NeuroToxicology 46 (2015) 145–154

Contents lists available at ScienceDirect

NeuroToxicology

Synaptic degeneration in rat brain after prolonged oral exposure to silver nanoparticles

Joanna Skalska [a], Małgorzata Frontczak-Baniewicz [b], Lidia Strużyńska [a,*]

[a] Laboratory of Pathoneurochemistry, Department of Neurochemistry, Mossakowski Medical Research Centre, Polish Academy of Sciences, 5 Pawińskiego Str., 02-106 Warsaw, Poland
[b] Electron Microscopy Platform, Mossakowski Medical Research Centre, Polish Academy of Sciences, 5 Pawińskiego Str., 02-106 Warsaw, Poland

ARTICLE INFO

Article history:
Received 6 June 2014
Accepted 7 November 2014
Available online 15 November 2014

Keywords:
Nanotoxicity
PSD-95
Synapsin I
Synaptophysin
Myelin-like bodies

ABSTRACT

Neurotoxicity of silver nanoparticles has been confirmed in both *in vitro* and *in vivo* studies. However, the mechanisms of the toxic action have not been fully clarified. Since nanoparticles are likely to have the ability to enter the brain and significantly accumulate in this organ, it is important to investigate their neurotoxic mechanisms. Here we examine the effect of prolonged exposure of rats to small (10 nm) citrate-stabilized silver nanoparticles (as opposed to the ionic silver) on synapse ultrastructure and specific proteins. Administration of both nanosilver and ionic silver over a two-week period resulted in ultrastructural changes including blurred synapse structure and strongly enhanced density of synaptic vesicles clustering in the center of the presynaptic part. Disturbed synaptic membrane leading to liberation of synaptic vesicles into neuropil, which testifies for strong synaptic degeneration, was characteristic feature observed under AgNPs exposure. Also a noteworthy finding was the presence of myelin-like structures derived from fragmented membranes and organelles which are associated with neurodegenerative processes. Additionally, we observed significantly decreased levels of the presynaptic proteins synapsin I and synaptophysin, as well as PSD-95 protein which is an indicator of postsynaptic densities. The present study demonstrates that exposure of adult rats to both forms of silver leads to ultrastructural changes in synapses. However, it seems that small AgNPs lead to more severe synaptic degeneration, mainly in the hippocampal region of brain. The observations may indicate impairment of nerve function and, in the case of hippocampus, may predict impairment of cognitive processes.

1. Introduction

Nanoparticle systems are comprised of ultra-fine materials ranging from 1 to 100 nm and possess properties different from properties of their bulk form (Wagner et al., 2006). These systems have many potential applications. Silver nanoparticles (AgNPs) are commonly used in a wide range of applications, including medical products, cosmetics and household goods, due to their strong antibacterial and antifungal activity (Chen and Schluesener, 2008; Kubik et al., 2005; Marambio-Jones and Hoek, 2010). This cytotoxic effect observed in microbes raises questions about potential toxic effects on cells in living organisms, including humans. Due to the widespread use of nanosilver in consumer products, there is a high potential risk of straightway oral, transdermal or inhalatory exposure (Christensen et al., 2010). Moreover, silver nanoparticles may be released into the environment with wastewater and contaminate natural water systems (Blaser et al., 2008), directly affecting aquatic organisms (Fabrega et al., 2011; Christen et al., 2013) and indirectly affecting humans through the food chain.

A number of studies on the toxicity of AgNPs to various cellular systems *in vitro* have shown that AgNPs influence the function of mitochondria, perturb cellular respiration, and increase free radical production, consequently leading to oxidative stress and cell death (AshaRani et al., 2009; Foldbjerg et al., 2009; Piao et al., 2011; Zieminska et al., 2014). In addition, various animal experimental models have also linked oxidative stress to AgNP-induced cytotoxicity (Eom et al., 2013; Struzynski et al., 2014; Wu and Zhou, 2013). In nanoAg-exposed fish (Scown et al., 2010) and rodents (Rahman et al., 2009) up-regulation of oxidative stress-related genes has been demonstrated. Generation of excessive reactive oxygen species by AgNPs was shown to lead finally to cell death trough the apoptotic pathway (Piao et al., 2011; Yin et al., 2013).

It is considered that AgNPs may enter the vertebrate brain via the upper respiratory tract and sensory nerves in the olfactory bulb

* Corresponding author. Tel.: +48 226086576.
E-mail address: lidkas@imdik.pan.pl (L. Strużyńska).

http://dx.doi.org/10.1016/j.neuro.2014.11.002
0161-813X/

while administered by inhalation (Oberdörster et al., 2009) or by permeating the blood–brain barrier (BBB) – during systemic or oral administration (Hoet et al., 2004; Sharma et al., 2009). However, the mechanisms of the neurotoxic effects of AgNPs, and nanoparticles in general, are still under investigation being of importance due to high susceptibility of the brain to oxidative stress. The ability of AgNPs to freely cross cell membranes, particularly the membranes of nervous cells, is of great concern in neuroscience and relevant to development of new therapeutic technologies, including drug delivery (Yang et al., 2010). It was shown that exposure to nanosilver induces destruction of the BBB (Yang et al., 2010), astrocyte swelling, and causes neuronal degeneration (Tang et al., 2009). Orally administered AgNPs affected also neurotransmitters (5-HT and dopamine) concentration in rat brain (Hadrup et al., 2012). Under pathological conditions, mainly those connected with enhanced permeability of cerebral microvessels, accumulation of AgNPs in brain parenchyma is augmented and may aggravate the existing brain pathology. AgNP-treated rats, further subjected to heat stress, showed greater BBB disruption leading to edema formation, impairment of cognitive and motor functions and brain damage (Sharma and Sharma, 2007).

It was previously shown that exposure to AgNPs inhibits extension of neurites and results in degeneration of neuritic processes of cultured neurons (Xu et al., 2013). However, until now no data have been provided concerning morphological changes in neurons after *in vivo* administration of low doses of small AgNPs.

The present paper describes the results of prolonged exposure of rodents to defined commercial AgNPs with a focus on brain tissue. The investigations are focused on ultrastructural observations combined with relevant biochemical analysis of synapse-specific proteins (synapsin I, synaptophysin and PSD-95) which have become established as molecular markers of synaptic density (Masliah and Terry, 1993).

The mechanism of AgNP-induced toxicity depends partly on release of Ag ions (Lubick, 2008; Meyer et al., 2010). However, nanoparticle-specific effects are not excluded (Yin et al., 2011; Zieminska et al., 2014). Thus, in this *in vivo* conducted study we compare the toxic effects of both forms of silver.

2. Materials and methods

2.1. Silver nanoparticles

Commercially-available AgNPs obtained from Sigma–Aldrich (CAS No. 730785) were used throughout the study. This material is defined by the manufacturer as a colloidal solution of nanoparticles 10 ± 4 nm in diameter, stabilized in sodium citrate. Sodium citrate prevents sedimentation and agglomeration of nanoparticles, thereby protecting their dispersed state. Nevertheless, additional characterization of the degree of dispersion and median diameter was performed using transmission electron microscopy (JEM-1200EX, Jeol) according to a standard method developed for non-biological preparations and commonly used during nanoparticles TEM analysis (Lee et al., 2013b), i.e. suspension of AgNPs in citrate was transferred onto formvar coated grids and dried in air. Nanoparticles were estimated, counted and measured under TEM equipped with digital camera MORADA using iTEM 1233 software (Olympus Soft Imaging Solutions GmbH, Germany).

2.2. Animals and experimental design

Male Wistar rats from Animal House of the Mossakowski Medical Research Centre, Polish Academy of Sciences (Warsaw, Poland) were used throughout the study. All experiments using animals were carried out in accordance with the international guidelines on the ethical use of animals. Experimental protocols were approved by the local Animal Care and Use Committee. Wistar rats are widely used in neurotoxicological studies, including those related to nanosilver-induced toxicity (Hadrup et al., 2012; Bidgoli et al., 2013). The body weight of the animals at the beginning of the study was 180–210 g ($N = 33$). All animals were housed conventionally with two per cage and kept in a temperature and humidity controlled environment under a 12 h light/dark cycle. During the experiment, the animals were allowed free access to drinking water and standard feed. We randomly divided rats into three groups, each consisted of 11 animals: a negative control group (treated with saline) and two silver-exposed groups, one treated with colloidal solution of citrate-stabilized silver nanoparticles (AgNPs), and another treated with ionic form of silver (silver citrate, Ag^+), Oral administration was applied as a relevant route of exposure because of extensive use of AgNPs in materials related to food packaging (Cushen et al., 2014).

Solutions of AgNPs stabilized in citrate buffer or silver citrate were administered once daily via the gastric tube at a dose of 0.2 mg/kg b.w. per day for 14 days (0.02 mg AgNPs or Ag^+/mL). The rats from the control groups received the same dose of saline. The dosing volume was 2 mL/kg b.w.

Exposure to any silver formulation did not affect either the body weight of rats or their general appearance.

2.3. Determination of silver with inductively-coupled plasma mass spectrometry (ICP-MS)

The animals were sacrificed on the day after the final dose and samples of brain tissue and blood were prepared for analyses of silver concentration by ICP-MS (Elan 6100 DRC Sciex Perkin Elmer, Canada). Blood was collected from the neck after decapitation and serum was prepared by centrifugation ($1000 \times g$, 4 °C, 10 min). After dilution of serum with 1% Triton solution, the concentration of silver was analyzed. The detection limit was 0.17 μg/L.

Additionally, the bioavailability of citrate-stabilized AgNPs was determined by measuring blood concentrations of silver 3 h, 5 h and 8 h after treatment with a single dose.

Forebrains were taken from three to four animals per group. The weighted tissue was lyophilized and digested with concentrated nitric acid. Thereafter, the concentration of silver in the digested fluid was analyzed. The detection limit was 0.241 μg/kg wet tissue.

Quantification results were verified by addition of an internal standard (with 3 concentration levels) using dissolved silver (ICP Multielement Standard, TraceCERT(R), Sigma Aldrich).

2.4. TEM analysis of brain samples.

After deep anesthesia with Nembutal (80 mg/kg b.w.), the animals were perfused through the ascending aorta initially with 0.9% NaCl in 0.01 M sodium-potassium phosphate buffer pH 7.4 and after with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Tissue was sampled from the forebrain cortex and the hippocampus of all rat groups. Samples of the dorsal hippocampus were collected according to the brain atlas of Paxinos and Watson (2007), from the CA1 region, level −4.16 to −4.30 from Bregma. Specimens were fixed in the above ice-cold fixative solution and post-fixed in 1% OsO_4 solution. Subsequently the material was dehydrated in the ethanol gradient and embedded in epoxy resin (Epon 812). Ultra-thin sections (60 nm) were stained with 9% uranyl acetate and lead nitrate and examined by transmission electron microscopy (JEM-1200EX, Jeol, Japan).

Additionally, another group of sections was left unstained during the processing. This step (contrasting with metal compounds; Pb, U) which is often associated with the occurrence of