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Involvement of *Caenohabditis elegans* MAPK Signaling Pathways in Oxidative Stress Response Induced by Silver Nanoparticles Exposure

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In the present study, toxicity of silver nanoparticles (AgNPs) was investigated in the nematode, *Caenohabditis elegans* focusing on the upstream signaling pathway responsible for regulating oxidative stress, such as mitogen-activated protein kinase (MAPK) cascades. Formation of reactive oxygen species (ROS) was observed in AgNPs exposed *C.elegans*, suggesting oxidative stress as an important mechanism in the toxicity of AgNPs towards *C. elegans*. Expression of genes in MAPK signaling pathways increased by AgNPs exposure in less than 2-fold compared to the control in *wildtype C.elegans*, however, those were increased dramatically in *sod-3 (gk235)* mutant after 48 h exposure of AgNPs (i.e. 4-fold for *jnk-1* and *mpk-2*; 6-fold for *nsy-1*, *sek-1*, and *pmk-1*, and 10-fold for *jkk-1*). These results on the expression of oxidative stress response genes suggest that *sod-3* gene expression appears to be dependent on p38 MAPK activation. The high expressions of the *pmk-1* gene 48 h exposure to AgNPs in the *sod-3 (gk235)* mutant can also be interpreted as compensatory mechanisms in the absence of important stress response genes. Overall results suggest that MAPK-based integrated stress signaling network seems to be involved in defense to AgNPs exposure in *C.elegans*.

***Key words*:** *Caenorhabditis elegans*, Silver nanoparticles, Mitogen activated protein kinase, Oxidative stress

INTRODUCTION

Due to the wide application of nanoparticles (NPs) in various fields, research on their toxicity has grown exponentially over the past few years (Campbell and Compton, 2010; Kahru and Dubourguier, 2010). Nevertheless, serious deficiencies still exist in the knowledge relating to this area, especially in nanoecotoxicology. Some of the studies previously conducted on NP toxicity have also reported oxidative stress as one of the most important toxicity mechanisms related to NP exposure (Limbach *et al.*, 2007; Monteiller *et al*., 2007).

Silver nanoparticles (AgNPs) are used in increasing numbers of products, and given the potentially high toxicity and specific concerns associated with the use of AgNPs, particular attention to their toxicity may be warranted. Recent literatures on the toxicity and risk of AgNPs suggest that, directly or indirectly, oxidative stress may be a potential toxic mechanism, as AgNPs exposure can trigger oxidative stress or exacerbate pre-existing oxidant reactions; thereby, inducing toxicity (Roh *et al.*, 2009; Limbach *et al.*, 2007).

However, even though numerous recent studies have suggested AgNPs may exert their toxicity via oxidative stress, the upstream signaling mechanism responsible for regulating the oxidative stress by AgNPs is still poorly understood, especially in non-mammalian models, such as *Caenorhabditis elegans*. The nematode *C. elegans* is an excellent model organism for research on and assessment of environmental contaminants, particularly, for the study of the toxicological relevance of chemical-induced molecular-level responses, as the comprehensive knowledge of the genome of *C. elegans* and functional genomics tools allow for a clearer insight into the operation of toxic mechanisms initiated by chemicals acting upon organisms, mechanisms that can have adverse effects at the organism level.

Our previous study on the ecotoxicity of silver nanoparticles (AgNPs) in the nematode, *Caenorhabditis elegans*, suggested that oxidative stress may be an important mechanism in the toxicity of AgNPs, and PMK-1 p38 MAPK is involved in it (Roh *et al*., 2009; Lim *et al*., 2012).

In the present study, as a continuation of our previous work, the involvement of oxidative stress as a toxic mechanism of AgNPs was investigated, focusing on mitogen-activated protein kinase (MAPK) pathways. MAPK, extracellular signal-regulating kinase (ERK), c-Jun N-terminal kinase

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(JNK) and p38 MAPK serve as transducers of extracellular stimuli, which allow cellular adaptation to environmental changes, and play key roles in many diverse physiological processes, including stress responses (Kyriakis and Avruch, 2001; Kim *et al*., 2009). Although, the roles of the p38 MAPK and JNK pathways in various stress responses of *C. elegans* have already been reported (Troemel *et al*., 2006; Back *et al*., 2010; Kim *et al*., 2002), they have not been approached in an toxicological context. In the present study, to test whether oxidative stress was directly involved in AgNPs toxicity reactive oxygen species (ROS) was measured in AgNPs exposed *C. elegans* and subsequently, the downstream genes, known to be regulated by the MAPK signaling pathway, were also investigated in *wildtype* and mutant *C. elegans* exposed to AgNPs.

MATERIALS AND METHODS

Maintain of C. elegans and treatment of AgNPs to C. elegans. *C. elegans* were grown in Petri dishes on nematode growth medium (NGM) and fed with OP50 strain *Escherichia coli*, according to a standard protocol (Brenner, 1974). Worms were incubated at 20°C, with young adults (3 days old) from an age-synchronized culture then used in all the experiments. Detailed information on the mutant strains used in this study is presented in Supporting Table 1. *Wildtype* and mutants were provided by the *Caenorhabditis* Genetics Center (www.CGC.org) at the University of Minnesota. For treatment to *C.elegans*, AgNPs were prepared and characterized as described previously (Roh *et al*., 2009). The dispersion of AgNPs was controlled by sonicating for 13 h (Branson-5210 sonicator, Branson Inc., Danbury, CT, USA), stirring for 7 days, and filtering through a cellulose membrane (pore size 100 nm, Advantec, Toyo Toshi Kaisha, Japan; Eom and Choi, 2009; Bae *et al*., 2010). The size distribution of the AgNPs was examined by Energy filtering transmission electron microscopy (LIBRA 120 TEM, Carl Zeiss, Oberkochen, Baden-Wurttemberg, Germany) and Photal dynamic light scattering (DLS-7000, Otsuka Electronics Co., Inc., Osaka, Japan). From these results, the range of the main size of the NPs distributed in the medium was about 30~50 nm (data not shown), even though AgNPs tended to agglomerate during the experiment. From stock solutions, experimental concentrations (0.1, and 1 mg/*l*) of AgNPs were prepared in k-media (0.032 M KCl and 0.051 M NaCl; Williams and Dusenbery, 1990).

ROS formation. To detect the levels of ROS, *wildtype* and mutants were exposed to 1 mg/*l* of AgNPs for different time intervals, then transferred to 0.5 m*l* of S buffer, containing 15 μM 2, 7-dichlorofluoroscein diacetate (DCFH-DA, Sigma, St. Louis, MO, USA), and then pre-incubated for 30 min at room temperature. To confirm the role of ROS, worms were pre-treated with a strong antioxidant, N-acetylcysteine (NAC), in the presence of AgNPs. ROS formation was reduced by pre-treatment with 300 μM NAC for 2 h before AgNPs exposure. The fluorescence was observed using a Leica DM IL microscope (Leica, Wetzlar, Germany), with images obtained using a Leica DCF 420C camera (Leica). Levamisole (2 mM, Sigma-Aldrich) was applied to *C. elegans*, with pictures of the live worms then taken.

Quantitative real-time PCR. For the quantitative real time-PCR (qRT-PCR) analysis, *wildtype* and mutants were exposed to 1 mg/*l* of AgNPs at different time intervals (from 4 to 48 h) and then analyzed using the IQTM SYBR Green SuperMix (Bio-Rad, Hercules, CA, USA). PCR was carried out on 12 selected genes, using a Chromo4 Real-Time PCR detection system (Bio-Rad). The primers were constructed based on sequences retrieved from the *C. elegans* database (www.wormbase.org, Supporting Table 2), the qRT-PCR conditions optimized, and efficiency and sensitivity tests performed for each gene prior to the main experiment. Three replicates were conducted for each qRT-PCR analysis.

Statistical analysis. Data are presented in arbitrary units compared to the control, with statistical differences between the wildtype and mutants relating to survival and reproduction determined by an analysis of variance (ANOVA) test, with a Dunnett's multiple comparison test. All statistical tests were performed using Statistical Package for Social Sciences 12.0 KO (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

AgNPs characterization. As the physicochemical attributes of NPs are critical parameters in determining their degree of toxicity, prior to the toxicity experiments, the characterization of AgNPs were investigated, as described previously (Roh *et al*., 2009). Particle characterization using TEM and DLS confirmed that the sizes of particles applied in the toxicity test were mainly within the range 20~30 nm and evenly distributed in the test medium, as described previously (Lim *et al*., 2012).

Formation of ROS by AgNPs exposure. Recent studies (AshaRani *et al*., 2009; Kawata *et al*., 2009), including our previous one (Roh *et al*., 2009), indicated that oxidative stress is a potential toxic mechanism of AgNPs. In our previous study, we investigated ROS formation, as direct evidence of oxidative stress, in AgNPs exposed wildtype as well as, *pmk-1(km25)* mutant (Lim *et al*., 2012). Increased ROS formation induced by AgNPs in *wildtype (N2) C. elegans* was rescued in the *pmk-1 (km25)* mutant, which suggests oxidative stress is an important mechanism of toxicity of AgNP and the PMK-1 p38 MAPK plays an important role in AgNPs toxicity(Lim *et al*., 2012). In this study, to investigate involvement of other MAPK genes in AgNPs

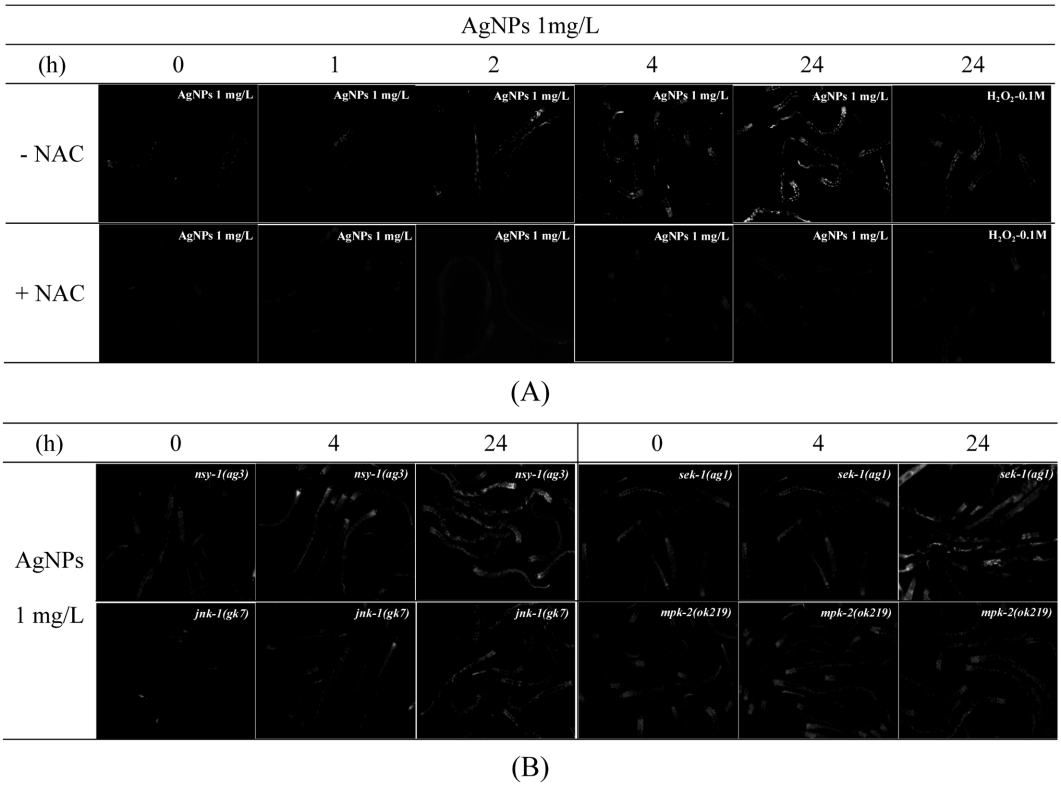

Fig. 1. The formation of ROS on exposure to AgNPs. The formation of ROS was observed in *wildtype* (A) and mutants (*nsy-1(ag3)*, *sek-1(ag1)*, *jnk-1(gk7)*, *mpk-2(ok219)*, (B). The worms were incubated with 15 μM of DCFH-DA at 37°C for 30 min, and then observed under fluorescence microscopy. To detect the level of ROS, the wildtype was exposed to 1 mg/l of AgNPs for 0, 1, 2, 4 and 24 h. 100 μM H_2O_2 was used as the positive control, with the antioxidant NAC pre-treated to confirm that the formation of ROS was the result of oxidative stress. The mutants were exposed to 1 mg/l AgNPs for 4 and 24 h. These pictures were taken under the 100 times magnification of the actual size.

induced oxidative stress, ROS formation was measured in four MAPK mutants strain, such as, *nsy-1(ag3)*, *sek-1(ag1)*, *jnk-1(gk7)*, *mpk-2(ok219)* (Fig. 1). Increases in ROS formation were observed 1, 2, 4 and 24 h after AgNPs exposure in *wildtype C. elegans*. The fluorescence intensity was more significant after 24 h exposure than during early exposure times. Increased fluorescence on AgNPs exposure was suppressed by pretreatment with NAC, an important antioxidant, which serves as a precursor for the synthesis of glutathione (Fig. 1A). This confirms that the AgNP-induced increase in ROS formation was due to oxidative stress. Subsequently, the degree of ROS formation was compared across the mutant strains 4 and 24 h after exposure to 1 mg/*l* of AgNPs (Fig. 1B). The responses of *nsy-1(ag3)*, *sek-1(ag1)* and *mpk-2(ok219)* to AgNPs exposure were similar to that of the *wildtype*. Slight decreased of ROS formation was observed in *jnk-1(gk7)* mutant.

Expression of genes in MAPK signaling pathway by AgNPs exposure. In our previous study, we reported that PMK-1 p38 MAPK was involved in AgNPs-induced oxidative stress (Lim *et al.*, 2012). In this study, we investigated involvement of *C. elegans* MAPK signaling pathways in response to AgNP exposure. The response of P38 MAPK signaling pathway, (i.e. *nsy-1* (MAPK kinase kinase, MAPKKK)/*sek-1* (MAPK kinase and MAPKK)/*pmk-1* (p38 MAPK)), JNK MAPK pathways, (i.e. *jkk-1* (MAPKK) and *jnk-1* (JNK MAPK)) and ERK MAPK pathway (i.e. *mpk-2* (ERK MAPK)), were investigated in AgNPs exposed *C. elegans*. To gain an insight of the role of MAPK pathways in oxidative stress, the response was also investigated also in *sod-3(gk235)* mutant (Fig. 2). In the *wildtype* exposed to AgNPs, the observed alterations in all the MAPK genes were less than 2 fold compared to the control; however, the expressions were highest after 24 h of exposure in all tested MAPK genes.

The pattern of MAPK mRNA expression in *wildtype* was different from those in *sod-3(gk235)* mutants (Fig. 2). A weak increase in MAPK mRNA was observed (mostly less than 2-folds compared to control) in the *wildtype*. In the *sod-3(gk235)* mutant, the degree of increased expression was as significant (about 2~6 fold compared to the control), except for the expression of *jkk-1*, where the increase reached about 10-fold that in the control after 48 h of expo-

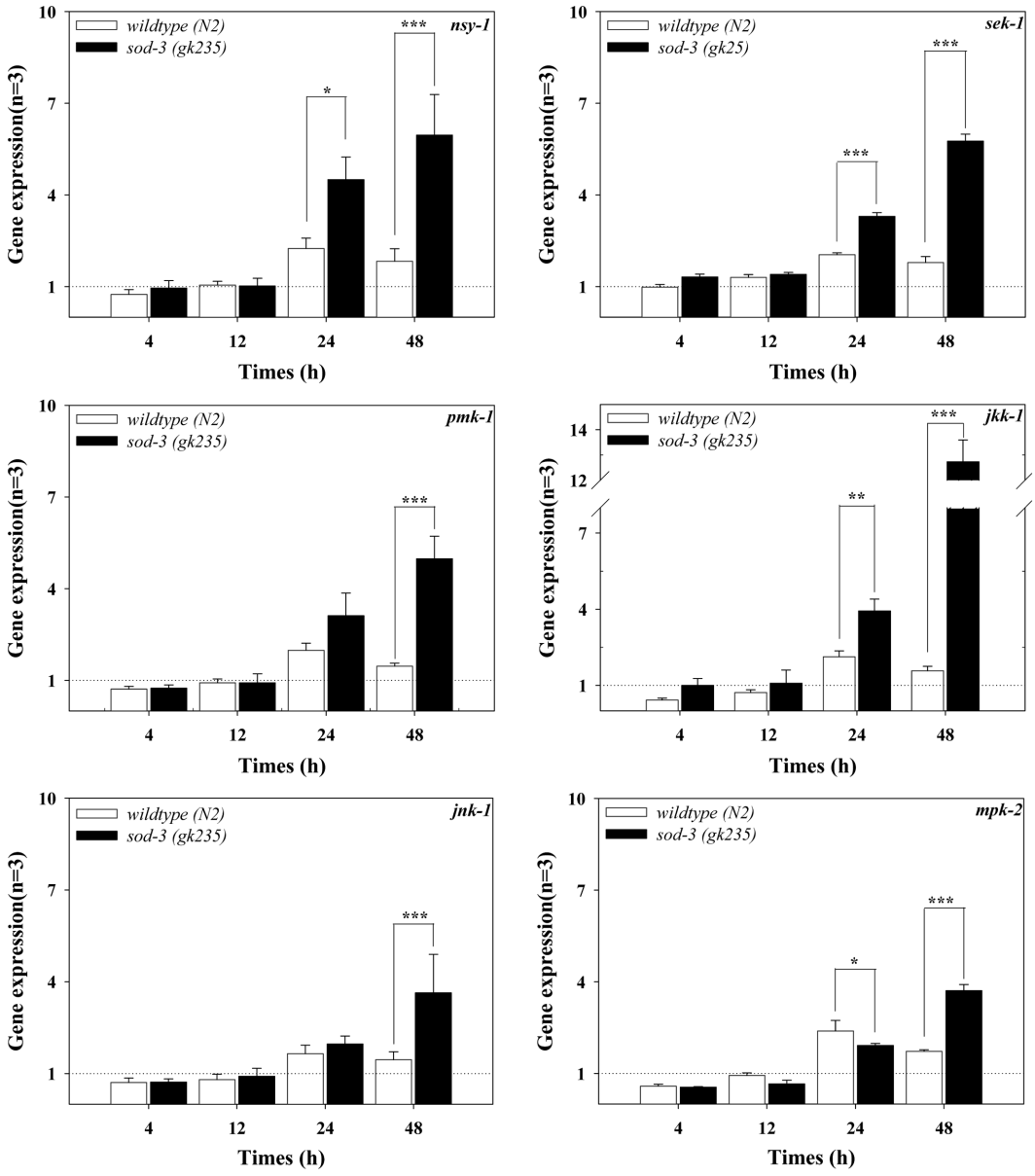

Fig. 2. *Expressions* of the genes in the MAPK signaling pathways in *wildtype* and *sod-3(gk235)* mutant. *C. elegans* was exposed to 1 mg/*l* of AgNPs for 0, 4, 12, 24 and 48 h, with qRT-PCR performed for the analysis of gene expressions. The results are expressed in relative units compared to the beginning of the experiment (0 h = 1). Statistically significant differences were analyzed between the response of the wildtype and mutants using a one-way ANOVA (number = 3; mean ± standard error of mean, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

sure. In the *sod-3(gk235)* mutant, a dramatic increase in the expression of the gene of JNK MAPK, *jkk-1*, as well as significant increases in the genes in the p38 MAPK pathway, *nsy-1*, *sek-1* and *pmk-1* (Fig. 2), suggest the importance of the p38 and JNK signaling pathways in the oxidative stress condition induced due to the exposure of *C. elegans* to AgNPs.

Oxidative stress response gene expression by AgNPs exposure. The expressions of oxidative stress responsive genes known to be regulated by the MAPK signaling pathway, such as *skn-1*, *sod-3* and *gst-4* were investigated in AgNPs exposed *wildtype* and *sod-3*(*gk235*) mutant at different exposure times (Fig. 3). Special attention was paid on the signaling cascade of *pmk-1*, *skn-1* and *gst-4*, as activation of the p38-Nrf-2-GST signaling pathway upon oxidative stress has been identified in mammalian systems (Giudice and Montella, 2006). Inoue *et al.* (2005) reported that the *C. elegans pmk-1*, p38 MAPK pathway regulates the oxidative stress response *via skn-1* on exposure to arsenate when used as a chemical stressor. An exposure time

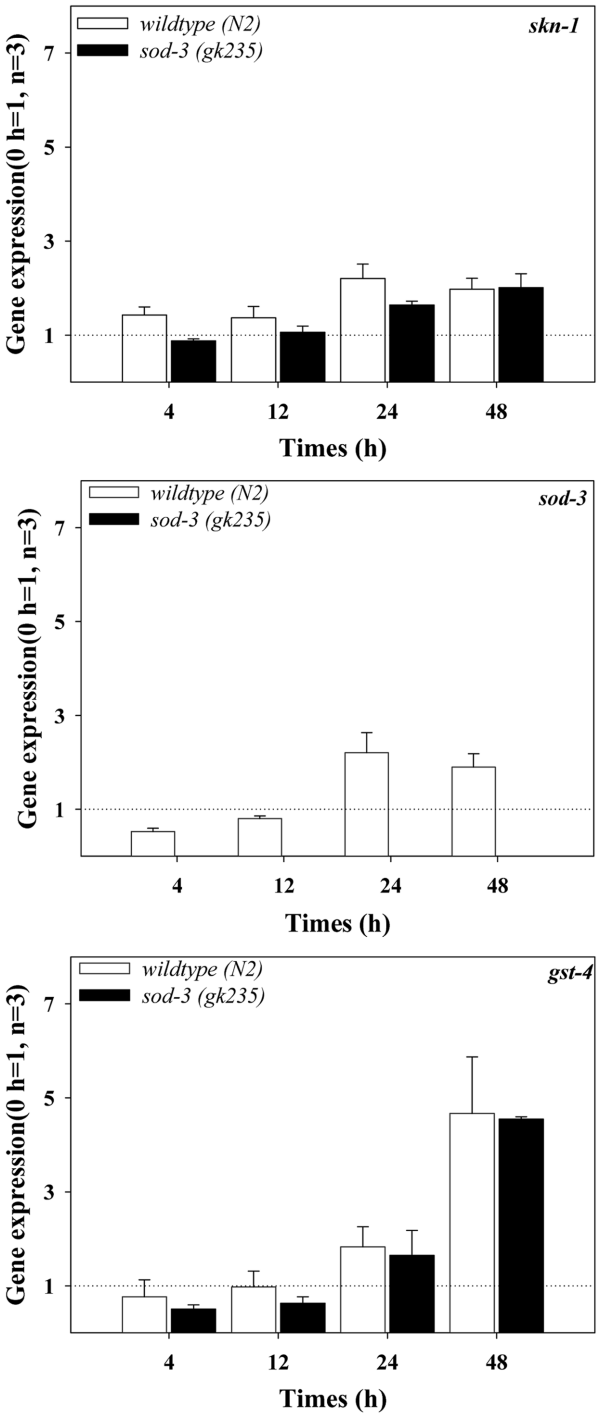

Fig. 3. Expressions of gene known to be regulated by MAPK signaling pathway (*skn-1*, *sod-3* and *gst-4*), in *wildtype* and *sod-3(gk235)* mutant. *C. elegans* was exposed to 1 mg/*l* of AgNPs for 0, 4, 12, 24 and 48 h, and qRT-PCR was performed for the analysis of gene expressions. The results are expressed in relative units compared to the beginning of the experiment (0 h = 1). Statistically significant differences were analyzed between the response of the wildtype and loss of function mutants using a one-way ANOVA (number = 3; mean ± standard error of mean, *p < 0.05, **p < 0.01, ***p < 0.001).

dependant increase in the expression of *gst-4* genes was observed in *wildtype* exposed to AgNPs. And the *skn-1* and *gst-4* gene expression did not show any statistical difference in the *wildtype* or in *sod-3(gk235)* mutant (Fig. 3). These results suggest that *skn-1* and *gst-4* may not be involved in the expression of the AgNPs induced *p38* mediated *sod-3* gene. Further investigations on the discovery of a new transcription factor potentially involved in AgNPs-induced p38 and *sod-3* activation, are warranted to fully elucidate the p38 MAPK mediated oxidative stress signaling in *C. elegans* on exposure to AgNPs. The high expressions of the *pmk-1* gene 48 h exposure to AgNPs in the *sod-3(gk235)* mutant can also be interpreted as compensatory mechanisms in the absence of important stress response genes.

In conclusion, *C. elegans* seems to possess a MAPK-based integrated stress signaling network, as the MAPK activation pathway can be part of a general stress response, from infection to chemical stresses (Pei *et al*., 2008). The overall results of the present study on AgNPs may provide further evidence for this hypothesis. However, the mechanism by which these responses occurred merits further investigation. Especially, how *C. elegans* MAPK signaling pathways are involved in defense mechanisms on exposure to AgNPs would be an interesting research topic in the emerging field of nanotoxicity. *C. elegans* has been used as a non-mammalian in vivo model for human toxicity screening. Therefore, it is important to identify how the mechanism of toxicity of AgNPs observed in *C. elegans* study can be extrapolated to a mammalian system. However, there is still a serious lack of information on the toxicity of AgNPs for any general correlation between the findings from *C. elegans* and in vitro and in vivo mammalian studies. This challenging task, which is not limited to nanotoxicity testing, may be achieved via comparative toxicity studies using various biological systems.

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