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EFFECT OF NANOPARTICLES ON MITOCHONDRIAL ACTIVITY OF COELOMIC CELLS OF *EISENIA FOETIDA*

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ABSTRACT

The mitochondrial activity controls life and death of cells. Dysfunction of mitochondria causes wide range of human diseases. Nanotechnology is rapidly expanding field of science with development of various nanaomaterials. In parallel to technological benefits from the impressive development of nanotechnologies, the arrival in the market of nano-products raises crucial issues dealing with human/environmental risk assessment and potential associated contamination. However, particles of nano-sized range have been present on earth for millions of year and have been used by mankind for thousands of years. But, recently their production and application has been increased because of our increasing ability to synthesize and manipulate their property. It is known that nanoparticles(NPs) able to enter in mitochondria and produce physical damage, contributing reactive oxygen species(ROS) and thereby modulate intracellular calcium concentrations, activate transcription factor etc. Our current understanding of the potential impact of nanomaterials and their potentiality to remediate/nullify their toxic forms by naturally available scavenger is limited. Nature has its own ways of resolving imbalances in the environment and organisms are one of the best tools of nature to eliminate toxic pollutants. The aim of present study is to analyse the effect of nanoparticles on mitochondrial activity of coelomic cells of earthworms to translate or improve bioremediation process.

INTRODUCTION

Nanotechnology is rapidly expanding field of science with development of various nano-products. Their applications have gained wide attention because of their novel properties including large surface area and high reaction activity. They are extensively used in various products *viz.* electronics, consumer products, optics, alternative energy, soil and water remediation, diagnostics and drug delivery devices. This technology has been increased globally due to their wide potential of application. The huge increase in manufacture and application raises concerns about their toxic effects and health implications of NPs (Oberdoster *et al*., 2005; Kreyling *et al*., 2006; Nel *et al*., 2006). Nanoparticles may be released from various products through normal use and then enter into wastewater stream. A major portion of these nanoparticles may release into sewage sludge those are disposed of in landfills, incinerated or applied to agriculture lands. Thus, soil system is an alternative sink for large portion of nanoparticles (Gottschalk *et al*., 2009). NPs may affect soil ecosystem *via:* 1) direct effect; 2) changes in the bioavailability of toxins or nutrients; 3) indirect effects resulting from their interaction with natural organic compounds; 4) interaction with toxic organic compounds which may amplify or alleviate their toxicity (Simonet and Valcarecel, 2009). Earthworms are considered as bio-indicators of soil health. As different species of earthworms (epigeic, endogeic, anecic) occupies the different ecological niches of soil ecosystem, thus all are not submitted to same response on exposure of NPs.

Nanoparticles can take several routes to enter into soil organism and their response depends on various factors like cellular uptake, receptors and degree of absorbance, aggregation and cellular interaction. However, specific response to an organism at cellular level may be in terms of effect of biotic uptake. The internalization of NPs depends on their size, shape and surface charge that interacts with the organism or the as well as type of organism. Their responses depend on cellular endocytosis (depend on acceptor activation) or direct membrane penetration (as reported in nanotubes and fullerenes) and extracellular medium (protein and lipid adsorption pattern). The researchers reported that nanoparticles with positively charged surfaces had higher cellular and

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mitochondrial uptake as the surface charge increased, with maximum cellular uptake at 34mV and mitochondrial uptake at 34mV (Bass *et al*., 2012). The present study is focused on behaviour of nanoparticles on mitochondrial activity of coelomic cells of earthworms.

MATERIALS AND METHODS

Collection of earthworms

Eisenia foetida were collected from vermibed Sagar, India (Latitude $23^0 50' 2''$N; Longitude $78^0 47' 1''$E; 550m elevation) during August - September, 2015. For identification, collected specimens were preserved in ethyl alcohol for molecular characterization, and also fixed in 4% formalin for morpho-anatomical study. Coelomic cells were extracted from live earthworms and sub-cultured in CO_2 incubator.

Isolation of Coelomocytes

Collected worms were thoroughly washed in running tap water before rinsing in distilled water and were not subjected to any control condition. Worms were placed on wet cotton to ensure complete defecation in order to avoid contamination during harvesting of coelomocytes. After 2-3 hrs, worms were wiped with cotton wool soaked with 70 % ethyl alcohol to avoid any further contamination. The surface cleaned worms were placed alternately in sterile petridish containing cold extrusion buffer (NaCl 71.2mM; Ethanol 5%; Guaicol-glycerol-ether 50.4mM; EGTA 5mM, pH 7.3) and distilled water at interval of one minute for 8-10 times. Coelomic fluid extruded out through dorsal pores due to external stress condition. After collection of coelomic fluid in cold extrusion buffer, worms were released in soil.

Culturing of Coelomocytes

The excreted coelomic fluid was pipette into tubes filled with LBSS solution (NaCl 71.5mM; KCl 4.8mM; $MgSo_4.7H_2O$ 1.1mM; KH_2PO_4 0.4mM, pH 7.3) and centrifuged at 4°C for 5 min. Loose pellets of coelomocytes were washed 2-3 times with cold LBSS solution. Cell count was maintained 10^7/ml with trypan blue exclusion. The isolated coelomocytes were loaded in petridish with DMEM supplemented with 10% FBS and incubated for 3 days in CO_2 incubator.

Cell Viability

Viability of cells was recorded at the time of isolation and after incubation for three days using haemocytometer and was examined in phase contrast/fluorescence microscope.

Exposure of nanoparticles on earthworms

Coelomic cells were seeded in 96-well plate at 5x 10^5 cells/ml and treated with 35, nm Zn0 nanoparticles at dose(mg/l) of 0.0(T1),2.0(T2),3.0(T3), 5.0(T4), 8.0(T5) for 48 hrs.

Flow Cytometeric assessment of coelomic cells

The uptakes of nanoparticles by coelomic cells were examined using flow Cytometeric method (Kumar *et al*., 2011). 50µl treated and control culture was added into 950µl PBS and than analyzed with flow cytometer (FACS Canto II BD Biosciences, San Jose, CA) using FACS Diva 6.1.2 software (BD Biosciences). In the dot plots, X-axis reflects the FSC intensity in logarithmic scale, and Y-axis corresponds to the SSC intensity in linear scale. The gating of the data was based on SSC and FSC of the control cells and control cells, respectively. This allowed us to differentiate the cells in which internalization of nanoparticles occurred from those where there was either no internalization or there was internalization along with adsorption. Dead cell discrimination of the treated cells was carried out according to the protocol described by Jung *et al*. (2015) using propidium iodide dye.

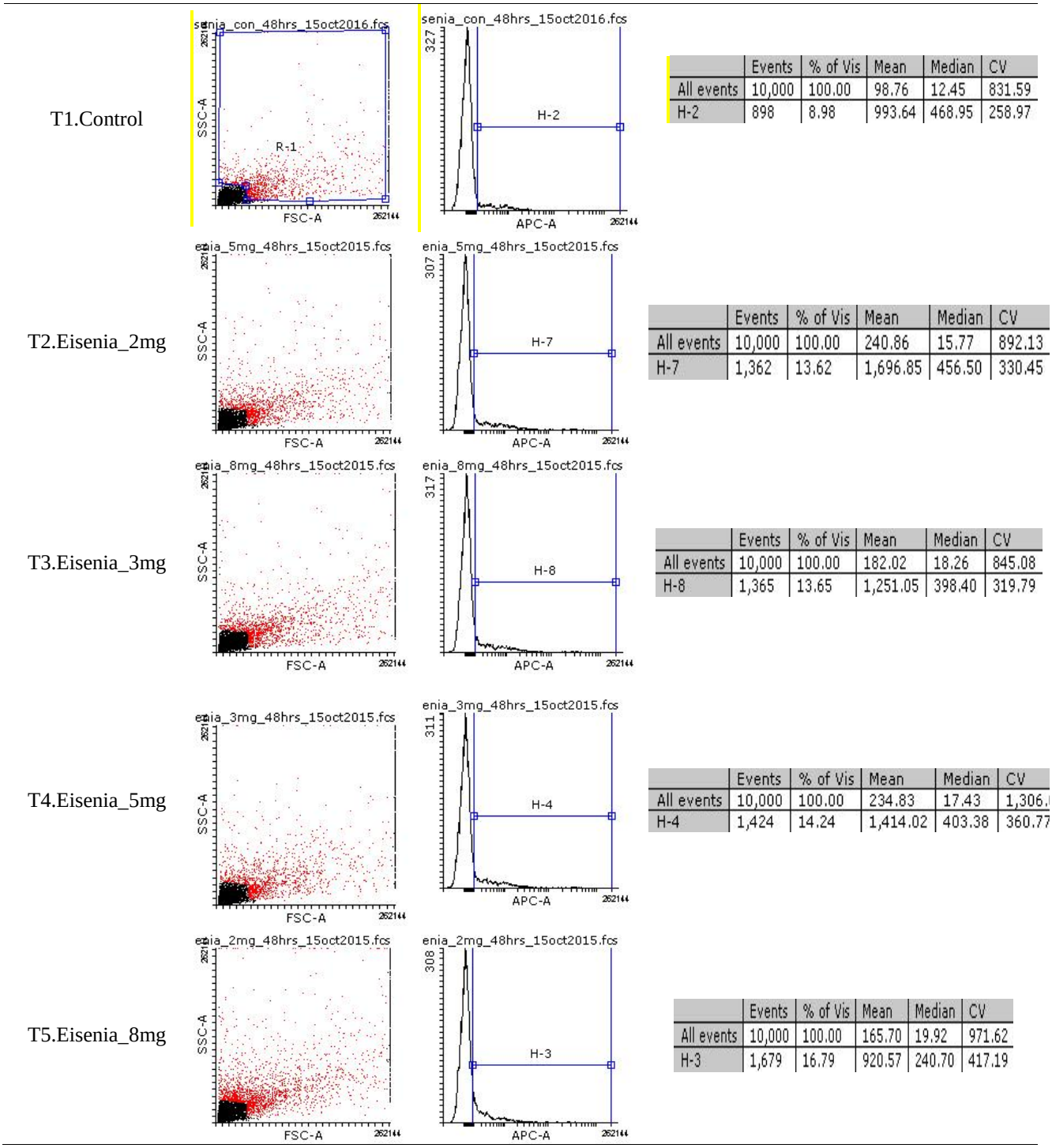
Examination of nanoparticles in coelomic cells

After exposure to nanoparticles coelomic cells were fixed in 2.5 % gluteraldehyde and made into embedded sections following routine techniques for Transmission Electron Microscopy (TEM) characterization.

RESULTS AND DISCUSSION

After exposure of NPs coelomic cells were examined at 48 hrs using flow cytometry and electron microscopy. Study demonstrated that cells after exposure of ZnO-NP exhibited an increase in intensity of the side scatter (SSC)) which was more pronounced with T4@8mg/l (16.79% increase) rather than T2, T3, T4 (13.62,13.65,16.79% increase respectively) when compared with the control (Fig. 1). The dose-dependent increase in SSC intensity of treated cells can be attributed to increase internalization of ZnO-NPs however maximum uptake of NPs reached upto only16.79% @ 8.0mg/l. At exposure of 2-5mg/l uptake of NPs not varied at large. Examination of coelomic cells under electron microscope revealed large agglomeration of NPs in coelomic fluid (Fig 2) in all treatments. The bioavailability of NPs was recorded very high throughout inter and intracellular spaces of coelomic cells in all exposures. However, there was no clear dose dependence for accumulation on a mass concentration basis, although on a particle number basis many more micro-size was present in intracellular spaces (Fig2C).

The accumulated pattern was nearly identical in all exposure (Fig 2B-E). This may be similar to observation of Unrine *et al*. (2010) for aggregation of Au nanoparticles at higher concentration which were more extensively aggregated in the soil pure water than 55nm particles and had a larger average hydrodynamic diameter. In all exposures no abnormal appearance of mitochondria was observed even distinct agglomerates of NPs observed in mitochondria (Fig 2). Coelomic cells of earthworms internalize NPs may be either by endocytosis or diffusion in present study. However, uptake of opsonized particulate substances and small solute volumes may internalized by mechanism of phygocytosis and, particles in large amounts of solute may taken up by pinocytosis (either macropinocytosis or receptor–mediated). As Kettler *et al*., (2014) suggested four mechanism of internalization of NPs by cells: 1) *macropinacocytosis*(non specific mechanism by which fluid contents are taken up in the same concentration as in the surrounding medium; 2) *clathrin-mediated endocytosis* (receptor-mediated endocytosis (RME) ; 3) *caveolae-mediated endocytosis* (uptake of RME vesicles); 4) *clathrin and caveolae* (independent endocytosis).



	Events	% of Vis	Mean	Median	CV
All events	10,000	100.00	98.76	12.45	831.59
H-2	898	8.98	993.64	468.95	258.97

	Events	% of Vis	Mean	Median	CV
All events	10,000	100.00	240.86	15.77	892.13
H-7	1,362	13.62	1,696.85	456.50	330.45

	Events	% of Vis	Mean	Median	CV
All events	10,000	100.00	182.02	18.26	845.08
H-8	1,365	13.65	1,251.05	398.40	319.79

	Events	% of Vis	Mean	Median	CV
All events	10,000	100.00	234.83	17.43	1,306.
H-4	1,424	14.24	1,414.02	403.38	360.77

	Events	% of Vis	Mean	Median	CV
All events	10,000	100.00	165.70	19.92	971.62
H-3	1,679	16.79	920.57	240.70	417.19

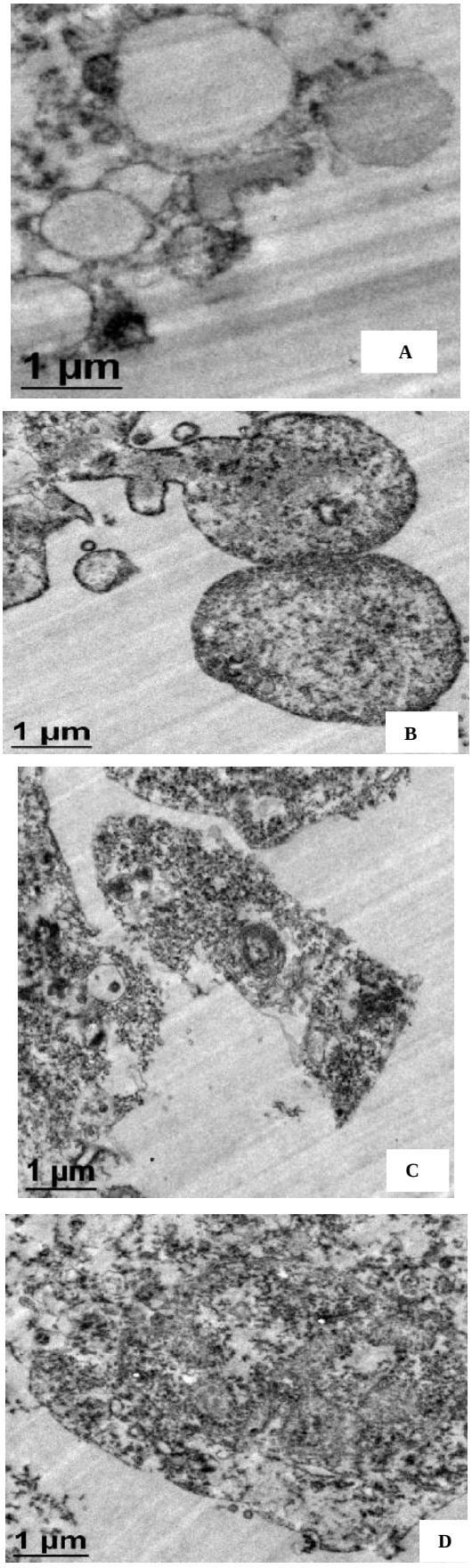
Figure 1 Scatter analysis of NPs uptake in coelomic cells of *Eisenia fetida* after exposure of 48hrs,(T1) Control;(T2) 2mg/l;(T3) 3mg/l;(T4) 5mg/l;(T5) 8mg/l

However, Internalization of NPs in cells is influenced by a large number of physical and chemical properties of the specific NP (like shape, surface charge, surface functional groups and NP hydrophilicity) and also the circumstances in the exposure medium of the cells.

Once NPs have entered in biological system of earthworms, they inevitable come into contact with huge variety of biomolecules including proteins, sugars and lipids that are dissolved in body fluids.

These biomolecules immediately coat the NP surface and form the so called 'protein corona' (Shang *et al.*, 2014) which determines biological identity of NPs (Lynch *et al.*, 2009). Now they selectively transported into and out of cells *via* endocytosis and exocytosis. In this process, an invagination forms in the cell membrane that is finally pinched off so as to generate a vesicle in the cytoplasm that contains the internalized material. These inward budding vesicles contain receptor protein that recognizes specific chemical groups on the molecules to be internalized. Thus, if protein absorbed to an NP

trigger cell surfaces receptors, they readily activated the cells uptake machinery.



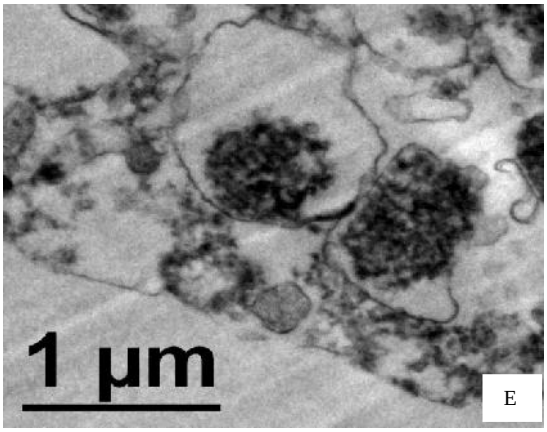


Fig 2 Transmission electron microscope image of coelomic cells of *Eisenia foetida* after exposure of ZnO NP(48 hrs).A. Control,B, 2.0 mg/l; C, 3.0 mg/l; D, 5.0 mg/l; E, 8.0 mg/l showing aggregates of NPs and structure of mitochondria

It is known that major site of ROS (reactive oxygen species) production in cell is mitochondria. The generation of ROS affect mitochondrial membrane potential and apoptosis. The mitochondrial membrane depolarization or free radical attack of membrane phospholipid may be resulted by ROS. It is reported in mammalian cells that deposition of NPs in the mitochondria can alter its normal functioning by disrupting the electron transport chain. There may be chances of elevated ROS level may also lead to activation of cellular stress dependent signalling pathways resulting in mitochondrial stress in present study. That may also interact with membrane-bound cellular receptors like growth factor receptors and integrins. However, uptake efficiency, internalization pathway selection, intracellular localization, cyto-toxicity of NPs may be affected by size of NPs. Van der Ploeg *et al*., (2013) reported reduction in growth and development, damaged cuticle with underlying pathologies of epidermis, muscles and gut barrier on exposure of fullerene NPs (C60) to *Lumbricus rubellus.* However, *Eisenia fetida* shown neither response of antioxidant enzyme expression or activity nor acute toxicity in C60 spiked soil (Li *et al*., 2011). In our earlier studies effect of ZnO-NPs on *E. fetida* were recorded in terms of reproductive behavior, antioxidant enzyme activities and accumulation of Zn^{++} remote from portal of entry (Gupta *et al*., 2014).

There may be chances primary NPs tend aggregate into cluster up to several microns in size in coelomic cells. These aggregates may be of homo (NP –NP attachment) and hetero-aggregates (dissimilar particles). Homo-aggregation may be due to fractal dimensions and hetero aggregation typically forms natural fractals, making aggregation state for difficult to predict. Naturally available organic matter within earthworms especially fulvic compound and humic substances can coat the surface of particles. Fulvic acid may alter the surface charge of particles and responsible to produce more stable form of aggregates. However, enhanced dispersion stability due to electrostatic forces is not often possible to predict. Natural organic matter in coelomic fluid of earthworms attaches to the surfaces of particles in a variety of ways *viz.*,irreversible adsorption onto the surfaces of iron oxidevis ligand exchange between carboxyl/hydroxyl functional groups of humic acids and iron oxide surfaces (Gu *et al*, 1994) or hydrophobic interactions with carbon bases Nanomaterials (Hyung *et al*.,

2007).This aggregation behaviour in earthworms' coelomic fluid affect fate, transport, reactivity, bioavailability of NPs and prevent them by alteration of mitochondrial functioning.

CONCLUSIONS

The ability to manipulate nanoparticles with naturally available organic substances in coelomic fluid of earthworms helps them to prevent from oxidative stress. Behaviour of NPs indicates that aggregation property with help of fulvic compounds and humic substances alter the activity of NPs in their coelomic fluid. This property of earthworms explores their importance as important receptor for NPs and their potential use as bioremediating agent for nanomaterials.

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