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Silver nanoparticles affect lens rather than retina development in zebrafish embryos

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ABSTRACT

Silver nanoparticles (AgNPs) have been reported to inhibit specification and differentiation of erythroid cells, chromatophores, and myofibrils during zebrafish embryogenesis. However, the knowledge of biological effects of AgNPs on eye development, especially on lens development is scarce. In this study, embryos were exposed to or injected with 0.4 mg/L AgNPs, and the results indicate that no obvious morphological changes in eye formation were observed in the stressed embryos compared to the controls. However, clefts and vacuoles were observed in lens of embryos from AgNPs stressed group. Additionally, the down-regulated expressions of different lens crystallin isoform genes and the normal expression of retinal genes were observed in AgNPs stressed embryos, suggesting AgNPs might inhibit the development of lens rather than the development of retina in zebrafish embryos. Moreover, no obvious cell apoptosis was observed, but normal nuclear DNA and RNA export was observed in lens cells. Together, the data in this study reveal that AgNPs damage the development of lens rather than retina resulting in eye abnormalities *via* some unknown mechanisms rather than *via* triggering cells apoptosis or blocking nuclear DNA or RNA export.

1. Introduction

Because of their attractive antibacterial properties, silver nanoparticles (AgNPs) are widely used in medical and living supplies such as clothing, plastics, medicine, and others fields (Gottschalk et al., 2009; Osborne et al., 2013). The risk quotients (RQ) of AgNPs (Gottschalk et al., 2009) to aquatic organisms even to human will increase constantly with the increasing consumption of commercial AgNPs. Thus, it is important to unveil the potential biological effects of AgNPs on organisms even on humans.

AgNPs were reported to disturb the early embryonic development in fish. AgNPs stressed embryos exhibited cardiac malformation, brain edema, eye abnormalities, and other developmental defects (Asharani et al., 2008; Bar-Ilan et al., 2009; Griffitt et al., 2008). Additionally, the molecular mechanism underlying some developmental abnormalities induced by AgNPs in fish embryos has been reported recently, and a previous study revealed that AgNPs inhibited erythrogenesis in developmental-stage-specific and cell-specific manners (Cui et al., 2016), suppressed specification and differentiation of melanophore and xanthophore lineages by releasing Ag^+ (Xu et al., 2017b), and induced dysfunctional locomotor behaviors and declined heartbeats in developing embryos (Xu et al., 2018). However, there is still a lack of a comprehensive understanding of the molecular mechanism of underlying AgNPs induced eye abnormalities during embryogenesis.

Zebrafish eyes are composed of lens, neural retina, and retinal pigmented epithelium cells. The zebrafish solid lens placode begins to form by the thickening of overlying surface ectoderm between 16 and 20 hpf (Greiling and Clark, 2009; Vihtelic, 2008), and the placode delaminates to yield the lens mass from 22 hpf (Greiling and Clark, 2009), then, cuboidal shaped lens epithelial cells, primary fiber cells, and secondary fiber cells form the spatial structure of the lens by 30 hpf (Greiling and Clark, 2009). However, the inner layer of the adjacent optic cup begins to generate the neural retina at 30 hpf, and newly differentiating rod and cone photoreceptors begin expressing opsin genes around 52 hpf (Dahm et al., 2007; Soules and Link, 2005). The lens is spherical and the visual system is functional till to 72 hpf during zebrafish embryogenesis (Greiling and Clark, 2009; Soules and Link, 2005). Lens tissue develops from ectoderm under the influence of retinal tissue (Iida et al., 2017; Kondoh et al., 2000), and it is reported that lens can arise from cultured embryonic neural retinal cells *via* inhibiting

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Notch signaling (Iida et al., 2017).

Lens has a relatively simple morphology composed of a monolayer of epithelial cells that cover the anterior surface and highly elongated fiber cells filling the lens core, and its fiber cells are majorly constructed with crystallin structural proteins. Mutations in different isoforms of crystallin in mouse usually lead to lens cataract (Brady et al., 1997; Cheng et al., 2016; Klopp et al., 1998). In humans, crystallin genes such as *CRYAA*, *CRYAB*, *CRYBA1*, *CRYBB1*, *CRYBB2*, *GRYGC*, *CRYGD* have been identified from patients with inherited cataract and are deemed to be cataract-related genes (Litt et al., 1997, 1998; Liu et al., 2006; Sun et al., 2005). Additionally, it is reported that HSF4 (heat shock transcription factor 4) promotes lens de-nucleation to protect lens transparency in zebrafish embryonic model (Cui et al., 2013), that this gene is a cataract-related gene in human patient (Cheng et al., 2016), and that its removal can lead to cataract development in mice (Shi et al., 2009), suggesting that there exists a gene regulation network during lens development and its cataract development in fish, in mammalian, even in human. Zebrafish mutants *lama1*^{arl} (*laminin alpha 1*) and *dsl* (*disrupted lens*) exhibit lens developmental defects (Vihtelic and Hyde, 2002; Vihtelic et al., 2001), and the *lama1* mutants exhibits defects in the lens cells without significantly affecting the organization and survival of retinal neurons at 5 dpf (Vihtelic, 2008). Moreover, the zebrafish *cdipt* (CDP-diacylglycerol: myo-inositol 3-phosphatidyltransferase) mutants exhibit eyeable lens opacity at 7 dpf (Murphy et al., 2011).

Cross-linking of lens crystallin proteins and metal ions and its potential implication of cataract development has been reported recently (Tweeddale et al., 2016). It was reported that AgNPs induced eye abnormalities during fish embryogenesis (AshaRani et al., 2009; Bar-Ilan et al., 2009). The development and maintenance of lens transparency is especially important for fish for visual food hunting. The risk quotients (RQ) of AgNPs to aquatic organisms have been paid more and more attentions with the increased consumption of AgNPs containing supplies in life, however, it is still unknown whether AgNPs induce lens defects during fish embryogenesis. Furthermore, to our knowledge, the cross-linking of AgNPs and lens cataract in vertebrates has not been investigated yet.

In order to unveil the developmental characteristics of zebrafish eyes, and the roles of crystallins in lens and its cataract process in zebrafish under AgNPs stressed environments, this study was aimed to explore the mechanism underlying AgNPs induced lens abnormalities with zebrafish embryos treated with AgNPs as previously performed (Cui et al., 2016; Xu et al., 2017). Then, the molecular characteristics, particularly, the expression of eye genes including lens and retina genes, and cell apoptosis as well as nuclear DNA and RNA export with AgNPs treatment will be examined in this study. In this study, we demonstrated that environmental pollute AgNPs specifically induced lens cataract without significantly affecting specification of retinal cells in fish, which will provide research foundation of animal model for assessment of aquatic vertebrates' hunting abilities under stressed conditions.

2. Materials and methods

2.1. AgNPs exposure and injection

Citrate-coated AgNPs suspension (40 nm, 20 mg/L) was purchased from Sigma-Aldrich (Cat # 730807) as reported previously (Cui et al., 2016; Xu et al., 2017b, 2018). It has been reported that AgNPs exhibited biological effects on zebrafish embryogenesis in a dosage dependent manner, and high percentage of defective embryos with no mortalities in embryos exposed to AgNPs at 0.4 mg/L (Cui et al., 2016; Xu et al., 2018). Thus, in this study, embryos from the same batch (spawned by several pairs of adult fish) and at the same developmental stages were collected and exposed to 0.4 mg/L AgNPs solution (40 nm, Cat # 730807, Sigma-Aldrich, U.S.A.), 0.024 mg/L silver ion solution (6% of 0.4 mg/L AgNPs, equal to the concentration of Ag^+ released from 0.4 mg/L AgNPs in pure water 16 h afterward), AgNPs supernatant solution (solvent control for AgNPs), and autoclaved pure water (the blank control) respectively. In this study, the 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) was used as the blank control to eliminate the influence from other ions in E3 medium as reported previously (Zhang et al., 2015), which was also used for dilution the aforementioned solutions as described previously (Cui et al., 2016; Xu et al., 2017b). The AgNPs supernatant solution (supernatant was collected after ultracentrifugation of AgNPs solution at 200,000 g for 2 h) was used as the solvent control for AgNPs to exclude the impacts of ions, small AgNPs particles, and solvent in the supernatant, and 0.024 mg/L silver ions was set to distinguish which source of AgNPs to exert its biological effects in this study as reported currently (Xu et al., 2018). Each exposure was initiated before sphere stage (4 hpf, hours post fertilization) and was performed as described in the previous studies with each treated group containing 50 embryos in 8 mL medium in a 6-mm dish (Cui et al., 2016; 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 24 hpf, 72 hpf, 96 hpf, 7 dpf, and 10 dpf for different tests such as phenotype observations, apoptosis assays, DNA and RNA export assays, immunofluorescence assays, qPCR, and WISH detections respectively.

In this study, in order to test the biological effects of AgNPs on embryonic development with reduced influences from egg chorion (Kim and Tanguay, 2014) or from out-embryonic environments (Liu et al., 2010) further, the embryos were injected with different concentration of AgNPs solution (5 nL/embryo) or supernatant solution (5 nL/embryo) at one cell stage (nearly 0.5 hpf). Embryos injected with 0.4 mg/L AgNPs exhibited high percentage of abnormalities but with no mortality (Xu et al., 2018), thus, 0.4 mg/L AgNPs was used for all of the injection experiments in this study as reported previously (Xu et al., 2017b, 2018). Nearly 200 embryos were injected for each group, and they were collected at 24 hpf for microarray detections, and gene expression profiles were compared between the AgNPs-exposed embryos and AgNPs-injected embryos as described in the recent study of chromatogenesis genes (Xu et al., 2017b).

2.2. Microscopy and histopathological analysis

Embryos from the blank control, the supernatant control, the Ag^+ group, or from the AgNPs exposed group, were observed and photographed under light microscopy (Leica M205FA) to examine eye development especially its lens development under AgNPs stressed conditions. For the hematoxylin and eosin (H&E) staining, embryos were collected at 72 hpf and at 10 dpf and fixed in PFA (4% paraformaldehyde in PBS) at 4 °C overnight. Then, the fixed embryos were washed with PBS 3 times and transferred and immersed in 30% saccharose-PBS buffer afterwards at 4 °C for overnight. Next, the permeated embryos were embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek, USA) for cryosectioning at 7 μm in thickness with frozen Microtomy (Leica), and the sections were collected on polylysine anti-off slides (Boster, USA) and used for H&E staining as described previously (Xu et al., 2017a). Briefly, the slides were placed in hematoxylin solution (hematoxylin, 1 g; ethanol, 50 mL; acetic acid, 5 mL; glycerol, 50 mL; potassium aluminum sulfate, 5 g; autoclaved ultrapure water, 50 mL) for 3.5 min, washed in running tap water for 20 min, next, the slides were counterstained with eosin from 15 s to 2 min depending on the age of the eosin, then, dehydrated in 95% and absolute alcohols until excess eosin was removed. Finally, the slides were cleared in xylene and mounted for observations. High-resolution images for H&E staining were obtained by using a microscope (ZEISS Axio Imager A2).