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Research paper

Particle coatings but not silver ions mediate genotoxicity of ingested silver nanoparticles in a mouse model

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ABSTRACT

Incorporation of silver nanoparticles (AgNPs) in toothpaste, food containers, dietary supplements and other consumer products can result in oral exposure to AgNPs and/or silver ions (Ag^+) released from the surface of AgNPs. To examine whether ingestion of AgNPs or Ag^+ results in genotoxic damage and whether AgNP coatings modulate the effect, we exposed mice orally to 20 nm citrate-coated AgNPs, polyvinylpyrrolidone (PVP)-coated AgNPs, silver acetate or respective vehicles at a 4 mg/kg dose (equivalent to 800× the EPA reference dose for Ag) for 7 days. Genotoxicity was examined in the systemic circulation and bone marrow at 1, 7, and 14 days post-exposure. We found that citrate-coated AgNPs induced chromosomal damage in bone marrow and oxidative DNA damage and double strand breaks in peripheral blood. These damages persisted for at least 14 days after exposure termination. Because oxidative DNA damage and strand breaks are repaired rapidly, their presence after exposure cessation indicates that citrate-coated AgNPs persist in the body. In contrast, PVP-coated AgNPs and silver acetate did not induce DNA or chromosomal damage at any time point measured. To determine whether coating-dependent genotoxicity is related to different AgNP changes in the gastrointestinal tract, we examined AgNP behavior and fate in an *in vitro* gastrointestinal digestion model using UV–visible spectroscopy and DLS. Citrate-coated AgNPs were more susceptible to agglomeration than PVP-coated AgNPs in digestive juices with or without proteins. In summary, AgNPs but not Ag^+ are genotoxic following oral ingestion. Nanoparticle coatings modulate gastrointestinal transformation and genotoxicity of AgNPs, where higher agglomeration of AgNPs in gastrointestinal juices is associated with higher genotoxicity in tissues. Since genotoxicity is a strong indicator of cancer risk, further long-term studies focusing on cancer are warranted.

1. Introduction

Silver nanoparticles (AgNPs) are the most commercialized engineered nanomaterial that is used in more than 30% of nanotechnology-enabled consumer products (PEN, 2015). Due to their unique antimicrobial and antifungal properties, AgNPs are used in personal care products, household items, food contact materials and textile fabrics (Hajipour et al., 2012; PEN, 2015; Tolaymat et al., 2010). Their unique optical properties are exploited in electronics, imaging, catalysis and biosensing (Tolaymat et al., 2010). There are numerous AgNP applications, including everyday items, which can lead to oral exposure to AgNPs and/or silver ions (Ag^+) released from the surface of AgNPs through the process of oxidative dissolution. For example, consumer products, including food storage containers (Echegoyen and Nerin, 2013), socks (Benn and Westerhoff, 2008; Geranio et al., 2009; Lorenz et al., 2012) and children's items (Quadros et al., 2013) have been shown to leach Ag (AgNPs and/or Ag^+) into water or food, drink and sweat simulating solutions. Incorporation of AgNPs in toothpaste and toothbrushes, food and beverage containers, kitchen utensils and dietary supplements can lead to ingestion of AgNPs and/or Ag^+. In addition to direct exposures, the use of AgNPs in laundry detergents, textile fabrics and home appliances can lead to environmental contamination, accumulation in soil or water and unintentional ingestion *via* edible plants

Abbreviations: AgNPs, silver nanoparticles; cit, citrate; PVP, polyvinylpyrrolidone; AgOAc, silver acetate; Ag^+, silver ions; 8-oxoG, 7,8-dihydro-8-oxoguanine; γ-H2AX, phosphorylated histone 2AX; DSBs, double strand breaks; MN, micronuclei; SPR, surface plasmon resonance; DLS, dynamic light scattering; UV–vis, UV–visible spectroscopy.

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or food animals (Blaser et al., 2008). Thus, the likelihood of exposure to AgNPs is high and gastrointestinal route is a primary route of exposure (Bergin and Witzmann, 2013).

Physicochemical properties of nanomaterials determine their biological effects. Of particular interest are the effects of nanoparticle surface coating and size. Surface coatings are used to stabilize AgNPs in solution by preventing their agglomeration, oxidation and Ag^+ release. The effects of AgNP coating and size have been examined on several toxicological parameters, in particular, cell death, oxidative stress and inflammation. Most studies were performed *in vitro* in cultured cells. In general, the smaller the AgNPs, the greater the effect was observed (Carlson et al., 2008; Kim et al., 2012; Liu et al., 2010; Miethling-Graff et al., 2014; Prasad et al., 2013). The effects of coatings have been investigated to a lesser extent. Citrate and polyvinylpyrrolidone (PVP) are two of the most commonly used coating agents (Huynh and Chen, 2011). Citrate provides electrostatic stabilization, is weakly bound and can be replaced by other molecules. In comparison, PVP stabilizes AgNPs by steric repulsion. It binds very strongly to metal surfaces and provides high AgNP stability in different solutions. Both coatings provide a negative surface charge to the nanoparticle. Studies that comparatively assessed citrate- and PVP-coated AgNPs found that coating, relative to size, has a small effect (Guo et al., 2016) or results were mixed (Prasad et al., 2013; Vecchio et al., 2014). Only a few studies examined the effect of coating *in vivo* in rodent animals. Anderson et al. reported that in rats exposed to AgNPs by intratracheal instillation, citrate-coated AgNPs resulted in a greater Ag retention in the lungs and a greater increase in lung macrophages at 21 days post-exposure compared to PVP-coated AgNPs (Anderson et al., 2015). Bergin et al. examined the effects of citrate- and PVP-coated AgNPs on body and organ weights, histopathology effects and fecal elimination kinetics in orally exposed mice, but did not observe changes in any of these parameters (Bergin et al., 2016). To date, the toxicological impact of nanoparticle coating is unclear.

While cytotoxic effects of AgNPs have been confirmed in many *in vitro* studies, understanding of AgNP-induced genotoxicity is limited. The importance of assessing genotoxicity is underscored by the fact that genotoxicity is a strong indicator of delayed *i.e.* long-term health effects, especially, cancer. Studies that utilized the Ames test measuring point mutations in bacterial stains of *S. typhimurium* and/or *E. coli* reported that AgNPs tested negative for mutagenicity in bacteria (Butler et al., 2015; Guo et al., 2016). The lack of mutagenicity was explained by the inability of bacteria to take up AgNPs (Butler et al., 2015; Guo et al., 2016). However, AgNPs induced mutagenic and clastogenic effects in mammalian cells as shown by mouse lymphoma and micronucleus assays, respectively (Butler et al., 2015; Guo et al., 2016; Vecchio et al., 2014). The effects of AgNPs were similar to those of soluble Ag salts (Ag acetate or Ag nitrate), indicating that both AgNPs and Ag^+ can induce genotoxic effects in cultured cells. In contrast to *in vitro* studies, animal studies that examined micronucleus formation in response to AgNP exposure reported mixed results (Dobrzynska et al., 2014; Kim et al., 2011; Kim et al., 2008; Kovvuru et al., 2015; Li et al., 2014; Patlolla et al., 2015). These studies were performed using different routes of exposure (intravenous, oral and inhalation), treatment durations and AgNP doses. In addition, AgNPs with different sizes and surface coatings or without coatings were used. These differences are likely to contribute to different study results. Further studies linking physiochemical properties of AgNPs to a specific genotoxic outcome and route of exposure are needed to understand whether and what kind of AgNPs pose genotoxic and cancer risks.

Our previous study showed that oral exposure of mice at a high dose (500 mg/kg) of PVP-coated AgNPs resulted in DNA damage and genomic instability in multiple tissues (Kovvuru et al., 2015). The current studies were conducted to understand whether genotoxicity can be induced at a significantly lower dose (4 mg/kg) and whether nanoparticle coating modulates the effect. In addition, to understand whether Ag^+ are genotoxic *in vivo*, we also examined the effect of silver acetate (soluble Ag salt that is used as a source of Ag^+). The current studies were performed at a dose equivalent to 800× the EPA oral reference dose (RfD) for Ag (Varner et al., 2010). RfD refers to a daily chronic oral exposure dose in humans that is considered to be safe, while 800× RfD represents the upper range of potential Ag exposure in humans that is associated with development of argyria (permanent discoloration of the skin) after ingestion of colloidal Ag solutions (Chung et al., 2010; Wadhera and Fung, 2005). Little is known how much Ag can be received from the use of AgNP-containing consumer products. A study that characterized AgNPs in selected consumer products estimated that oral exposure to AgNPs when drinking milk formula from a sippy cup is 1.53 μg Ag/kg (Tulve et al., 2015). Significantly higher exposure levels can be anticipated from unregulated use of AgNP dietary supplements and could potentially reach 800× RfD. In addition, irrespective of whether or not human exposure data is available, dose selection in animal studies involves considerations of interspecies differences, in particular, higher susceptibility to toxicants, longer exposure durations and large inter-individual differences in humans *versus* laboratory rodents. These considerations imply that in order to identify a possible adverse health effect in a small group of animals and translate findings to human populations, doses that are several orders of magnitude higher than human exposure levels are used in animal studies.

In addition to oral exposure studies in whole animals, we examined the behavior and fate of citrate-coated AgNPs and PVP-coated AgNPs in an *in vitro* gastrointestinal digestion model (Brandon et al., 2006; Versantvoort et al., 2005; Walczak et al., 2013). We found that citrate-coated AgNPs were more susceptible to agglomeration than PVP-coated AgNPs in digestive juices, with or without proteins, and induced genotoxic effects in the systemic circulation and bone marrow. In contrast, ingestion of PVP-AgNPs or Ag^+ did not result in genotoxicity.

2. Materials and methods

2.1. Nanoparticles and reagents

AgNPs manufactured by nanoComposix (San Diego, CA) were supplied by the National Institute of Environmental Health Sciences Centers for Nanotechnology Health Implications Research (NCNHIR) consortium. Particles were provided as 20 nm citrate- or PVP-stabilized aqueous 1 mg/ml dispersions (BioPure™). Citrate-coated AgNPs were in 2 mM sodium citrate (pH 7.0) and PVP-coated AgNPs were in water. Ag acetate (AgOAc) and sodium citrate were purchased from Sigma-Aldrich (St. Louis, MO). Chemicals used for the preparation of artificial gastrointestinal juices were obtained from Sigma-Aldrich.

2.2. AgNP characterization

Physicochemical characterization of AgNPs was performed by the manufacturer (nanoComposix) and by the Nanotechnology Characterization Laboratory at the National Cancer Institute. AgNPs were also characterized in-house with dynamic light scattering (DLS) and UV–visible (UV–vis) spectroscopy prior to use. DLS was performed on samples diluted 1:100 in deionized water with a Zetasizer Nanoseries (Malvern Instruments, Westborough, MA) to characterize AgNP size distribution. UV–visible spectroscopy was performed on samples diluted 1:30 in deionized water with a NanoDrop1000 Spectrophotometer v3.8 (Thermo Fisher Scientific, Franklin, MA) to monitor AgNP colloidal stability.

2.3. Mice and treatments

C57BL/6J p^{un}/p^{un} mice (Jackson Laboratory, Bar Harbor, ME), congenic to C57BL/6J strain, were housed in the virus-free animal facility at the University at Albany Cancer Research Center under standard conditions. All procedures were approved by the institutional animal use and care committee. Seven to 10 week-old mice in equal proportions of males and females were used. There were 3 treatments that