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Ag-NPs induce apoptosis, mitochondrial damages and *MT3/OSGIN2* expression changes in an *in vitro* model of human dental-pulp-stem-cells-derived neurons

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ABSTRACT

Silver nanoparticles (Ag-NPs) are one of the most popular nanotechnologies because of their unique antibacterial and antifungal properties. Given their increasing use in a wide range of commercial, biomedical and food products, exposure to Ag-NPs is now a reality in people's lives. However, there is a serious lack of information regarding their potential toxic effects in the central nervous system. In this study, we investigated the biocompatibility of "homemade" Ag-NPs in an *in vitro* model of human neurons derived from dental pulp mesenchymal stem cells. Our results showed that acute exposure to Ag-NPs cause cytotoxicity, by triggering cell apoptosis, damaging neuronal connections, affecting the mitochondrial activity and changing the mRNA expression level of *MT3* and *OSGIN2*, two genes involved in heavy metals metabolism and cellular growth during oxidative stress conditions. Further studies are needed to understand the molecular mechanisms and the physiological consequences underlying Ag-NPs exposure.

1. Introduction

Silver nanoparticles (Ag-NPs), one of the most commonly used metal-derived nanomaterials, own a broad-spectrum of antibacterial and antifungal properties that are promoting their use in many life's domains. Given the high interfacial reactivity and relatively low manufacturing costs, Ag-NPs are currently used in a variety of items of commercial goods, biomedical products (such as medical implants, catheters and wound dressings), as well as in food processing and bioengineering (Ju-Nam and Lead, 2008; Kwi Jong et al., 2006; Quang Huy et al., 2013; Shahverdi et al., 2007; Sharma et al., 2009). Although exposure to Ag-NPs is becoming a reality in people's lives, there is still a lack of information regarding both the biological safety and the potential toxicological implications, particularly in the central nervous system.

In the last years, a number of *in vivo* and *in vitro* studies have started to describe the neurotoxic properties of Ag-NPs. They can easily cross the blood-brain barrier and accumulate in rodent brain after an oral or intravenous administration (Karmakar et al., 2014; Park et al., 2010; Trickler et al., 2010; Xu et al., 2015). This blood-brain barrier dysfunction may facilitate further penetration of Ag-NPs into brain tissues, causing both synaptic (Skalska et al., 2015) and neuronal (Tang et al., 2009) degeneration. Moreover, such accumulation may increase under pathological conditions (e.g., meningitis, stroke, or systemic inflammation) (Xu et al., 2013). *In vivo* experiments have also shown that Ag-NPs cause cognitive impairment, motor deficits and cellular alterations in brain (Panyala et al., 2008), while several *in vitro* studies have reported the cytotoxic or genotoxic effect of Ag-NPs in different continue or primary type of cell cultures (AshaRani et al., 2008; Foldbjerg et al., 2011; Park et al., 2011, Dhawan and Sharma, 2010; Kahru and Dubourguier, 2010; Lima et al., 2012).

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Despite this evidence, our knowledge about the toxic effects of Ag-NPs on human neurons is still limited. Nowadays, human adult stem cells represent a suitable *in vitro* neuronal model for the investigation of ion metals' toxic mechanisms (Bonaventura et al., 2015). One promising source of mesenchymal stem cells that can be easily differentiated into neurons is represented by dental pulp stem cells (DPSC). These highly proliferative cells can be isolated with a minimally-invasively protocol and simply expanded *in vitro*. They express stem cell markers, such as Sox2, Oct4, Nanog, as well as CD90, CD73, and CD105, and retain the ability to differentiate into multiple cell lineages including neuronal cell types (Ferro et al., 2012; Goorha and Reiter, 2017). Since DPSC derive from the embryonic neural crest, they represent a useful source of primary cells for modeling neurological disorders at the molecular level (Kawashima, 2012; Waddington et al., 2009).

The aim of the present work was to assess the biocompatibility of laboratory-synthesized Ag-NPs (hereafter defined "homemade") in an *in vitro* model of human neurons derived from DPSC. In particular, we developed an *in vitro* protocol to differentiate mesenchymal cells in neuronal-like cells and investigated the effect of an acute Ag-NPs exposure in terms of viability, apoptosis, neuronal network connections, mitochondrial activity and variations of oxidative stress-related genes.

2. Materials and methods

2.1. Ag-NPs generation and characterization

Silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$), ascorbic acid, polyvinylpyrrolidone (PVP) and trisodium citrate (TSC) were supplied by Sigma-Aldrich. All chemicals were used as received without further purification. Double distilled MilliQ water was used for the synthesis of the nanoparticles. A two-step procedure was carried out (Fig. 1). The first step consisted in the preparation of the silver seeds. A 20 ml volume of aqueous solution containing $AgNO_3$ (2.9×10^{-4} M) and TSC (2.5×10^{-4} M) was prepared and cooled in an ice-bath. An aqueous solution of $NaBH_4$ (0.1 M, 0.6 ml) was added dropwise with vigorous stirring and the solution became bright yellow immediately. The second step consisted in the heterogeneous nucleation and growth of the particles from seed solution. Aqueous PVP (1%, 10 ml), seed solution (100 μL), TSC (2.5×10^{-2} M, 300 ml) and ascorbic acid (0.1 M, 50 μl) were combined. To this solution, $AgNO_3$ (0.01 M, 5×50 μl) was slowly added with vigorous stirring until a yellow solution was obtained.

The synthesis procedure used in the present paper allowed us to synthesize different shape particles: sphere, prolate ellipsoids, oblate ellipsoid, prisms, ellipsoidal platelet and triangular platelet. The shape of silver nanoparticles has a large influence in the optical properties (plasmon resonance) as well as in silver ion release. This is due to the sharp or rounded nanoparticle edge.

The Ag-NPs were characterized using a Scanning Electron Microscopy (SEM, SOPRA 25 ZEISS). Images were acquired at working distance of 4 mm, using an electron beam of 3 keV and an SE detector. Scanning was performed at intermediate/low speed, averaging each line scan 10 times. Optical UV–Vis spectroscopy was carried out by a Perkin-Elmer Lambda 35 spectrometer in the wavelength range 260–900 nm using a 1 cm path cell. The Dynamic Light Scattering (DLS) measurements were carried out by a homemade apparatus as described elsewhere (Zimbone et al., 2012a; Zimbone et al., 2012b). The sample was lighted with a 660 nm diode laser power (10 mW). The incident light was vertically polarized, and measurements were carried out at 90°. The analysis of the fluctuations (due to the Brownian motion) of the scattered light was performed by the intensity auto-correlation function. The diffusion coefficient and hydrodynamic diameter (d_H) were thus measured.

2.2. Dental pulp stem cells extraction

DPSCs were isolated from five extracted teeth of five patients as described elsewhere (Gronthos et al., 2000). Informed consent was obtained from patients for the use of biological material and for the access to medical records for research purposes. Briefly, freshly extracted teeth were immediately cracked open and the pulp tissue removed, minced into small fragments of 1 mm, and then digested in 3 mg/ml collagenase type I (Gibco- Invitrogen, Carlsbad, CA) for 1 h at 37 °C. The obtained tissue pellet was suspended in Dulbecco's modified Eagle's Medium (DMEM) containing penicillin

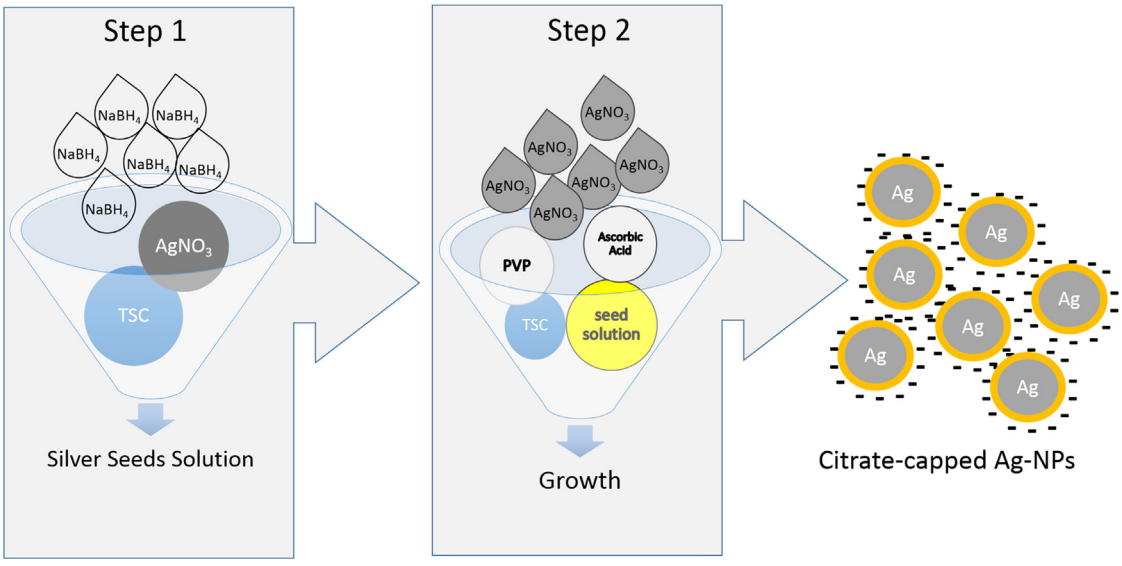

Fig. 1. Workflow of Ag-NPs synthesis. The procedure consists of two steps: Step 1) Preparation of silver seeds in 20 ml volume of aqueous solution containing $AgNO_3$ (2.9×10^{-4} M) and TSC (2.5×10^{-4} M). An aqueous solution of $NaBH_4$ (0.1 M, 0.6 ml) was added dropwise with vigorous stirring until the solution became bright yellow. Step 2) Growth heterogeneous nucleation and of Ag-NPs from seed solution. Aqueous PVP (1 wt %, 10 ml), seed solution (100 μl), TSC (2.5×10^{-2} M, 300 ml) and ascorbic acid (0.1 M, 50 μl) were mixed together. $AgNO_3$ solution (0.01 M, 5×50 μl) was added slowly with vigorous stirring until a yellow sol is obtained (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).