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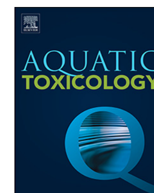

Silver nanoparticles impair zebrafish skeletal and cardiac myofibrillogenesis and sarcomere formation

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ABSTRACT

Metal nanoparticles from industries contaminate the environment and affect the normal development of fish even human health. However, little is known about their biological effects on fish embryogenesis and the potential mechanisms. In this study, zebrafish embryos exposed to/injected with silver nanopaticles (AgNPs) exhibited shorter body, reduced heartbeats, and dysfunctional movements. Less, loose, and unassembled myofibrils were observed in AgNPs-treated embryos, and genes in myofibrillogenesis and sarcomere formation were found to be down-regulated in treated embryos. Down-regulated calcium (Ca^{2+}) signaling and *loci*-specific DNA methylation in specific muscle genes, such as *bves*, *shroom1*, and *arpc1a*, occurred in AgNPs-treated embryos, which might result in the down-regulated expression of myofibrillogenesis genes and muscle dysfunctions in the treated embryos. Our results for the first time reveal that through down-regulating Ca^{2+} signaling and myogenic *loci*-specific DNA methylation in zebrafish embryos, AgNPs might induce defects of myofibril assembly and sarcomere formation *via* their particles mostly, which may subsequently cause heartbeat reduction and behavior dysfunctions.

1. Introduction

Nanomaterials have been widely used in biotechnology and life science in recent years. Over 300 t of AgNPs is produced each year and the amount is increasing with time (Nowack et al., 2011). Some previous studies have predicted the risk quotient (RQ) of AgNPs to aquatic organisms in environmental compartments (Gottschalk et al., 2009). However, their potential effects on human and environmental health still remain largely unknown.

AgNPs has been revealed to cause thickening of epithelial gill tissue, reduce the diffusion conductance of gill, induce oxidative stress, and alter gene expression profiles in cells of liver, gill, and peripheral blood in juvenile and adult fish (Bar-Ilan et al., 2009; Choi et al., 2010; Osborne et al., 2015). Recently, we report that AgNPs inhibits fish erythrogenesis and chromatogenesis in stage-specific and cell-specific manners (Cui et al., 2016; Xu et al., 2017b). Additionally, AgNPs has been reported to affect oxidative phosphorylation and cardiac muscle contraction (van Aerle et al., 2013) and to induce cardiac malformation during fish embryogenesis (Browning et al., 2013; Lee et al., 2013; Lee et al., 2007). However, little information is available about the molecular characteristics and the potential mechanisms underlying AgNPs-induced developmental defects of cardiac malformation and behavior dysfunction in an *in vivo* animal model.

Genes specific in skeletal and cardiac muscle system have been revealed to have important functions in regulating larvae behaviors and cardiac functions (Granato et al., 1996; Ha et al., 2013). Genes labeling slow fibers, such as slow myosin heavy chain 1 (*smyhc1*), *myhz5*, *tnni1a*, *tnni1d* (Devoto et al., 1996), are responsible for triggering the first locomotion and powering continuous stationary activity. Genes of fast *myhz* isoforms and troponin proteins such as *tnnt3* (Hsiao et al., 2003; Nord et al., 2014), which label fast fibers, are probably essential for powering burst-swimming in newly hatched larvae (Buckingham, 2006).

Gene expression patterns during myogenesis can be modified by epigenetic factors, and epigenetic reprogramming of the zygote and primordial germ cells is sensitive to environmental exposure to affect behaviors and cause other defects in organisms (Bohacek and Mansuy, 2015). Exposure to endocrine disruptors between E8 and E14, or to alcohol between E7 and E12, induces embryonic differentially methylated regions (DMRs) and the corresponding behavior disorders

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(Manikkam et al., 2012). Additionally, endocrine active compound bisphenol-A (BPA) (Bastos Sales et al., 2013) and metal arsenic (As) (Li et al., 2009) have also been proven to induce epigenetic alterations. Recently, AgNPs is reported to block the transcription of globin genes by altering the methylation status of histone 3 in mammalian cells (Qian et al., 2015), and to affect the epigenome of HT22 cell by increasing the levels of 5-mC, DNMT1, DNMT3a, and DNMT3b (Mytych et al., 2017). However, there have been no reports about the AgNP-mediated changes in DNA methylation pattern (Mytych et al., 2017), and very few studies have been focused on the roles of AgNPs in epigenetic reprogramming during embryogenesis, especially in myogenesis of vertebrates.

Zebrafish has been widely used in developmental biology, genetics, and pharmaceutical screening for a long time due to its unique advantages over other *in vivo* vertebrate models (Zhang et al., 2015). Recently, it has been used to assess the biological effects of contaminants because its embryos and larvae are sensitive *in vivo* systems to monitor the effects of environmental pollutants on cell specification and developmental process, and the transparency of its eggs allows easy and convenient observation (Cui et al., 2016). In this study, we tested the molecular characteristics and the potential mechanisms underlying the induction of defects by AgNPs in zebrafish embryos, particularly whether the expression of myogenic genes and Ca^{2+} signaling genes as well as the methylation of *loci*-specific DNA were affected by AgNPs treatment.

2. Materials and methods

Some detailed descriptions of materials and methods are presented in supplementary material.

2.1. Characterization of AgNPs in egg water

Citrate-coated AgNPs suspension (40 nm, 20 mg/L) was purchased from Sigma-Aldrich (Cat # 730807) as reported previously (Cui et al., 2016). In the present study, AgNPs, supernatant controls, and dissolved silver ions (Ag^+) solutions (AgNO3) were diluted with autoclaved pure water instead of E3 water (5.0 mM NaCl; 0.17 mM KCl; 0.33 mM CaCl; 0.33 mM MgSO4; 0.05% methylene blue, pH ~ 7.4) to eliminate the influence from other ions in E3 medium (Zhang et al., 2015). Media with diluted AgNPs for embryo exposure were collected at 24 hpf for Tecnai G2 Twin transmission electron microscope (TEM) (HITACHI-7650, Japan) detection, and at 0 hpf, 24 hpf, or 96 hpf for hydrodynamic diameter and zeta potential characterizations using a Zetasizer (Malvern ZETASIZER Nano-ZS) as reported previously (Cui et al., 2016), and the absorption spectra were measured *via* a UV–vis spectrophotometer (Multiskan FC, Thermo Fisher Scientific, U.S.A.) as reported previously (Cui et al., 2016).

2.2. Maintenance of fish stocks and collection of embryos

Adult zebrafish (AB strain) and adult transgenic *mylz2*-GFP fish (GFP labeling fast fibers) (Ju et al., 2003) were maintained in a circulation filtration system (circulating water with the exclusion of salt, organics, bacteria, and particles) and were fed with hatched fairy shrimp (without silver or other ions) three times per day (Zhang et al., 2015). Male and female zebrafish were kept separately until mating and spawning. After natural spawning, embryos were pooled and washed, and then fertilized embryos were collected under a dissection microscope (SMZ168, Motic, China). The collected embryos were staged by morphological features (Kimmel et al., 1995), and were used for different bioassays as described below. The ages of embryos and larvae were presented as hours post-fertilization (hpf) or days post-fertilization (dpf).

2.3. AgNPs treatment

40 nm 0.4 mg/L AgNPs was used to treat zebrafish embryos in this study, and the AgNPs supernatant solution (supernatant was collected after ultracentrifugation of AgNPs solution at 200,000*g* for 2 h) was used as the control for AgNPs to exclude the impacts of ions, small AgNPs particles, and solvent in the supernatant (Cui et al., 2016; Xu et al., 2017b). 0.024 mg/mL Ag+ solution (6% of 0.4 mg/L AgNPs, equal to the concentration of Ag+ released from 0.4 mg/mL AgNPs 16 h afterword) was set to distinguish which source of AgNPs to exert its biological effects in this study as we reported previously (Cui et al., 2016; Xu et al., 2017b). Embryos at same stages collected from the same batch were divided into parallel groups and were treatment with AgNPs (0.4 mg/L), supernatant, and Ag^+ solution (0.024 mg/L) separately. The exposure was conducted in a 28 ± 0.5 °C incubator with each group containing 50 embryos in 8 mL medium, and embryos were treated before 4 hpf as performed previously (Xu et al., 2017b). Each group was biologically repeated 2–3 times in this study. Each medium was changed every 24 h, and the exposed embryos were collected at the indicated stages for different tests including qRT-PCR and WISH.

Embryos injected with 0.4 mg/L AgNPs (5 nL/embryo) or supernatant (5 nL/embryo) in this study, and the injected embryos were used to assess the biological sensitivities of zebrafish embryos to different treatments as we reported previously (Xu et al., 2017b).

2.4. Microscopy, locomotor behaviors, and heartbeat rate

Embryos from the controls, AgNPs-exposed group, and AgNPs-injected group were observed and photographed under light microscopy (Leica M205FA) to examine the morphology, assess locomotor behaviors, and to count the heartbeats. The calculation was based on the presence or absence of the defective phenotypes. The swimming activities of embryos were tested by high-speed video (Olympus SZX16), and then the quantification of larval locomotor activities at 4 dpf was performed using the Video-Track System (ViewPoint Life Science, Montreal, Canada) as described previously (Zhang et al., 2015). The heartbeats of embryos were counted at 60 hpf and analyzed using hypergeometric distribution analysis by software of R-console. Fluorescent GFP in *mylz2*-GFP fish was observed and photographed by microscopy (Leica M205FA) with fluorescent filter, and the fluorescent intensity of the positive cells in embryos was analyzed by software of Image J [https://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health (NIH), Bethesda, MD, USA].

2.5. Histopathological analysis

Embryos at 48 hpf from the AgNPs-exposed group and the supernatant control group were fixed in PFA (4% paraformaldehade in PBS) overnight and then the fixed embryos were washed with PBS (pH 7.0) for three times before immersed in 30% saccharose-PBS buffer overnight at 4 °C. The permeated embryos were embedded in O.C.T (Optimal Cutting Temperature, Germany) and cross sectioned at 7 μm in thickness with a cryostat refrigerated Microtome (Leica). The slices were collected on polylysine anti-off slides (Boster, USA), and were dried at room temperature for 1–2 h. After that, the slices were used for H&E staining by standard techniques as performed previously (Xu et al., 2017a). Staining sections were observed and photographed directly under microscope (ZEISS Axio Imager A2).

Moreover, embryos at 48 hpf from supernatant group and AgNPs group were used for TEM dissection to examine the structure and organelles of muscle cells in AgNPs-treated embryos. Samples for TEM observations were prepared following standard procedures (detailed description was presented in supplementary materials) (Niu et al., 2014).

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