HHS Public Access

Author manuscript

Sci Total Environ. Author manuscript; available in PMC 2024 September 03.

Published in final edited form as:

Sci Total Environ. 2020 July 10; 725: 138523. doi:10.1016/j.scitotenv.2020.138523.

Epigenetic effects induced by silver nanoparticles in *Caenorhabditis elegans* after multigenerational exposure

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Abstract

Multigenerational effects of silver nanoparticles (Ag-NPs) on reproduction of the soil nematode *Caenorhabditis elegans* have been observed previously. However, mechanisms of this reproductive sensitivity are unknown. Here we examine whether epigenetic changes occur as a result of multigenerational exposure to Ag-NPs and whether such modifications can be inherited by unexposed generations. Changes at histone methylation markers, histone 3 lysine 4 dimethylation (H3K4me2) and histone 3 lysine 9 trimethylation (H3K9me3), known to affect reproduction, as well as changes in the expression of the genes encoding demethylases and methyltransferases associated with the selected markers, were investigated. We exposed *C. elegans* at EC_{30} to $AgNO_3$, pristine Ag-NPs, and its environmentally transformed product, sulfidized Ag-NPs (sAg-NPs). Histone di-methylation levels at H3K4me2 increase in response to pristine Ag-NP exposure and did not recover after rescue from the exposure, suggesting transgenerational inheritance. Compared to pristine Ag-NPs, exposure to transformed sAg-NPs significantly decreased H3K4me2 and H3K9me3 levels. These changes in the histone methylation were also supported by expression of *spr-5* and *jmjd-2* (H3K4me2 and H3K9me3 demethylases, respectively) and *set-30* (H3K4me2 methyltransferase). Our study demonstrates that multigenerational exposure to Ag-NPs induces epigenetic changes that are inherited by unexposed offspring. However, environmental

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Credit Author Statement

Anye Wamucho: Methodology, Investigation, Writing - Original draft, Formal Analysis. **Allison Heffley: Investigation. Olga Tsyusko:** Supervision, Conceptualization, Methodology, Writing - Reviewing and Editing.

Declaration of interests

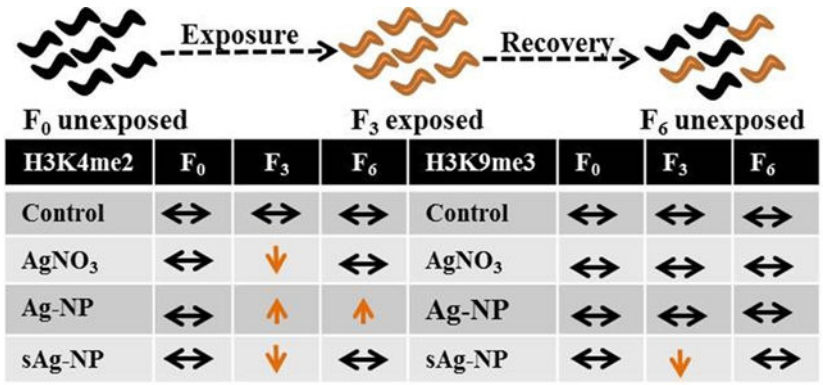
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflicts of Interest

The authors declare that they have no conflicts of interests with respect to this study.

transformations of Ag-NPs may also reduce toxicity via epigenetic mechanisms, such as changes at histone methylation.

Graphical Abstract



Keywords

Demethylase; Gene expression; Histone methylation; Methyltransferase; Nanomaterials; Nematode

1. Introduction

Silver NPs are valued for their antimicrobial, optical and conductive properties (Mélanie et al., 2009; Rao et al., 2002). This has resulted in the widespread application of Ag-NPs in the industrial and biomedical sectors (Haider and Kang, 2015; Juganson et al., 2015; Salata, 2004). After detailed analysis of consumer products in the market advertised to contain nanomaterials from several countries, Ag-NPs was identified as the most used nanomaterial in the consumer products recorded (Vance et al., 2015). There has been an increasing concern over the release of silver nanoparticles (Ag-NPs) into the environment from the many consumer products containing Ag nanomaterials (Juganson et al., 2015; Vance et al., 2015). Silver nanoparticles can be introduced in the environment during production, as well as during the usage and disposal of consumer products, thereby increasing the environmental concentration of Ag. Most of the Ag-NPs released from consumer products end up in wastewater treatment plants (WWTP), where they mainly partition into sewage sludge (Kaegi et al., 2011; Mueller and Nowack, 2008). The sludge may then be applied on land as fertilizer for agricultural purposes, thus introducing Ag-NPs into soils. A global life cycle release of several engineered nanomaterials showed that most of the engineered nanomaterials eventually end up in landfills (63–91 %) and soils (8–28 %), followed by the aquatic environment (0.4–7 %), and then air (0.1–1.5 %) (Keller et al., 2013).

The release of Ag-NPs into soil environment is ongoing process and it is inevitable that soil organisms will encounter these NPs. Thus, examining effects and mechanisms of exposure to Ag-NPs over multiple generations in a soil invertebrate such as a nematode, *Caenorhabditis elegans*, represents an environmentally realistic scenario. Nematodes inhabit soil and water environments and are among the most abundant, in terms of numbers, soil-

dwelling animal organisms with ability to adapt to a wide range of environmental conditions (Neher and Powers, 2005). Model nematode *C. elegans* is a free living nematode, about 1 mm in length, with a short life cycle (3 days) and prolific reproduction (up to 300 offspring). For over two decades it has been accepted as a powerful model for ecotoxicity studies including nanotoxicity (Choi et al 2014).

Potential harmful effects of Ag-NPs in the environment have been investigated in different soil organisms (Diez-Ortiz et al., 2015; Jesmer et al.; Shoults-Wilson et al.; Starnes et al., 2015; Velicogna et al., 2016; Waalewijn-Kool et al., 2014; Yang et al., 2014). However, most Ag nanotoxicity studies have focused on acute or sub-chronic exposures. Adequate analysis of the risk posed by Ag-NPs should include more realistic chronic exposures at sub-lethal concentrations, with environmentally transformed forms of Ag-NPs taken into consideration. Prominent environmental transformation of pristine Ag-NPs includes oxidation which results in the release of Ag^+ ions and sulfidation to form sulfidized Ag-NPs (sAg-NPs) (Levard et al., 2012; Lowry et al., 2012).

Schultz et al. (Schultz et al., 2016) performed one of the most extensive multigenerational exposure study in which the nematode *C. elegans* exposed to $AgNO_3$ and Ag-NPs, showed heightened reproductive sensitivity in subsequent generations. Multigenerational reproductive effects of Ag-NPs have also been observed in *Drosophila melanogaster* (Panacek et al., 2011; Raj et al., 2017). Despite these multigenerational effects, the mode of toxicity transfer from exposed parental generations to subsequent generations has not been thoroughly investigated. The contribution of germline mutations to the previously observed multigenerational toxicity in response to Ag-NPs and Ag ions was investigated in our most recent study (Wamucho et al., 2019). In that study we showed that there was a pattern for increase in the germline mutations observed in all treatments with transformed sAg-NPs inducing significant increase in the number of transversion. This result could not explain the previously observed multigeneration toxicity, where pristine Ag-NPs and $AgNO_3$ induced stronger reproductive toxicity than sAg-NPs. Therefore the results from the genomic mutation study (Wamucho et al., 2019) suggest that other mechanisms of inheritance, such as epigenetics, must be involved in the observed multigenerational toxicity in response to Ag-NPs and $AgNO_3$. Additionally, a previous study with human cell lines showed changes in epigenetic patterns, such as DNA methylation, histone methylation and miRNA expression, in response to Ag-NP exposure (Wong et al., 2017).

Epigenetic changes such as histone modifications (methylation, acetylation, and phosphorylation), DNA methylation, and non-coding RNAs, which do not involve changes in the underlying DNA sequence, can be inherited. Epigenetic markers regulate vital processes such as development and aging in *C. elegans* (Gonzalez-Aguilera et al., 2014). Transgenerational fertility defects in *C. elegans* are known to correlate with changes in certain histone methylation profiles as well as DNA methylation (Greer et al., 2014; Greer et al., 2015). Environmental stressors also induce epigenetic changes that are inherited by subsequent unstressed generations (Kishimoto et al., 2017; Klosin et al., 2017; Yu and Liao, 2016). As such, the heightened reproductive sensitivity observed in subsequent generations after exposures to $AgNO_3$ and Ag-NPs may be explained by changes in heritable epigenetic markers. Previous sub-chronic exposure revealed differences in bio-distribution of Ag and

transcriptomic profiles in response to $AgNO_3$, Ag-NPs, and sAg-NPs (Starnes et al., 2016; Starnes et al., 2015). Thus, different epigenetic responses are expected after *C. elegans* exposure to the different Ag forms.

Changes in epigenetic markers such as histone 3 lysine 4 dimethylation (H3K4me2) and histone 3 lysine 9 trimethylation (H3K9me3), have been shown to correlate with transgenerational fertility in *C. elegans*. An increase in H3K4me2 and a decrease in H3K9me3 were shown to increase sterility in *C. elegans* in a transgenerational manner (Greer et al., 2014). Methyltransferases and demethylases associated with these epigenetic markers, which catalyze the addition or removal of methyl groups respectively, have been predicted or identified. Among them are H3K4 mono/dimethyltransferases, *set-17* and *set-30*, and H3K4me2 demethylase, *spr-5* (Greer et al., 2014).The demethylase for H3K9me3 is *jmjd-2* and the methyltransferase is *set-25* (Gonzalez-Aguilera et al., 2014; Greer et al., 2014). Though *set-26* was initially shown to trimethylate H3K9 *in vitro* (Greer et al., 2014) but not *in vivo*, it is involved in such vital biological processes as transgenerational fertility, life span, development and aging, and also seems to repress H3K4me3 levels (Gonzalez-Aguilera et al., 2014; Ni et al., 2012; Wang et al., 2018). In this study, we investigated the epigenetic changes in histone methylation markers, H3K4me2 and H3K9me3, after multigenerational exposure of *C. elegans* to pristine and transformed (sulfidized) Ag-NPs (sAg-NPs) as well as $AgNO_3$. The $AgNO_3$ treatment was included as a positive control for complete dissolution of Ag-NPs. Analyses were carried out on nematode populations prior to exposure (F_0), after three generations of exposure (F_3), and three generations of rescue from exposure (F_6) to determine if these epigenetic changes after exposures, if any, are inherited by subsequent unexposed generations. The expression levels of the genes encoding the specific demethylases and methyltransferases were also investigated to determine if they correlate with the histone methylation levels.

2. Materials and Methods

2.1. Silver nanoparticle synthesis and characterization

Polyvinylpyrrolidone (PVP) coated Ag-NPs were synthesized as previously described (Cheng et al., 2011). The same batch of Ag-NPs used by Starnes et al. (Starnes et al., 2015) and Schultz et al. (Schultz et al., 2016) was used in this study. Sulfidation was carried out by combining Ag-NPs with Na_2S at a 2:1 molar ratio of S to Ag. The mixture was incubated at room temperature for 4 h open to the atmosphere. Tube was capped and sealed and incubated at room temperature for an additional week. The sAg-NPs were separated from the reaction solution and triple washed with 18MΩ deionized water. Complete sulfidation was confirmed by powder X-ray diffraction (X'Pert Pro, Malvern PANalytical, Malvern, UK).

Characterization of Ag-NPs was described in our previous studies (Schultz et al., 2016; Starnes et al., 2015). The transmission electron microscopy (TEM) primary particle sizes were reported to be 58.3 ± 12.9 nm for PVP coated Ag-NPs and 64.5 ± 19.4 nm for sAg-NPs (Starnes et al., 2015). Modified simulated soil pore water (SSPW: Na 4 mM, Mg 0.5 mM, Al 1 μM, K 1.0 mM, Ca 1.25 mM, NO_3 3.5 mM, SO_4 0.5 mM, PO_4 1.0 μM, and

I=10.3 mM) was used for all exposures to mimic natural soil environment for *C. elegans* (Schultz et al., 2016; Tyne et al., 2013).

Upon addition of Ag-NPs and sAg-NPs into the SSPW(Tyne et al., 2013), the volume weighted sizes were determined by dynamic light scattering as 66.26 ± 34.34 and 60.73 ± 20.67 nm, respectively. The zeta potential of Ag-NPs and sAg-NPs in the SSPW were −5.3 mV and −15.7 mV, respectively. The stability of these Ag nanoparticles over period of exposure of 72 h have been investigated and described in our most recent study (Wamucho et al. 2019). According to that study, after the 72 h period, the volume weighted sizes were 68.87 ± 30.38 nm for Ag-NPs and 68.64 ± 20.78 nm for sAg-NPs. After 72 h zeta potential of Ag-NPs and sAg-NPs in the SSPW were −5.3 and −15.7 mV, respectively. From our previous multigenerational study, which also used the same Ag-NPs in the same media (SSPW), the dissolution determined via ultrafiltration was 1.5 ± 0.1% for Ag-NPs and 0.023 ± 0.002% for sAg-NPs (Schultz et al., 2016).

2.2. Nematode exposures

Caenorhabditis elegans (N2) was acquired from Caenorhabditis Genetics Center (University of Minnesota, USA). The exposures were carried out on a population of nematodes which were propagated for multiple generations (Fig. 1). Epigenetic markers (H3K4me2 and H3K9me3) and gene expression levels of the demethylases and methyltransferases associated with these epigenetic markers were analyzed in different generations prior to exposure (F_0), after three generations of exposure (F_3), and after three generations of rescue from exposure (F_6).

Age synchronization using NaClO/ NaOH solution was performed and eggs were placed in 10 cm SSPW agar plates to start the unexposed F_0 populations of four replicates (about 4000 nematodes per replicate) per treatment group (control, $AgNO_3$, pristine Ag-NPs, and sAg-NPs). Uracil deficient *E. coli* strain, OP50, suspended in 6 ml of SSPW at an optical density (540 nm) of 0.35 was added (10 μl/ml) to each plate as food source and incubated at 20 °C. The amount of *E.coli* used provided optimal feeding conditions for the nematodes. After 96 h, starting from the egg stage, gravid adults were washed off the plate into 15 ml centrifuge tubes using SSPW and split in half. Half of the nematodes were saved for histone and RNA extraction and half used for age synchronization to obtain eggs for the next generation (F_1). Exposures were started at the F_1 generation with the SSPW/OP50 food source dosed with equitoxic concentrations of $AgNO_3$ (0.07 mg/L), Ag-NPs (1.5 mg/L), and sAg-NPs (6 mg/L). These concentrations correspond to EC_{30s} for reproduction, as determined from concentration-response experiments previously (Schultz et al., 2016) and also reconfirmed in this experiment (Fig. S1). The EC_{30} concentrations were selected to examine epigenetic and gene expression changes at sub-lethal equitoxic concentration in all treatments. The SSPW/ OP50 solution without NPs or $AgNO_3$ was used for controls throughout the experiment. Exposures were carried out for three generations (F_1, F_2, and F_3). Each generation was exposed for 96 h starting at the egg stage after which age synchronization was performed to obtain eggs for each subsequent generation. The 6 ml of SSPW (for control) or exposure solutions in SSPW were deposited on the agar plates. When exposures are performed in liquid media starting from eggs, there is a delay in *C. elegans* development. Instead of 64 h

required for *C. elegans* to reach stage of gravid adults, when maintained on a solid K-agar media, it took 96 h to reach this stage in liquid (SSPW) media. After the last exposure (F_3), nematodes were washed off the plate with SSPW and half of the nematodes saved for histone and RNA extraction. The other half was used for age synchronization to obtain eggs to start the F_4 generation at which point rescue from exposure started by feeding nematodes with SSPW/OP50 solution without NPs or $AgNO_3$. Rescue was carried out for three generations (F_4, F_5, and F_6) at which point the experiment was terminated. The F_6 population were washed off the plate with SSPW and used for histone and RNA extraction.

2.3. Histone and RNA extractions

Nematodes were washed three times using DI water with gentle centrifugation at 800 rpm for 1 min to remove residual bacteria. After the final wash, the nematodes were split in two groups for histone and RNA extractions. Nematodes for histone extraction were homogenized by sonication on ice after which the EpiQuik total histone extraction kit (EpiGentek, Farmingdale, NY) was used following manufacturers recommendations. Extracted total histone was quantified by use of the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Rockford, IL). RNA extraction was performed using RNeasy Mini Kit with on-column DNase 1 digestion for 15 min at room temperature (QIAGEN, Hilden, Germany) and quantified using a UV-visible spectrophotometer.

2.4. Total H3, global H3K4me2 and global H3K9me3 quantification

Total H3 levels were measured with enzyme-linked immunosorbent assay (ELISA)-based colorimetric EpiQuik Total Histone H3 Quantification Kit (EpiGentek, Farmingdale, NY) using 250 ng of total histone proteins. Global H3K4me2 and global H3K9me3 levels were measured with EpiQuik Global Di-Methyl Histone H3K4 Quantification Kit EpiQuik and Global Tri-Methyl Histone H3K9 Quantification Kit (EpiGentek, Farmingdale, NY), respectively, using 1000 ng of total histone. The tri- and dimethylated histones were captured by anti-dimethyl H3K4 or anti-trimethyl H3K9 antibody and detected with labeled detection antibody. The absorbance were measured at 450 nm using SpectraMax i3. Measurements were performed using three biological replicates per treatment with two technical replicates per sample. Global levels of H3K4me2 and H3K9me3 were normalized to the total H3 levels before analysis. The calculations of the concentrations were performed using a Four Parameter Logistic curve fit, implemented into MyAssays analysis open source software available at myassays.com.

2.5. Quantitative Real-Time Polymerase Chain Reaction Analysis

Expression levels of H3K4me2 demethylase (*spr-5*) and H3K4 mono/dimethyltransferases (*set-17* and *set-30*) were investigated. H3K9me3 demethylase (*jmjd-2*) and H3K9 methyltransferase (*set-25*) expression levels were also analyzed as well as expression levels of *set-26*. The TaqMan primer/probe assay IDs and their amplification efficiencies are shown in Table S1. Y45F10D.4 gene, a putative iron sulfur cluster assembly enzyme, was used as the reference gene due to its highly stable expression levels (Zhang et al., 2012). Its expression levels were also stable among all Ag treatments used in this study with 0.5–1Ct difference (Fig. S2).

cDNA synthesis was carried out with 500 ng of total RNA by using RevertAid First Strand Synthesis Kit (ThermoFisher Scientific, Vilnius, Lithuania). qRT-PCR reactions were carried out in 10 μL volumes with TaqMan fast advanced master mix, TaqMan gene expression assays for each gene, and cDNA diluted 1:100 (*spr-5,set-17,set-30*) or 1:19 (*jmjd-2, set-25, set-26*). The optimal cDNA dilution factor was determined based on the dilution amplification curves from the efficiency tests. StepOne Plus system (Applied Biosystems) was used for all amplifications with a program of 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. All treatments for each gene were run in biological and technical triplicates. Negative controls and minus reverse transcriptase (-RT) negative controls were run for every gene/sample to check for DNA contamination. The data were exported into GenEx software (MultiD), and after normalization to the reference gene, the expression levels of the target genes relative to controls were calculated following the Pfaffl method (Pfaffl, 2001). The patterns of the gene expression levels were compared to their respective histone methylation levels observed.

2.6 Data analysis

Statistical analyses were conducted in SAS. The data are presented as mean and standard errors of the mean. The statistical significance among treatments was determined after applying one-way ANOVA with Tukey's post hoc test. The differences were accepted as statistically significant at $p < 0.05$.

3. Results and Discussion

This study was designed to investigate the potential for changes in epigenetic modifications and their transgenerational inheritance in *C. elegans* exposed to $AgNO_3$, Ag-NPs, and sAg-NPs. We anticipate that our results provide insight into the mechanisms of previously observed transgenerational toxicity to Ag-NPs and $AgNO_3$ (Schultz et al., 2016). Overall, our results indicate that all three different Ag treatments induce differential epigenetic changes in global levels of H3K4me2 and H3K9me3 in *C. elegans* and this may have different implications in regards to toxicity. Differential gene expression patterns identified previously after sub-chronic exposures of *C. elegans* within single generation to these Ag treatments, suggested different mechanisms of toxicity (Starnes et al., 2016). Epigenetic modifications, such as histone methylation, are known to regulate gene expression (Dong and Weng, 2013; Liu et al., 2011; Pokholok et al., 2005), and it is possible that the differential histone methylation patterns observed after exposure to different forms of Ag play a role in such differential gene expression. In addition, the differences in epigenetic changes observed in response to pristine and transformed Ag-NPs (sAg-NPs) in the current study correlate with the different effects on reproduction identified in our previous multigenerational study (Schultz et al., 2016). This suggests that the epigenetic mechanism, such as histone methylation, is likely involved in the multigenerational reproductive toxicity. We have also observed that changes in histone methylation persist even in the nematode populations after rescue from pristine Ag-NP treatment (Fig. 2) suggesting that these modifications have been inherited by the unexposed generations. Our histone methylation results correlate with the changes in the expression of the genes, shown or predicted to play

a role in these epigenetic modifications, thus providing additional support for the results of this study.

3.1 Global H3K4me2 levels and expression of H3K4me2 demethylase and methyltransferase

Global H3K4me2 levels prior to exposure (F_0), after three generations of exposure (F_3), and after three generations of rescue from exposure (F_6) are shown in Fig. 2a. Exposure to $AgNO_3$ and sAg-NPs resulted in a significant decrease ($p = 0.030$ and $p = 0.001$ respectively) in H3K4me2 levels compared to the unexposed F_0 with recovery after rescue from the exposure at F_6 ($p = 0.0674$ and $p = 0.146$ respectively). For Ag-NP treatment, an increase in H3K4me2 was observed after three generations of exposure compared to the unexposed F_0, though not significant ($p = 0.334$). Interestingly, H3K4me2 level was significantly higher in F_6 ($p = 0.043$) after three generations of rescue from Ag-NP exposure compared to the unexposed F_0 and not significantly different ($p = 0.585$) from the F_3 generation suggesting transgenerational inheritance of the H3K4me2 levels.

An increase in histone methylation levels may be explained by a down-regulation in the demethylase and/or up-regulation in the methyltransferase associated with the histone methylation mark. However a decrease in histone methylation may be explained by an up-regulation in the demethylase and/or a down-regulation in the methyltransferase. The expression level of *spr-5*, an H3K4me2 demethylase, is shown in Fig. 2b. No significant changes in *spr-5* expression levels was observed for $AgNO_3$ for all three generations analyzed despite the significant decrease in H3K4me2 levels observed after exposures at F_3 when compared to F_0. Though no significant down-regulation ($p = 0.792$) in *spr-5* was observed after exposures to Ag-NP at F_3, a significant down-regulation ($p = 0.045$) in *spr-5* levels was observed after rescue from exposure (F_6) when compared to the unexposed F_0, which corresponds to the higher levels of H3K4me2 observed at F_6. This also suggests transgenerational gene regulation, which can be achieved through the transfer of small non-coding RNAs to subsequent generations through the germline that may affect gene expression in subsequently unexposed populations (Rechavi and Lev, 2017) but this was not investigated in this study. There was a borderline significant up-regulation ($p = 0.053$) in *spr-5* levels after three generations of exposure (F_3) to sAg-NPs when compared to F_0. The *spr-5* expression levels recovered with a significant decrease ($p = 0.024$) from the F_3 levels at F_6 to unexposed F_0 levels after rescue from the exposure. This correlates with the H3K4me2 levels which was significantly decreased after exposure and returned to the F_0 levels after rescue.

When comparing treatments to control group, there were significant differences in H3K4me2 levels. However, since each treatment started with a different population of nematodes with potentially different histone methylation backgrounds, unexposed F_0 within each treatment served as its respective “control’. In addition, there were no significant differences among F_0, F_3, and F_6 generations in the levels of H3K4me2 in our main control group.

Expression levels of *set-17* and *set-30*, H3K4 mono/dimethyltransferases, were also tested as their expression levels may explain the observed H3K4me2 levels in addition to the

spr-5 expression levels. No significant differences in expression levels were observed for *set-17*, previously identified as mono/dimethyltransferase for H3K4 (Greer et al., 2014), after exposure to any of the three Ag forms or after recovery from the exposure (Fig. 2c). For *set-30*, no significant changes were observed among generations for $AgNO_3$ and sAg-NP treatments after exposure and recovery. However, for Ag-NP treatment, a significant up-regulation ($p = 0.001$) in *set-30* expression levels was observed after exposures (F_3) as seen in Fig. 2d. Though recovery from the increased levels was observed at F_6 with a significant decrease ($p = 0.03$) when compared to F_3, the expression levels remain significantly higher ($p = 0.019$) than F_0, again suggesting transgenerational gene regulation. These expression levels of *set-30* correspond with the H3K4me2 levels observed for the Ag-NP treatment.

For the Ag-NP and sAg-NP treatments, correlation observed between the gene expression levels of the demethylases and methyltransferases with the global H3K4me2 levels provides another level of evidence to support the H3K4me2 levels observed. However, despite the significant decrease in H3K4me2 levels after exposures to $AgNO_3$ treatment, none of the gene expression levels corresponded to the H3K4me2 trend. Given the good correlation between the expression levels of demethylases and methyltransferases for Ag-NP and sAg-NP treatments, it is possible that genes encoding other enzymes, which have not been identified, may be at play in the response of *C. elegans* to $AgNO_3$. Our previous findings suggested that some of the mutigenerational reproductive toxicity was due to release of Ag^+ ions from pristine Ag-NPs while mainly particle specific toxicity was indicated for sAg-NPs (Schultz et al., 2016). The epigenetic changes observed in this study seem to indicate that some of these modifications in pristine Ag-NP treatment are also particle-specific, which is possible, given their low dissolution of 1.5% in the SSPW exposure media.

Exposure of a single generation of *C. elegans* to another environmental toxin arsenic in arsenite form was shown to cause an increase in H3K4me2 levels and down-regulation of *spr-5* in up to three subsequent unexposed generations (Yu and Liao, 2016), which also suggests inheritance of H3K4me2 levels or transgenerational regulation of *spr-5* expression levels. In our study, this transgenerational effect was observed only for Ag-NP exposures. The trend in H3K4me2 was different for Ag-NPs compared to sAg-NPs. This suggests that an environmental transformation, such as sulfidation of pristine Ag-NPs, is likely to reduce their toxic effects by affecting histone methylation differently than pristine Ag-NPs. Genomic regions with high H3K4me2 levels have been linked with active transcriptional activity (Bernstein et al., 2002; Pokholok et al., 2005) and as such, the changes observed may affect gene expression in these nematodes.

3.2. Global H3K9me3 levels and expression of H3K9me3 demethylase and methyltransferase

Global H3K9me3 levels for the F_0, F_3, and F_6 populations are shown in Fig. 3a. Exposure to $AgNO_3$ did not cause any significant changes among the generations. In the case of Ag-NP exposure, an increase was observed at F_3 when compared to F_0 but was not significant ($p = 0.114$) and the levels recovered to almost F_0 levels after recovery from exposure (F_6). sAg-NP exposure caused a significant decrease ($p = 0.002$) in H3K9me3 levels after exposures (F_3) when compared to the unexposed F_0. Recovery was observed at F_6 with a

borderline significant increase ($p = 0.052$) in H3K9me3 levels when compared to F_3 and not significantly different ($p = 0.316$) from F_0 levels.

A significant down-regulation ($p = 0.006$) in H3K9me3 demethylase, *jmjd-2*, expression levels was observed after exposure to Ag-NPs at F_3 (Fig. 3b). Even though a significant recovery ($p = 0.027$) was observed at F_6, the levels still remained significantly lower ($p = 0.029$) compared to $F_{0,}$ again suggesting transgenerational gene regulation for Ag-NP exposure. Though no significant differences in H3K9me3 levels were observed for Ag-NP exposure, the trend observed correlates with the *jmjd-2* expression levels. For transformed sAg-NPs, a significant up-regulation ($p = 0.019$) in *jmjd-2* levels was observed after exposure in F_3 when compared to the unexposed $F_{0,}$ which also supports the decrease in H3K9me3 levels. The *jmjd-2* levels recovered in F_6 with a significant increase ($p = 0.016$) when compared to F_3. The recovery overcompensates such that the *jmjd-2* levels in F_6 were significantly lower than the F_0 levels. This again correlates with the H3K9me3 levels observed in response to the sAg-NP exposure. In addition to serving as demethylase for H3K9me3, *jmjd-2* is also demethylase for H3K36me3 (Camacho et al., 2018). The H3K36me3 levels were not measured in this study but the changes in the *jmjd-2* levels may be indicative of the impact of Ag-NPs on H3K36me3 levels. The trends observed for the different treatments suggest that an environmental transformation of Ag-NPs alters their toxicity and toxicity mechanisms. For $AgNO_3$ treatment, an insignificant down-regulation ($p = 0.099$) was observed after exposures in F_3 compared to the F_0 generations. No significant differences were detected among any of the $AgNO_3$ generations for *jmjd-2* expression levels as expected, based on the H3K9me3 levels, suggesting that exposure to $AgNO_3$ does not affect H3K9me3 levels.

The expression levels of H3K9 methyltransferase *set-25* are shown in Fig. 3c. No significant differences were observed in the expression levels after exposures at F_3 compared to the unexposed F_0 levels for all treatments. This suggests that the changes in H3K9me3 levels observed after exposures to Ag-NPs and sAg-NPs are primarily due to the changes observed for *jmjd-2*. Despite the significant down-regulation of *jmjd-2* in F_3 for Ag-NPs, the insignificant increase in H3K9me3 levels might be explained by the slight but insignificant down-regulation of *set-25* ($p = 0.091$) observed after exposures. Surprisingly, after rescue from exposure at F_6, there was a significant up-regulation of *set-25* for $AgNO_3$ when compared to F_0 ($p = 0.013$) and F_3 ($p = 0.008$) but this does not correlate with H3K9me3 levels. The recovery population of the Ag-NP exposed worms also showed a significant up-regulation of *set-25* when compared to F_3 ($p = 0.019$) but not F_0 ($p = 0.077$). Some redundancy in the activity of the methyltransferases might be expected and may also play a role in histone methylation at other lysine sites, as has been shown for human histone methyltransferases (Volkel and Angrand, 2007). It has been shown previously that *set-25* can mediate all three modifications at H3K9 including me1, me2 and me3(Padeken et al., 2019). Therefore, it is likely that *set-25* expression levels in F_6 in $AgNO_3$ and Ag-NP treatments is due to the effect of the exposures on the methylation levels at the other two marks. This suggests that the changes observed in the H3K9me3 levels are primarily due to *jmjd-2* expression levels as the expression pattern clearly matches the H3K9me3 changes observed for Ag-NP and sAg-NP exposures.

Though *set-26* was shown to have H3K9 methyltransferase activity *in vitro* (Greer et al., 2014), it does not have the same activity *in vivo.* However, it plays a role in the regulation of lifespan, transgenerational fertility, development, and ageing (Ni et al., 2012; Wang et al., 2018). No significant differences in terms of the gene expression levels were observed among generations for the $AgNO_3$ and Ag-NP treatments (Fig. 3d). Despite the increase observed at F_6 for controls and $AgNO_3$, there was a large amount of variation among replicates and the increases were not significant when compared to the corresponding F_0 ($p = 0.253$ and $p = 0.311$ respectively) or F_3 generations ($p = 0.263$ and $p = 0.479$ respectively). Exposure to sAg-NPs caused a significant up-regulation of *set-26* ($p = 0.022$) after exposures at F_3 compared to F_0 (Fig. 3d). After recovery from exposure (F_6), there was a significant down-regulation ($p = 0.029$) when compared to F_3 but no significant difference ($p = 0.661$) compared to the unexposed F_0. This gene expression pattern of *set-26* does not correspond with H3K9me3 levels observed for sAg-NPs suggesting that *set-26* indeed may not have H3K9 methyltransferase activity *in vivo*. Interestingly, in our previous multigenerational study by Schultz et al. (Schultz et al., 2016), sAg-NPs showed a significant decrease in lifespan and *set-26* is known to correlate negatively with lifespan (Ni et al., 2012).

4. Conclusions

The results of this study demonstrate that changes in epigenetic modifications, such as global levels of H3K4me2 and H3K9me3, are likely to play an important role in toxicity when *C. elegans* is exposed to Ag nanomaterials. These epigenetic changes can be inherited and may explain, at least in part, some of the transgenerational toxicity that has been observed (Schultz et al., 2016). The results also show that environmental transformation of Ag-NPs to sAg-NPs has an effect on the biological responses of *C. elegans*. While pristine Ag-NPs seem to cause an increase in levels of the histone methylation markers assessed, sAg-NPs have an opposite effect by decreasing the levels of the histone methylation markers. When compared to pristine Ag-NPs, $AgNO_3$ may have a different mode of toxicity despite both treatments inducing transgenerational reproductive toxicity. This study examined changes in the global levels of histone methylations and future studies of the locus-specific histone methylation patterns as well as other histone methylation marks, implicated in transgenerational inheritance are warranted to gain a deeper understanding of epigenetic memory of the reproductive stress induced by the exposure to Ag nanomaterials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

The authors gratefully acknowledge assistance of Jason Unrine, Shristi Shrestha, Stuart Lichtenberg, Tyler Bair, Jieran Li, and Zeinah El Baddar. *Caenorhabditis elegans* strains were provided by the Caenorhabditis Genetics Center, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). This work was supported by the U.S. National Science Foundation (NSF) and U.S. Environmental Protection Agency (EPA) under EF-0830093, DBI-1266252, and CBET-1530594. This work was also supported by the USDA National Institute of Food and Agriculture Multistate Project NC 1194. Any opinions, findings, conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the NSF or the EPA. This work has not been subjected to EPA review and no official endorsement should be inferred. AH was supported

by Summer Undergraduate Research in Environmental Sciences (SURES) funded by the National Institute of Environmental Health Sciences (NIEHS) R25ES027684.

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Highlights

- Multigenerational exposure to Ag-NPs induces epigenetic changes
- Changes in histone methylation differ between pristine and sulfidized Ag-NPs
- Pristine Ag-NPs increase histone methylation at H3K4me2 marker
- The histone methylation at H3K4me2 is inherited by unexposed offspring

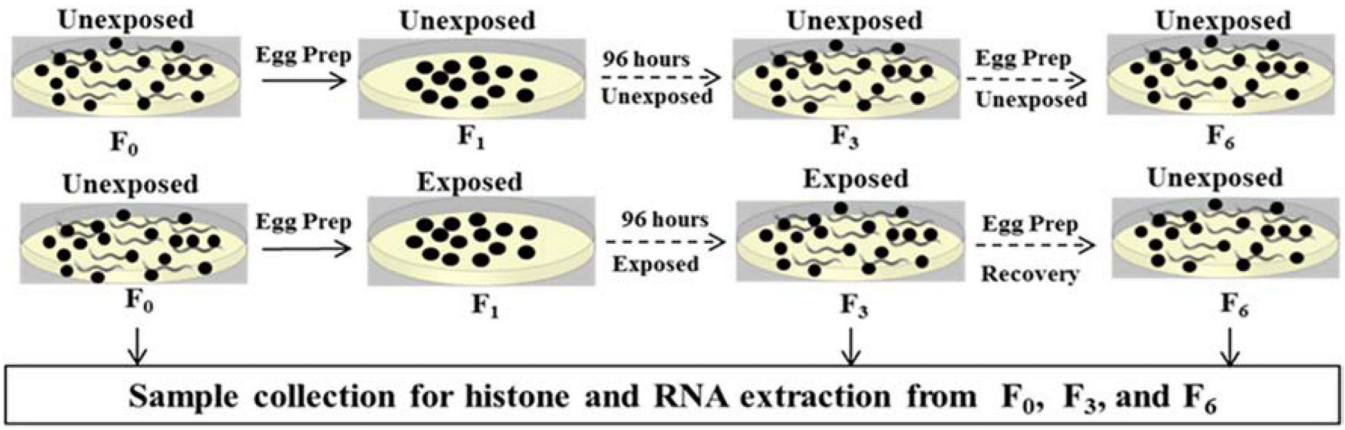

Fig. 1.

Exposure of *Caenorhabditis elegans* to $AgNO_3$, pristine Ag nanoparticles (Ag-NPs) and sulfidized Ag-NPs (sAg-NPs). Top panel illustrates multigenerational control group and bottom panel illustrate Ag exposures. Total histone and RNA were extracted from generations F_0, F_3 and F_6.

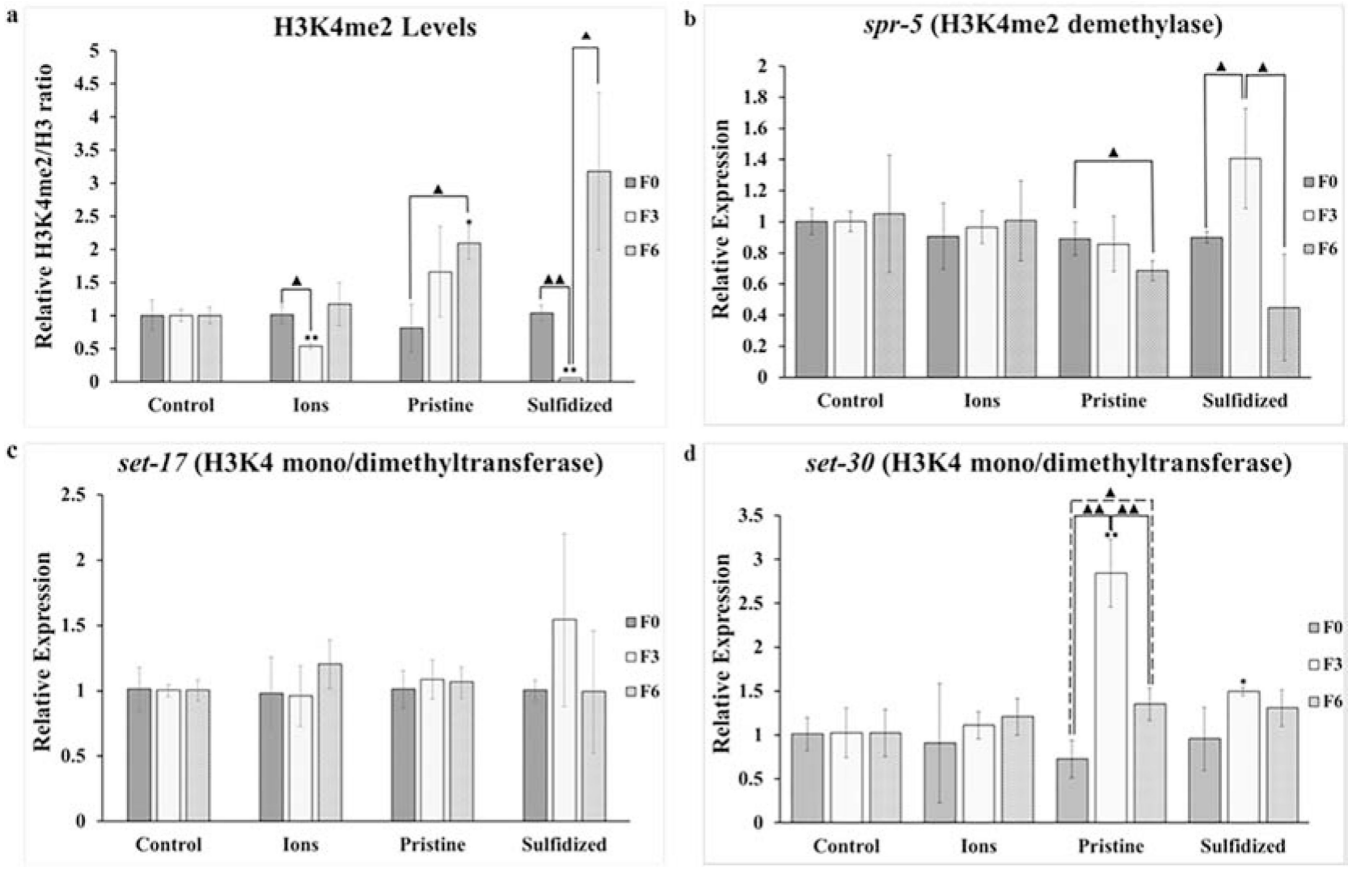

Fig. 2.

Changes in global levels of histone di-methylation at H3K4 marker (a) and levels of gene expression at *spr-5* (b), *set-17* (c), and *set-30* (d) after exposure of *Caenorhabditis elegans* to $AgNO_3$ (Ions), pristine Ag nanoparticles (Ag-NPs) and sulfidized Ag-NPs (sAg-NPs). Bars represent standard error of the means. *, ** signifies difference with control at $p \leq 0.05$ and $p \leq 0.01$ respectively.▲, ▲▲signifies difference between generations of the same treatment at $p \leq 0.05$ and $p \leq 0.01$, respectively.

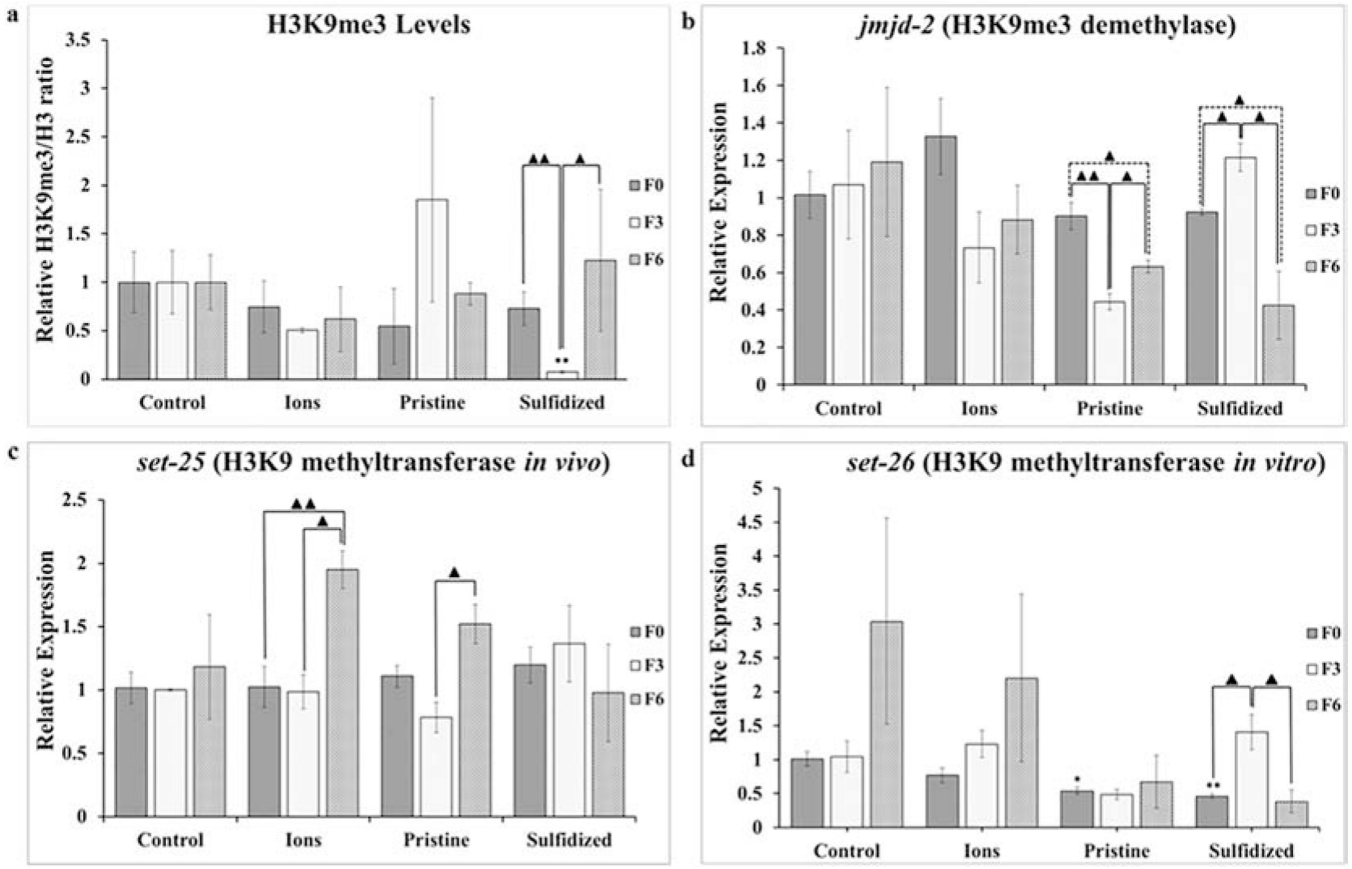

Fig. 3.

Changes in global levels of histone tri-methylation at H3K9 marker (a) and levels of gene expression at *jmjd-2* (b), *set-25* (c), and *set-26* (d) after exposure of *Caenorhabditis elegans* to $AgNO_3$ (Ions), pristine Ag-NPs (Ag-NP) and sulfidized Ag-NPs (sAg-NP). Bars represent standard error of the means. *, ** signifies difference with control at $p \leq 0.05$ and $p \leq 0.01$ respectively.▲, ▲▲signifies difference between generations of the same treatment at $p \leq 0.05$ and $p \leq 0.01$ respectively.