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Developmental Exposure to Silver Nanoparticles at Environmentally Relevant Concentrations Alters Swimming Behavior in Zebrafish (*Danio rerio*)

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Abstract

Silver nanoparticles (Ag-NP) are ubiquitous in household and medical products because of their antimicrobial activity. A consequence of the high volume of Ag-NP production and usage is increased amounts of Ag-NPs released into the environment. Their small size (between 1–100 nanometers) results in unique physiochemical properties that may increase toxicity relative to their bulk counterpart. Therefore, the goal of this study is to assess the potential toxicity of environmentally relevant concentrations of Ag-NPs in zebrafish (*Danio rerio*). Wild-type tropical 5D zebrafish embryos were exposed to Ag-NPs from 4 to 120 hours post fertilization (hpf) at 0.03, 0.1, 0.3, 1, 3 ppm (mg/L). Inductively-coupled plasma mass spectrometry (ICP-MS) confirmed concentration-dependent uptake of Ag into zebrafish as well as bioaccumulation over time. A morphological assessment revealed no significant hatching impairment, morphological abnormalities, or mortality at any concentration or time point examined. However, assessment of photomotor behavior at 3 days post fertilization (dpf) revealed significant hyperactivity in the 0.3, 1, and 3 ppm Ag-NP treatment groups. At 4 dpf, significant hyperactivity was observed only in the 3 ppm treatment group, while 5 dpf larvae exposed to Ag-NPs displayed no significant abnormalities in photomotor behavior. These findings suggest that non-teratogenic concentrations of Ag-NPs are capable of causing transient behavioral changes during development.

Keywords

silver; nanoparticles; neurotoxicity; zebrafish; development; behavior

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Conflict of Interests

The authors declare that they have no conflicts of interest.

Data Availability Statement

Data pertaining to this manuscript are deposited in figshare at DOI:xxxx.

1. INTRODUCTION

Nanoparticles, defined as any material with at least one dimension between 1 to 100 nanometers, have become ubiquitous in the environment. Their small size results in unique physiochemical properties that allow them to behave differently from their bulk counterpart, including an increased ability to interact with macromolecules and cross otherwise impenetrable biological barriers (Horie 2012). These properties make nanoparticles highly desirable for medical and industrial applications, but may also increase their potential for toxicity (Zoroddu 2014).

Silver nanoparticles (Ag-NP) are highly efficacious antimicrobials, making them one of the most widely used nanomaterials (Nowack 2011). Ag-NPs are used extensively in both household and medical items such as clothing, mattresses, children's toys, medical ointments, tube linings, and surface coatings. The high rate of Ag-NP usage and disposal has resulted in increased release into aquatic environments (Benn 2008, Geranio 2009). Recent reports demonstrating that Ag-NPs may be significantly more toxic than their bulk counterpart (Abramenko 2018, Recordati 2016) highlight the need to investigate the potential ecological impacts of these nanoparticles.

While there are limited studies evaluating Ag-NP levels in aquatic systems, Ag-NPs have been reported in surface waters in the United Kingdom at concentrations above 30 ppb ($\mu\text{g/L}$) (O'Brien 2010), and in wastewater treatment plant effluent in Germany at concentrations above 1 ppb (Li 2013). Because of the rapidly increasing use and production of Ag-NPs (Ge 2014), environmental Ag-NP levels in both Europe and the United States are expected to exceed 4 ppm (mg/L), with the potential for annual exponential increases (Fabrega 2011). Consistent with this prediction, a recent publication reported environmental concentrations of Ag-NPs in Malaysia as high as 10 to 20 ppm (Syafiuddin 2018). Despite this, the United States Environmental Protection Agency does not monitor the use of Ag-NPs and regulates them as bulk silver (Nowack 2011). Therefore, understanding the potential toxicity associated with these compounds, especially at environmentally relevant concentrations, is of increasing importance.

The zebrafish is a vertebrate model that has been used extensively to evaluate nanoparticle toxicity (Ginzburg 2018, Harper 2015, Nix 2018, Sheng 2016, Wehmas 2015). It was recently reported that exposure of embryonic zebrafish to Ag-NPs at concentrations far greater than those observed in the environment caused morphological and physiological abnormalities (Cunningham 2013, Gao 2015). Zebrafish models have also been used to show that Ag-NPs accumulate in brain tissue (Asharani 2008) and alter the expression of genes related to central nervous system (CNS) development (Xin 2015). Because of their ability to penetrate the chorion (Lee 2007, Lee 2013), Ag-NPs pose a significant threat to early-stage zebrafish embryos when many neurodevelopmental processes are at their peak (Miller 2018).

In addition, developmental exposure to Ag-NPs has been associated with functional impairments in zebrafish, including altered expression of genes related to sensorimotor function (Osborne 2016), as well as disruptions in normal swimming behavior (Asmonaite

2016, Powers 2011). However, because of the limited number of studies, variability in experimental designs, and differences in nanoparticle properties, it is important that the functional consequences of developmental exposures to Ag-NPs, particularly at environmentally relevant levels, be confirmed by independent laboratories. Therefore, the goal of this study is to evaluate whether environmentally relevant Ag-NP concentrations produce functional behavioral abnormalities in developing zebrafish. To address this, embryonic zebrafish were exposed from 0 to 5 days post-fertilization (dpf) to vehicle or increasing concentrations of Ag-NPs, and monitored for hatching success, morphology, and mortality. At 3, 4, and 5 dpf, hatched larvae were assessed for photomotor function using a light-dark photomotor assay. Our results suggest that embryonic exposure to Ag-NPs at relatively low concentrations that cause no significant hatching impairment, morphological abnormalities, or mortality, can alter normal swimming behavior.

2. MATERIALS AND METHODS

2.1 Animal husbandry and care

Fish husbandry, spawning, and all experiments with zebrafish were performed in accordance with the University of California, Davis Institutional Animal Care and Use Committee (IACUC) protocol 19391. Wildtype tropical 5D adult zebrafish (*Danio rerio*; obtained from Dr. Robert Tanguay, Oregon State University) were maintained in a standalone aquatic flow-through system (Aquaneering) in deionized water further purified by reverse osmosis at 28 ± 0.5 °C. The “system” water was supplemented with 20 g/L NaHCO_3 to maintain a pH of 7.2 ± 0.4 and 40 g/L sea salt solution (Instant Ocean) to maintain conductivity at $600 \mu\text{S} \pm 100$. Adult fish were kept on a 14:10 light:dark cycle and fed twice daily with Gemma Micro fish food (Skretting). Embryos were obtained by natural group spawning, collected within 30 min of fertilization, and kept in an incubator at 28.5 ± 0.5 °C until exposed to vehicle or Ag-NPs beginning at 4 hours post fertilization (hpf).

2.2 Nanomaterials and exposure

Ag-NPs were manufactured by QSI-Nano (QuantumSphere Inc.) and obtained from the University of California Center for Environmental Implications of Nanotechnology (UC CEIN). Detailed characterization of these particles has demonstrated a particle size ranging from 20–40 nm and an average diameter of 25 nm (Jin 2010). This particle size was determined in a medium with very similar salt concentration, pH, and conductivity as the medium used in the present study to expose the fish. Stock solutions of Ag-NPs at 1 ppt, 50 ppm, and 1 ppm were dissolved in 10 ppm alginate (Millipore Sigma) in deionized water and sonicated for 45 min. Alginate was used to bind to the surface of the Ag-NPs and prevent aggregation of the particles over time. At 4 hpf, embryos were individually plated into 96-well plates filled with standardized Embryo Medium (Westerfield 2007). All embryos were exposed from 4 to 120 hpf to either vehicle (alginate at 3 ppm) or Ag-NPs at 0.03, 0.1, 0.3, 1, or 3 ppm (Figure 1A, 1B). Embryos were maintained on a 14:10 light:dark cycle and incubated at $28.5^\circ \text{C} \pm 0.5$ in 100 μL of total treatment solution with 1 embryo per well.

2.3 Analysis of tissue concentrations of Ag

Zebrafish samples from vehicle, 0.03, 0.3, and 3 ppm Ag-NP exposure groups were analyzed at 3 and 5 dpf to quantify tissue concentrations of Ag. Samples were analyzed in triplicate with 22 to 26 larval zebrafish/sample obtained from 3 separate spawnings. Zebrafish samples, triplicate method blanks (200 μ L 18.2 M Ω /cm water), and triplicate NIST1640a digestion quality control standards (200 μ L NIST1640a Trace Elements in Natural Water - National Institute of Standards and Technology) were digested by adding 30 μ L concentrated TraceMetal Grade HNO₃ (Fisher Scientific) and 100 μ L concentrated TraceMetal Grade HCl (Fisher Scientific) then heating in a hot block that was ramped up to 95 °C over 30 min, followed by digestion at 95 °C for 1 h. 550 μ L 30% ULTREX II H₂O₂ (J.T. Baker) was added incrementally to room temperature samples with heating between additions. The following increments were used: 50 μ L at 4 °C for 15 min, 100 μ L at 67 °C for 10 min, 100 μ L at 85 °C for 45 min, 150 μ L at 95 °C for 25 min, and 150 μ L at 95 °C for 1 h. The samples were allowed to cool, then brought to a final volume of 1 mL with 18.2 M Ω -cm water prior to analysis by the Interdisciplinary Center for Plasma Mass Spectrometry at the University of California at Davis (ICPMS.UCDavis.edu) using an Agilent 8900 ICP-MS (Agilent Technologies).

2.4 Mortality and malformation assessment

Hatching success was monitored from 0 to 5 dpf to evaluate whether Ag-NP exposure resulted in hatching delays or impaired hatching. Impaired hatching was defined as the failure of an embryo to completely hatch from its chorion by 3 dpf. Embryos and hatched larvae were evaluated daily under a dissection microscope for mortality and malformations, primarily spinal curvature and yolk-sac edema (n=48/group from 3 separate spawning events). There were no statistically significant differences between control fish from different spawning events (data not shown). All assessments were conducted by researchers blinded to experimental group.

2.5 Photomotor assessment

Individual larval zebrafish were assessed for locomotor response to changing light conditions at 3, 4, or 5 dpf. Larvae in 96-well plates were placed in a DanioVision behavior system (Noldus) and subjected to a 35 min light/dark locomotor test (n=48/group from 3 separate spawning events). Temperature of the system and plates was maintained at 28.5 $\pm$ 0.5 °C using a Noldus Temperature Control Unit. The behavioral test consisted of a 10 min light period (Light 1; ~1900 lux) to allow for acclimation and to record baseline swimming behavior, followed sequentially by a 5 min dark period (Dark 1; ~0 lux) to stimulate increased swimming behavior, a 5 min light period (Light 2; ~1900 lux) to stimulate freezing behavior, and a 15 min dark period (Dark 2; ~0 lux) to observe increased swimming behavior following acclimation to the dark condition (Figure 1C). Larval behavior was recorded by the DanioVision camera with an infrared filter to allow visibility in both light and dark conditions. Total distance moved was automatically tracked using the EthoVisionXT software v 10.1 (Noldus), and all data were exported to GraphPad Prism v 7.03 for analysis. Dead and/or malformed larvae were excluded from the behavior analysis.

There were no statistically significant differences between control fish from different spawning events (data not shown).

2.6 Statistical analysis

Total distance moved for individual experimental groups was calculated for each test period (Light 1, Dark 1, Light 2, and Dark 2). Photomotor behavior and morphology/mortality data were analyzed using a one-way ANOVA to compare data across all treatments (vehicle control, 0.03, 0.1, 0.3, 1, and 3 ppm Ag-NP), and when applicable, *post-hoc* Bonferroni-Holm multiple comparisons test was used to identify statistically significant differences between groups. Significance was determined at $p < 0.05$. All statistical analysis was conducted using GraphPad Prism v 7.03.

3. RESULTS

3.1 Ag uptake

ICP-MS was used to measure total Ag in the tissue of embryonic zebrafish exposed to Ag-NPs for up to 5 dpf (Figure 1). Ag concentrations were measured in vehicle control fish at the time of dosing (Time 0) to obtain background levels, and in 3 and 5 dpf zebrafish exposed to vehicle or Ag-NPs at 0.03, 0.3, or 3 ppm. Quantifiable Ag uptake was observed in the 0.3 and 3 ppm groups at 3 and 5 dpf (Figure 2A, 2B). At both time points, the 3 ppm group showed higher Ag levels than the 0.3 ppm group, demonstrating concentration-dependent uptake. Higher levels of Ag were detected at 5 dpf than at 3 dpf within experimental groups, demonstrating accumulation of Ag in fish tissue over time.

3.2 Mortality and malformations

At 3 dpf, incidence rates for unhatched, malformed, or dead embryos remained 3%, 0%, and 5%, respectively, across all concentrations (Figure 3). At 4 dpf, new cases remained 3%, 3%, and 0% for unhatched, malformed and dead embryos, respectively. At 5 dpf, no new cases of unhatched, malformed, or dead embryos were observed. These rates were not statistically different from vehicle at any time point or concentration of Ag-NP examined (Figure 3).

3.3 Behavioral assessment

The total distance zebrafish moved was calculated for each light and dark period (Figure 1C). Data from Light 1 period was not statistically analyzed as it represents acclimation of the zebrafish to the test chamber. Statistics were performed using data generated during the Dark 1 period, representing the first activity period and acclimation to the dark condition, and the Light 2 period, representing freezing behavior. The primary period of interest was the Dark 2 period which assess total movement following acclimation to both dark and light periods.

No significant differences between experimental groups were observed in the Dark 1 (Figure 4A, 4B) or Light 2 periods (Figure 5A, 5B). This suggests that there were no differences in baseline activity or freezing behavior between vehicle and Ag-NP-treated larvae. However, effects of Ag-NPs were observed in the Dark 2 period (Figure 5C). At 3 dpf, significant

hyperactivity was observed in larvae exposed to 0.3, 1, and 3 ppm Ag-NP relative to vehicle controls. At 4 dpf, significant hyperactivity was observed only in larvae exposed to 3 ppm Ag-NP. At 5 dpf, the total distance moved by Ag-NP-exposed larvae was no longer significantly different from vehicle controls.

4. DISCUSSION

In this study, relatively low concentrations of Ag-NPs were used to assess the toxicity of environmentally relevant concentrations. The concentrations used in the present study are more than 6 times lower than those reported in the environment of Malaysia, and also below predicted levels in the United States and Europe. Of note, Ag-NPs were observed to disrupt photomotor behavior at concentrations that caused no mortality, morphological abnormalities, or hatching impairment, highlighting the potential for Ag-NPs to induce behavioral changes in the absence of overt toxicity. This observation is consistent with the finding that behavior is among the most sensitive endpoints in zebrafish toxicity screening (Gerhardt 2007).

The present study found significant concentration-dependent hyperexcitability in zebrafish following developmental exposures to Ag-NPs. This phenotype is consistent with recent literature describing hyperactivity in zebrafish following relatively low-concentration (~3 ppm) Ag⁺ exposure (Powers 2011). In addition, several other studies have reported hyperexcitability in zebrafish following low-concentration exposure to diverse chemicals, including lead (Chen 2012), bifenthrin (Frank 2018), ethanol (Blaser 2011, Irons 2010), *d*-methamphetamine (Irons 2010), and bisphenol-A (Saili 2012). Interestingly, a recent study evaluating behavioral deficits following Ag-NP exposure reported hypoactivity in exposed zebrafish (Asmonaite 2016). Although the concentrations used were similar to those in the present study (2.15 ppb-2.15 ppm), a critical difference is that the authors reported hypoactivity only at concentrations that also caused teratogenicity. Overtly toxic concentrations of Ag-NPs have been reported to degrade the motor neurons of exposed zebrafish (Muth-Kohne 2013), which potentially explains Ag-NP-induced hypoactivity. The authors did not measure zebrafish tissue concentrations of Ag, so it is possible that the teratogenicity and hypoactivity they observed reflect greater Ag uptake than was observed in our model. Additional differences between our study and theirs include the zebrafish strain used and the age at which the behavioral tests were performed. It is well documented that temporal, age-related, and genetic factors can influence zebrafish behavior in response to chemical exposure (MacPhail 2009, Padilla 2011).

The behavioral abnormalities in our model were most robust at 3 dpf and diminished over time, suggesting that the effects are transient. This does not rule out the possibility that other behavioral abnormalities manifest at later times in development. For example, in other zebrafish models of chemical-induced hyperactivity, exposed animals also showed impaired learning and memory (Chen 2012, Knecht 2017, Saili 2012). Hyperactivity has also been observed in various zebrafish models of intellectual disability (Chen 2018, Kim 2014, Lange 2018). Therefore, it may be that the hyperactivity observed in zebrafish exposed to Ag-NPs is in fact an impaired acclimation to the Dark 2 period. This interpretation is consistent with the absence of behavioral differences during the Dark 1 period as well as the diminishment

of the phenotype over time, potentially suggesting a delayed learning process in Ag-NP exposed larvae compared to vehicle controls. However, further experiments are needed to evaluate whether Ag-NP exposure impairs learning and memory processes.

Alternatively, Ag-NPs may be inducing hyperactivity via effects on the sensorimotor system. Ag-NPs have been reported to alter transcription of genes associated with photoreception and CNS development at concentrations of 0.01–2 ppm (Cambier 2018, Xin 2015), as well as to induce apoptosis in developing zebrafish at 50–60 ppm (Chakraborty 2016, Verma 2018). Ag-NP exposure has also been shown to disrupt development of the lateral line system (LLS), a critical component of sensorimotor function in the zebrafish CNS. More specifically, they have been shown to alter gene transcription in the neuromasts (Osborne 2016) and impair rheotaxis at 0.2–1 ppm, demonstrating a functional consequence of LLS disruption (McNeil 2014). These adverse effects induced by Ag-NPs represent potential mechanisms contributing to the hyperexcitability observed in our model.

5. CONCLUSIONS

Developmental exposure to Ag-NPs can cause hyperexcitability in developing zebrafish at concentrations that do not cause mortality, malformations, or hatching impairments. Of note, this behavioral phenotype was observed at concentrations below those found in some parts of the aquatic environment. Due to the rapidly increasing use of Ag-NPs, this study highlights the importance of continued mechanistic research into the behavioral effects of Ag-NP exposure.

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Figure 1. Experimental design.

(A) Schematic illustrating the exposure paradigm used to evaluate the morphological and behavioral effects of Ag-NPs in developing zebrafish. (B) Ag-NP concentrations tested in larval zebrafish. (C) Representative trace of typical zebrafish behavior in the photomotor behavioral assay. dpf = days post-fertilization; L1 = Light phase 1; D1 = Dark phase 1; L2 = Light phase 2; D2= Dark phase 2.

Figure 2. Uptake of Ag-NPs is concentration-dependent.

(A) Total silver ion concentrations ($\mu\text{g Ag}^+/\text{mg tissue}$) in 3 and 5 dpf zebrafish exposed to vehicle or Ag-NPs at 0.03, 0.3, or 3 ppm beginning at 0 dpf. Data presented as mean $\pm$ SE.

(B) Total silver ion concentrations in vehicle controls at baseline (Time 0) and in exposure groups at 3 and 5 dpf. Data presented as mean $\pm$ SD. N=3 replicate samples/group with 22–26 fish/sample. **= $p < 0.01$; ***= $p < 0.001$; # = below limit of quantitation (~ 0.5 ppb).

Figure 3. Ag-NP exposure does not induce significant hatching impairments, malformations, or mortality.

Incidence rates of unhatched, malformed, and dead zebrafish embryos following exposure to increasing concentrations of Ag-NPs or alginate vehicle (veh). Embryos were scored at 3, 4, and 5 dpf. No significant differences between groups were identified using one-way ANOVA. N=48/group from 3 separate spawning events.

Figure 4. Ag-NP exposure does not alter locomotion in the Light 1 (L1) or Dark 1 (D1) phase. (A) Behavioral traces showing mean movement of zebrafish larvae at each 1 minute time bin during the L1 acclimation phase (0–10 min) and the D1 phase (10–15 min). (B) Quantitative summary of photomotor activity showing total distance moved during the D1 phase. No significant differences between groups were observed at any D1 period as determined by one-way ANOVA. Data presented as mean $\pm$ SE. N=48/group from 3 separate spawning events.

Figure 5. Ag-NP exposure induces significant hyperactivity in the Dark 2 testing phase.

(A) Behavioral traces showing mean movement of zebrafish larvae at each 1 min time bin during the Light 2 (L1) phase and the Dark 2 (D1) phase (20–35 minutes). Quantitative summary of the total distance moved during the (B) L2 phase and (C) D2 phase for each experimental group. Significant hyperactivity was observed in the D2 phase in zebrafish exposed to Ag-NPs at 3 and 4 dpf as determined by one-way ANOVA and *post-hoc* Bonferroni-Holm multiple comparisons test. Data presented as mean $\pm$ SE. N=48/group from 3 separate spawning events. *= $p<0.05$; ***= $p<0.001$.