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Oxidative stress-mediated inhibition of intestinal epithelial cell proliferation by silver nanoparticles

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ABSTRACT

Given the increasing use of silver nanoparticles (Ag NP) by the food and food packaging industries, this study investigated potential consequences of Ag NP ingestion in intestinal epithelial C2BBe1 cells. Treatment of proliferating cells (<10,000 cells/cm^2) with 0.25 μg/cm^2 (1.25 μg/mL) of 23 nm Ag NP for 24 h induced 15% necrotic cell death and an 80% reduction in metabolic activity and decreased the GSH/GSSG ratio, indicating oxidative stress. G2/M phase cell cycle arrest and complete inhibition of cell proliferation was also induced by Ag NP treatment. Simulated *in vitro* digestion of Ag NP prior to cell exposure required the use of slightly higher doses to induce the same toxicity, likely due to slower Ag dissolution. Treatment of cells with silica, titania, and ZnO NP partially inhibited cell proliferation, but inhibition at low doses was unique to Ag NP. These data suggest that Ag NP induces oxidative stress, cell cycle arrest, and the inhibition of cell proliferation. However, toxicity and induction of oxidative stress were not observed in confluent cells (>100,000 cells/cm^2) treated with 10 μg/cm^2 (40–50 μg/mL) Ag NP, indicating that these cells are less sensitive to Ag NP.

1. Introduction

Nanotechnology is being increasingly exploited by a variety of industries for the unique properties materials display at the nanoscale. Silver nanoparticles (Ag NP) are used for their increased catalytic activity. Optical properties of Ag NP are also being exploited in chemical and biological sensors (El-Nour et al., 2010). Another property of Ag NP is their strong antimicrobial activity which has found application in water purification, wound dressings, prevention and treatment of infection, and other medical uses (Alexander, 2009). The Project on Emerging Nanotechnologies, which attempts to inventory consumer products containing nanotechnologies, currently lists 438 products containing silver nanoparticles (Anon., 2015). These include textiles such as sheets, towels, and socks, curling irons, air purifiers, a stuffed animal, and toothbrushes, all utilizing the antimicrobial properties of Ag NP. The food and food packaging industries are also interested in utilizing Ag for its antibacterial properties and 41 of the products listed by the Project on Emerging Nanotechnologies are in the Food and Beverage category. These include Ag supplements, cookware, utensils, and appliances coated with Ag NP, and Ag NP incorporated into food storage containers to prevent food spoilage. In addition to direct consumption of Ag NP used in foods, some fraction of Ag NP incorporated into food packaging and food contact materials may be transferred to food, further increasing Ag NP consumption.

It has been suggested that Ag NP mediate their antimicrobial effects through oxidative stress, which damages bacterial membranes. Electron spin resonance has been used to detect radicals on the surface of 13.5 nm Ag NP (Kim et al., 2007). While this microbial toxicity has great potential utility, the same mechanism

Abbreviations: NP, nanoparticle; TEM, transmission electron microscopy; GSH, reduced glutathione; GSSG, oxidized glutathione; ROS, reactive oxygen species; PBS, phosphate-buffered saline; DRIFTS, diffuse reflectance infrared Fourier transform spectroscopy; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; LDH, lactate dehydrogenase; NAC, N-acetylcysteine; XPS, X-ray photoelectron spectroscopy; HBSS, Hank's Balanced Salt Solution; BE, binding energy; ICP-MS, inductively coupled plasma mass spectrometry.

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may contribute to cytotoxicity to mammalian cells. In medical applications, care can be taken to use doses of Ag NP that will be toxic to microbes but not human cells. However, the intake of Ag NP is less controllable when these nanoparticles are being ingested in food and food contact materials.

Ingested Ag NP will be transported through the digestive tract, exposed to digestive enzymes, and excreted from the body if not absorbed in the intestines, as occurs with most non-nutrient components of food. Intestinal epithelial cells within the intestines are responsible for transport of nutrients from the intestinal lumen to the bloodstream to be used by the rest of the body. Nanoparticles are likely to come into the most contact with epithelial cells after ingestion. In order to gain access to the circulation to be more widely distributed throughout the body, Ag NP will have to first be transported across the intestinal epithelium. *In vivo* studies with PLGA, polystyrene, and thiol-organosilica nanoparticles between 50 and 100 nm have shown that nanoparticles are internalized by and transported across the intestinal epithelium into the circulation more readily than larger particles between 100 and 10,000 nm (Awaad et al., 2012; Desai et al., 1996; Jani et al., 1990; Zane et al., 2015). Studies in which 10–60 nm Ag and other NP were orally administered to rodents have also shown incorporation of nanoparticles into intestinal epithelial cells and transport of nanoparticles across the epithelium (Jeong et al., 2010; Platonova et al., 2013; van der Zande et al., 2012). Thus, interaction of Ag NP with intestinal epithelial cells will be important for the systemic effects of Ag NP ingestion, and intestinal epithelial cells serve as a relevant *in vitro* model to study impact of nanoparticle ingestion.

As the use of Ag NP has increased, there has been concern about environmental accumulation of Ag NP and the potential health impacts of Ag NP in humans. This has led to studies in various systems exploring Ag NP cytotoxicity and the mechanism of response to Ag NP in several cell models. These studies have described an oxidative stress-mediated mechanism of cytotoxicity in cells which leads to DNA damage, mitochondrial damage, cell cycle arrest, and apoptosis (AshaRani et al., 2009b; Chairuangkitti et al., 2013; Eom and Choi, 2010). Studies in intestinal epithelial cell models have reported Ag NP toxicity including decreased cell viability, formation of reactive oxygen species (ROS), decreased mitochondrial function, increased IL-8 generation by cells, and DNA damage (Abbott Chalew and Schwab, 2013; Aueviriyavit et al., 2014; Böhmert et al., 2012; Sahu et al., 2014). However, there has been less thorough investigation of the mechanism of Ag NP toxicity in intestinal epithelial models and no investigation of Ag NP effects on cell proliferation. It is known that oxidative stress plays a role in inflammation and disease progression in the intestines, so an increase in oxidative stress by Ag NP in the intestines may be particularly problematic for those with intestinal pathologies (Circu and Aw, 2012).

In this study, we utilize the intestinal epithelial C2BBe1 model to investigate the mechanism of ~23 nm Ag NP cytotoxicity on proliferating and confluent cells. The biological end points included cellular oxidative stress, cell cycle arrest, and inhibition of cellular proliferation. Inhibition of cell proliferation at low doses (0.25 μg/cm^2) was unique to Ag NP and not observed after SiO_2, TiO_2, or ZnO NP treatment.

2. Materials and methods

2.1. Nanoparticles

Zinc oxide, titania, and silica nanoparticles were purchased from Sigma–Aldrich (St. Louis, MO). The specifications of the ZnO particles were size ⩽100 nm, with a specific surface area of 15–25 m^2/g (Catalog No. 544906). For TiO_2, the particle size was specified as 21 nm, with surface area of 35–65 m^2/g, purity of ⩾99.5%, and a trade name of Aeroxide P25 (Catalog No. 718467). Silica particle specification included size of 12 nm, surface area of 175–225 m^2/g, and purity of 99.8% (Catalog No. 718483).

2.2. Silver nanoparticle synthesis

Silver nanoparticles (~23 nm) were prepared as follows. A 200 mL solution of 0.25 mM $AgNO_3$ and 0.25 mM trisodium citrate was prepared in purified water and stirred for 30 min. Six mL of a 10 mM $NaBH_4$ solution was slowly added while stirring. The solution was stirred for 30 min, then left at room temperature for 24 h. Particles were washed twice by centrifugation at 209,000*g* for 30 min, removal of supernatant, and redispersion in purified water.

2.3. Simulated gastrointestinal digestion of Ag

Pepsin, pancreatin, and bile salts were used to simulate the gastric and small intestinal digestive environments *in vitro* (McCracken et al., 2013). The concentrations used were based on *in vitro* digestion methods used in previously published studies (Connolly et al., 2010; Glahn et al., 1996; Mandalari et al., 2008; Reboul et al., 2006). Briefly, pepsin (stomach, 146 U/mL, Sigma–Aldrich), pancreatin (intestinal enzyme mixture, 2 mg/mL, Sigma–Aldrich), and bile extract (porcine, 0.024 mg/mL, Sigma–Aldrich) were dissolved in water and adjusted to a pH of 2 (pepsin) or 7 (pancreatin and bile extract).

Silver nanoparticles (50 mg/L) were incubated sequentially in the pepsin, pancreatin, and bile extract solutions for one hour each at 37 °C. Subsequent to each incubation, the nanoparticles were pelleted by centrifugation (209,000*g* for 30 min) and resuspended in the next solution. After incubation in the bile extract, nanoparticles were centrifuged, resuspended in phosphate-buffered saline (PBS) and used for biological studies. Ag NP treated in this manner are hereafter referred to as "digested NP" while untreated NP are referred to as "pristine".

2.4. Particle size and surface charge

A Zetasizer Nano ZS (Malvern, Westborough, MA) was used to measure the zeta potential of the pristine and digested silver nanoparticles. The Nano ZS uses a 633 nm laser as its light source. For zeta potential measurements, a forward angle of 12° was used for collecting light. The default Smoluchowski model in the software program was used. Each measurement included 20 runs, and monomodal analysis provided by the vendor was used for analysis. Samples were titrated versus pH using an attached MPT-2 Autotitrator. The titrator was supplied with 0.1 M HCl and 1.0 M HCl. Three replicate measurements were taken at each pH with a two minute pause between all measurements.

2.5. TEM images of pristine silver nanoparticles

Images were taken using a Tecnai F20 Transmission Electron Microscope. A 10 mg/L solution of pristine silver nanoparticles in ethanol was prepared and sonicated for 30 min. The solution was dropped onto lacey carbon copper TEM grids (Ted Pella, Inc., Redding, CA) and allowed to dry for several hours.

2.6. Infrared spectroscopy

Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) was performed on pristine silver nanoparticles and digested silver nanoparticles. For the digested silver, after the final digestion step, the particles were washed twice with water. The