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Nanosilver toxicity in gills of a neotropical fish: Metal accumulation, oxidative stress, histopathology and other physiological effects

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ABSTRACT

The widespread and increasing use of silver nanoparticles (AgNP) as biocide results in an unknown release into the aquatic environment. In order to contribute to the understanding of their potential toxicity, the aim of this study was to analyze branchial multiple biomarkers on the neotropical fish *Prochilodus lineatus*. We exposed fish to sublethal AgNP concentrations: 0 (control), 2.5 and 25.0 μg L^{-1}. After 5 and 15 days, we analyzed in gills total Ag accumulation, oxidative stress markers (antioxidant enzymes, lipid peroxidation and antioxidant capacity against peroxyl radicals), aspartate and alanine aminotransferases activities (ALT, AST) and histopathologies (morphometric analysis, proportion of the secondary lamellae available for gas exchange, reaction indexes, and organ index -I_{gills}-) that included mucus cell count (MCc). The Ag accumulation after 15 days was five times higher than after 5 days in the case of 25.0 μg AgNP L^{-1}-exposure. Regarding oxidative stress, all enzymes activities were inhibited after 5 days at both AgNP concentrations. ALT activity decreased and a reduction in the antioxidant capacity was evidenced after 2.5 μg AgNP L^{-1} and 15 days. LPO levels and AST activity increased after the highest time of exposure and AgNP concentration, and the same occurred with I_{gills}. MCc increased after 15 days at both AgNP concentrations. The results confirmed that the presence of low AgNP concentrations, in short and subchronic exposures, generates alterations in stress biomarkers and in the structure of this vital organ that are the gills.

1. Introduction

Silver has been used as an antibiotic since the ancient time, and it has had greater applications in medicine, optics, sensing, paintings and cosmetics (Chen and Schluesener, 2008; McShan et al., 2014). The exceptional characteristics of nanosilver particles (AgNP) as biocide have made them the largest and fastest growing class of manufactured nanomaterials in commercial applications (Pinto et al., 2010). The Nanodatabase (www.nanodb.dk) listed 353 consumer products containing AgNP in 2017. The use of AgNP is proliferating, and it is expected that the environment will be increasingly exposed to these materials.

When AgNP arise the aquatic media, they release Ag^+ that is one of the most toxic metal forms for organisms in natural water systems (Ratte, 1999). As well as most nanoparticles, AgNP physico-chemistry suggests they are likely to aggregate depending on concentration and type of organic matter, and solids in suspension (Handy et al., 2008). Unfortunately, precise estimations of the emissions from silver-containing materials are hampered by lack of available information about content and form of the silver in the products (Geranio et al., 2009). The release of AgNP can happen at any stage of the product life-cycle: production, transport, storage, usage and disposal (Ribeiro et al., 2014). The predicted environmental concentrations for AgNP in the aquatic environment are in the low μg L^{-1} or ng L^{-1} (Gottschalk et al., 2013). Therefore, environmental concerns have risen due to there is evidence that AgNP induce deleterious effects in aquatic systems as well in aquatic life (Choi et al., 2010).

Many investigations have proved that fish are valuable organisms to asses toxicological effects caused by AgNP (Govindasamy and Rahuman, 2012; Lee et al., 2012; Wu and Zhou, 2012; Massarsky et al., 2013; Bacchetta et al., 2016; Martin et al., 2016). Particularly, the gills constitute a multifunctional organ (respiration, ionoregulation, acid-

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base regulation, nitrogenous waste excretion) accounting for well over 50 per cent of the total surface area of the animal. They are the major site of uptake for most waterbone toxicants and also the first, and most important, site of toxic impact for many of them. Essential physiologic processes are performed by the gills and they are sensitive to both structural and biochemical disturbance of the branchial epithelium (Schlenk and Benson, 2001).

Nanosilver can be taken up by many different cells and become internalized inside the cell. High levels of Ag accumulation from AgNP has been reported in the liver, gills, kidney, intestine and muscle of fish (Scown et al., 2010; Wu and Zhou, 2012; Bacchetta et al., 2016; Martin et al., 2016). The main deleterious effect reported by AgNP is oxidative stress due to they enter the cell through diffusion or endocytosis and generate reactive oxygen species, leading to damages to proteins and acids inside the cell, and finally inhibition of cell proliferation (McShan et al., 2014). Lipid peroxidation, impairment of antioxidant enzyme system and glutathione depletion caused by AgNP has been reported in gills and liver of many fish species (Farmen et al., 2012; Govidasamy and Rahuman, 2012; Wu and Zhou, 2012; Griffitt et al., 2013; Martin et al., 2016). Regarding a higher level of damage, it has been demonstrated that AgNP caused histopathological alterations in liver, muscle and gills of fish (Wu et al., 2010; Govindasamy and Rahuman, 2012; Wu and Zhou, 2013), including mucus cells proliferation (Lee et al., 2012). Finally, other responses such as metallothioneins induction, DNA damage and gene expression have been associated with toxicity generated by AgNP (Choi et al., 2010; Gagné et al., 2012; Martin et al., 2016).

The neotropical fish *Prochilodus lineatus* has been widely used in experimental designs for being sensitive to variations in water quality and tolerant to laboratory conditions (Camargo and Martinez, 2006; Cazenave et al., 2014; da Silva and Martinez, 2014; Vieira et al., 2016). Concern and reports about toxicity mechanism related to AgNP exposures have risen through the years; however assays that include aquatic organisms exposed to chronic conditions remain scarce. Massarsky et al. (2014) considered essential to predict environmental concentrations and thus the risk associated with AgNP. In order to contribute to the understanding of their potential toxicity, the aim of this study was to analyze multiple responses in gills of a neotropical fish (*Prochilodus lineatus*) exposed to sublethal AgNP concentrations.

2. Materials and methods

2.1. Nanosilver suspension, preparation and characterization

A colloidal suspension of 1% w/v AgNP was provided by Nanotek S.A., under the brand name nanArgen®. The main ingredient of the product (> 99.9%) is metallic silver (CAS Number 7440-22-4), with an average particle size of 20–40 nm (Material Safety Data Sheet, MSDS).

The capping agent is made of glucose oligomers, mainly nanocrystalline cellulose (CAS Number 9004-34-6). NP were characterized by Scanning Electron Microscopy (SEM), Energy Dispersive Spectroscopy (EDS) and Transmission Electron Microscopy (TEM). A drop of the NP suspension was air dried onto a carbon film-coated grid. Afterwards, SEM was assessed in a Zeiss EVO 40 (Carl-Zeiss, Oberkochen, Germany) at 20000X. In addition, Energy Dispersive Spectroscopy (EDS) was employed for the chemical characterization of the nanoparticulate silver suspension.

The size and the shape of the particles were investigated by TEM. Briefly a drop of the sample in aqueous solution was deposited on 400-mesh carbon-coated copper grids. After one minute, the liquid was blotted with filter paper (Whatman no. 4). TEM was performed at room temperature using a JEOL 1011 electron microscope operating at 100 kV.

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

2.2. Fish and exposure conditions

Juvenile *Prochilodus lineatus* (n = 120; 6.81 ± 0.08 cm standard length; 6.58 ± 0.23 g) were obtained from a local fish farm. For acclimation purpose, fish were held in 120-l tanks containing well aerated dechlorinated water for two weeks, and fed once daily ad libitum with dry commercial pellets. The test water conditions were: pH 6.58; conductivity 178 mS/cm^3; total hardness 48.01 ppm CO_3Ca; calcium 16.0 ppm Ca^{++}, magnesium 2.0 ppm Mg^{++}, alkalinity 60.0 ppm CO_3Ca; 61.50 CO_3H^-.(levels of CO_3^{2} not detected). Fish feeding was suspended 24 h before the fish dissection. The laboratory conditions corresponded to 12:12 h light-dark cycles and temperature of 25 ± 1 ºC. All experiments were conducted in accordance with national and institutional guidelines (CONICET, 2005) for the protection of animal welfare. Aquaria were covered with a thin black plastic that prevented stress by management practices in adjacent aquaria.

The selected concentrations were based on previous studies (Bacchetta et al., 2016, 2017) and on the LC50–96 h value calculated previously (data not published), which was stated as 53.84 µg AgNP L^{-1} (confidence interval: 35.29 - 82.18). Fish (2 fish per 10-L aquarium) were exposed to the following AgNP concentrations: 0 (control), 2.5 and 25.0 µg AgNP L^{-1}. All treatments were replicated twenty times. Experiments were performed under semi-static conditions and solutions were renewed every 48 h by transferring the fish to another aquarium that contained the renewed AgNP concentration. During assays, fish were fed once a day in the morning (8 a.m., approximately). After 5 and 15 days, the animals were anesthetized and measured, weighted, sacrificed and dissected. For histopathological analyses, the right second branchial arch from each individual was fixed in paraformaldehyde (PAF) 4%. The remaining gill tissue was immediately frozen and stored at −80 ºC until biochemical determinations were determined.

2.3. Exposure media and tissue silver content

The measurement of total recoverable Ag in water samples was performed according to the method 200.2 described by the United States Environmental Protection Agency (US EPA, 1994), with modifications. Water samples were taken from each aquarium at: 0 h (beginning of the assay, without fish), 12 h, 24 h, 36 h and 48 h (re-dosing time). Briefly, a 100 ml aliquot from each sample was transferred to a Griffin beaker, and 2 ml of (1 + 1) nitric acid were added. The volume of the samples aliquots was reduced to 20 ml by heating evaporation, and heated at 85 °C for 30 min, covering the beakers lips.

Tissues samples preparation was carried out according to the method 200.3 proposed by the US EPA (1991), with modifications. Samples of gills were digested by adding concentrated nitric acid, heating to 95 °C, and leaving to cool. This process was repeated until all tissue was in the solution. Then, 30% hydrogen peroxide was dosed.

In accordance with the method 200.9 (US EPA, 1994), Ag was then quantified in water and tissue samples using a graphite furnace atomic absorption spectrophotometer (GF AAS, Perkin Elmer AAnalyst 800) equipped with an auto sampler and which limit of detection on the digested samples was 1 µg Ag L^{-1}. Lectures were made using a calibration curve with Merck® certificated pattern. One aliquot was injected into the GF AAS, and three readings of each run were recorded. The mean of the reading was used to calculate the amount of silver in the aliquot of digested water and tissue.

2.4. Antioxidant and oxidative damage determinations

Antioxidant enzymes extracts from gills tissue were prepared from