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Evaluation of the toxic impact of silver nanoparticles on *Japanese* medaka (*Oryzias latipes*)

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ABSTRACT

The increased use of nano-sized metallic materials is likely to result in the release of these particles into the environment. It is, however, unclear if these materials are harmful to aquatic animals. Furthermore, because the dissolution of such nanomaterials will occur, it is probable that some of the adverse effects resulting will result from the dissolved metal species. In this study, therefore, we investigated the health and environmental impact of silver nanoparticles (Ag-NPs) on *Japanese* Medaka by studying changes in the expression of stress-related genes using real time RT-PCR analysis and compared these results with those of Medaka exposed to soluble silver ions. The stress-related genes selected here were metallothionein, HSP 70, GST, p53, CYP 1A and the transferrin gene. The expression levels of each gene were determined using two different Ag-NPs dosages and were quantified by measuring the mRNA concentrations in liver extracts with the Taqman-based Real-Time PCR method. The results suggest that these two silver forms have distinguishable toxic fingerprints between them. While the Ag-NPs led to cellular and DNA damage, as well as carcinogenic and oxidative stresses, genes related with metal detoxification/metabolism regulation and radical scavenging action were also induced. In contrast, the ionic silver led to an induction of inflammatory response and metallic detoxification processes in the liver of the exposed fish, but resulted in a lower overall stress response when compared with the Ag-NPs.

1. Introduction

Manufactured nanomaterials are materials with diameters of nanometer size, while nanotechnology is one of the fastest growing sectors of the hi-tech economy. The application of nanotechnology has recently been extended to areas in medicine, biotechnology, materials and process development, energy and the environment. The broad application of this technology is in part due to the finding that, as the size of the particles were reduced, many new properties have been realized in various scientific fields, such as pharmacology, electronic engineering, magnetic fields and semi-conductors (Nam et al., 2006; Cha and Myung, 2007). Clearly, interest in the potential benefits of nanomaterials and a greater production of these materials has naturally led to an increased concern about the potential toxic effects resulting from their usage or unintentional release into the environment (Service, 2004; Moore, 2006; Nel et al., 2006; Nowack and Bucheli, 2007). A number of nanotoxicological studies so far have focused on atmospheric contamination and the respiratory effects in mammals or *in vitro* assays with mammalian cells (Warheit et al., 2006; Grassian et al., 2007; Lewinski et al., 2008). However, the use of nanomaterials is also likely to result in their release into aquatic environments and may pose risks to aquatic ecosystems (Moore, 2006; Handy et al., 2008a; Farre et al., 2009). Unfortunately, to date, no accepted risk assessment method or test guideline for nanomaterials toxicity tests in aquatic environment exists (Handy et al., 2008b)

While regulations have been issued to protect aquatic organisms from soluble toxic metals, it is crucial to determine whether the possible toxicity of metallic nanomaterials is quantitatively or mechanistically distinguishable from soluble metals. This situation is exemplified by the application of nano-sized silver particles, which are widely used due to their antibacterial and odor-fighting properties and, as such, have been extensively applied in detergents and wound dressings that end up in the environment during waste disposal (Asz et al., 2006). Few researchers, however, have investigated the toxicity of metallic nanomaterials in aquatic environments (Asharani et al., 2008; Griffitt et al., 2009). In this study, therefore, the adverse effects of the silver nanoparticles (Ag-NPs) on *Japanese* Medaka fish were assessed using six reliable and representative fish biomarkers, *i.e.*, the metallothionein (MT), heat shock protein 70 (HSP70), glutathione S transferase (GST), p53, cytochrome p450 1A (CYP1A) and transferrin (TF) genes. The metallothionein protein is a cysteine-rich (30–33%), low molecular

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weight (6–7 kD), metal binding protein which involved in homeostatic control of essential metals (e.g., Cu and Zn), detoxification of excess heavy metal ions (Cd, Hg, and Ag) and antioxidant defenses (Brouwer et al., 1989; Roesijadi, 1992; Viarengo et al., 1999). It is considered a good biomarker for metal exposure since differential expression levels have been reported in many organisms that were exposed to heavy metal contaminants (Lemoine et al., 2000; Van Cleef-Toedt et al., 2001; Sato and Kondoh, 2002; Cho et al., 2005). Likewise, higher level expression of HSP70s, a class of molecular chaperones, is often associated with a cellular response to a harmful stress or to adverse life conditions. Due to the high conservation of these proteins among eukaryotes and prokaryotes, they are very useful biomarkers that have been used extensively to monitor the impact of environmental factors on various species (Wirth et al., 2003; Mayer and Bukau, 2005). GST is a family of phase II detoxification enzymes that catalyze the conjugation of glutathione to a wide variety of endogenous and exogenous electrophilic compounds, such as therapeutic drugs, environmental toxins and products of oxidative stress (Leaver et al., 1997; Barata et al., 2005; Hayes et al., 2005). As well, GST proteins may also bind toxins and function as transport proteins. The p53 gene is a critical biomarker for analyzing the carcinogenicity and DNA damage resulting during an exposure to environmental toxicants since its product plays a key role in cell cycle arrest, apoptosis and DNA repair. Meanwhile, CYP1A induction is activated by the aryl hydrocarbon receptor (AHR) pathway, and its protein plays an essential function in the biotransformation and detoxification of endogenous and exogenous compounds. Cytochrome P450 1A is a widely accepted environmental biomarker, useful for monitoring the biological effects of several xenobiotic groups (oil compounds, dioxins, PCBs, PAHs, etc.), including heavy metals, that may be present in aquatic environments (Lewis et al., 2006). Lastly, transferrin is a blood plasma protein which plays an essential function in the transport of iron through the blood to the liver, spleen and bone marrow. Because iron metabolism is vital for cell proliferation and is associated with the innate immune system, TF is regarded as an immune system-related gene. However, in contrast with MT, the binding of iron to the regulatory regions of TF inhibits this genes expression (Welch, 1992).The goal of this study, therefore, is to measure expression levels of these six select biomarker genes to investigate whether silver nanoparticles are toxic to Medaka and, if so, to determine the toxic mode caused by dispersed Ag particles and how this may differ from the toxicity of Ag^+ ions.

2. Materials and methods

2.1. Chemicals and particle characterization

The silver nanoparticle (Ag-NPs) powder (99% pure, cat.# 484059-5G) and $AgNO_3$ (99% pure, cat.# 204390-10G) were purchased from Sigma–Aldrich, Korea. Approximately 500 mg of the Ag-NPs were sonicated for 2 min, with a 2 s pause, for 1 h followed by continuously stirring for 2 weeks in 1 L Milli-Q water. Thereafter, the suspended solution was filtered with a 200 nm nylon membrane filter (Whatman®, England). To determine the differences in the toxic responses to Ag-NPs and Ag^+ ions, $AgNO_3$ was dissolved in Milli-Q water and used at an equivalent elemental Ag mass. Prior to use, the size, zeta potential and surface areas of the Ag-NPs were characterized. The distribution, size and shape of the particles were obtained by transmission electron microscopy (TEM) and X-ray diffraction (XRD) analysis. The zeta potential of the suspended particle solution was measured using an electrophoretic light scattering spectrophotometer (ELS-8000, OTSUKA Electronics). Likewise, the concentration of the Ag-NP stock solution was determined, after filtration, using inductively coupled plasma-optical emission spectroscopy (ICP-OES) utilizing a Varian model vista PRO.

2.2. Fish housing

Japanese Medaka fish (*Oryzias latipes*, 4–5 month old), strain d-rR, are of the orange-red variety and were provided by Environmental Toxicology Laboratory, National Institute of Environmental Research, Korea. Fish were raised on an artificial dry feed (Tetramin Bits, Germany) (*ca.* 2% body weight), which was added daily to the water batch using fresh water. Furthermore, the fish were reared under a cycle of 16 h illumination/8 h dark per day with constant aeration. The same brood of fish was used for each test. The fish were acclimated for at least 2 wks and went 48 h without feeding prior to exposure to the tested chemicals.

2.3. Exposure and sampling method

Initially, acute toxicity tests were carried out to determine the dose dependent response curves for lethality when Medaka are exposed to Ag-NPs or $AgNO_3$ following the guidance for fish acute toxicity tests (Dir 92/69/EEC (O.J. L383 A, 1992)) and (EPA712-C-96-118, 1996). To avoid metabolic and microbial breakdown of the chemicals, a flow-through system was established where seven fish from same brood of the holding stock were randomly selected and exposed in a 2 L diamond glass beaker containing the desired concentration of the test chemical. Solutions with the same exposure concentrations were prepared daily and pumped into each beaker at a flow rate of 1 L/24 h to renew 50% of aquaria water each day. The Masterplex pump (system model No. 7553–70) and Masterplex tube (L/S 14) were used to supply the fresh solution. The pumping system was calibrated both before and after the test. The exposure solution pHs were always in range of 7.0–8.0 and the aquarium temperature was around 25 °C. Oxygen was dissolved *via* aeration using an air pump, sparger and PTFE-membrane filter (0.20 μm). The controls were carried out in the same manner but by pumping in only water. All tests were performed at the same time and in triplicate for biological validation.

To study changes in the gene expression levels, the exposure method was similar with the acute toxicity tests mentioned above but eight fish were included in each beaker. Two concentrations of Ag-NPs, 1 μg/L (low dosage) and 25 μg/L (high dosage), were selected for the tests. The higher dosage, 25 μg/L, was chosen based upon the findings in the acute toxicity tests, which showed this to be the lowest concentration causing loss in viability (LOEC) while the lower concentration, 1 μg/L, was selected to evaluate the environmental toxicity of Ag-NPs at a low level exposure. Additionally, 1.58 and 39.46 μg/L Ag-NO_3 were also tested. The concentrations were selected to offer an equivalent mass of Ag as in the low and high Ag-NP exposures, respectively. Two fish from each test and control group of a replication were randomly selected after a 1, 2 and 4-day exposure, kept on ice to kill it and dissected to extract the liver immediately. Two fish were taken from 10-day samples as well for the 1 μg/L tests. The extracted livers were collected in sterile 1.8 mL Effendorf tubes, chilled on ice and immediately used for RNA isolation. The tissues from each tube were pooled and the total RNA was isolated using the RNA RNeasy Mini kit (Qiagen, USA). The quantity and quality of the RNA was determined using a ND-1000 UV–vis spectrophotometer (Nanodrop, Wilmington, USA). The total RNA was diluted to the same concentration for each sample using ultra-pure nuclease-free water and kept at −70 °C until used.

2.4. Reverse transcription and real time RT-PCR

Specific primers and Taqman probes for the MT, HSP70, GST, p53, CYP1A and TF genes were designed and purchased from Applied