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Genotoxicity of ferric oxide nanoparticles in *Raphanus sativus*: Deciphering the role of signaling factors, oxidative stress and cell death

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ABSTRACT

We have studied the genotoxic and apoptotic potential of ferric oxide nanoparticles (Fe_2O_3-NPs) in *Raphanus sativus* (radish). Fe_2O_3-NPs retarded the root length and seed germination in radish. Ultrathin sections of treated roots showed subcellular localization of Fe_2O_3-NPs, along with the appearance of damaged mitochondria and excessive vacuolization. Flow cytometric analysis of Fe_2O_3-NPs (1.0 mg/mL) treated groups exhibited 219.5%, 161%, 120.4% and 161.4% increase in intracellular reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\Psi m$), nitric oxide (NO) and Ca^{2+} influx in radish protoplasts. A concentration dependent increase in the antioxidative enzymes glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) and lipid peroxidation (LPO) has been recorded. Comet assay showed a concentration dependent increase in deoxyribonucleic acid (DNA) strand breaks in Fe_2O_3-NPs treated groups. Cell cycle analysis revealed 88.4% of cells in sub-G1 apoptotic phase, suggesting cell death in Fe_2O_3-NPs (2.0 mg/mL) treated group. Taking together, the genotoxicity induced by Fe_2O_3-NPs highlights the importance of environmental risk associated with improper disposal of nanoparticles (NPs) and radish can serve as a good indicator for measuring the phytotoxicity of NPs grown in NP-polluted environment.
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Introduction

The advantages of nanoparticles (NPs) are potentially enormous. Despite less information on the risks of NPs to human health and environment, NPs have already gained entry into the composition of different consumer goods. Increased application of NPs raises the possibility of their entry to different levels of ecosystem either intentionally by the spread of sewage sludge or unintentionally due to accidental spills during manufacturing, atmospheric emissions and transport (Fabrega et al., 2011). For instance, TiO_2, MnO, FeO and ZnO-NPs have already been found in the running waters (Pradhan et al., 2014). A profound study has been done on the adverse effects of plant-metal interaction, nevertheless the toxicological response of plant with NPs exposure is still less studied (Ma et al., 2010). Some evidences on NPs-phytotoxicity demonstrated that Ag and cerium oxide nanoparticles (nCeO-NPs) induce toxicities in *Oryza sativa* and *Phaseolus vulgaris* (Thuesombat et al., 2014; Majumdar et al., 2014). *Vicia faba* exposed to multiwalled carbon nanotubes showed nutrient imbalance, damaged leaves and oxidative stress (Wang et al., 2014). *Glycine max* cultivated in $nCeO_2$ and nZnO-NP amended soil exhibited an alteration in its nutritional constituents (Peralta-Videa et al., 2014). TiO_2-NP uptake and translocation in *Allium cepa* were found responsible for enhancing intracellular reactive oxygen species (ROS) generation, cytogenetic anomalies and deoxyribonucleic acid (DNA) damage (Pakrashi et al., 2014). *Triticum aestivum* seeds treated with Fe_2O_3-NPs showed reduction in the rate of seed germination and root biomass (Feizi et al., 2013). Recently, Fe_2O_3-NPs have been reported to retard the root and shoot lengths in *Vigna radiata* seedlings (Kumar et al., 2015). Tobacco BY-2 cells treated with modified magnetic NPs (Fe_2O_3-NH_2 and Fe_2O_3-OH) resulted in a concentration dependent loss of cell viability and protein content (Krystofova et al., 2013).

Phytotoxicity of NPs is primarily related with their unique characteristics of increased surface area, ROS generation and adsorbing tendency on cell wall, where they facilitate the release of toxic ions (Ma et al., 2010). NPs-phytotoxicity is not only a resultant of NPs uptake and dissolution of metal ions from the rhizosphere solution but also from NPs itself (Andreotti et al., 2015). ROS can attack virtually all biological macromolecules, which induce serious damage to the DNA and cell components, resulting irreparable metabolic dysfunctions and cell death (Karuppanapandian et al., 2011). Under stressed condition, ROS plays an active role in stimulating secondary signaling messengers *viz.* NO and Ca^{2+}, which are well known to trigger cell death in plants (Gilchrist, 1998). NO interferes with mitochondria functionality and exerts imbalance between ROS generation and scavenging (Zottini et al., 2010). Crosstalk between NO and Ca^{2+} provides a molecular basis for the indirect regulation of physiological processes in plant (Courtois et al., 2008). Several features of plant cell death resemble with those observed in animals by sharing similarities in DNA sequences, apoptosis-related genes, processing of the DNA and ruptured nuclei (Collazo et al., 2006). However, mechanism of DNA damage, ROS generation, mitochondrial impairment, activation of plant cell signaling pathways and cell death upon NPs exposure is scarce. These data gaps motivated this study to investigate Fe_2O_3-NPs toxicity in popularly consumed edible tuberous root (radish).

Fe_2O_3-NPs due to its magnetic properties have gained immense potential in biomedical and *in vivo* clinical applications (Gupta et al., 2007). Fe_2O_3-NPs have been reported to improve the efficiency of anticancer drugs and act as a good candidate for targeted drug carrier (Lin et al., 2007). Fe_2O_3-NPs combined with different chemotherapeutics have provided new clinical options in the treatment of lymphoma (Jing et al., 2010). Within the clinical exposure settings citrate-coated Fe_2O_3-NPs have an important role in blocking blood vessels by its low solubility, which give rise to precipitation and agglomeration under physiological conditions. Therefore, Fe_2O_3-NPs disposal through wash off and wastewater discharges raises concern on its entry to different levels of ecosystems (Alidoust and Isoda, 2014). In this context, it is important to understand how plants will respond to NPs exposure and regulates the molecular mechanism of cell death pathways. Therefore, this study is aimed to investigate the (1) phytotoxicity of Fe_2O_3-NPs in radish, (2) NPs translocation and subcellular changes, (3) intracellular ROS generation and mitochondrial dysfunction, (4) biochemical enzymatic changes, (5) DNA damage and (6) flow cytometric assessment of cell cycle, NO generation, Ca^{2+} influx and esterase activities.

1. Materials and methods

1.1. Nanoparticles characterization

Fe_2O_3-NPs (2 mg/mL) were sonicated in ultrapure water for 10 min at 50 W and the solution was dropped on copper grids of a transmission electron microscope (TEM). Total five grids of Fe_2O_3-NPs were prepared and subjected to TEM analysis at 200 keV. Fe_2O_3-NPs were further characterized by analyzing the surface topography of powdered Fe_2O_3-NPs using an atomic force microscope (AFM) (Veeco Instruments, USA) in non-contact tapping mode. The topographical images were obtained in tapping mode with a resonance frequency of 218 kHz. Crystal structure of Fe_2O_3-NPs was characterized by X-ray diffraction (XRD) (Rigaku X-ray diffractometer, Japan) using Cu$K\alpha$ radiation (α = 1.54056 Å) in the range of $20° \leq 2\theta \leq 80°$ at 40 keV. Scherrer's relationship was used to calculate the particle size (D), using the formula $D = 0.9\lambda/\beta\cos\theta$, where, ($\theta$) is the Bragg's diffraction angle, (β) is the broadening of diffraction line measured as half of its maximum intensity in radians and (λ) is the wavelength of X-ray. Line width of XRD peaks was used to estimate the particle size. Fe_2O_3-NPs were further analyzed in liquid environment by measuring the dynamic light scattering (DLS) and zeta (ζ)-potential by the use of Zetasizer 2000 (ZetaSizer-HT, Malvern, UK). Briefly, Fe_2O_3-NPs stock suspension of 15 μg/mL was prepared in ultrapure water, sonicated for 15 min at 40 W and the solution was analyzed for DLS and ζ-potential, values presented were the average of 10 readings.

1.2. Comparative effect of Fe_2O_3-NPs and bulk Fe_2O_3 on root elongation

We have selected Fe_2O_3-NPs and its bulk counterpart Fe_2O_3 to determine the phytotoxicity in radish. Seeds were surface

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