Influence of Nanosilver on Endothelial Function and Vascular Reactivity of Isolated Rabbit Carotid Artery

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Summary: There is paucity of information on the effects and mechanism of action of Nanosilver on vascular tone and endothelial function in spite of the upsurge in nanotechnology application in biomedicine. The present study determined the effect of Nanosilver on vascular reactivity and endothelial function on isolated rabbit carotid artery in standard laboratory 20 mL organ bath procedures containing physiological salt solution (PSS) bubbled with 95% O₂, 5% CO₂. Isometric contractions were recorded electronically with a 4-channel Grass Polygraph and maintained at 37°C and pH 7.4. Cumulative dose response tests to α-receptor agonist phenylephrine (PE) was examined separately, in normal PSS (control) and following 20 minutes exposure to varying concentrations of Nanosilver solution [(NAGs) (1.25 and 2.50)] μg/mL in endothelium intact (+E) (control) and endothelium denuded (-E) rings. Contractile responses were analysed with reference to maximal contractions induced by 8 x 10⁻³ M K⁺ in normal PSS. In another experiment, arterial rings were precontracted with EC₂₀ M PE, high and/or low (8, 2 x 10⁻³) M K⁺/PSS. At stable contractions, cumulative relaxation responses to NAGs was studied. Relaxation responses were analysed with reference to maximal contraction induced by EC₂₀ M PE and/or K⁺ depolarization in normal PSS. Following 20 minutes exposure to NAGs, dose relaxation response to acetylcholine (ACH) was also examined in normal PSS (control), and pre-incubated L-NAME (NO synthase inhibitor) and indomethacin (cyclooxygenase inhibitor) precontracted arterial rings to further determine mechanisms of action. Data were presented as Means ± SEM. Graphs and statistical analysis were done using GraphPad prism version 7.03 and Student t-test. P-values (P< 0.05) were considered statistically significant. The results showed that nanosilver decreased maximum contraction (Emax) and induced attenuated receptor mediated contraction is endothelium-dependent and showed a biphasic dose-dependent response. In conclusion, Nanosilver causes attenuation in carotid arterial smooth muscle reactivity with a biphasic dose-dependent relaxant effect and multiple endothelium-dependent pathways mode of action.

Keywords: Nanosilver, Vascular reactivity, Carotid arterial rings, Vascular endothelium.

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Manuscript Accepted: December, 2018

INTRODUCTION

Nanoparticles are heterogeneous substances with a size range of between 1 - 100 nm in at least one dimension and characterized by a high surface area-to-mass ratios resulting in better activity (Hector et al., 2009, Tiwarri and Behari, 2011). Nanoscience and nanotechnology have received much significance recently owing to their unique probiotic properties with proven wide range of biomedical health benefits and potential uses in commercial applications (Esenaliev, 2000; Czajka, 2005; Wagner et al., 2006; Emerich and Thanos, 2007;). Nanosilver particles (NAGPs) is one of the fastest-growing nanomaterials consumer products, industrials and biomedical applications owing to their unique properties particularly in catalytic activity and enhanced surface area to mass ratios and in permeating cellular membranes. Consequently, NAGPs have been extensively used for biomedical applications including but not limited to: antiviral, anti-angiogenic, antitumor, biosensors and bioimaging; as well as wound dressing, silver impregnated catheters, vascular prosthetics, clothing undergarments, air filters, laundry detergents, toiletries and water taps (Thote and Gupta, 2005; Holland et al., 2015; Zhang and Kun, 2016).

Regardless of their conventional benefits, current studies have shown conflicting reports on the nanosilver effects on organ function and metabolism as well as very subtle possible tissue adverse effects particularly in cardiovascular toxicity in varied concentrations, size, biological target exposure time and tissue variations (Schrand et al., 2010; Trickler et al., 2010; Kang et al., 2011; Haase et al., 2012; Grosse et al., 2013, Puja et al., 2015). Furthermore, possible mechanisms through which NAGPs may interact with
biological surfaces are only beginning to emerge. Considering the conflicting reports, paucity of information on the effect of nanosilver on cardiovascular health and vascular smooth muscle responses in particular, this present study was designed to evaluate the effects of nanosilver on vascular smooth muscle reactivity and endothelial function in isolated rabbit carotid artery.

MATERIALS AND METHODS

Tissue preparation and Protocol
Arterial segments of the carotid artery were obtained from adult New Zealand rabbits which were sacrificed by stunning and bleeding and placed in physiological salt solution (PSS) of the following composition (mM): NaCl 119, KCl 4.7, NaHCO₃ 24.9, NaH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 1.6, glucose 11.5. The arteries were cleaned of adhering connective tissues and cut into 2-3 mm rings. The rings were suspended between 2 L-shaped wire loops in 20 ml organ baths containing PSS. The upper loop was attached to a Grass Model FT03 force transducer connected to a Grass Model 7P polygraph (Grass Instruments Co., Quincy, MA, USA) while the lower loop was fixed to the base of the organ bath. An initial load of 1 g was applied. The PSS was bubbled throughout with 95% O₂ - 5% CO₂ gas mixture with the pH and temperature maintained at 7.4 and 37°C respectively. An equilibration period of 60 minutes was allowed; following this, carotid rings were stimulated twice with 8 x 10^{-3} M K⁺ PSS, at 20-minute interval. The average of these contractions represented the maximum (100%) agonist/KCl which subsequent contractions to phenylephrine were evaluated (as previously reported Uche and Ebeigbe, 2015).

Concentration-response to agonists
Cumulative concentration-response tests (1x10^-⁹ to 2.5 x10^-⁴ M) to the agonist PE were examined in normal PSS (control) (n = 6) (where n is the number of animals from which arterial rings were obtained for each protocol); as well as following 20 minutes exposure to varying concentrations of nanosilver solution (1.25 µg/ml and 2.50 µg/ml) in arterial carotid rings (+E or -E). Contractile responses were analysed with reference to maximal contractions induced by 80 mM K⁺ PSS (n = 6).

Relaxation-response and mode of action
Arterial rings were precontracted with EC₇₀ M PE and/or high (80 mM K⁺) PSS. At stable contraction, cumulative relaxation response tests (0.25 to 5.0 µg) to nanosilver were studied in normal PSS (control) (n = 6); in +E and -E rings.

Role of endothelium
To further elucidate the possible mechanisms of action of Nanosilver, the role of vascular endothelium in vascular reactivity by NAGs was studied in intact (+E) and endothelium denuded (-E) HM; PE or high-K⁺ precontracted arterial rings. Endothelium removal was effected mechanically (Furchgott and Zawadzki, 1980) by gently rubbing the inner lining surface of the rings with a pair of forceps (Ebeigbe et al., 1990). The effectiveness of de-endothelialisation was confirmed by lack of relaxation response to 10⁻⁵ M Acetylcholine (Ach) and more than 70% relation in phenylephrine - precontracted endothelium-denuded and intact arterial rings respectively (Ebeigbe et al., 1990; Olele et al., 1998). Also, relaxation response tests to acetylcholine (1x10⁻⁹ to 1x10⁻³) was examined in normal PSS (control) and following 20 minutes exposure to NAGs (1.25 µg/ml only and/or 5 minutes pre-exposure to L-NAME (1x 10⁻⁵ M) and indomethacin (3 x 10⁻⁶ M) or its vehicle Na₂CO₃; final bath concentration less than 0.03%) in arterial rings. Relaxation responses were analysed with reference to maximal contraction induced by EC₇₀ M PE in normal PSS.

Chemicals
The following drugs and chemical reagents were used: Phenylephrine hydrochloride (Sigma USA), Nanosilver solution (Mineral for life Ltd, Abuja, Nigeria), L-NAME and Indomethacin (Sigma USA); and prepared fresh by dissolving in distilled water and NaHCO₃.

Statistical Analysis
Data are presented as Means ± SEM (standard error of means); n represents the number of rabbits from which arterial rings were obtained. EC₅₀ (concentrations producing 50% maximal response) and IC₅₀ were determined graphically. Comparison of the means was effected using the Student’s t-test, ANOVA and Graph pad prism version 7.03 statistical package. P - Values less than 0.05 (P<0.05) were considered statistically significant for two independent variables (test and control).

RESULTS

Effect of Nanosilver on dose-response to agonists
Exposure of arterial rings to varying NAGs concentrations resulted in phenylephrine-induced attenuated contractions. NAGs exposure resulted in significant (p<0.05) right-ward shift of the curves and attenuated maximal contraction in both +E and -E rings (fig. 1).

Comparative PE EC₅₀ (M) contraction in varying NAGs concentration
Comparative EC₅₀ (M) values of α-adrenergic phenylephrine-induced contraction in +E and -E arterial rings exposed to varying concentrations of NAGs showed significant decreased Emax contraction (P<0.05).
Nanosilver influences vascular reactivity and endothelial function

Fig. 1. Cumulative concentration responses to phenylephrine following exposures to varying Nanosilver concentration in normal PSS in carotid rings: n = 6; means ± SEM. NAGs exposure resulted in significant (p<0.05) right-ward shift of the curves and attenuated maximal contraction in both +E and -E rings.

Fig. 2. Comparative EC\textsubscript{50} (M) values of α-adrenergic receptor-PE-induced contractions in +E and -E arterial rings exposed to varying concentrations of NAGs showing significantly decreased E\textsubscript{max} contractions (p<0.05).

Fig. 3. NAGs-induced biphasic relaxation response following EC\textsubscript{70} M PE precontraction and greater relaxation effect in low (2 x 10^{-2} M K\textsuperscript{+}) precontractions whereas PE and 8 x 10^{-2} M K\textsuperscript{+} precontractions were abolished in -E carotid rings.

Fig. 4. Endothelium-dependent ACh-induced dose-dependent relaxation response curves to agonist in normal PSS (control) and attenuation in relaxation following exposures to Nanosilver and pre-incubation in L-NAME (NO synthase inhibitor) and indomethacin (cyclooxygenase inhibitor) solution.

Fig. 5. Comparative IC\textsubscript{50} (M) values of ACh-endothelium dependent induced relaxation in PE-precontracted arterial rings exposed to vasoactive vascular endothelial agents.

Dose-relaxation response to NAGs
Carotid arterial rings were each precontracted with 10^{-7} M PE, high and/or low (8, 2 x 10^{-2}) M K\textsuperscript{+}. When the contractions were stable, NAGs (0.25 to 5.0) µg/mL was added cumulatively and the effects examined. The resultant relaxation responses were determined and expressed as percentage (%) of the initial tension. Nanosilver induced relaxation is endothelium dependent. (fig. 3).

NAGs in ACh-induced relaxation
Cumulative addition of ACh to 10^{-7} M PE-induced contractions in normal PSS (control) and following 20 minutes exposure to 1.25 µg/mL Nanosilver, and pre-incubation in L-NAME (1x10^{-6}) M or indomethacin (3x10^{-6}) M carotid arterial rings and maintained throughout the protocol. There was a significant shift of the test response curves to the right of the control.
showing attenuated relaxation and Nanosilver endothelium non-specific mode of action (fig. 4).

Acetylcholine IC$_{50}$ (M) relaxation in varying NAgs concentration.
IC$_{50}$ (M) values were graphically determined from the cumulative dose relaxation responses. The IC$_{50}$ (M) values of ACh-endothelium dependent induced relaxation with nanosilver (NAgs), L-NNAME (nitric oxide synthase inhibitor) and indomethacin (an inhibitor of cyclooxygenase) in phenylephrine-precontracted arterial rings showed no significant relaxant differences suggesting non-specific endothelium mediated responses.

Discussion
This study has identified the effects of nanosilver solution [(NAgs (10ppm)] on endothelial function and vascular reactivity in rabbit isolated carotid artery. The results of the present study indicate that the contractile responses to α-receptor agonist phenylephrine (PE) in isolated rabbit carotid artery in both intact endothelium (+E) and endothelium denuded (-E) rings were attenuated and maximal contraction decreased following exposure to 1.25µg/mL nanosilver solution compared to the contractile response of the control carotid arterial ring in normal PSS. The EC$_{50}$ values of phenylephrine contractions in control and test +E or -E arterial rings were: $7.0 \times 10^{-7}$, $5.04 \times 10^{-5}$, $2.03 \times 10^{-5}$, $8.02 \times 10^{-5}$ and $8.01 \times 10^{-5}$ respectively (figure 2). However, attenuation in contractile response was significantly greater in endothelium denuded than intact endothelium arterial rings suggesting greater direct diffusivity effect of Nanosilver molecules across plasmalemma membrane. This observation is in tandem with previous reports alleging of an emerging risk represented by the wide diffusion of nanoparticles, such as the silver nanoparticles, as well as their worldwide diffusion for industrial processes and treatments (Marzhan et al., 2013); and the ability of nanosilver particles to cross the capillary wall (Bachler et al., 2013, Holland et al., 2015). Exposure to silver nanoparticles has been associated with inflammatory, oxidative stress, genotoxic, and cytotoxic consequences (Marzhan et al., 2013).

We also demonstrated that exposure to 1.25µg/mL nanosilver solution significantly attenuated endothelium-dependent vascular smooth muscle relaxation induced by ACh in carotid artery in normal PSS dose-dependently; whereas pre-incubation with NO synthase inhibitor [(L-NNAME (1x 10^{-5}M))] and cyclooxygenase inhibitor [(indomethacin (3 x 10^{-6} M))] followed the same tenet but with no significant change in the response curves compared to ACh-induced relaxation in NAgs incubated carotid rings. The IC$_{50}$ values of acetylcholine-induced relaxations in pre-incubated L-NNAME (endothelium nitric oxide synthase inhibitor) and indomethacin (an inhibitor of cyclooxygenase) arterial rings showed no significant difference comparatively (figure.4) Therefore mechanisms of Nanosilver modulatory relaxant effect may be mediated through endothelium-related-multiple pathways. Acetylcholine-induced vascular relaxation is well known to be endothelium-dependent (Furchgott and Zawadzki, 1980, Ebeigbe and Aloamaka, 1985; Obiefuna et al., 1991). Several other vasorelaxant agents are equally known to mediate their effects via interaction with vascular endothelium in both receptor operated channels and voltage-sensitive channels- mediated contractions (Bolton,1979; Van de Voorde and Leusen, 1983; Ebeigbe and Aloamaka, 1985). Previous reports on vascular reactivity have shown that vascular endothelium modulates VSM responses, regulates and maintain vascular tone and homeostasis, as well as vascular resistance (Ajay et al., 2007); possibly by secretion of vast array of endothelium-derived factors including: Nitric oxide (NO), prostacyclin, endothelin-derived hyperpolarization factor (EDHF) (Furchgott and Zawadzki, 1980, Mori et al., 2006, and Kroetsch and Bolz, 2013). The exact nature of EDHF has not been fully characterised however, the following constituents have been identified including epoxyeicosatrienoic acids (EETs), K+, gap junction, and hydrogen peroxide (Mori et al., 2006). Hence different EDHF might exist in different forms and the contribution of each component to endothelium-dependent relaxation might vary, depending on the species tested and the vessel type used (Mori et al., 2006).

Nonetheless, these three different endothelium-derived vasodilators, nitric oxide, prostacyclin and EDHF play a vital role in the regulation of vascular tone. Vascular tone is a key determinant of local organ blood flow and peripheral resistance (Mori et al., 2006). Additionally to elucidate further mode of action, following precontractions by EC$_{50}$M PE and/or 8.2 x 10$^{-2}$ M K$^+$ PSS with no significant difference in Emax, cumulative addition of NAgs elicited a greater biphasic concentration-dependent relaxation with receptor-mediated contraction compared to high- K$^+$ in intact endothelium arterial rings; suggesting a greater effect of NAgs on mechanism associated with Ca$^{2+}$ influx and that nanosilver may elicit contractile responses in higher concentrations (figure 3 ). NAgs-induced relaxant effects were however abolished in endothelium denuded arterial rings in both receptor and non-receptor mediated contractions in normal PSS whereas there was greater NAgs relaxation effect in low potassium depolarization compared to high K$^+$ and &-receptor mediated phenylephrine contractions (figure 3). It is suggestive that different EDRFs-related multipathways probably mediate the vasorelaxant effect of NAgs. However, we observed no effect of Nanosilver on basal tone. To our knowledge, literature is scarce on the effects of nanosilver particles and silver ion on vascular reactivity in vitro and vascular.
endothelial modulators effects on endothelial function. Therefore, these observations are quite novel in nanomaterial vascular reactivity. In conclusion, the results of this study show that nanosilver solution via a direct action on vascular smooth muscles, inhibits contractile responses and attenuates ACh-induced endothelium dependent relaxation in a dose-dependent manner in isolated rabbit carotid arterial rings. The inhibitory effect of nanosilver is probably not unconnected with the release of endothelium-derived relaxation factors.

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