oxidative stress caused by Ag^{+} ions in *C. riparius*.

In the present study, the mRNA expression of *C. riparius* CAT gene significantly increased after exposure to different concentrations of AgNPs. In order to counteract the excess formation of H_2O_2 by the action of different SODs by conversion of superoxide radicals, formed as a result of AgNPs exposure, might have resulted in the up-regulation of CAT mRNA in *C. riparius*. Up-regulation of CAT mRNA has also been reported in other species upon exposure to environmental pollutants, such as cadmium (Cd), causing oxidative stress. Induction of CAT mRNA after exposure to Cd has been reported in river pufferfish (*Takifugu obscures*) (Kim et al., 2010b). Up-regulation of CAT mRNA was also observed in *Daphnia magna* after exposure to Cd (Kim et al., 2010c). Recently, we observed significant up-regulation of CAT mRNA in *C. riparius* after exposure to oxidative stress causing chemicals paraquat and Cd (Nair et al., 2011b).

In this study, the mRNA expression of *C. riparius* PHGPx1 after AgNPs exposure was significantly up-regulated. In mammals, PHGPx is considered the primary component in the oxidative stress response and enzymatic defense for the protection of bio-membranes against oxidative stress (Ursini et al., 1995; Imai and Nakagawa, 2003). In *C. riparius*, PHGPx1 expression was up-regulated as a result of oxidative stress (Nair et al., 2012). It has also been reported from several species that the expression of PHGPx was induced due to oxidative stress caused by environmental stressors, such as metals and H_2O_2 (Sugimoto and Sakamoto, 1997; Li et al., 2000; Dash et al., 2006; Navrot et al., 2006; Bae et al., 2009). Similar to the present results, the modulation of PHGPx gene due to oxidative stress has been reported from *Drosophila melanogaster*, a model insect belonging to the dipteran order (Li et al., 2000). In *Apis cerana cerana*, the expression of the AccPHGPx1 gene was modulated upon exposure to oxidative stress causing chemicals (Wang et al., 2010). These results support the idea that *C. riparius* PHGPx1 may also participate in the protection against oxidative stress caused by exposure to AgNPs in *C. riparius*.

In this study, we investigated the expression of *C. riparius* TrxR1 after AgNPs and Ag^+ ion exposure and found that the mRNA expression level of TrxR1 was significantly up-regulated after exposure to all tested AgNP concentrations. Induction in the mRNA expression of TrxR1 has been reported from different systems as a result of oxidative stress. In *A. cerana cerana*, ROS produced by heat and UV induced the mRNA expression of TrxR1 (Heck et al., 2003; Yang et al., 2010). In vascular endothelial cells, TrxR1 expression was induced by Cd (Sakurai et al., 2005). Dimopoulos et al. (2002) reported the induction of the Trx system in another dipteran insect, *Anopheles gambiae*, including the TrxR gene, due to oxidative stress. Therefore, based on the results obtained in the present study, we predict that TrxR1 plays an important role in protection against oxidative stress caused by AgNPs in *C. riparius*. As TrxR is an antioxidant enzyme having an activity to scavenge ROS (Mustacich and Powis, 2000), AgNP-induced TrxR1 expression might be one of the cellular defense mechanism against AgNPs in *C. riparius*.

In this study, we observed that induction of *C. riparius* GST genes after exposure to AgNPs and Ag^+ ions. However, the GST mRNA expression levels caused by AgNP exposure were several times greater than those induced by Ag^+ ion exposure. AgNPs treatment up-regulated the expression of GST isoforms, which are involved in antioxidant mechanisms in *Tigriopus japonicus* (Lee et al., 2008). Similar to our results, in *D. melanogaster*, several GSTs participate in defense mechanisms against oxidative stress (Sawicki et al., 2003). The induction of GST expression by the AgNPs and Ag^+ ions suggests that this protein plays some significant role in the detoxification of the AgNPs and Ag^+ ions in *C. riparius*. One detoxification route of Ag^+ ions from biological systems is elimination via conjugation to GSH, which is catalyzed by GSTs (Almofti et al., 2003). The induction of GST isoforms after exposure to Ag^+ ions might be due to the removal of Ag^+ ions by binding to thiol groups of GSH mediated by GSTs (Passow et al., 1961; Gritzka and Trump, 1968; Rangachari and Matthews, 1985; Kone et al., 1988; Almofti et al., 2003).

5. Conclusion

We tested the response of antioxidant and detoxification genes in *C. riparius* exposed to AgNPs and Ag^+ ions. From the present study results we assume that exposure to AgNPs leads to excess oxidative stress in *C. riparius* due to its interactions with various biological systems and sub cellular compartments of the cell. The modulation in the expression of different genes coding for oxidative stress-related defense mechanisms showed its biological role in dealing with the AgNP-mediated stress response in *C. riparius*.

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