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Neurotoxicity and biochemical responses in the earthworm *Pheretima hawayana* exposed to TiO_2NPs

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ABSTRACT

Serious concerns have been expressed about potential risks of manufactured TiO_2NPs. In this research, toxicity of nanoparticulate and bulk TiO_2 were examined to the earthworm *Pheretima hawayana*. The 24-h median lethal concentration (LC_{50}) and sublethal endpoints were assessed. Both NPs and their bulk counterparts were toxic. The 24-h LC_{50} for TiO_2NPs (145.36 mg kg^{-1}) was highly toxic than that of bulk TiO_2 (357.77 mg kg^{-1}). The aim of the present work is to evaluate the suitability of *P. hawayana* and its biochemical responses to be used as a bioindicator organism and biomarkers of TiO_2 toxicity. Earthworms were exposed to three sublethal concentrations of TiO_2NPs (1, 10 and 100 μg kg^{-1}) for 28 days to test acetylcholinesterase (AChE), antioxidant enzymes (superoxide dismutase: SOD and catalase: CAT) activities and MDA content. The response of the antioxidant enzymes combined with AChE inhibition and MDA accumulation indicated that TiO_2NPs could induce significant impairments to the earthworms at the actual environment tested concentrations. The results pointed out the high sensitivity of the antioxidant and oxidative stress related responses to TiO_2NPs exposure, demonstrating their usefulness in environmental monitoring and risk assessment. The study highlights also the usefulness of earthworm *P. hawayana* as potential bioindicator species for assessing the risk of nanoparticles environmental contamination.

1. Introduction

Nanotechnology is a major scientific and economic growth area and the nanoscale chemicals and materials are increasingly used in a wide range of industries. The industrial and consumer production of nanoparticles will inevitably lead to their pervasion into all ecosystems (Pu et al., 2014; Guo et al., 2015; Tedesco et al., 2015). European Government and Council regulations concerning the Registration, Evaluation, Authorization and Restriction of Chemicals, REACH (2008) defineds nanomaterials as a natural incidental or manufactured material containing particles in an unbound state or as an aggregate. Others have defined NPs as particulate matter with at least one dimension less than 100 nm (Riu et al., 2006; Magaye et al., 2012). Nanoparticles have unique physicochemical properties such as tiny size, large surface area, reactivity, charge, shape and media interaction. One of these nanoparticles, TiO_2NPs have been widely used in sun care (Serpone et al., 2007) as well as on self-cleaning coating (Cai et al., 2006) due to their high stability, anticorrosive and strong catalytic activity (Shi et al., 2013). Certain novel properties of NPs lead to adverse biological effects and tend to create toxicity (Oberdörster et al., 2005; Nel et al., 2006). The rapid increase in production and use of NPs has raised concerns about their potential to cause harm to human and non-target organisms hence, several studies have been conducting to assess its possible harmful effects (Zhu et al., 2006). NPs may enter the human body through the lungs (respiratory tract), skin (dermal penetration), intravenous injection and ingestion. There has been great argument about whether or not NPs can cross the blood–brain barrier (Begley, 1996). However nanosizing allows particulates to interact, disrupt and penetrate the neuronal cell membranes into the brain (Sharma and Sharma, 2010).

Several studies have shown that nanoparticles stress in living organisms often results in the production of reactive oxygen species (ROS), including free radicals, hydrogen peroxide, and singlet oxygen. Exposure to TiO_2NPs may lead to oxidative stress (Jemec et al., 2008; Su et al., 2014); physiological alterations (Ratnasekhar et al., 2015; Srpčič et al., 2015) and lipid peroxidation (Su et al., 2014). Many endogenous antioxidant enzymes, such as SOD and CAT, will scavenge excess ROS so as to alleviate their deleterious effects of oxidative stress and lipid peroxidation. Malondialdehyde (MDA) is a final product of lipid peroxidation, which is often used as an indicator of oxidative damage. Therefore, changes in ROS, MDA levels and enzymatic activities may indirectly indicate the toxic effects of contaminants on living organisms (Li and Huang, 2014).

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Reactive oxygen species (ROS) induced by functionality on the surface of nanoparticles plays an important role in the common mechanism of their toxicity (Oberdörster et al., 2005; Nel et al., 2006) and triggers inflammation in the organs in which nanoparticles are deposited. ROS are reportedly associated with neurodegenerative disorders such as Parkinson's disease (Matės et al., 1999). AChE is the enzyme that degrades the neurotransmitter acetylcholine in cholinergic synapses of the nervous system (Čolović et al., 2013). AChE activity in soil invertebrates may be indicative of exposure to TiO_2NPs, therefore, may provide an early warning for potential adverse effects that may occur later in population and community level. AChE activity has been characterized biochemically in various macroinvertebrates (Rickwood and Galloway, 2004; Rault et al., 2007; Essawy et al., 2009).

Gottschalk et al. (2009) modeled environmental concentration of engineered nanomaterials of TiO_2 for different regions. They suggested that typical TiO_2NPs concentration in Europe are 1.28 μg kg^{-1} in soil, 89.2 μg kg^{-1} in sludge treated soil, whereas values in the USA are approximately half of those mentioned above. Biochemical responses versus environmental stress are sensitive, informative, and reproducible in living organisms. Enzyme activity responses of organisms to environmental contaminants are regarded as established early warnings indices of pollution in the environment.

The traditional sentinel species for soil toxicity testing is the earthworm which may represent 60–80% of the total soil biomass (Bouche, 1992). They are important organisms for soil formation and organic matter breakdown in most terrestrial environments. Earthworms are root feeders and ingest massive volumes of soil to extract decaying organic matter. There is therefore potential for large scale exposure of earthworms to any nanoparticles in the soil. New practical techniques and biological approaches to soil monitoring, such as the measurement of biochemical and cellular responses to pollutants on organisms living in the soil, have become a mandatory requirement for the assessment of the quality of this environmental compartment (Kammenga et al., 2000).

Pheretima hawayana is a common earthworm in Egypt. As anecic earthworms, they could create permanent, vertical burrow down to a three m depth while endogeic and epigeic earthworms burrow horizontally. Also, they collect their food from the letter layer at the soil surface, so their burrows connect soil surface with deep soil layers (Palm et al., 2013).

Up to now, through the literature retrieval there were no studies on neurotoxicity and antioxidant defense system exposed to TiO_2NPs using *P. hawaiana* as a model sentinel species. The aim of this study was to better understand the effect of TiO_2NPs on *P. hawayana* in order to provide information on their subchronic toxic action in soil ecosystem. The changes of enzymatic activities; AChE, SOD and CAT as well as energy reserve levels (glycogen, lipid and protein) in the *P. hawayana* tissues after sublethal exposure to TiO_2NPs were detected. MDA content was also measured in earthworm tissue to determine whether lipid peroxidation (LPO) was engendered by TiO_2NPs.

2. Materials and methods

2.1. Chemicals

Titanium dioxide (TiO_2) nanoparticles (Product No. 718467-100G and APS ≥ 21 nm) purchased from Sigma-Aldrish, Egypt. NP diameter ranged from 20 to 40 nm. Bulk TiO_2 (200 nm) and other chemicals were of highest purity and were purchased from Al Gomhoria Pharm. Ind., Cairo, Egypt.

2.2. Test soil

To better reflect exposure in the fields, non-contaminated natural soil was used to maintain the earthworms in the laboratory. Test soil was a loamy, medium-acidic and lightly humic sand. The soil was characterized for (pH, 6.2), organic matter content (OM, 2.7%), water holding capacity (WHC, ml H_2O kg^{-1} dry soil), sand (66%), silt (20%), and clay (14%), following the methods of Allen (1989). Prior to the experiment, the soil was oven dried at 80 °C overnight to eliminate undesired soil fauna.

2.3. Earthworms sampling

Adult earthworms of *P. hawayana* (1000 ± 150 mg) were manually collected from untreated nursery plants topsoil at a depth of 0–20 cm near Belbeis city (30° 25′N, 31° 34′E), Sharkia Governorate, Egypt during spring and autumn seasons 2013. Earthworms were transferred in plastic bags to the laboratory and maintained under laboratory conditions in plastic boxes (30x30 × 15 cm) with 5 kg moistened native soil/box. The animals were fed abundantly with cow-dung, dried at 90 °C and ground to pass 2 mm sieve and then rewetted prior to addition as food, from healthy animals. Earthworms were supplied with a 12 h:12 h light/dark photoperiod cycle at room temperature (22 ± 2 °C).

2.4. Acute toxicity assay

The earthworms were acclimatized to the test soil for 7 days prior to testing.

Preliminary experiments were carried out to establish the effective range of TiO_2NPs and bulk TiO_2. Concentrations of the test compound used in short-term definitive tests were between the highest concentration at which there was 0% mortality and the lowest concentration at which there was 100% mortality. Appropriate amounts of TiO_2NPs and bulk TiO_2 were thoroughly suspended (Waalewijn-Kool, et al., 2012) in distilled water and rapidly spiked into air-dried sterilized soil, achieving the following nominal concentrations, 0 (control), 10, 50, 100, 200 and 400 mg kg^{-1} dry soil. The experiment was conducted in precleaned 1 L plastic boxes containing 500 g dry soil and 100 ml of test solution with specific contaminant concentration and was kept at 50% moisture content. Test solutions were added dropwise to the soil samples and stored in the dark overnight to reach equilibrium before being homogenized. Fifteen earthworms were added to each box and each treatment had triplicates. All exposures were conducted at room temperature (± 22 °C) and a 12 h:12 h light/dark photoperiod cycle. Ten gram air-dried, ground cow-dung were added to a hole in the soil surface of each replicate. After 24 h, the surviving worms were counted. Death was determined as a lack of response to gentle probing with a forceps. LC_{50} of the test TiO_2 (nanoparticles and bulk) was calculated from the data obtained in acute toxicity bioassays following the probit analysis method as described by Finney (1971). The probit analysis was carried out using Statview v.5 (SAS software Inc., USA). During bioassay, the mortality in treated groups was corrected by using the formula of Abbott (1987) for control mortality. Abbott's formula: Corrected % mortality = (% alive control − % alive treated) × 100%/(% alive control).

2.5. Sublethal toxicity assay

In a separate set of experiments, relevant sublethal concentrations (10, 50 and 100 μg kg^{-1} dry soil) of TiO_2NPs were applied to the earthworms for 28 d as in the same procedures mention before to study possible biochemical changes in *P. hawayana*. The earthworms were divided into four groups, with 40