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Genotoxicity and oxidative stress in fish after a short-term exposure to silver nanoparticles

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ABSTRACT

This study examined the effects of waterborne silver nanoparticles (AgNPs) on juvenile fish *Piaractus mesopotamicus* ("pacú"), and analyzed toxicological endpoints such as metal burdens, oxidative stress and genotoxicity in a short-term assay. Fish were individually exposed to 0 (control), 2.5, 10, and 25 μg AgNPs/L. After 24 h, silver accumulation was greater in the brain than the liver and gills at all silver concentrations. Fish exposed to higher AgNPs concentrations showed major alterations in oxidative stress markers. An increase in lipid peroxidation (LPO) levels was observed in the liver of fish exposed to 10 μg AgNPs/L with no changes in the antioxidant enzymes activities. In the case of the 25 μg AgNPs/L treatment, a hepatic activation of the enzymatic antioxidant defense occurred, and LPO levels resulted unaltered. On the other hand, the brain presented the highest LPO levels at both 10 and 25 μg AgNPs/L exposures. The AgNPs toxicity was also evidenced by the DNA damage in fish erythrocytes at higher concentrations. Summarizing, a short exposure to sublethal concentrations of AgNPs is enough to generate deleterious effects on fish, including DNA damage.

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1. Introduction

There are many consumer products and applications utilizing silver nanoparticles (AgNPs). "The Nanodatabase" is an inventory of commercially available products that claim to contain engineering nanoparticles in the European consumer market. This inventory is updated daily, and currently lists 2362 products of which 353 are registered as silver containing products (The Nanodatabase, 2017). Several AgNPs applications are mainly related to the exceptionally broad spectrum of silver bactericidal activity (Kim et al., 2007; Marambio-Jones and Hoek, 2010). The low cost of manufacturing AgNPs has also made them the largest and fastest growing class of nanomaterials in product applications such as plastics, metals, textiles, and in medical and veterinary devices (Ahamed et al., 2010; Messaoud et al., 2010; Rather et al., 2011; Rhim et al., 2013). Moreover, the nanoparticle-based vaccines and the use of nanoparticles as tools for diagnosing bacterial, fungal and viral diseases, is an emerging field in fish research (Shaalan et al., 2016). However, the potential environmental impact of AgNPs has not been fully understood yet (Massarsky et al., 2014a), and their toxic properties deserve further analysis.

AgNPs may be discharged to the environment by several routes: manufacturing, incorporation into goods, and goods recycling or waste (Fabrega et al., 2011). Recent studies have reported that AgNPs may be released from biocidal plastics, textiles, paints and

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other home products (Benn et al., 2010; Lorenz et al., 2012; Voelker et al., 2015), reaching the aquatic environment. Such AgNPs input occurs mainly by their releasing in wastewaters which reaches sewage treated plants (Kaegi et al., 2011). According to Blaser et al. (2008) silver residues from Europe, Asia and North America reached 190–410 t/year and between 11.5–31.7% of those residues had been treated in wastewater plants and were found in receiving natural water. Although most AgNPs underwent a wastewater treatment process, a significant proportion (about 10%) was released in the effluent (Gottschalk et al., 2009). Environmental concentrations of AgNPs in surface water are still unknown but according to the estimates the concentrations may range 40–320 ng/L (Blaser et al., 2008) and 0.09-2.63 ng/L (Gottschalk et al., 2009). In a recent study, Bruneau et al. (2016) investigated the fate, bioavailability of AgNPs and their effects on fish using municipal effluents. This study consisted of juvenile rainbow trout exposure to 40 μg AgNPs/L or 4 μg $AgNO_3$/L in diluted (10%) municipal wastewater for 96 h, yielding that both forms of silver produced immunotoxic effects and AgNPs in wastewater were bioavailable to fish despite of their formation of aggregates.

The AgNPs toxicity is closely related to their transformation in biological and environmental media, including surface oxidation, silver ion release, and interaction with biological macromolecules (McShan et al., 2014). AgNPs can interact with membrane proteins and activate signaling pathways, leading to inhibition of cell proliferation (Braydich-Stolle et al., 2010; Roh et al., 2012). These nanoparticles can also enter the cell through diffusion or endocytosis causing a mitochondrial dysfunction (Bressan et al., 2013) and generating reactive oxygen species (ROS) inside the cell. Previous studies demonstrate that oxidative stress could be involved in the toxicity of AgNPs. Some authors have correlate ROS accumulation with the activation of antioxidant enzymes and the depletion of glutathione content in vitro in rat liver cells (Hussain et al., 2005), mouse germline stem cells (Braydich-Stolle et al., 2005), human cells (Piao et al., 2011; Rosarin et al., 2012), and fish cells (Massarsky et al., 2014b; Taju et al., 2014). In addition, Asharani et al. (2009) have suggested that the disruption of the mitochondrial respiratory chain by AgNPs increases ROS production and interrupts ATP synthesis, leading to DNA damage. These AgNPs deleterious effects on DNA have already been demonstrated *in vitro* with human and other mammalian cells (Ahamed et al., 2008; Singh et al., 2010). Some studies have investigated *in vivo* the effects of AgNPs in fish. Results indicate that these nanoparticles are accumulated in the gills and liver tissue affecting the ability of fish to cope with low oxygen levels and inducing oxidative stress (Bilberg et al., 2010; Scown et al., 2010). Massarsky et al. (2013) reported decreased glutathione content (GSH) in zebrafish embryos exposed to AgNPs. Such depletion has been observed by other authors along with an increase in lipid peroxidation levels in embryos and adults medaka (Wu and Zhou, 2013a,b).

The alkaline (pH 13) version of the comet assay (Singh et al., 1988) is commonly used as it is highly sensitive and detects a broad spectrum of DNA lesions. Such assay has made it possible to evaluate DNA alterations induced by xenobiotics and has been successfully applied in fish erythrocytes exposed to different genotoxic agents (Ali et al., 2008; Cavalcante et al., 2008; Frenzilli et al., 2009; Vanzella et al., 2007). One advantage of this technique lies in the fact that it can be applied regardless of both the chromosomes size and number, and the mitotic activity. The latter is particularly important in fish because the metabolic rate fluctuates considerably with temperature making it difficult to isolate mitotically active tissue (Simoniello et al., 2009). The use of enzymes increases both the sensitivity of the assay (in terms of the ability to detect a wider range of damage overall), and more importantly its specificity. Knowledge concerning mechanisms of uptake and toxic effects of nanomaterials including AgNPs in waterborne exposure scenarios (Stone et al., 2010) is still scarce. Chae et al. (2009) and Farmen et al. (2012) carried out short-term assays in which fish were exposed *in vivo* to AgNPs (between 20 and 100 μg/L) and the gene expression related to stress biomarkers in their liver and gills was analyzed, reporting changes in heat shock protein 70, metallothionein A, Na/K ATPase, glutathione S-transferase, cytochrome P450 1A, and transferrin genes. Most studies about genotoxic effects of AgNPs are restricted to *in vitro* ones in fish cells (Munari et al., 2014; Taju et al., 2014; Wise et al., 2010). *Piaractus mesopotamicus* ("pacú") is one of the most important species for Argentinian fish farming due to its fast growth rate, easy adaptation to artificial feeding and high consumer appreciation. Besides, this is a neotropical species widely distributed in South America which have been selected due to its favorable experimental properties (it can be obtained from local fish farms and has an easy adaptation to laboratory conditions), and their sensitiveness to pollutants exposure (Bacchetta et al., 2014; de Moraes et al., 2015; Sampaio et al., 2012).

Thus, this study aimed to analyze several toxicological endpoints such as metal burdens, oxidative stress and genotoxicity in *P. mesopotamicus* exposed *in vivo* to waterborne AgNPs in a short-term assay. We hypothesize that AgNPs enter and are bioaccumulated in fish organs after 24 h of waterborne exposure. The presence of AgNPs leads to an activation of the antioxidant defense system and the occurrence of lipid and DNA damage if antioxidant enzymes were unable to overcome oxidative stress.

2. Materials and methods

2.1. AgNPs suspension, preparation and characterization

A colloidal suspension of 1% w/v AgNPs was provided by Nanotek S.A., which manufactures the product under the brand name nanArgen®. According to the Material Safety Data Sheet (MSDS) of nanArgen®, the main ingredient of the product (> 99.9%) is metallic silver (CAS Number 7440-22-4), with an average particle size of 50 nm. To synthesize the nano-sized silver colloid, silver nitrate was dissolved in Milli pore water to a concentration of 0.20 M and mixed with an aqueous solution of 0.1 M polyvinyl pyrrolidone (PVP) as the stabilizing agent. Next soluble nanocrystalline cellulose in a 0.02 M solution was added as a reducing agent. The reaction mixture was then placed in a pressurized reactor and held at 130 °C for 30 min. All reagents and solvents were used without any further purification. To evaluate particles size and surface charge of AgNPs suspension, dynamic light scattering (DLS) and zeta potential measurements were carried out using a Zeta-Sizer Malvern (Model Nano-ZS). Transmission electron microscopy (TEM) was used to visualize and confirm the DLS results; TEM was performed in a JEOL JEM 1010 equipment. Energy dispersive spectroscopy (EDS) was employed to chemical characterization of the nanoparticulate silver suspension. Preparation of colloidal AgNPs samples for EDS included initial filtration, dilution 1:100, centrifugation and redispersion of 50 ml aliquots in 100 ml of pure water. The EDS analysis of nanArgen® stabilized on a calcium carbonate crystal was performed as follows: no peaks were omitted in the spectrum and all elements were analyzed (normalised).

In parallel, the release of Ag+ ions from AgNPs was evaluated. For this purpose, the AgNPs suspension was filtered at different times using Vivaspin™ ultrafiltration devises (30 kDa MWCO, Sartorius Stedim Biotech Gmbh) and the filtrate analyzed by Atomic Absorption Spectrometry in a VGP 210 atomic absorption spectrometer (BuckScientific, East Norwalk, CT, USA) by the electrothermal atomization method using pyrolytic graphite tubes.

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