Cytotoxicity of zinc oxide (nano)particles in a rat intestinal cell model: effect on cellular glutathione and mitochondria

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Abstract

Zinc oxide nanoparticles (NPs) have a variety of commercial and biomedical applications ranging from UV filtration in sunscreen to anti-cancer, fungal and anti-microbial agents. Human oral exposure to zinc oxide NPs may occur following accidental or intentional ingestion, hand-to-mouth activity, or mucociliary transport following inhalation. This study assessed the cytotoxicity of two different sized zinc oxide particles (10 and 150 nm) in rat intestinal cells (IEC-6). The 10 nm particle is classified as a nanoparticle. Previously we have reported that the cytotoxicity of these two particles and zinc ions are time- and dose-dependent in these cells. Dissolution of the particles and $ZnSO_4$ in media with 10% serum protein was assessed over 24 h. Zn concentration in incubation filtrate was determined by ICP-OES and was assumed to be free zinc ion. Intracellular glutathione (GSH) concentrations and mitochondrial area were assessed in the IEC-6 cells using spectroscopic methods after a 4-h exposure to the particles (0.1 – 100 ug/mL) or $ZnSO_4$ (100 ug/mL). Dissolution studies in media showed that both particles formed soluble Zn ions, and that media components bind these ions. At 24 h, approximately 40% of $ZnSO_4$, 40% of 10 nm ZnO and 30% of 150 nm ZnO was detected as free Zn ion in the filtrate. In H_2O, 100% $ZnSO_4$ was detected in filtrate at 24 h, showing complete dissolution. A significant dose-dependent decrease in cellular GSH and mitochondrial area was detected in the IEC-6 cells after a 4-h exposure to both particles and $ZnSO_4$. Both cellular GSH and mitochondrial area in treated cells decreased up to 40% relative to non-treated cells. The results suggest that ZnO particles, regardless of size, form zinc ions in media. Decreased cellular GSH may result from the reaction of this thiol with reactive oxygen species, which may be formed by zinc ions. The increased oxidative stress in the treated cells may also be damaging to the mitochondria. An imbalance of a cellular antioxidant such as GSH caused indirectly by zinc ions formed from ZnO particles may result in impairment of organelles such as the mitochondria resulting in cell death. (This abstract does not represent US EPA policy.)

Introduction

Particles and materials that have at least one dimension less than 100 nm are termed nanoparticles (NPs) and nanomaterials (NM)s, respectively. NPs and NMs exist in nature but can also be prepared or engineered (ENMs). ENMs include metals and metal oxides, such as silver and gold NPs and zinc oxide and copper oxide NPs, respectively.

ZnO NPs have a variety of commercial and biomedical applications ranging from UV light filtration in sunscreens to anti-cancer, fungal and anti-microbial agents.

ZnO NPs are toxic in rodents and in vitro cellular systems (Vandebriel and De Jong, *Nanotechnol Sci Appl* 5:61-72, 2012). Following inhalation or tracheal instillation of ZnO NPs in rats, inflammation of the lung develops. In cellular systems, including Caco-2 cells, a human intestinal model, cytotoxicity, formation of reactive oxygen species, DNA damage and other effects are noted after exposure to ZnO NPs.

Human oral exposure to ZnO NPs may occur following accidental or intentional ingestion, hand-to-mouth activity, or mucociliary transport following inhalation. As ZnO NPs are used in food packaging, leaching of the NPs from the packaging to food may occur. ZnO NPs are also found in dietary supplements.

Chemical and physical factors of NPs, such as their size and dissolution to ions may impact their toxic potential.

Objective

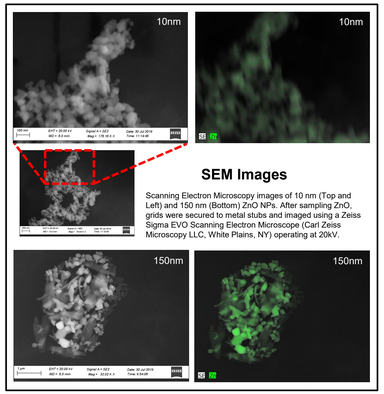
To assess the effect of size of ZnO particles on cytotoxicity in regard to glutathione levels and organelle health in a rat 2-dimensional model system of the small intestine. The dissolution of the ZnO NPs to ionic Zn was also examined.

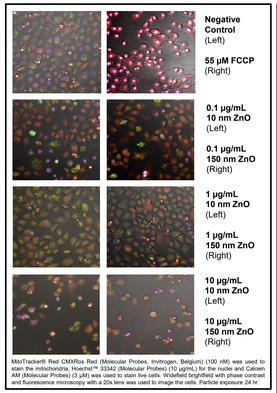
Note: The contents of this poster do not necessarily represent U.S. EPA policy.

Materials and Methods

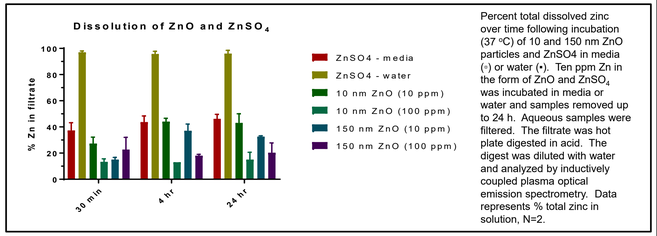
- Rat small intestine epithelial cells (IEC-6) from Advanced Tissue Culture Collection (Manassas, VA) were seeded at approximately 3 x 10^4 or 6 x 10^4 cells/well in a 96-well microtiter plate and placed in an incubator (37 °C, 5% CO_2, 95% relative humidity) for 24 hr before NP exposure.
- Zinc oxide (ZnO) particles (10 and 150 nm particle size) were from Meliorum Technologies (Rochester, NY). The particles were suspended (1 mg/ml) in cell culture media with 10% fetal bovine serum. The particles were dispersed with a probe sonicator (3 x 3 secs @ 4.5W).
- Transmission electron microscopy (TEM) of the particles was performed by nanoComposix (San Diego, CA). Briefly, a dilute solution (1 mg/mL, deionized water) of particles was dropcast onto a carbon-coated copper TEM grid (Ted Pella) and dried under vacuum. Images were generated using a JEOL 1010 Transmission Electron Microscope operating at 100 kV and captured on an AMT XR41 CCD camera. Scanning electron microscopy (SEM) of the particles was performed by Michael E. Bishop (ORISE-EPA). After sampling the particles grids were secured to metal stubs and imaged using a Zeiss Sigma EVO Scanning Electron Microscope (Carl Zeiss Microscopy LLC, White Plains, NY) operating at 20kV.
- For the dissolution study, 30 mL of completed media was spiked with $ZnSO_4$, 10 nm ZnO particles, or 150 nm ZnO particles at a concentration of 10 and 100 µg/mL and incubated at 37 °C for up to 24 h. A 5-mL aliquot was removed at 0.5, 4 and 24 hr. Each sample was centrifuged through a 10 kDa filter (Amicon Ultra-15, 10 K, Millipore, Bedford, MA) at 5911g for 20 min. An aliquot of each filtrate was hot plate digested at 60°C with 1 mL concentrated HNO_3 overnight. The digested filtrate was diluted with deionized water and directly analyzed for copper by inductively coupled plasma optical emission spectroscopy (ICPOES) (Thermo Scientific, Waltham, MA, USA).
- Glutathione (GSH) concentration in IEC-6 cells treated with ZnO particle (0.1 – 100 µg/mL) for 4 hr was measured using the GSH- Glo™ Glutathione Assay kit from Promega Corp. (Madison, WI).
- IEC-6 cells were plated to 30,000 cells per well in black 96-well plates, TC-treated, and left overnight to attach in an incubator as described above. The following day, cells were treated with ZnO particles (0.1 – 100 ug/mL) or ZnSO4 (100 ug/mL) for 4 hours and then rinsed once with incomplete media (i.e., no FBS or pencillin/streptomycin). One hour prior to staining, 80 µM FCCP from Abcam (Cambridge, MA) in incomplete media was added to one column of cells to serve as a positive control.
- Cellular and organelle health (mitochondria and nucleus) in IEC-6 cells was assessed using fluorescent microscopy. MitoTracker® Red CMXRos Red (Molecular Probes, Invitrogen, Belgium) (100 nM) was used to stain the mitochondria, Hoechst™ 33342 (Molecular Probes) (10 µg/mL) was used to stain the nuclei and Calcein AM (Molecular Probes) (3 µM) was used as a live-cell stain.
- All three stains were added to incomplete media, pre-warmed to 37 °C and 50 µL was added to each well. The plate was returned to the incubator for 5 min. Cells were rinsed once with incomplete media and 4% paraformaldehyde (PFA) at 4 °C was added for 5 min to fix the cells and prevent further cellular activity. The PFA was rinsed from the wells once with incomplete media and 50 µL incomplete media was returned to the wells. After fixing, plates were sealed with adhesive film.
- Analysis of the staining to assess cellular and organelle health was performed on a Thermo-Fisher Cellomics Array Scan XTI using a Zeiss 20X EC Plan NeoFluar objective along with the Cellomics High Content Screening (HCS) Studio software (Thermo-Fisher Scientific, Waltham, MA) where fluorescence in the mitochondria of each cell was compared to a negative and positive control. The Organelle Health Assessment Assay in the Cellomics HCS software was selected (Cellomics BIoAPP Guide: Organelle Health)

Results

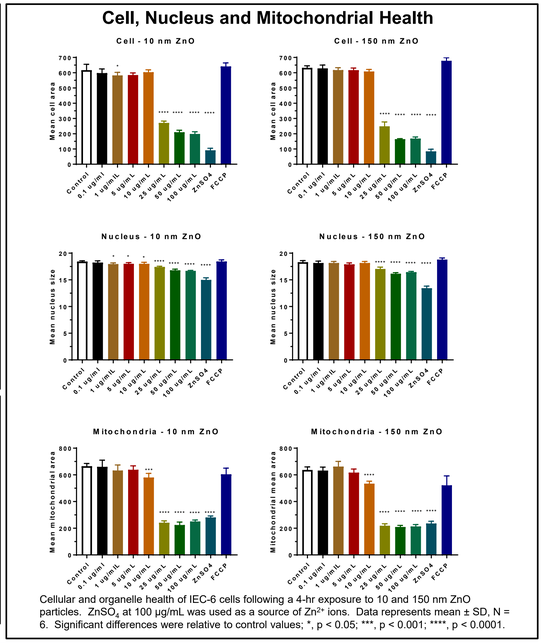




MitoTracker® Red CMXRos Red (Molecular Probes, Invitrogen, Belgium) (100 nM) was used to stain the mitochondria, Hoechst™ 33342 (Molecular Probes) (10 ug/mL) for the nuclei and Calcein AM (Molecular Probes) (3 µM) was used to stain live cells. Widefield brightfield with phase contrast and fluorescence microscopy with a 20x lens was used to image the cells. Particle exposure 24 hr.

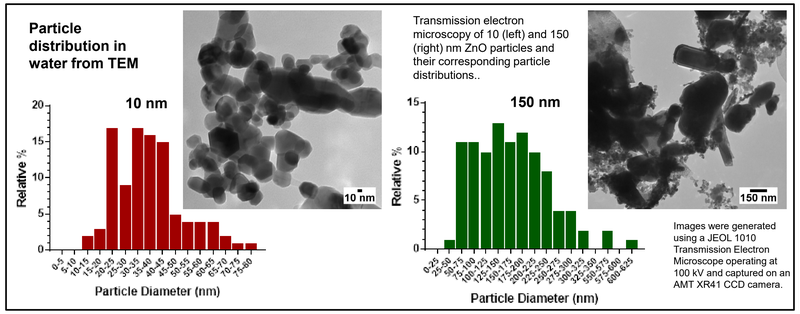


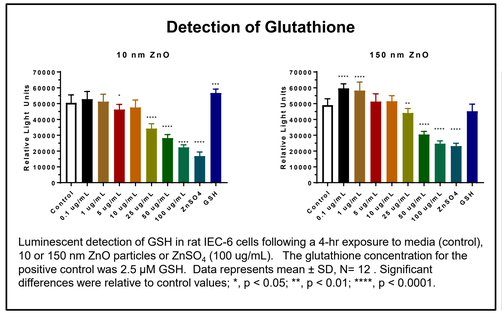
Percent total dissolved zinc over time following incubation (37 °C) of 10 and 150 nm ZnO particles and ZnSO4 in media (-) or water (+). Ten ppm Zn in the form of ZnO and $ZnSO_4$ was incubated in media or water and samples removed up to 24 h. Aqueous samples were filtered. The filtrate was hot plate digested in acid. The digest was diluted with water and analyzed by inductively coupled plasma optical emission spectrometry. Data represents % total zinc in solution, N=2.



Cellular and organelle health of IEC-6 cells following a 4-hr exposure to 10 and 150 nm ZnO particles. $ZnSO_4$ at 100 µg/mL was used as a source of Zn^{2+} ions. Data represents mean ± SD, N = 6. Significant differences were relative to control values; *, p < 0.05; ***, p < 0.001; ****, p < 0.0001.

Results





Luminescent detection of GSH in rat IEC-6 cells following a 4-hr exposure to media (control), 10 or 150 nm ZnO particles or $ZnSO_4$ (100 ug/mL). The glutathione concentration for the positive control was 2.5 µM GSH. Data represents mean ± SD, N= 12 . Significant differences were relative to control values; *, p < 0.05; **, p < 0.01; ****, p < 0.0001.

Summary

- TEM images show non-uniform structures of the particles and a large particle size distribution.
- SEM of the 10 and 150 nm ZnO particles show they tend to aggregate in media. Agglomerates seem to be at least 10 times the particle size.
- Over a 24-hr period, the dissolution studies show that at a minimum 10-40% of the initial ZnO particles were ionized in media. $ZnSO_4$ in water completely ionized to Zn^{2+}, while in media, only about 40% of the initial $ZnSO_4$ appeared to have ionized. This suggests Zn^{2+} binds to media components.
- Increasing concentrations of ZnO particles decrease levels of cellular glutathione.
- Increasing concentrations of ZnO particles show a reduction in mean cell area as well as a reduction in nucleus size
- Mean mitochondrial area also decreases as concentration of ZnO particles increases.
- Mitochondrial effects seem to be greater than nuclear.
- The effects observed do not appear to be dependent on the size of the ZnO particles (10 and 150 nm) studied.