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Responses to silver nanoparticles and silver nitrate in a battery of biomarkers measured in coelomocytes and in target tissues of *Eisenia fetida* earthworms

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ABSTRACT

The current use and development of applications with silver nanoparticles (Ag NPs) could lead to potential inputs of these NPs to soils. Consequently, it is crucial to understand the ecotoxicological risks posed by Ag NPs in the terrestrial compartment. In the present investigation, the effects produced by PVP-PEI coated Ag NPs were assessed in *Eisenia fetida* earthworms in comparison with the soluble form ($AgNO_3$). Earthworms were exposed for 1, 3 and 14 days to a range of sublethal concentrations of Ag (0, 0.05 and 50 mg/kg) and at each exposure time, apart from mortality and weight loss of individuals, metallothionein (MT) protein concentration and catalase (CAT) activity were quantified in earthworm tissues. In addition, cellular and molecular level endpoints (cell viability, absolute and relative trophic indices and transcription levels of catalase-*cat*- and metallothionein-*mt*-) were measured in coelomocytes extruded from exposed earthworms. Despite the lack of effects in traditional endpoints (mortality and weight loss), Ag NPs and $AgNO_3$ posed changes at lower levels of biological complexity (biochemical, cellular and molecular levels). Both Ag forms induced similar changes in the metal detoxification mechanism (MT, *mt*) and in the antioxidant response system (CAT, *cat*) of *E. fetida*. In contrast, Ag form dependant cytotoxicity and subpopulation ratio alterations (eleocytes/amoebocytes) were recorded in extruded coelomocytes. Complementarily, the use of coelomocytes to assess molecular level endpoints represented a relevant alternative for development of non-invasive biomarkers.

1. Introduction

The wide range of current and potential future applications exhibited by silver nanoparticles (Ag NPs) has made them one of the most commonly used nanomaterials (Dubey et al., 2015; Vance et al., 2015). Due to these applications and to the massive disposal of sewage sludge released from Waste Water Treatment Plants (WWTP, one of the major sources of Ag NPs in biosolids), Ag NPs might have the potential to severely affect soil health (Shoults-Wilson et al., 2011; Tourinho et al., 2012). However, the potential risk of Ag NPs in soils has been poorly investigated in comparison with aquatic environments. Even if fewer studies have involved the effects of Ag NPs on terrestrial organisms, the number of studies carried out with earthworms has increased during the last five years (Diez-Ortiz et al., 2015a, 2015b; García-Velasco et al., 2016; Gomes et al., 2013, 2015; Hayashi et al., 2012; Kwak and An, 2015; Shoults-Wilson et al., 2011; Tsyusko et al., 2012).

Earthworms play an important role in terrestrial ecosystems (*e.g.* decomposition and nutrient recycling) and therefore, the study of effects exerted by Ag NPs on them is crucial to understand the potential impacts of NPs in soils. In this context, standard toxicity tests (OECD, 1984, 2004) with *Eisenia fetida* earthworm are aimed to address traditional endpoints such as survival or weight loss in order to calculate different toxicity indices (LC_x and EC_x). Furthermore, tissue, cellular or molecular level biomarkers could be also quantified in target tissues of *E. fetida* in order to assess the exposure degree or the toxic effects of pollutants. For instance, metallothioneins (MTs), low molecular weight proteins, with high cysteine content (up to 30%) that enables to bind a variety of metal atoms (Asensio et al., 2007; Brulle et al., 2006), participate in homeostasis of essential metals and in the

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detoxification of toxic trace metals (Brulle et al., 2006) and may prevent oxidative stress (Ribeiro et al., 2015). Ag NPs are known to cause oxidative stress in terrestrial invertebrates by the production of highly reactive oxygen species (ROS) that can damage cell components including DNA, proteins and membranes (Yang et al., 2011). Cells, in order to protect themselves from ROS, have developed complex defense systems including non-enzymatic scavengers and antioxidant enzymes such as catalase (CAT). A change in CAT activity is an indicator of a cellular lesion after exposure to chemicals, and thus it is considered as an early environmental stress biomarker (Asensio et al., 2013; Gomes et al., 2015).

Biomarkers can be measured in earthworm tissues or even in target cells as coelomocytes. Coelomocytes are the immune cells of earthworms and play a pivotal role in recognition and elimination of foreign materials and are involved in clotting and wound healing (Cooper, 2002; Kurek et al., 2007). Coelomocytes compose a heterogeneous cellular group that circulates in fluid-suspension in the coelomic cavity. Based on cytomorphometric, ultrastructural and cytochemical properties three cell types are distinguished: eleocytes (derived from the chloragogenous cells that surround the digestive epithelium), hyaline amoebocytes and granular amoebocytes or granulocytes (for detailed descriptions see Adamowicz (2005)). Changes in coelomocytes viability and subpopulation ratios in earthworms exposed to xenobiotics or subjected to different types of stress reflect alterations in the earthworms immune response and in the general health status (Di Marzio et al., 2005; Homa et al., 2003; Irizar et al., 2015b). Hence, these cellular parameters have been proposed as biomarkers of general stress in soil toxicity assessment (Homa et al., 2003; Irizar et al., 2015b; Olchawa et al., 2006). Regarding lower levels of biological organization, Ag NPs are known to alter the transcription of genes involved in the abovementioned pathways in *E. fetida*: oxidative stress, detoxification and immune signaling (Hayashi et al., 2013; Tsyusko et al., 2012). Transcription levels of target genes such those encoding CAT or MT have been easily measured in earthworm tissues (Asensio et al., 2007; Brulle et al., 2006; Irizar et al., 2014b). However, the utilization of immune cells (coelomocytes) to assess molecular level endpoints would represent a relevant alternative for the development of non-invasive biomarkers in more controllable and reproducible test systems than whole animals.

The aim of the present investigation was to assess the toxicity of PVP-PEI coated Ag NPs in earthworms, *E. fetida*, in comparison with the soluble form of the metal ($AgNO_3$). For this purpose, earthworms were exposed for 1, 3 and 14 days to a range of sublethal concentrations of Ag (0, 0.05 and 50 mg/kg) in the form of Ag NPs and $AgNO_3$. At each exposure time, apart from mortality and weight loss of individuals, MT protein concentration and CAT activity were quantified in earthworm tissues. In addition, cellular and molecular level endpoints (cell viability, absolute and relative trophic indices and transcription levels of *cat* and *mt* genes) were measured in coelomocytes extruded from exposed earthworms.

2. Materials and methods

2.1. Test species

Eisenia fetida earthworms (350–500 mg fresh weight) used for the experiments were healthy adults, clitellated and obtained from the stock population provided by a commercial dealer (LOMBRICOR S.C.A., Córdoba, Spain). Earthworms were maintained in the laboratory under controlled conditions of temperature (19 ± 2 °C), darkness and constant humidity. As food source medication-free horse manure was provided when required.

2.2. Test substances

Polyvinylpyrrolidone-polyethylenimine (PVP-PEI, 3.35:1) coated silver nanoparticles (NP Ag-2106W) were purchased from NANOGAP (SUB-NM-POWDER, S.A., A Coruña, Spain). Ag NPs were water dispersed (10 g Ag/L with 104 g PVP-PEI/L), 5.08 ± 2 nm average size and with a Z-potential of 18.6 ± 7.9 mV. Particle size distribution and zeta potential determinations through Dynamic Light Scattering were provided by NANOGAP CoA. High grade (> 99% purity) $AgNO_3$ was purchased from Sigma-Aldrich.

2.3. Artificial soil preparation, contamination and characterization

The OECD artificial soil was prepared following the OECD guideline 207 (OECD, 1984). The artificial soil contained 70% sand (50% of particles were between 50 and 200 μm), 20% kaolin clay and 10% sphagnum peat sieved at 2 mm. pH was adjusted to 6.0 ± 0.5 by addition of 0.01% calcium carbonate. Dry constituents were mixed, placed in glass containers and moistened to 40% of their water holding capacity (WHC, 21.91%) with suspensions of Ag NPs and solutions of $AgNO_3$ in distilled water or with distilled water in the case of the control group. Two sublethal concentrations (0.05 and 50 mg Ag/kg soil) were chosen according to previous experiments (García-Velasco et al., 2016). After spiking with the corresponding silver form, experimental soils were thoroughly mixed to ensure a homogeneous distribution of the metal. Then soils were stabilized during 3 days before adding earthworms previously acclimated (24 h) to OECD soil. Earthworms (n = 20) were exposed to unpolluted soil (control) and to soils spiked with Ag NPs or $AgNO_3$ during 1, 3 and 14 days. At the end of each Ag exposure, weight loss was assessed in earthworms and Ag quantification and pH measurements were carried out in experimental soils. The real concentration of Ag in soils was quantified following the EPA 3051A method and analyzed in Inductively Coupled Plasma Mass Spectrometry (ICP-MS, 7700-Agilent Technologies) in the Central Analysis Service (SGIker) of the University of the Basque Country following USEPA directions (USEPA, 2007). Detection limit (DL) was 0.03 mg/kg. For the measurements of the pH an adaptation of the ISO 10390: 2005 “Soil Quality – Determination of pH in water” was followed.

2.4. Concentration of MTs

MTs concentration was determined in earthworms by the spectrophotometric method described by Viarengo et al. (1997). In order to perform pools, the post-clitellar portion of 3 earthworms were weighed and homogenized in three volumes of 0.5 M sucrose and 20 mM Tris–HCl buffer (pH 8.6) containing 0.006 mM leupeptine and 0.5 mM phenylmethylsulfonilfluoride, as an antiproteolytic agents, and 0.01% β-mercaptoethanol, as a reducing agent. Homogenates were ultracentrifuged (30.000 × *g*, 20 min, 4 °C) and precipitated with ethanol/chloroform. Three pools were done per treatment and exposure time. MTs concentration was quantified by spectrophotometric titration of the sulfhydryl residues using the Ellman's reagent (5,50-dithiobis-2-nitrobenzoic acid) with reduced glutathione (GSH) as standard. Samples were centrifuged for 5 min (530 × *g*, 4 °C) and the supernatant (300 μL) was added in 96-well microplate wells. Each sample was replicated four times. Finally, absorbance was measured at 412 nm in a microplate reader Multiskan Thermo Scientific Spectrophotometer. Data were expressed as μg MTs/g earthworm wet weight (ww).

2.5. CAT activity

CAT (EC 1.11.1.6) activity was determined measuring decrease of absorbance at 240 nm due to hydrogen peroxide consumption (Claiborne, 1985). The pre-clitellar portion of 5 earthworms were weighed and homogenized in five volumes of homogenization buffer (TVBE pH 7.4) in order to obtain pools. Two pools per treatment and exposure time were used. Absorbance was measured in 96-well UV Flat Bottom microplates and using a microplate reader Multiskan Thermo