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Effects of ZnO nanoparticles in plants: Cytotoxicity, genotoxicity, deregulation of antioxidant defenses, and cell-cycle arrest

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ABSTRACT

Cytotoxicity, genotoxicity, and biochemical effects were evaluated in the plants *Allium cepa, Nicotiana tabacum*, and *Vicia faba* following exposure to ZnO nanoparticles (np; diameter, ~85 nm). In the root meristems of *Allium cepa* cells, we observed loss of membrane integrity, increased chromosome aberrations, micronucleus formation, DNA strand breaks, and cell-cycle arrest at the G2/M checkpoint. In *Vicia faba* and *Nicotiana tabacum*, we observed increased intracellular ROS production, lipid peroxidation, and activities of some antioxidant enzymes. TEM images revealed gross morphological alterations and internalization of the np. Our findings provide evidence of ZnO np toxicity, characterized by deregulation of components of ROS-antioxidant machinery, leading to DNA damage, cell-cycle arrest, and cell death. These plants, especially *Allium cepa*, are reliable systems for assessment of np toxicology.

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1. Introduction

Nanotechnology and nano-enabled products have recently received much attention, due to their remarkable properties. Nanomaterials (NM) have been defined as materials (either manufactured or occurring naturally) containing particles either in the unbound state or as aggregates/agglomerates, with 50%+ of the particles in the range of 1–100 nm diameter. In addition to their small size, these particles are characterized by their large surface areas, high aspect ratios, and unusual surface properties. These properties may also result in high reactivity and toxicity; hence, np should be treated as different entities from the bulk forms of the same substances.

With continuing advances in nano-based industry, revenue generation is projected to be as high as US $4 trillion by the end of 2018. According to a recent market survey [1], the market share of zinc oxide np is exceeded only by silver, titanium dioxide, and carbon nanotubes. ZnO np are widely used in several products, owing to their excellent UV absorption and reflective properties [2]. In addition, ZnO np are excellent candidates for application in the agriculture and food sector, as pesticides, fungicides, and fertilizer [3–5]. A literature search with the terms "Nanoparticle" and "Pesticide" in Pubmed returned 328 results, of which 9 included ZnO as one of the components. A similar search in Scopus returned 508 results, of which 18 reported the use of ZnO. The ever-increasing production and wide range of applications increase the risk of environmental exposure. However, information on the ecotoxicological effects of ZnO np is very limited across all taxa.

Recently, Ma et al. [6] reviewed the toxicity of ZnO np in different species. Of the studies discussed, only a limited number were performed on plants; most of these [7–10] were limited to endpoints such as seed germination, root elongation, and biomass. Kumari et al. [11], however, reported the genotoxicity of ZnO np in *Allium cepa*. Similar studies have revealed significant toxicity of several other nanoparticles, including metals and metal oxides [12–15]. Phytotoxicity of TiO_2 np has been observed in various plant species, including *Allium cepa*, *Vicia narbonensis*, and *Zea mays* [16–18]. In addition to physiological endpoints, genotoxicity has been observed in these studies. Significant physiological-biochemical and cyto-genotoxic alterations induced by Al_2O_3 np have been well documented [19–21].

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Investigation of ZnO np toxicity is essential for risk assessment and management. In the present study, we have evaluated the toxicity of this material in the plants *Allium cepa, Nicotiana tabacum*, and *Vicia faba*, with a focus on np characterization and measurements of cytotoxicity, genotoxicity, oxidative stress, and antioxidant enzymes. The effect of ZnO np on cell cycle progression was also studied.

2. Materials and methods

2.1. Study design

We used three plant species, *Allium cepa*, *Vicia faba*, and *Nicotiana tabacum*. Concentration selection was based on preliminary cytotoxicity tests (Evans blue assay) and published literature [11,22]. Genotoxicity was studied using the comet, chromosome aberration, and micronucleus assays. Biochemical endpoints were evaluated to test the effects of ZnO np on oxidative stress and activities of antioxidant enzymes.

2.2. Nanoparticle characterization

Zn np were obtained from Sigma–Aldrich (St. Louis, MO, USA), particle size <100 nm: purity: 99.5%; specific surface area: 15 $m^2\ g^{-1}$. Primary particle size and morphology were analysed using Transmission Electron Microscope (Jeol JEM-2100 LaB6, 200 kV) and Scanning Electron Microscopy coupled with Energy-dispersive X-ray spectroscopy (EDX) (Hitachi S-415A electron microscope at 25 kV). ZnO np were suspended in filter-sterilized water and dispersed by ultrasonic vibration (100 W, 30 KHz; Sonics and Materials Inc., Newtown, CT, USA) for 30 min, on ice. Hydrodynamic diameter was measured using dynamic light scattering (90 Plus Particle Size Analyzer, Brookhaven Instruments Corporation, Holtsville, NY, USA). DLS measurements were performed for all concentrations of ZnO np (0.2, 0.4 and 0.8 g/L) used for the experiments. The amount of Zn^{2+} ion leached from the ZnO NPs was measured in filter-sterilized water, using atomic absorption spectroscopy (AAnalyst 400, Perkin-Elmer, Waltham, MA, USA).

2.3. Test system and treatment schedule

Three model plant systems were used. *Allium cepa* was the primary system for all of the assays, but certain assays were also performed in *Vicia faba* (Evans blue cytotoxicity, chromosome aberration, micronucleus assay) and *Nicotiana tabacum* (comet assay) for confirmation. *A. cepa* bulbs were set to germinate in sterilized moist sand and allowed to grow until roots reached length = 2–3 cm *V. faba* seeds were germinated on sand and allowed to grow for 7 d until roots reached length = 2–3 cm *N. tabacum* seeds were germinated in garden soil. The plantlets were used when they reached the fourth-leaf stage.

The plants were treated hydroponically. The roots were exposed to ZnO np (0.2, 0.4, and 0.8 g/L) prepared in water, in well-aerated glass vials, for 24 h. Additionally, bulk ZnO particles were used as a control. The experiments were conducted at room temperature ($25 \pm 1\ ^\circ C$) and the roots were processed for different assays.

2.4. Cytotoxicity (Evans blue dye)

Loss of cell viability was studied using the Evans blue staining method [23]. *A. cepa* and *V. faba* roots were stained with Evans blue (0.25% w/v) for 15 min and subsequently washed with distilled water for 30 min. Triton X-100 was used as positive control. Bulk particles of ZnO were used as an additional control. The roots were then macro photographed to estimate cell death. Additionally, 5 root tips (1 cm) were soaked in *N*,*N*-dimethylformamide for 1 h at room temperature and the absorbance was measured at 600 nm.

2.5. Genotoxicity

2.5.1. Chromosome aberration and micronucleus assays

The clastogenic/aneugenic effects of ZnO np were evaluated using chromosome aberration and micronucleus tests in *A. cepa* and *V. faba* root cells. Slides were prepared from each of the roots following the squash technique of Sharma and Sharma [24] with modifications described earlier [15]. Maleic hydrazide was used as a positive control.

2.5.2. Comet assay

The alkaline comet assay was performed to evaluate DNA damage. Nuclei isolated from *A.cepa* roots and *N. tabacum* leaves and roots were processed according to methods described previously [15]. For analysis, 75 nuclei (25 nuclei per replicate) per concentration were analysed and the results represented means of the comet parameter, tail DNA (%). EMS was used as a positive control. Additionally, the genotoxicity of bulk ZnO particles was evaluated in *A. cepa*.

2.6. Oxidative stress response and antioxidant enzyme activity in Allium cepa

2.6.1. H_2O_2 generation

H_2O_2 content in root tissue was determined according to the method of Loreto and Velikova [25]. Root tissues (1 g) from control and treated plants were homogenised in 0.1% (w/v) trichloroacetic acid at 4 °C. The homogenate was centrifuged at 12,000 rpm for 15 min and the supernatant was mixed with 10 mM sodium phosphate buffer (pH 7.0) and 1 M potassium iodide. The H_2O_2 content of the supernatant was measured spectrophotometrically at 390 nm.

2.6.2. Lipid peroxidation

Lipid peroxidation was determined according to the method of Ohkawa et al. [26] with minor modification, as previously described [16]. Thiobarbituric acid-reactive substances content was determined using extinction coefficient = 155 $mM^{-1}\ cm^{-1}$ and expressed as nmol mg^{-1} fresh weight.

2.6.3. Intracellular ROS production by DCFH-DA staining

Analysis of ROS production were evaluated by staining the roots with 2,7-dichlorofluorescin diacetate (DCFH-DA), 25 μM, for 15 min [15]. H_2O_2-treated roots were used as positive control. Confocal laser scanning was used to obtain high content image s(BD Pathway TM 855). The fluorescence intensity was quantified using the image processing and analysis software Image J.

2.6.4. Antioxidant enzyme activities

For catalase (CAT, E.C. 1.11.1.6) and guaiacol peroxidase (GPOD, E.C. 1.11.1.7), root tissues (100 mg) were homogenised in tris buffer. The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C. Catalase activity was measured according to the method of Aebi [27]. The decrease in absorbance as a result of H_2O_2 (ε = 39.4 $mM^{-1}\ cm^{-1}$) decomposition was recorded at 240 nm and expressed as nmol H_2O_2 mg $protein^{-1}\ min^{-1}$. GPOD activity was determined in the enzyme extract according to the method of Hemeda and Klein [28]. Increase in absorbance at 470 nm due to formation of tetraguaiacol (ε = 26.6 $mM^{-1}\ cm^{-1}$) is expressed as μmol mg $protein^{-1}\ min^{-1}$.

2.6.5. Total thiol, protein thiol, and GSH content

Total thiol, protein, and nonprotein thiol contents of the homogenates were measured using Ellman's reagent [29]. For total

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