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Full Length Article

Silver nanoparticles exhibit coating and dose-dependent neurotoxicity in glutamatergic neurons derived from human embryonic stem cells

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ABSTRACT

Silver nanoparticles (AgNPs) are used extensively as anti-microbial agents in various products, but little is known about their potential neurotoxic effects. In this study, we used glutamatergic neurons derived from human embryonic stem cells as a cellular model to study 20 nm citrate-coated AgNPs (AgSCs) and Polyvinylpyrrolidone-coated AgNPs (AgSPs) induced neurotoxicity. AgSCs significantly damaged neurite outgrowths; increased the production of reactive oxygen species and Ca^{2+} influxes; reduced the expression of MAP2, PSD95, vGlut1 and NMDA receptor proteins at concentrations as low as 0.1 μg/ml. In contrast, AgSPs exhibited neurotoxicity only at higher concentration. Furthermore, our results showed that AgSCs induced glutamate excitotoxicity by the activation of calmodulin and the induction of nitric oxide synthase; increased the phosphorylation of glycogen synthase kinase-3 α/β at Tyr^{216} and Tau at Ser^{396} and reduced the expression of Tau46, which are typically observed in Alzheimer's disease. This study indicated that stem cells can provide an excellent platform for studying nanoparticle induced neurotoxicity.

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1. Introduction

Nanoparticles (NPs) are ultra-fine materials (1–100 nm in length or diameter) that have gained enormous popularity in modern technology due to their wide variety of potential applications in the biomedical, optical, and electronic fields (Hoet et al., 2004; Kirkpatrick and Bonfield, 2010). Silver nanoparticles (AgNPs) possess potent antibacterial and antifungal characteristics and are one of the most widely used metal NPs with applications in medical equipment, textiles, cosmetics, and plastics (Ahamed et al., 2010; Chen and Schluesener, 2008; Yang et al., 2010).

Due to their infinitesimal size and unique chemical properties, AgNPs have the potential to affect cellular structures and functions. For example, AgNPs can pass through biological membranes, be transported into mitochondria (Foley et al., 2002), and affect currents through ion channels (Kirchner et al., 2005). Additionally, NPs are redox active and can induce the production of intracellular reactive oxygen species (ROS) and superoxide anions, causing oxidative stress in human cells (Colvin, 2003). AgNPs can be constructed with different shapes, sizes and surface properties by varying the type of capping agent used to prevent aggregation. Nanoparticle coatings are intended to promote stability and dispensability through surface polarity that prevents agglomeration, and Citrate and polyvinylpyrrolidone (PVP) coated are the most commonly employed coating/stabilizing agents (El Badawy et al., 2010; Tolaymat et al., 2010). In this study, 20 nm electrostatically stabilized citrate-coated AgNPs (AgSCs) with a zeta potential of −48 mV and 20 nm sterically stabilized polyvinlypyrrolidone (PVP) with a zeta potential of −37 mV were chosen to study. The zeta potential is a key indicator of the stability of colloidal dispersions. The magnitude of the zeta potential indicates the degree of electrostatic repulsion between adjacent particles. The colloids with low zeta potentials tend to coagulate or flocculate. The colloids with a high zeta potential will resist aggregation and confer stability. So AgSC (−48) with high zeta potential (negative or positive) disperses better in the solution than AgSP (−37). Several studies confirmed citrate coated AgSCs elicit greater cytotoxicity due to its higher dissolution compared to the PVP coated AgSPs (Angel et al., 2013; Tejamaya et al., 2012).

Over the past several years, several groups have explored the potential neurotoxicity of AgNPs in animal models (Rungby and Danscher, 1983a, 1983b; Tang et al., 2009), primary neuronal cell cultures (Haase et al., 2012; Xu et al., 2013), and neuronal cell line

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models (Hadrup et al., 2012). For instance, studies reported that animals treated with AgNPs or CuO exhibited cognitive impairment, motor deficits and cellular alterations in the brain (An et al., 2012; Liu et al., 2012). In mixed primary neuronal cell cultures of the mouse frontal cortex, AgNPs induced an acute intracellular rise in calcium that was followed by a strong oxidative stress response and cytotoxicity in both neurons and glial cells (An et al., 2012; Haase et al., 2012). Other studies revealed that AgNPs could alter excitatory glutamatergic synaptic transmission and receptor functions (Haase et al., 2012). AgNPs also changed cellular excitability by affecting the voltage-gated sodium (Liu et al., 2009) and potassium channels (Liu et al., 2011) in primary CA1 neurons from mice. But most studies on AgNPs' effects on neuronal cells have been conducted in neuronal cell lines and in mixed or primary neuronal cultures. Here we report the use of human embryonic stem cell (hESC)-derived glutamatergic neurons (hGN) as a cellular model to evaluate the neurotoxicity of two different coating nanoparticles at different concentration, and investigate the molecular mechanism of neurodegeneration associated with AgNP exposure.

2. Materials and methods

2.1. Human embryonic stem cell (hESC) culture

Human embryonic cells (H9 lines, National Stem Cell Bank code WA09) were ordered from Wicell and maintained in mTeSR1 medium (STEMCELL Technologies). The colonies were maintained in an undifferentiated state in Matrigel-coated (BD Biosciences) T25 flasks.

2.2. Differentiation of hESCs into glutamatergic neurons (hGNs)

Undifferentiated hESCs were treated with collagenase IV (2 mg ml^{-1}, Life Technologies). The fragmented colonies were equally distributed onto low adhesion suspension culture plates (Olympus) for the generation of neurospheres under 10% CO_2 in knockout serum replacement medium with additional supplements (SKSRM) as we recently described (Begum et al., 2015). Briefly, the neurospheres were maintained for 7 days in neuronal induction medium containing neuronal maintenance medium (NMM) supplemented with 10 μM SB431542 (Tocris Bioscience) and 1 μM dorsomorphin (Tocris Bioscience). Over the 7 days of neuronal induction, the KSRM media was gradually replaced with NMM media. NMM is a 1:1 mixture of supplemented DMEM/F12 (Life Technologies) and supplemented neurobasal (Life Technologies) medium. The DMEM/F12 medium was supplemented with 1x N2, 5 μg/ml insulin (Sigma-Aldrich), 2 mM glutamax (Life Technologies), 100 μM nonessential amino acids (Life Technologies), 100 μM 2-mercaptoethanol (Sigma), 50 U ml^{-1} penicillin and 50 mg ml^{-1} streptomycin (Life Technologies). The neurobasal medium was supplemented with 1x B-27 (Life Technologies), 2 mM glutamax (Life Technologies), 50 U ml^{-1} penicillin and 50 mg ml^{-1} streptomycin. After neuronal induction, the neurospheres were mechanically fragmented and resuspended in NMM, equally distributed onto polyornithin/laminin or Matrigel-coated plates and maintained for 1–3 weeks with NMM with a medium change every other day until hGN appeared in the culture and functional synapses were generated (Begum et al., 2015).

2.3. Exposure of hGNs to AgNPs

The 20 nm citrate-stabilized colloidal silver nanoparticles (AgSCs) and the polyvinlypyrrolidone (PVP)-stabilized colloidal silver nanoparticles (AgSPs) were generous gifts from the National Institute of Environment and Health Sciences (NIEHS). The materials were characterized by the Nanotechnology Characterization Laboratory (NCL, Supplementary Table 1). The average primary particle size and the size distribution were determined using high-resolution transmission electron microscopy (TEM). The silver concentration was determined by inductively coupled plasma mass spectrometry (ICPMS). Both samples had the same silver concentration (1.1 mg/g). The physical and chemical characterization data of these nanoparticles can be found in the booklet provided by NIEHS (NCL-NIEHS201305A). The AgNPs were supplied in water at a concentration of 1 mg/ml. The dosing solutions were prepared in cell culture media (NMM) for the treatments. To dissolve the AgNPs in medium, different concentrations of AgNP-containing media were sonicated twice for 1 min at room temperature at 40 W to prevent agglomeration. The dosing of AgNPs was based on our previous study. Different AgNPs mass concentrations (0, 0.1, 1, 5, 10, 30 and 50 μg/mL) had been used to test the apoptosis response with Caspase 3/7 cell viability kit (Thermofisher) first, and then the min inhibitory concentration (IC_{25}), half maximal inhibitory concentration (IC_{50}), and max inhibitory concentration (IC_{100}) was selected as particular doses based on the administered dose-response relationships curve. So three concentrations of AgNPs (0.1, 1.0 and 5.0 μg/ml) were chosen added to the hGNs, followed by a 72 h incubation to determine changes in neuronal morphology, toxicity, ROS production and the expression of specific markers (i.e., MAP2, vGLUT1, PSD95, pGSK, pTau, and Tau46).

2.4. Lactate dehydrogenase (LDH) assay

The LDH cytotoxicity assay was used to evaluate the neurotoxicity of AgNPs for hGN. After 72 h of incubation with AgNPs, the lactate dehydrogenase (LDH) levels leaking into the media were measured as an index of toxicity using a LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific) with the absorption read at 490 nm as previously described (Begum et al., 2015). Each data point was determined in triplicate, and the S.D. values did not exceed 5%.

2.5. Reactive oxygen species (ROS) assay

AgSP and AgSC (0.1, 1.0 and 5.0 μg/ml)-treated and untreated hGNs were incubated with 5 μM CellROX Deep Red Reagent (GIBCO, Cat # C10422) for 30 min in the dark at 37 °C with 5% CO_2 in NMM and then washed 3 times in PBS (pH 7.2–7.6). The CellROX Deep Red Reagent is a cell-permeant dye with absorption/emission maxima of ~644/665 nm. Images were taken with an Evos fluorescence microscope (Life Technologies, Cy5 channel).

2.6. Neurite outgrowth assay

The hGNs were grown in 24-well and flat bottom 96-well plates for the staining and quantification of cells, respectively. To visualize and quantify the relative neurite outgrowth and cell viability with or without AgNPs, a neurite outgrowth cell kit (Life Technologies) was used to stain the cells according to the manufacturer's directions. Briefly, AgSPs and AgSCs were added to hGNs in three different concentrations (0.1, 1, and 5 μg/ml) for 72 h. After incubation, the cells were washed with PBS and aspirated. Then, 1X working stain solution containing the cell membrane stain and cell viability indicator was added to the cells followed by the incubation for 20 min at 25 °C. Then, the stain was immediately removed and the wells were washed with PBS. Next, 1X background suppression dye was added to each well and images were captured with an EVOS fluorescence microscope. Another set of stained cells in 96-well plates was immediately read with a microplate fluorescence reader (POLARstar Omega, BMG Labtech). Cell viability was determined by the excitation at 495 nm