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Nanoparticulate-specific effects of silver on teleost cardiac contractility☆

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ABSTRACT

Silver nanoparticles (nAg), due to their biocidal properties, are common in medical applications and are used in more consumer products than any other engineered nanomaterial. This growing abundance, combined with their ability to translocate across the epithelium and bioaccumulate, suggests that internalized nAg may present a risk of toxicity to many organisms in the future. However, little experimentation has been devoted to cardiac responses to acute nAg exposure, even though nAg is known to disrupt ion channels even when ionic Ag^+ does not. In this study, we examined the cardiac response to nAg exposure relative to a sham and an ionic $AgNO_3$ control across cardiomyocyte survival and homeostasis, ventricular contractility, and intrinsic pacing rates of whole hearts. Our results suggest that nAg, but not Ag^+ alone, inhibits force production by the myocardium, that Ag in any form disrupts normal pacing of cardiac contractions, and that these responses are likely not due to cytotoxicity. This evidence of nanoparticle-specific effects on physiology should encourage further research into nAg cardiotoxicity and other potential sublethal effects.

1. Introduction

Nanoparticles (NPs), commonly defined as having at least one dimension between 1 and 100 nm, possess high surface area to volume ratios which lend high reactivity to materials that are relatively inert at bulk scales. This high reactivity has enabled a technological revolution leading to the incorporation of nanomaterials into hundreds of consumer and industrial products, with nanoparticulate silver (nAg) appearing more frequently in consumer products than any other nanomaterial (Vance et al., 2015). In addition to a wide range of textile, food contact, and other sanitary applications, nAg has been under development for medical use for over a decade (Project on Emerging Nanotechnologies, 2013; Vance et al., 2015). Like its constituent atoms, nAg possesses antibacterial and antifungal activity through membrane damage and permeabilization (Lok et al., 2006; Rai et al., 2009, 2012; Li et al., 2010; Mukherjee et al., 2014), and also possibly through oxidative damage (Kim et al., 2007). This activity is effective against both Gram-positive and -negative bacteria (Morones et al., 2005), making nAg a promising candidate for antibacterial applications. nAg has been used in creams and dressings to promote healing (Furno et al., 2004; Ip et al., 2006; Leaper, 2006; Tian et al., 2007; Lorenz et al., 2012; Ge et al., 2014; Brennan et al., 2015), bone and dental cements (Furno et al., 2004; Ge et al., 2014; Brennan et al., 2015), and in coatings for cardiovascular prosthetics and catheters (Furno et al., 2004; Eby et al., 2009; Ge et al., 2014). In addition to its antibacterial applications, nAg is also being courted for targeted drug delivery applications (Ge et al., 2014; Mukherjee et al., 2014). Finally, dietary supplementation of colloidal silver for a variety of indications has continued despite no supporting clinical or physiological evidence (Fung and Bowen 1996; FDA, 1999). nAg is well-known to cross vertebrate epithelial barriers, as reviewed by Bergin and Witzmann (2013), and *in vitro* models have validated the permeability of gut (Bouwmeester et al., 2011) and fish gill (Yue et al., 2017) epithelia to nAg. Following uptake, NPs could bioaccumulate and become toxic to internal organs (Blinova et al.,

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2010; Angelica and Fong, 2013; Ates et al., 2013; Ramskov et al., 2014; Walters et al., 2014), although additional data are needed to better inform this risk (Callaghan and MacCormack, 2017). The availability of nAg to a wide variety of tissues underscores the need for a comprehensive analysis of its toxic potential at the organ level.

The potential for nAg-induced oxidative stress has been shown in both mammalian (Ramirez-Lee et al., 2014) and fish models (Choi et al., 2010; Scown et al., 2010; Bilberg et al., 2010). NP-mediated oxidative damage manifests in the accumulation of oxidized lipids (Federici et al., 2007; Hao and Chen, 2012; Dieni et al., 2014; Bessemer et al., 2015) and proteins (Hao et al., 2013; Gormley et al., 2016). In addition to indirectly mediating cellular oxidation, NPs can physically interact with cell membranes and proteins (Morones et al., 2005; MacCormack et al., 2012; Dieni et al., 2013). Together, these effects can interfere with ion transport pathways, as seen in epithelial cells directly exposed to nAg (Schultz et al., 2012). Muscle contractile activity is dependent on coordinated ion flux across membranes, so these findings suggest that heart function may be compromised by exposure to blood borne nAg. Experimentation in rats showed that nAg exacerbated cardiac infarct size post-ischaemia-reperfusion, and was associated with increased levels of pro-inflammatory cytokines (Holland et al., 2015), although the mechanism underlying these effects was unclear. A previous study observed a decline in echocardiographic function in broiler chickens fed nAg (Raieszadeh et al., 2013), however the lack of ionic control in the study confounds the isolation of a nano-specific effect from the inherent toxicity of Ag^{+}.

In this study, we exposed a variety of isolated heart preparations to nAg and Ag^{+} to observe NP-specific effects on metrics of cardiomyocyte (CM) survival, cardiac contractility, and energy metabolism. Teleost fish hearts were chosen as a model system since their contractility is largely reliant on trans-sarcolemmal Ca^{2+} flux, as opposed to mammalian models that use sarcoplasmic reticular (SR) Ca^{2+} stores to generate force following Ca^{2+}-induced Ca^{2+} release. Our findings demonstrate that while both nAg and $AgNO_3$ lower the intrinsic pacing rate of the heart and resting ventricular tension, only nAg decreases systolic force production, suggesting NP-specific mechanisms of toxicity.

2. Animals, materials, and methods

2.1. Nanomaterials and characterization

Silver nanospheres (Impurities: Bi $\leq$ 0.002%, Cu $\leq$ 0.01%, Fe $\leq$ 0.004%, Pb $\leq$ 0.001%, and Sb $\leq$ 0.003% as per certificate of analysis) capped with polyvinylpyrrolidone with an advertised diameter of 80 nm were purchased from Nanostructured and Amorphous Materials Inc. (Houston, TX, lot #0476-120215). This composition was previously found to have 27.3% metallic silver, 6.1% silver and 53% carbon on its surface by X-ray photoelectron spectroscopy (Flory et al., 2013), albeit in nAg of a different nominal diameter.

Dissolution was assessed in triplicate by dialyzing a 250 μL of a 1 mg mL^{-1} nAg suspension as previously described (Shultz et al., 2012; Bessemer et al., 2015) using hydrated G2 Slide-A-Lyzer cassettes (2k MWCO, Thermo Fisher Scientific, Waltham, MA) in 250 mL of incubation media (containing, in mmol L^{-1}: 155 NaCl, 5 KCl, 1 NaH_2PO_4, 2 $MgSO_4$, and 10 HEPES pH 7.6) with samples collected after 1 h. Total dialysate Ag levels were measured using USEPA method 200.8 (Determination of metals in water and wastes by inductively coupled plasma-mass spectrometry), Rev. 5.4. Measurements using Ag-107 and Ag-109 yielded similar results, suggesting the absence of spectral interferences in the measurement. All final measurements were taken using Ag-107, as recommended. Y-89 was used as an internal standard, and the method was validated using certified reference material CRM-TMDW-A (High-Purity Standards, Chaleston, SC). The lower limit of detection of the method was approximately 0.0016 μg L^{-1}.

Hydrodynamic diameter and zeta (ζ) potential were assessed by dynamic light scattering using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern UK). A 1 mg mL^{-1} nAg stock suspension was prepared in Cortland saline (composition below), sonicated for 30 s at 50 W using a wand-type sonicator (F60 Sonic dismembrator, Fisher Scientific, Waltham, MA), and filtered through a 0.2 μm syringe filter. Samples were assayed in triplicate at 25 °C and measurements met all quality control thresholds of the instrument.

Transmission electron micrographs (TEM) were taken to qualitatively assess nAg structure and size. Dry nAg powder was diluted in diH_2O and sonicated at 50 W for 20 min. A drop of the suspension was placed on both i) a carbon coated TEM grid for low magnification images to assess particle size and morphology, and ii) a lacey carbon support film for high magnification images to demonstrate nAg crystallinity.

Experimental stock solutions of nAg of concentrations described below were prepared immediately prior to use by adding dry nAg powder to diH_2O before dispersal using a wand-type sonicator for 30 s at full power.

2.2. Animals

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Fraser's Mills Fish Hatchery (Fraser's Mills, NS) and killifish (*Fundulus heteroclitus*) were collected by beach seine (Little Shemogue, NB). Fish were held in 750 L tanks of semi-recirculating and filtered freshwater (rainbow trout) or 15 ppt brackish water (killifish; total system volume of 2400 L at 16 ± 0.5 °C) and fed *ad libitum* with sinking pellets (Corey Aquafeeds, Fredericton, NB). Fish were not fed the day before an experiment. All procedures were performed with the approval of the Mount Allison University Animal Care Committee (protocol #15-15).

To initiate an experiment, fish were placed in an anaesthetic bath (300 mg L^{-1} tricaine methanesulfonate buffered with 600 mg L^{-1} $NaHCO_3$) until ventilation ceased. Fish were sacrificed by spinal transection and the heart was quickly excised and placed in chilled Cortland saline containing (in mmol L^{-1}): NaCl (143). $CaCl_2$ (0.88), $MgSO_4$ (0.90), KCl (3.35), NaH_2PO_4 (2.25), $NaHCO_3$ (5.50), and HEPES (0.010), in preparation for isolated cardiomyocyte, ventricular muscle strip, or whole heart preparations.

2.3. Rainbow trout cardiomyocyte isolation and treatment

Ventricular cardiomyocytes were isolated from rainbow trout using an established protocol (Legate et al., 1998), with slight modifications as described previously (Callaghan et al., 2017). Briefly, finely-chopped ventricles were rinsed for 15 min in 4 °C Ca^{2+}-free isolation solution (containing, in mmol L^{-1}: 100 NaCl, 10 KCl, 1.2 KH_2PO_4, 4 $MgSO_4$, and 10 HEPES pH 6.9) with additional 30 mmol L^{-1} taurine and 20 mmol L^{-1} glucose. Media was then refreshed with additional 0.75 mg mL^{-1} collagenase type 1A, 0.5 mg mL^{-1} trypsin type IX.S, and 0.75 mg mL^{-1} BSA for 1 h, followed by gentle trituration. The resulting single-cell suspension was passed through a 41 μm nylon net filter and the filter rinsed with isolation medium. Filtrate was centrifuged at 4 °C for 5 min at 1500 *g*. The supernatant was discarded by pipette, and the pellet resuspended in 250 μL incubation medium also containing additional 0.125 mmol L^{-1} $CaCl_2$ for 5 min, with successive additions of 250 μL incubation medium with 0.25, 0.5, 1, and 2 mmol L^{-1} $CaCl_2$. The preparation was centrifuged at 4 °C for 5 min at 1500 *g* and resuspended in incubation media containing 2 mmol L^{-1} $CaCl_2$ to a final concentration of 50 mg cells mL^{-1}. An aliquot of cells mixed

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