Ecotoxicology and Environmental Safety 148 (2018) 620–631

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Immunotoxicity of copper nanoparticle and copper sulfate in a common Indian earthworm

Arunodaya Gautam[a], Abhishek Ray[a], Soumalya Mukherjee[a], Santanu Das[a], Kunal Pal[b], Subhadeep Das[c], Parimal Karmakar[b], Mitali Ray[a], Sajal Ray[a,*]

[a] Aquatic Toxicology Laboratory, Department of Zoology, University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, West Bengal, India
[b] Nanobiotechnology Laboratory, Department of Life Science and Biotechnology, Jadavpur University, 188 Raja Subodh Chandra Mallick Road, Kolkata 700032, West Bengal, India
[c] Proteomics Laboratory, Department of Botany, University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, West Bengal, India

ARTICLE INFO

Keywords:
Copper nanoparticles
Copper sulfate
Metaphire posthuma
Nitric oxide
Phenoloxidase
Superoxide anion

ABSTRACT

Copper oxide nanoparticles and copper sulfate are established contaminants of water and soil. *Metaphire posthuma* is a common variety of earthworm distributed in moist soil of Indian subcontinent. Comparative toxicity of copper nanoparticles and copper sulfate were investigated with reference to selected immune associated parameters of earthworm. Total count, phagocytic response, generation of cytotoxic molecules (superoxide anion, nitric oxide), activities of enzymes like phenoloxidase, superoxide dismutase, catalase, acid phosphatase, alkaline phosphatase and total protein of coelomocytes were estimated under the exposures of 100, 500, 1000 mg of copper oxide nanoparticles and copper sulfate per kg of soil for 7 and 14 d. A significant decrease in the total coelomocyte count were recorded with maximum depletion as 15.45 ± 2.2 and 12.5 ± 2 × 10^4 cells/ml under the treatment of 1000 mg/kg of copper nanoparticles and copper sulfate for 14 d respectively. A significant decrease in generation of nitric oxide and activity of phenoloxidase were recorded upon exposure of both toxins for 7 and 14 d indicating possible decline in cytotoxic status of the organism. A maximum inhibition of superoxide dismutase activity was recorded as 0.083 ± 0.0039 and 0.055 ± 0.0057 unit/mg protein/minute against 1000 mg/kg of copper nanoparticles and copper sulfate treatment for 14 d respectively. Activities of catalase and alkaline phosphatase were inhibited by all experimental concentrations of both toxins in the coelomocytes of earthworm. These toxins were recorded to be modifiers of the major immune associated parameters of *M. posthuma.* Unrestricted contamination of soil by sulfate and oxide nanoparticles of copper may lead to an undesirable shift in the innate immunological status of earthworm leading to a condition of immune compromisation and shrinkage in population density of this species in its natural habitat. This article is the first time report of immunological toxicity of nanoparticles and sulfate salt of copper in *M.posthuma* inhabiting the soil of India, an agriculture based country.

1. Introduction

Earthworm is considered as a candidate species for monitoring the toxicity of environmental xenobiotics and evaluation of chemical toxicity in soil (Brulle et al., 2006). They serve as an important component of food chain and diet for many organisms. Due to their edaphic mode of habitat, they are in intimate contact with the aquatic and solid phases of soil and can accumulate environmental pollutants within their body (Panzarino et al., 2016). Thus, it is apprehended that this organism bears the risk of physiological toxicity by diverse xenobiotics including different nanoparticles and sulfate salt of copper distributed in soil. Earthworms process a large volume of soil, therefore, are prone to toxic exposure of environmental pollutants than any other terrestrial invertebrates. They act as an influencing agent of pH, texture of soil and an enhancer of soil fertility and porosity.

Nanoparticles, by definition, are substances in which the diameter does not exceed beyond 100 nm. Different types of soil contaminants like nanoparticles and sulfate of copper generated from industrial, agricultural and pharmacological industries contaminate the terrestrial and aquatic ecosystems. In comparison to the aquatic environment, the

* Corresponding author.
E-mail addresses: ashiskumargautam3@gmail.com (A. Gautam), abhishekzooa05@gmail.com (A. Ray), mukherjee.soumalya259@gmail.com (S. Mukherjee), santanuzoo.snail@gmail.com (S. Das), kunalpal685@gmail.com (K. Pal), subhabiology@gmail.com (S. Das), pkarmakar_28@yahoo.co.in (P. Karmakar), mitalirayin@yahoo.co.in (M. Ray), raysnailmail@rediffmail.com (S. Ray).

https://doi.org/10.1016/j.ecoenv.2017.11.008
Received 21 May 2017; Received in revised form 31 October 2017; Accepted 3 November 2017

toxicological effects of nanotoxin in soil are less investigated (Garciá-Velasco et al., 2016). In recent years, studies on nanotoxin have gained a special scientific attention of the immunotoxicologists due to their widespread distribution on the environment and their ability to modulate the immune parameters of the organisms. Toxicity of copper nanoparticle in earthworm is in report (Unrine et al., 2010). However, the report of immunotoxicity of copper oxide nanoparticles in *Metaphire posthuma* (Annelida: Clitellata: Oligochaeta) is absent in the current scientific literature. Copper sulfate was used as a biocide previously. Due to its high solubility in water, it can easily contaminate the environment. It is also used as algicide to control the growth of phytoplanktons in the aquatic ecosystem. The adverse effect of this metallic salt on fish is in report (Karan et al., 1998). Present investigation is aimed to assess the toxicities of copper oxide nanoparticles and copper sulfate on the selected immunotoxicological parameters of *M. posthuma* from a comparative viewpoint.

The coelomic cavity of earthworm is filled with fluid containing mobile coelomocytes which are functionally involved in different aspects of cellular and humoral immunity like phagocytosis, generation of cytotoxic molecules and secretion of antimicrobial molecules for the purposes of killing and deactivation of environmental pathogens and parasites (Cholewa et al., 2006). Total and differential coelomocyte counts are considered as sensitive biomarkers of toxicity of environmental pollution in earthworm (Cooper, 1996). According to the author these parameters act as physiological marker of health of earthworm inhabiting the polluted environment. Phagocytosis in annelids has been claimed as a multiphase immune reaction and is involved in the process of self-nonself discrimination (Adamowicz and Wojtaszek, 2001). Oxygen dependent respiratory burst activity resulted in generation of superoxide anion, a potential cytotoxic agent which is generated within the immunocytes for the killing and neutralisation of pathogenic microorganisms and toxins. Nitric oxide, another important cytotoxic agent and a signaling molecule, is generated as a reactive nitrogen intermediate during the conversion of L-arginine to L-citrulline by nitric oxide synthase. Coelomic fluid of earthworm contains enzymes such as antimicrobial proteases that are involved in the process of prophenoloxidase cascade activation which requires the involvement of generation of reactive oxygen species (ROS) (Kauschke et al., 2007). Active phenoloxidase is a key enzyme for melanisation. It catalyses the oxidation of phenols into quinones, which subsequently polymerise to melanin- deposited as brown bodies for the purposes of pathogen destruction (Valembois et al., 1994).

Antioxidant enzymes like superoxide dismutase and catalase are primary defense molecules that protect the biological system from oxidative damage (Roubalová et al., 2015). Superoxide dismutase biochemically transforms superoxide anion into hydrogen peroxide and water and neutralizes the cellular oxidative stress. Catalase, another important antioxidant enzyme, enzymatically transforms hydrogen peroxide into water and thus protects the cell from hydrogen peroxide mediated toxicity. Acid phosphatase, a lysosomal hydrolytic enzyme, is considered as a potential biomarker of environmental toxicity. Stein and Cooper (1978) reported the existence of acid phosphatase activity in chloragosomes and lysosomes in earthworm, *Lumbricus terrestris*. According to Englemann et al. (2004), elimination of pathogenic microorganism in earthworm is facilitated by the combined effects of cell mediated phagocytic response and activation of humoral factors such as induction of activity of acid phosphatase. Involvement of the activity of alkaline phosphatase in the digestion and humification of soil organic matter in earthworm is in report (Prabha et al., 2007).

In this present investigation, total coelomocyte count, phagocytic response, generation of superoxide anion, nitric oxide and activities of phenoloxidase, superoxide dismutase, catalase, acid and alkaline phosphatases and total protein content were estimated in the coelomocytes of *M. posthuma* under the sublethal concentrations of copper oxide nanoparticles and copper sulfate. The current analyses would provide important ecotoxicological information regarding the comparative toxicity of copper oxide nanoparticles and copper sulfate in *M. posthuma*. Moreover, the selected immune associated parameters of *M. posthuma* might bear the potential to act as biomarkers of contamination of soil by copper oxide nanoparticle and copper sulfate.

2. Materials and methods

2.1. Physical characterization of copper nanoparticle

The copper oxide (II) nanoparticles (CAS number: 1317-38-0) were purchased from Sisco Research Laboratories Private Limited, India. The hydrodynamic particle diameter and distribution of copper oxide nanoparticle was determined by dynamic light scattering using a Malvern Zetasizer (NanoZS90, Malvern Instruments Limited, Worcestsershire, U.K.). The nanoparticle solution was prepared by dispersing 2 mg of copper nanoparticle in 1 ml of millipore water prior to sonication for 30 min. Surface charge of the particle was measured by Malvern Zetasizer (NanoZS90, Malvern Instruments Limited, Worcestsershire, U.K.). The physical nature of copper oxide nanoparticle, in powdered conditions, was measured by Expert Pro (Phillips) X ray diffractometer using CoKα radiation (α = 0.178897 nm). Samples were scanned from 20° to 80° of 2θ increment at 0.04° with 2 s counting time (Laha et al., 2014).

The size and structure of the nanoparticle were studied by high resolution transmission electron microscope JEOL JEM 2100 HR with EELS, Japan operating at 200 keV. 0.5 mg dry nanopowder was dispersed in 1 ml of deionised water and sonicated in room temperature for 10 min at 30 W. Prior to sonication the sample was prepared in carbon coated copper grids and air dried before TEM analysis. The particle topology was determined by high resolution field emission scanning electron microscope JEOL JSM-7600F, Japan operating at 30 keV (Pramanik et al., 2015).

2.2. Collection, transportation and laboratory acclimation of experimental earthworm

Adult healthy specimens of *M. posthuma* were manually collected from the selected fields (22° 30′ N, 88° 22′ E) located in the district of Kolkata of the state of West Bengal, India. Well clitellated earthworms having a length of about 6 cm and weighing about 250–300 mg were collected by digging the topsoil (up to 20 cm) followed by sorting. The field stations from where the earthworms were collected did not exhibit any history of industrial or agricultural contamination. The specimens were transported to the laboratory within 1 h of collection with adequate volume of soil and transferred to the wide mouth glass containers (1 L) supplied with the soil collected from the field stations. Experimental earthworms with uniform length were acclimated in the controlled laboratory condition in natural soil for 48 h. A uniform dark light cycle with a ratio of 12:12 h was maintained throughout the period of acclimation and experiment. To keep the experimental soil moist, water was sprinkled at a regular basis and dry non contaminated dairy manure was added as food. The experiment on earthworm species was designed according to the guideline and institutional norms of animal ethics and handling of the department of Zoology of the University of Calcutta.

2.3. Test soil

Experiment was carried out in accordance to the Organization for Economic Co-operation and Development (OECD) test guideline 222, where the use of natural soil was practiced. After collection from the field, the plant roots and large lumps were manually discarded from the soil and the soil samples were spread on a rectangular glass tray for drying. Sufficient amount of water was added to bring the soil sample to 45% of water holding capacity and kept in dark at 25 °C for 1 week before adding toxicants. The physical parameters of the experimental