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TITLE: The influence of silica nanoparticles on small mesenteric arterial function, *ex vivo* and *in vivo.*

ABSTRACT

Silica nanoparticles (SiNPs) are attractive drug delivery platforms and diagnostic tools, however, recent reports suggest that they may be detrimental to conduit arterial function. We aimed to examine whether SiNPs influence the function of small size arteries, which play an essential role in controlling blood perfusion into tissues, using an *ex vivo* model. We show that while exposure to SiNPs under static conditions, attenuated dilator responses *ex vivo*, attenuation was only evident at lower agonist concentrations, when exposed under flow conditions or *in vivo*. Pharmacological inhibition studies suggest that SiNPs may interfere with the endothelial dependent hyperpolarising factor (EDHF) vasodilator pathway. The dosage dependent influence of SiNPs on arterial function will help identify strategies for their safe clinical administration.

Key words: silica nanoparticles, vasodilation, mesenteric artery, vasoconstriction, EDHF, Nanomedicine

Word count (excluding abstract and references)=

1. INTRODUCTION

There is a growing interest in the fabrication of silica nanoparticles (SiNPs) for applications in cell tracking, imaging diagnostics and medical therapeutics, as drug delivery platforms, due to their tuneable size, stability under flow conditions, and its ability to decompose into silicic acid (DIACONU et al., 2010) (Kim et al., 2011, Motwani et al., 2011, Jokerst et al., 2013, Liberman et al., 2014) [reviewed in 4]. However, there are limited studies investigating their influence on blood vessel function. The lining endothelial cell (EC) layer displays the first contact surface for nanoparticles when intravenously injected for diagnostic and/or therapeutic purposes. Furthermore, *in vivo* inhalation studies have demonstrated the translocation of nanoparticles into the systemic circulation, where they can be detected in tissues distal to the exposure site (Du et al, year?). A number of studies have suggested that SiNPs may be cytotoxic to ECs, depending on their size, shape, and interaction with target molecules (Kim et al., 2015). For example, amorphous SiNPs have been shown to induce endothelial dysfunction in human umbilical vein ECs (Corbalan et al., 2011) as well as the disruption of vascular homeostasis (Corbalan et al., 2012). These effects were mediated via a nitric oxide (NO)/peroxynitrite imbalance associated with an increased peroxynitrite ($ONOO^-$) production leading to a low [NO]/[$ONOO^-$] ratio (Corbalan et al., 2012) and platelet aggregation within the blood stream via adenosine diphosphate and matrix metalloproteinase 2-dependent mechanisms respectively. As ECs are key modulators of vessel function and contractility, the above studies highlight the need to understand the effect of SiNP uptake on the physiological function of vessels.

The effects of nanoparticles on blood vessels can be distinguished in large and small blood vessels, due to their varied structural and physiological characteristics and the profile of mediators released by the lining ECs. We have previously demonstrated that SiNPs of 100 and 200 nm in size have no overall detrimental effect on conduit arterial function, but is influenced by nanoparticle charge, dosage and dye encapsulation (Akbar et al., 2011, Farooq et al., 2013). The influence of SiNPs on the function of small size arteries remains poorly understood, yet this is key to establishing SiNP biosafety, as the function of these small arteries is important in the regulation of blood perfusion into organs and tissues. The vascular endothelium plays an important role in the modulation of vascular tone through the release of

vasoconstrictor and vasodilator factors. A number of molecules are implicated in contributing to the vasodilator response in arteries, including nitric oxide, prostanoids, and endothelial derived hyperpolarising factors (EDHFs). EDHFs are represented by a number of candidate molecules, including epoxyeicosatrienoic acid (EETs), hydrogen peroxide, and potassium ions through the activation of small (SK_{Ca}) and intermediate (IK_{Ca}) conductance calcium-activated potassium channels [reviewed in Luksha et al; atherosclerosis, 2009]. While the major dilator component is attributed to nitric oxide in conduit size arteries, EDHF is the principal dilator in small (~200-300 μm diameter; e.g. second and third-order) mesenteric arteries (Hilgers and Webb, 2007). Other components such as nitric oxide and prostanoids from the cyclooxygenase pathway (Honing et al., 2000) may have a minor role in contributing to small vessel dilation, particularly when EDHF action is compromised. The more pronounced role of EDHF in small mesenteric arteries compared with medium sized and large conduit arteries such as the superior mesenteric artery and the aorta is attributed to the action of intermediate and small conductance calcium-sensitive potassium channels (IK_{Ca} and SK_{Ca}), in acetylcholine-induced relaxations (Hinton and Langton, 2003, Hilgers et al., 2006).

In rat mesenteric arteries, the stimulation of ECs results in the activation of endothelial calcium-sensitive potassium channels and leads to the generation of the hyperpolarising event. The resulting efflux of potassium ions (K^+) from ECs via SK_{Ca} and IK_{Ca} channels (Edwards et al., 1998), regarded as the hyperpolarisation current, propagates or spreads passively via myoendothelial gap junctions (Sandow et al., 2002) to accumulate in the myoendothelial space where it stimulates the sodium ion (Na^+)/K^+ ATPase pumps and voltage-gated potassium channels (K_v; e.g. $K_v1.5$ and $K_v2.1$ (Hald et al., 2012)) in vascular smooth muscle cells (VSMCs) (Edwards et al., 1998). This results in the EDHF-attributed hyperpolarisation in VSMCs. Hence, the EC-VSMC electrical coupling (Little et al., 1995) is characterised by endothelial potassium channels that influence smooth muscle cell contractile activity by reducing Ca^{2+} influx via voltage-operated Ca^{2+} channels and by suppression of key enzymes involved in agonist-induced transduction pathways (Itoh et al., 1992). The aim of the present study was to investigate the influence of SiNPs on small mesenteric arterial function, after acute exposure *ex vivo*, under both static and flow conditions, and following intravenous (iv) injection *in vivo*. Furthermore, to explore the mechanism of

attenuated dilator responses by SiNPs, inhibition studies were carried out using specific pharmacological inhibitors of the various dilator pathways.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

All agonists and reagents were obtained from Sigma (Poole, UK). Physiological salt solution (PSS) composition [mM]: 119 NaCl, 4.7 KCl, 1.2 $MgSO_4 7H_2O$, 25 $NaHCO_3$, 1.17 $KHPO_4$, 0.03 K_2EDTA, 5.5 glucose, 1.6 $CaCl_2 2H_2O$; pH 7.4. High potassium PSS (KPSS, 60 mM KCl) composition [mM]: 78.2 NaCl, 60 KCl, 1.2 $MgSO_4 7H_2O$, 25 $NaHCO_3$, 1.17 $KHPO_4$, 0.03 K_2EDTA, 5.5 glucose, 1.6 $CaCl_2 2H_2O$; pH 7.4. Water purified by Milli-Q purification system was utilised for all studies.

2.2. Synthesis and characterisation of silica nanoparticles

Mono-dispersed dye-encapsulated fluorescent SiNPs was synthesised using the modified Stöber sol-gel method. (Stöber et al., 1968, Ismail et al., 2010). The initial stages in the synthesis involved the incorporation of the rhodamine B isothiocyanate (RBITC) core within silica. Briefly 40 mg of RBITC was stirred with 600 µL of 3-aminopropyltrimethoxysilane (APS) at room temperature (~20^oC) under nitrogen atmosphere for 3 hours. Ammonium hydroxide (NH_4OH), absolute ethanol, water and TEOS were magnetically stirred for 15 minutes. 50 µL of APS coupled with RBITC was added. The reaction solutions were centrifuged at 6000 rpm for 20 minutes and the supernatant solution discarded; sedimented nanoparticles were re-dispersed in absolute ethanol by vortex mixing and ultra-sonication. The solution was re-centrifuged at 6000 rpm for a further 20 minutes and the supernatant discarded again. Nanoparticles were re-dispersed in distilled water and then characterised using the Malvern Zeta-sizer and scanning electron microscopy.

2.3. Vascular function studies

Male Wistar rats (150-250 g) were humanely euthanised in accordance with the 'Animals (Scientific Procedures) Act 1986' and Institutional guidelines. Animals were killed by stunning and cervical dislocation of the neck and the mesentery removed and placed in ice cold PSS. Small mesenteric arteries (2^{nd} – 3^{rd} order, 145-300 µm initial diameter, 3-4 mm in length) were finely dissected (the fat tissue layer and connective tissue surrounding each vessel were also removed), isolated and mounted between two glass cannulae (tied using fine nylon thread) on a pressure myograph chamber (Living Systems, Burington, USA). The mounting of the mesenteric artery on a pressure myograph chamber is a very delicate procedure and requires extensive practice to avoid any damage to the endothelial cell layer. Two knots of thread were tied on both cannulae, which were used to tie the mounted vessel and keep it in place. Arteries were initially pressurised to an intravascular (luminal) pressure of 60 mmHg and maintained at that pressure using a pressure servo-control unit (Living Systems, Burlington, USA); the system tubing and valve systems was initially checked for leaks to ensure a sealed pressurised circuit. All vessels were constantly superfused in a continuous source of PSS at 37℃, pH 7.4, and gassed with 95% O_2-5% CO_2 (from a standard gas cylinder), with the superfusate entering and leaving the chamber via luer connections in the side of the chamber. This was done under a Nikon inverted microscope to allow for optimal resolution in visualising the tissues. Lumen and internal diameters of the vessel were constantly measured using a video dimension analyser, with data recorded on the computer using Chartlab 5 software (Powerlab system, AD Instruments, UK) for later analysis. Arteries were superfused at the end of each experiment with Ca^{2+}-free PSS containing 2 mM EGTA for 20-30 minutes to obtain passive diameters and allow the vessel to relax completely.

The viability of the mesenteric arteries was assessed for their ability to constrict to a high potassium solution (KPSS, 60 mM KCl). The influence of SiNPs on mesenteric arterial function was assessed in response to vasoconstrictors and vasodilators before (in non-treated control vessels), and 30 minutes subsequent to intraluminal infusion in PSS (control) or SiNPs. The intraluminal infusion was conducted as follows: The pressure servo-control unit was turned off and a 1 mL syringe was inserted into one end of the 3-way luer connection in the side of the pressure myograph chamber. A 0.5 ml of diluted SiNP solution (in PSS) was slowly

administered via the syringe. The 3-way luer valve was closed off and pressure returned to 60 mmHg, using the pressure servo unit. For the flow experiments, SiNPs were infused at a flow rate of 10-20 µL/min, over the 30 min incubation period, using a microfusion pump connected to the end of one of the glass cannulae. Vasoconstrictor responses (phenylephrine [Phe], 10^{-9}-10^{-4} M) and endothelial-dependent (acetylcholine [ACh], 10^{-13}-10^{-3} M) and independent (sodium nitroprusside [SNP], 10 and 100 nM and papaverine [PAPA], 100 µM) dilator responses were assessed before (initial responses), and 30 minutes subsequent to intralumenal infusion of PSS or SiNPs (second responses). The vasodilator responses were assessed on vessels that have been precontracted in a submaximal dose of Phe. Responses after incubation in PSS were used as controls to compare with SiNPs responses. Two dosages of SiNPs were used; high dose (at 5.32 x 10^{11} NPs/mL), and low dose (at 1.01 x 10^{11} NPs/mL).

2.4. In vivo animal studies

Animal procedures were conducted in compliance with institutional guidelines and UK Home Office requirements (Project License 70/6616). Animals were given food and water ad libitum and housed in a 12 hours/12hours light dark cycle. The rat was anaesthetised in an induction chamber using 2% isoflurane. Maintenance of anaesthesia was achieved by the use of 1.5-2.0% isoflurane via a concentric oronasal mask connected to an anaesthetic circuit. Core body temperature was maintained at 37.0 ± 0.5℃ using a heating pad and a rectal temperature probe. The *in vivo* study involved an intravenous injection of SiNPs at a concentration of 10 mg/Kg of animal weight into the tail vein of a 300 g unconscious male Wistar rat. For this purpose, 3 mg of SiNPs was suspended in 100 µL of PSS and injected into the animal. SiNPs were given at the same dosage used in our *ex vivo* study (5.32 x 10^{11} NPs/mL dosage), taking into consideration the blood volume, thus 5.32 x 10^{11} NPs/mL of SiNPs per 1 mL of animal blood (0.2 mg/mL). After injection, the rat was monitored for a period of 2 hours and then terminated by cardiac catheterisation and scarified. At the end of the *in vivo* experiment, the rat mesentery, and other vessels (aortic) were dissected-away and placed in ice-cold PSS. Other tissues such as the brain, lung, heart, stomach, liver, spleen, small intestine, large intestine, kidneys, bladder, tail and thyroid were also excised. A small length of mesenteric vessel was excised and placed on ice for further physiological analysis. A first- or second order

artery from the mesentery (200-400 µm initial diameter, 2-3 mm in length) from the SiNP-injected rat was dissected out using fine forceps and mounted between two glass cannulae fixed in place on a modified pressure myography chamber at 60 mmHg intra-vascular pressure and constantly superfused with gassed PSS at 37°C, pH 7.4, as described above. The viability of the mesenteric artery was determined by its ability to constrict to KPSS (60 mM KCl). Constrictor responses to Phe (10^{-9}-10^{-4} M) was initially assessed, followed by the endothelial-dependent (ACh, 10^{-13}-10^{-3} M) and independent (SNP, 10 and 100 nM; PAPA, 100 µM) dilator responses in Phe-preconstricted vessels.

2.5. Tissue fixation and transmission electron microscopy

At the end of the functional studies, vessels were carefully untied from the glass cannulae and immediately fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3 over 2 hours at 22°C. The samples were postfixed with reduced osmium (OsO_4 1% + $K_4Fe(CN)_6$ 1.5%) for 1 hour, then dehydrated in a series of alcohols, infiltrated with TAAB LV resin and polymerised for 24 hours at 60°C, as described previously (Akbar et al., 2011). Ultrathin 70 nm sections were cut with Leica 'Ultracut S' ultramicrotome and placed on copper grids. The grids were observed in Tecnai 12 Biotwin transmission electron microscope at 80 kV.

2.6. Determination of SiNP uptake and biodistribution

Inductively coupled plasma mass spectrometry (ICP-MS; Perkin Elmer, UK) was used in order to quantify and calculate the relative amount of the silica (Si) metal in a sample containing the *ex vivo* or *in vivo* -relevant amount. The sample was diluted in 5 mL dH_2O and subjected to analysis using ICP-MS. Additionally, to verify SiNP uptake by vessels and other organs, ICP-MS was used to measure Si concentration. Briefly, vessels were weighed and placed into 500 µL lysate buffer solution (containing 0.5 g sodium dodecyl sulphate (SDS; Sigma, Japan), 0.29 g NaCl, 0.39 g tris, 0.03 g Tris(hydroxylmethyl) and aminomethane, and 250 µL of the protease inhibitor cocktail in 25 mL of dH_2O) and allowed to digest over 48 hours at room temperature. The lysate was subsequently mixed in 1 mL high purity (70%) nitric acid in glass tubing and placed in an oil bath at 115°C, for 2-3 hours. The solution was made up to 5 mL in distilled water and analysed. A standard curve was used to

quantify the amount of metal in the tissues, using standards (Sigma, UK), in the same background solution as that of the tissue solutions. The lower limit of Si quantification was 50 ng/mL. These results were used to quantify the actual concentration of SiNPs within solutions in mg/g of animal tissue by considering the dilution factor. The uptake concentrations were expressed as mean percentage of the actual dose injected (100%).

2.7. Inhibition studies

A series of inhibition studies were carried out, firstly to characterise the dilator component in small mesenteric arteries, followed by co-incubation of vessels with SiNPs in the presence of the inhibitors. *Ex vivo* vessels were mounted as described previously and responses to ACh were assessed before and 30 minutes after incubation in inhibitors including; N_{ω}-nitro-L-arginine (L-NNA, 100 μM), indomethacin (10 μM), apamin (100 nM) and TRAM-34 (1 μM) (potassium channel inhibitors; Sigma-Aldrich, Poole, UK). Inhibitors were intraluminally infused into the mesenteric artery as well as superfused for 30 minutes. Dilator responses were assessed 30 minutes after incubation in the inhibitors, in the continued presence of inhibitors inside the vascular lumen and in the superfusate. Subsequently, vessels were co-incubated in both L-NNA and SiNPs (5.32×10^{11} NPs/mL in PSS) for 30 minutes to assess the effects on function. In a separate set of experiments, vessels were co-incubated in SiNPs and superoxide dismutase (SOD), used at 300 U/mL.

2.8. Statistical analysis

A generalised linear mixed model (GLMM) approach was performed for the statistical analysis of the data obtained using the IBM statistical package for social sciences (SPSS) version 19.0 (Breslow and Clayton, 1993). The model is an extension of the generalised linear model in which the linear predictor contains random effects, in addition to the usual fixed effects for sample effects regression analysis of grouped data. This model provides an extension of the linear mixed models to non-normal data (in our case % response values). This fixed-effects model of analysis of variance was applied due to the existence of different parameters or independent variables across different sets of experiments that can influence the dependent variable. The dependent variable represents the dilator response elicited by ACh and

SNP which is expressed as percentage relaxation (corresponding to the increase from the pre-constricted maximal Phe value), or the constrictor response by Phe. The independent variables include: the number of the mesenteric vessel segments used, incubation type of the vessels [PSS, SiNPs at high dosage, SiNPs at low dosage, SiNPs with L-NNA, SiNPs with SOD, inhibitors (L-NNA, indomethacin, apamin and TRAM-34; alone or in combination] and chemical concentration (10^{-13}-10^{-3} M) of the agonist used. As each vessel in this current study was repeatedly assessed (before as well as after incubation) for the different agonist concentration, the co-variant was also taken into account. The co-variant is the weight of each vessel i.e. how many occasions it was measured for either % contraction or relaxation. This allowed for the estimation of the ranges of response variable values that the independent factors would generate in a particular set of experiments. This whole analysis determined two factors, which are the 'incubation type' and the 'agonist concentration' to be responsible for the main significant effects contributing to the variation in vascular % responses (contraction or relaxation). This justified the use of repeated measures (one-, two-, three- or multi-variate) analysis of variance (ANOVA) for multiple sample mean comparisons due to the vessels being repeatedly measured for their responses to different agonist concentrations. The ANOVA tests were performed to specify which incubation type is contributing to the significance of results (% response measurements). The pairwise adjustments identified the particular dose(s) that lead to the significance of results among the different incubation types.

The number of mesenteric vessel segments from different animals are expressed as n, represents the number of vessels utilised for each set of experiments (1 vessel per animal). Data is represented as mean ± standard error of mean (SEM). Concentration response curves were assessed using the appropriate ANOVA test with subsequent pairwise comparisons to determine the main effects of dose and incubation as well as the interaction of effects. Bonferroni adjustments were used to correct for multiple comparisons. If a main effect was observed, Student's paired or unpaired *t*-test (two tailed) was used to determine the locations of differences as appropriate. $p<0.05$ was considered to be statistically significant. The maximum agonist effect (E_{max}) and concentration inducing 50% of E_{max} (EC_{50}) were determined

from each concentration response curve and pD_2 was calculated as the -log(EC_{50}). E_{max} and pD_2 values were calculated using the GraphPad Prism Software 7.

3. RESULTS

3.1. Characterisation of SiNPs

Transmission electron microscopy (TEM) and scanning electron microscopy images demonstrated the mono-dispersed and spherical morphology of SiNPs with an overall size of 97.85 ± 2.26 nm (**Figure 1**). The Zeta potential measurement of SiNP samples in water was -41.0 ± 1.04 mV and that in PSS was -12.0 ± 0.38 mV (**Figure 1**). The fluorescence spectra of SiNPs showed a peak obtained at its expected wavelength (550-690 nm), confirming the presence of the RBITC dye (**Fig 1C**).

3.2. Cellular uptake of SiNPs into the mesenteric arteries

Examination of the TEM sections demonstrates the presence of SiNPs inside ECs of the mesenteric vessels after a 30 minute incubation in SiNPs (at 5.32 x 10^{11} NPs/mL) at 37ºC *ex vivo* (**Figure 2A and B**). They were unevenly distributed and dispersed throughout the cytoplasm of ECs, but none were found in the nucleus. SiNPs were located either singly or in small clusters and were predominantly membrane-bound within vesicle structures that may be of endosomal or lysosomal origin suggesting that an endocytic pathway of uptake may be involved (**Figure 2A and B**). A greater number of SiNPs were identified inside ECs after exposure to high dose vs low dose SiNPs. SiNPs were also identified (but with reduced number in comparison to *ex vivo* exposure), within the cytoplasm of ECs from the *in vivo* injected rats with none being identified in the nucleus, smooth muscle cell layer or the adventitia (**Figure 2C and D**). Uptake of SiNPs into the mesenteric vessels was also confirmed by ICP analysis (Section 3.6)

3.3. The influence of SiNPs on vasoconstrictor responses

All vessels constricted to high potassium salt solution (60 mM KCl). Incubation in SiNPs *ex vivo*, had no overall influence on the contractile responses (the mean percentage constriction was 78.49 ± 7.13% vs. 65.80 ± 7.04% after incubation in PSS and SiNPs, n=7 and 10 respectively, unpaired *t*-test, NS; **Figure 3A**). This

finding was similar to that obtained in the *in vivo* injected rats (The mean percentage constriction was 78.49 ± 7.13% vs. 71.29 ± 17.39% after incubation in PSS and SiNP injection, n=7 and 4 respectively, unpaired *t*-test, NS; **Figure 3A**). All vessels constricted to Phe, in a dose dependent manner. Incubation in SiNPs *ex vivo*, at 5.32 x 10^{11} NPs/mL, had no effect on the pD_2 values for Phe-induced constriction in mesenteric arteries despite the significant reduction in the maximal constriction responses (For SiNPs incubated *ex vivo*, at 5.32 x 10^{11} NPs/mL ; Phe E_{max} 39.73 ± 5.40%; pD_2 0.22 ± 1.18 μM vs. E_{max} 96.51 ± 5.90%; pD_2 0.51 ± 0.58 μM for control responses ($p<0.05$); **Table 1**). The incubation in SiNPs *ex vivo*, at 5.32 x 10^{11} NPs/mL, led to a significant reduction in the contractile responses of vessels to Phe, at specific concentrations (1, 10 and 100 μM). At 10 μM Phe, the mean percentage constriction was 98.55 ± 1.45% vs. 36.51 ± 11.17% after incubation in PSS and SiNPs ex *vivo*, n=4 and 5 respectively, unpaired *t*-test, $p<0.0001$; **Figure 3B**). There was no overall change in the Phe constrictor responses in mesenteric arteries after *ex vivo* infusion at the lower dosage of 1.01 x 10^{11} NPs/mL (Phe E_{max} 103.60 ± 3.59%; pD_2 0.37 ± 0.29 μM, vs. E_{max} 96.51 ± 5.90%; pD_2 0.51 ± 0.58 μM for control responses ($p<0.05$)**Table 1**), or after the *in vivo* injection of SiNPs, as compared to control (At 100 nM Phe, the mean percentage constriction was 15.85 ± 4.05% vs. 19.48 ± 13.57% after incubation in PSS and SiNP injection *in vivo*, n=4 and 3 respectively, unpaired *t*-test, NS; **Figure 3B**).

3.4. The influence of SiNPs on vasodilator responses; Endothelial-dependent (ACh) Responses

All pre-constricted vessels dilated to ACh in a dose dependent manner. Incubation in SiNPs *ex vivo*, at 1.01 x 10^{11} NPs/mL (lower dosage) had no overall detrimental influence on ACh responses (**Figure 4**). However, exposure to SiNPs at 5.32 x 10^{11} NPs/mL (higher dose), *ex vivo* led to a significant attenuation in the dilator responses to ACh following Phe pre-constriction, at specific concentrations (100 nM to 1 mM) (**Figure 4**). At 1 μM of ACh, the mean percentage dilation was 96.15 ± 3.11%, 27.82 ± 5.13% after incubation in PSS and SiNPs respectively (n=6 and 11 respectively, unpaired *t*-test, $p<0.001$; **Figure 4**). There was a significant rightward shift in the concentration-response curve and reduced the maximal dilation to ACh (ACh E_{max} 43.67 ± 4.33%; pD_2 1.14 ± 8.66 μM, vs E_{max} 97.03 ± 3.38%; pD_2 0.02 ± 0.04 μM for control responses ($p<0.05$); **Table 1**). When infused under flow conditions *ex vivo* (at

a final dosage of 5.32 x 10^{11} NPs/mL) SiNPs had no overall effect on the magnitude of dilator responses at higher concentrations of ACh following Phe pre-constriction (1–100 μM of ACh); but led to a significant decrease in the magnitude of dilator responses at ACh concentration of 100 nM (**Figure 4**). Although the incubation in SiNPs at the lower dosage and under flow conditions produced a rightward shift in pD_2, the maximal dilation levels were unaffected (**Table 1**).

In contrast, injection of SiNPs *in vivo*, at a final dosage of 5.32 x 10^{11} NPs/mL, had no overall effect on the magnitude of dilator responses at high concentrations of ACh following Phe pre-constriction of mesenteric arteries (1-100 μM of ACh; **Figure 4**). *In vivo* injected SiNPs produced a significant reduction in the magnitude of ACh-induced dilation only at lower concentrations of ACh (10 and 100 nM; similar to *ex vivo*) following Phe pre-constriction of mesenteric arteries (**Figure 4**), with a right ward shift in pD_2 (Table 1). Hence, the infusion of SiNPs under flow conditions and their injection *in vivo* displayed less detrimental effect on vasodilator responses compared to *ex vivo* exposure under static conditions.

3.5. The influence of SiNPs on vasodilator responses; Endothelial-independent responses

The endothelial-independent dilator responses to the NO donor, SNP were unaffected by incubation in SiNPs at 5.32 x 10^{11} NPs/mL at SNP concentrations of 10 and 100 nM (At 10 nM ACh, the mean percentage dilation was 79.44 ± 6.52% vs. 60.56 ± 18.51% after incubation in PSS and SiNPs, *ex vivo*, n=4 and 6 respectively, unpaired *t*-test, NS; **Figure 5A**). In contrast, SNP responses were significantly affected by SiNPs that were injected *in vivo* at specific SNP concentrations (10 and 100 nM) (At 10 nM concentration of SNP, the mean percentage dilation was 79.44 ± 6.52% vs. 2.80 ± 2.14% after incubation in PSS and SiNP injected *in vivo*, n=4 and 3 respectively, unpaired *t*-test, $p<0.001$; **Figure 5A**). The maximal concentration of SNP also displayed a significant reduction in dilation in SiNP-injected rats (At 100 nM concentration of SNP, the mean percentage dilation was 99.31 ± 5.72% vs. 15.21 ± 13.40% after incubation in PSS and SiNP injected *in vivo*, n=4 and 3 respectively, unpaired *t*-test, $p<0.001$; **Figure 5A**). The dilator responses to an endothelial-independent smooth muscle cell relaxant (PAPA; at 100 μM) were unaffected by the infusion of SiNPs *ex vivo* (At 100 μM concentration of PAPA, the

mean percentage dilation was 89.04 ± 12.53% vs. 105.07 ± 2.79% after incubation in PSS and SiNPs respectively, n=3, unpaired *t*-test, NS; **Figure 5B**). Likewise, the vasodilator responses to PAPA were unaffected by SiNP injection *in vivo* (At 100 μM concentration of PAPA, the mean percentage dilation was 89.04 ± 12.53% vs. 104.53 ± 4.32% after incubation in PSS and SiNPs respectively, n=3, paired *t*-test, NS; **Figure 5B**).

3.6. SiNP biodistribution within tissues and organs

The ICP elemental analysis confirmed the internalisation of SiNPs within various tissues and organs after their injection *in vivo* (**Figure 6**). A small fraction of 5.6 ± 2.56% of the SiNPs ($5.60 \times 10^{-4} \pm 2.56 \times 10^{-4}$ mg/g of animal tissue) administered *in vivo* were localised within various tissues including; lungs, heart, aorta, mesentery, liver, spleen and kidney (**Figure 6**). Despite the minimal concentration of SiNPs internalised into different tissues, the largest fraction of uptake was observed within the mesenteric arterial tissue with the latter accounting for nearly 5% of the SiNPs injected *in vivo* (**Figure 6**).

3.7. The molecular mechanisms of SiNPs-induced attenuated dilation

Findings from the present study suggest that exposure of small mesenteric arteries to SiNPs at the higher dosage under static conditions, over a 30 minute acute period, has a detrimental effect on endothelial-dependent (ACh) dilator responses. In order to elucidate the mechanisms involved, a series of experiments were conducted including; the characterisation of the dilator component in the absence and presence of SiNPs and examination of the effect of co-incubation with superoxide dismutase (a reactive oxygen species [ROS] scavenger). We examined two theories concerning 1) the involvement of ROS in mediating SiNP-induced impairment of vascular function and 2) the influence of SiNPs on vasodilator pathways, by using specific dilator pathway inhibitors (L-NNA, Indomethacin, as well as apamin and TRAM-34 which are small and intermediate potassium channel inhibitors, respectively, used to inhibit the EDHF pathway).

In control vessels, the inhibition of nitric oxide synthesis (using L-NNA) *ex vivo* had no overall effect on the ACh-induced dilator responses in Phe pre-constricted vessels (**Figure 7A**). This is despite a significant decrease in dilation to ACh at 10

nM (the mean percentage dilation was 39.30 ± 11.25% vs. 8.78 ± 4.86% after incubation in PSS and L-NNA respectively, n=6, paired *t*-test, $p<0.05$; **Figure 7A**), while at 1 nM, submaximal (100 nM) and higher (1 μM and 10 μM) ACh concentrations, dilations were preserved (**Figure 7A**). Incubation in the SK_{Ca} and IK_{Ca} channel-selective inhibitors; apamin (at 100 nM) and TRAM-34 (at 1 μM), respectively, produced a significant rightward shift in the concentration-response curve, without affecting the maximal relaxation to ACh (ACh E_{max} 106.20 ± 3.58%; pD_2 0.27 ± 0.24 μM for apamin ($p<0.05$, n=4); E_{max} 99.37 ± 2.79%; pD_2 0.27 ± 0.18 μM for TRAM-34 ($p<0.05$, n=4) vs. E_{max} 97.03 ± 3.38%; pD_2 0.02 ± 0.04 μM for control responses (n=6), respectively; **Table 1**). However, when the apamin was added in combination with TRAM-34, this produced a further rightward shift in the concentration-response curve and reduced the maximal relaxation (For apamin and TRAM-34; ACh E_{max} 19.78 ± 4.16%; pD_2 31.19 ± 7.20 μM, $p<0.01$, n=4; **Table 1**). The co-incubation of mesenteric arteries in apamin and TRAM-34 significantly reduced and almost abolished the vasodilator responses to ACh, with this being further reduced when an inhibitor cocktail containing L-NNA, apamin and TRAM-34 was added (**Figure 7A**). To investigate whether a NO-independent pathway was involved in mediating the small degree of dilation that is present following the inhibition in L-NNA, apamin and TRAM-34, we used a cyclooxygenase inhibitor; indomethacin. The inhibition of the COX with indomethacin had no overall effect on the ACh-induced dilator responses in Phe pre-constricted vessels; (At 100 nM ACh, the mean percentage dilation was 78.77 ± 8.58% vs. 84.41 ± 10.97% after incubation in PSS and indomethacin, n=6 and 4 respectively, unpaired *t* test, $p<0.05$; **Figure 7A**). The incubation in the inhibitor cocktail containing L-NNA, apamin + TRAM-34 or L-NNA, indomethacin, apamin and TRAM-34 abolished the vasodilator responses to ACh (For all inhibitors; ACh E_{max} 0.63 ± 0.33%; pD_2 1.52 x 10^3 ± 1.45 x 10^3, $p<0.001$, n=5; **Table 1 and Figure 7A**).

There was a significant rightward shift in the concentration-response curve with reduced maximal dilation to ACh The co-incubation of SiNPs in the presence of L-NNA completely abolished the dilator responses to ACh at all concentrations tested (**Figure 7B**). The most drastic effects were observed at the submaximal (100 nM), maximal (1 μM) and highest (10 μM) ACh concentrations (At 100 nM ACh, the mean

percentage dilation was 78.77 ± 8.58% vs. 3.09 ± 1.48% after incubation in PSS and SiNPs with L-NNA respectively, n=6 and 7 respectively, unpaired *t* test, p<0.001; **Figure 7B**). (ACh E_{max} 3.34 ± 0.77%; pD_2 23.99 x 10^3 ± 0.74 x 10^3 μM, p<0.0001, n=7; **Table 1**).

When vessels were co-incubated in SiNPs (5.32 x 10^{11} NPs/mL) and superoxide dismutase (SOD) dilation of Phe pre-constricted vessels was not restored but led to further reduction in the ACh dilator responses, especially at the highest ACh concentration (At 10 μM ACh, the mean percentage dilation was 96.68 ± 7.82% vs. 35.58 ± 3.51% after incubation in PSS and SiNPs with SOD respectively, n=6 and 5 respectively, unpaired *t*-test, p<0.001; **Figure 7B**).

4. DISCUSSION

In the present study, we demonstrate that SiNPs can influence the functional responses of small mesenteric arteries after acute exposure, *ex vivo*, and after iv administration *in vivo*. Using our *ex vivo* model, we show that these effects are dose dependent but are less detrimental after exposure under flow vs static conditions. Using pharmacological inhibition studies, our findings suggest that SiNPs may interfere with the EDHF vasodilator pathway.

The acute exposure of vessels to SiNPs led to their cellular uptake into the cytoplasm of ECs, either being freely localised or inside vesicles, without affecting cellular integrity. Some SiNPs seemed to form aggregates near the surface of ECs and were seen inside vesicle structures near the outer edges of the cell. This was evident *ex vivo* under static and flow conditions and after *in vivo* exposure, though there were less SiNPs taken up by ECs *in vivo*. Under flow conditions, *ex vivo*, many nanoparticles were seen outside the cell. Due to the predominant negative charge on the SiNPs at physiological pH, this may have caused their repulsion from EC surfaces, perhaps via their negative membrane phospholipidic groups (Gamucci et al., 2014). We assume that SiNPs have either become neutralised or gained positive charge or both by the ionic species found in PSS and/or ECs secretions, ECM proteins *in vivo*, forming a protein corona (Gamucci et al., 2014). The internalisation

of SiNPs by ECs has previously been documented both *in vitro* and *in vivo*. Barbé et al. reported the internalisation and passage of SiNPs through small intercellular openings in normal blood vessel walls *in vivo* (Barbé et al., 2004), while Kasper et al. found that amorphous SiNPs (30, 70 and 300 nm) at 2 x 10^{11} NPs/mL were localised within human microvascular ECs, inside specialised flotillin-1- and flotillin-2-bearing endocytic vesicles (Kasper et al., 2013). The uptake of SiNPs may largely be related to an engulfment through the endocytosis pathway.

While acute exposure to SiNPs *ex vivo* had no overall influence on contractile responses to high potassium solution, we show that SiNPs can significantly reduce Phe-induced constrictor responses of mesenteric arteries, at maximal Phe concentrations. Phe acts via α1-adrenoceptors located on the membrane surface of the vascular smooth muscle cells (VSMCs) to activate a Ca^{2+}-dependent sensitising pathway during the sustained late-phase contraction (Kitazawa and Kitazawa, 2012). The action of Phe is mediated via protein kinase C (PKC) leading to the phosphorylation and inhibition of myosin light chain phosphatase and subsequent sacroplasmic Ca^{2+} release and voltage-dependent Ca^{2+} influx (Kitazawa and Kitazawa, 2012). The compromise of the Phe-induced constriction and the unsustained constriction of the vessels at maximal Phe doses after SiNP exposure *ex vivo* may be due to a number of reasons, including the random blockage of Phe receptor, acting as a non-competitive antagonist as indicated by the reduced E_{max} and pD_2 values, and/or the enzymes, mediators and adaptor proteins involved in mediating the action of Phe. SiNPs injected *in vivo*, had no overall effect on Phe-induced contraction (at the higher doses) contrary to the results found after *ex vivo* infusion of SiNPs. This may be due to the corona formation after the SiNPs are injected into the blood (Tenzer et al., 2011), thus preventing their interaction with the α1-adrenergic receptors. The ability of PAPA to induce relaxation of VSMCs after the *in vivo* SiNP injection (attributed to its direct action on the smooth muscle cell layer) suggests that SiNPs had no overall effect on the contractile machinery, or the integrity of the VSMCs. Another plausible reason for the reduced Phe constriction may relate to the possibility that SiNPs may interfere with the action of potassium channels. A direct influence of nanoparticles on potassium channels has recently been documented. Soloviev et al (2015) used a whole cell patch clamping technique to demonstrate potassium channel activation (large conductance, $BK_{Ca,}$ which are

found on the surface of VSMCs) by gold nanoparticles. Addition of gold nanoparticles to whole isolated aortic vessels led to a decrease in the amplitude of Norepinephrine induced contractions, in a concentration dependent manner (Soloviev et al, 2015).

Acute exposure to SiNPs under static conditions (at the higher dosage) significantly reduced ACh-induced dilation of mesenteric vessels *ex vivo.* Although SiNPs injected *in vivo*, induced significant reduction in dilator responses at the lower ACh doses, there was no overall detrimental reduction in dilation at higher ACh doses. This may relate to corona formation after the nanoparticles are injected into the animal (Tenzer et al, 2011). A recent study by Nemmar et al. suggested a significant reduction in the endothelium-dependent relaxation (at ACh concentration of 0.1 μM) of small rat mesenteric arteries (third-branches) following exposure to amorphous SiNPs (of 50 and 500 nm size respectively, at 50 μg/mL) *ex vivo*, using the wire myography system (Nemmar et al., 2014). Previous findings from our group, using large aortic vessels, also demonstrate that SiNPs have a direct influence on dilator function. While acute exposure to 100 and 200 nm nonmodified SiNPs (at 1.1×10^{11} NPs/mL) had no detrimental effect on conduit arterial function, the positively charged SiNPs had a greater degree of attenuation on dilator responses (Akbar et al., 2011). Furthermore, using 30 and 70 nm SiNPs, we demonstrate that attenuation of endothelial-dependent vasodilator responses is related to nanoparticle surface area rather than size (Farooq et al., 2013). In the present study, we show that the attenuation in endothelial-dependent dilation *ex vivo*, is dosage dependent. This may relate to the number of nanoparticles being taken up by the lining ECs, however, it is recognised that SiNPs do not need to be internalised to affect vascular function, possibly interacting with cell surface receptors and channel proteins (Soloviev et al, 2015; Yan et al, 2013).

The SiNP surface has previously been shown to generate ROS, that could quench nitric oxide hence affecting vasodilation (Farooq et al., 2013) and/or cause nitroxidative/oxidative stress (Corbalan et al., 2011). Previous studies have reported effects of SiNPs on inducing EC dysfunction through oxidative stress via JNK-, p53-

and NF-κβ-related pathways (Liu and Sun, 2010) or the induction of antioxidant enzymes (catalase, glutathione transferase and thioredoxin reductase) by 100 nm SiNPs through ROS-dependent pathways (Passagne et al., 2012). Furthermore; an impairment of EDHF-related vasodilation in the mesenteric arteries of the rat via ROS-dependent mechanism has also been suggested previously (Zhao et al., 2013). The co-incubation of vessels in SiNPs and SOD did not restore vasodilation, as we have previously demonstrated with large vessels (Farooq et al., 2013), suggesting that the mechanism for SiNP impairment of vasodilation is ROS-independent and may involve alternative mechanisms.

Our inhibition studies demonstrate that in control Phe-preconstricted vessels, the vasodilator response was influenced by all inhibitors tested, thus demonstrating that both nitric oxide and EDHF contribute to the vasodilator component in mesenteric arteries. Therefore, the incubation of mesenteric arteries in L-NNA may have partially compromised vasodilation as a result of reduced nitric oxide bioavailability (Joshi and Woodman, 2012). The co-incubation of SiNPs in the presence of L-NNA completely abolished the dilator responses to ACh. This suggests that SiNPs may directly affect the EDHF pathway. The more pronounced role of EDHF in small mesenteric arteries (specifically second to fourth order arteries) compared with medium sized and large conduit arteries such as the superior mesenteric artery and the aorta is attributed to the action of intermediate- and small-conductance Ca^{2+}-activated potassium channels in ACh-induced relaxations (Hilgers et al., 2006). The dilation of mesenteric arteries in the presence of the inhibitor cocktail (L-NNA, apamin and TRAM-34) was not sufficient to completely abolish the dilator response of the mesenteric arteries and was able to maintain a very minimal dilation which may be due to the contribution of prostanoids (Honing et al., 2000) from the cyclooxygenase pathway. Previous reports also suggest the participation of connexins in mediating the endothelial-dependent hyperpolarisation via myoendothelial gap junctions in different vascular beds (de Wit and Griffith, 2010). These gap junctions may have alternative potassium channels to the apamin/TRAM-34-sensitive calcium-activated potassium (K^+) ones and/or rely on other pathways that lead to vasodilation including cAMP (Chaytor et al., 2002). Consequently, the attenuation in dilation may be due to 1) the indirect or the direct interaction of SiNPs

with specific extracellular, intracellular and downstream factors involved in the EDHF-dependent pathway in ECs and 2) the inability of nitric oxide to compensate for the loss in EDHF action due to the delayed nitric oxide production and/or the influence of SiNPs on eNOS or other factors involved in nitric oxide synthesis and action, as has been demonstrated after TiO_2 NP inhalation, which caused disruption in nitric oxide signalling and resultant blunted arteriolar dilation (Nurkiewicz et al., 2009, LeBlanc et al., 2009). Additionally, the effect of SiNPs on EC function may be related to the interaction, disruption and/or blockage of muscarinic (M)-3 ACh receptors on the EC surface, voltage-gated channels, and/or trafficking and transport of the vasodilator machinery. Our findings are further supported by evidence suggesting that cardiovascular toxicity triggered by SiNPs occurs mainly in vascular endothelium rather than cardiomyocytes, where the SiNPs induced damage to cytoskeletal organisation, attenuating expression of cellular adhesion molecules and contributing to disturbance of EC homeostasis and influence on VEGF2/MAPK/Erk1/2/mTOR signalling pathways (Duan et al., 2014). Nanoparticles have also been shown to alter the activity of potassium channels. Although there is a lack of understanding of the direct influence of SiNPs on potassium channels within ECs, one study has demonstrated that treatment of HUVECs with SiNPs (~50 nm diameter), *in vitro*, led to a decrease in cell survival rate, increased inflammatory cytokines and increased the potassium channel activity in the opening rate and current intensity (Yang et al, 2013).

The *ex vivo* exposure to SiNPs had no influence on the NO-independent dilator pathway for mesenteric vessels indicating that smooth muscle sensitivity to NO was unaffected. This finding was supported by other studies from our group, in large vessels described earlier. In contrast, the *in vivo* injected SiNPs induced a significant reduction in SNP induced dilation, as we have previously demonstrated for the QDs (Shukur et al., 2013). The mechanism for this is unclear, however, it is noteworthy that ingestion of silica over an 8 day period has been documented to modify the characteristics of endothelial dilation in rat aorta, with signification reduction in the SNP induced dilation and attenuation of SMC responsiveness to nitric oxide (Oner et al, 2006). This has important medical implications on SNP-based drugs used in cardiovascular disease patients.

A comparison between *ex vivo* and *in vivo* findings suggest that while under static conditions SiNPs attenuate endothelial dependent dilation, when introduced under flow conditions *ex vivo,* responses were similar to those after their *in vivo* injection. *In vivo*, the injected SiNPs are likely to adsorb proteins from the serum, forming a 'corona' thus altering surface characteristics of the SiNPs, which may lead to reduced uptake by the lining ECs, in comparison to uptake *ex vivo* (Ang et al., 2014). Whilst these effects may relate to the number of nanoparticles being taken up by ECs, the influence of cyclic stretch on the uptake and cytotoxicity has been controversial. In one study, culturing human umbilical vein ECs under physiological cyclic stretch conditions did not show enhanced uptake and/or increased cytotoxicity of amorphous SiNPs when compared to static culture conditions (Freese et al., 2014). In contrast, Hu and Liu (2015) have demonstrated enhanced cellular uptake of 100 nm polystyrene nanoparticles under cyclic stretch using bovine aortic endothelial cells (relevant to physiological strain levels) (Hu and Liu, 2015).The uptake of SiNPs by ECs was also confirmed by ICP analysis demonstrating that SiNPs had largely accumulated in mesenteric arteries *in vivo*. The mesentery represents the highest proportion of ECs, in comparison to other tissues e.g. the heart. A similar *in vivo* study by Borak et al. found that only 36% of the introduced SiNPs (150 nm) were excreted after four days of iv injection, suggesting a longer term retention of these nanoparticles by tissues (Borak et al., 2012). The dosage of SiNps used in our *in vivo* study is comparable to that used by other biocompatibility and biodistrinution studies. Ivanov et al. (20??), used, SiNPs (10 and 13 nm) at a dosage of 7 mg/Kg to assess their hematological and biochemical parameters in rats 5, 21 and 60 days after intravenous administration. The study by Lee et al. injected 16 mg/Kg of mesoporous SiNPs with an average size of 50–100 nm for *in vivo* optical imaging. A similar dosage of 20 mg/Kg of SiNPs was also injected into mice to study their *in vivo* biodistribution and pharmacokinetics (Yu et al, year ??).

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5. CONCLUSION

In conclusion, we demonstrate that exposure to SiNPs (infused intralumenally or injected intravenously), can alter physiological function of rat mesenteric vessels, depending on nanoparticle dosage. In the present study, an attempt was made to

elucidate the mechanism of attenuated endothelial dependent dilator responses by SiNP uptake, using inhibition studies. The compromise in dilator responses *ex vivo* may be attributed to SiNP effects on the EDHF related vasodilator pathway. Our findings highlight the importance of assessing the biosafety of nanoparticles for use in imaging diagnostics and medical interventions in order to minimise their toxicological influence on vessel contractility and function.

6. Summary points:

1. Mono-dispersed dye-encapsulated silica nanoparticles [SiNPs] (97.85 ± 2.26 nm diameter) were fabricated and characterised and their influence on small mesenteric arterial function assessed *ex vivo,* under static and flow conditions. The influence of intravenous injection of SiNPs, *in vivo*, was also assessed.
2. The technique of pressure myography was used to assess the vasoconstrictor (phenylephrine [Phe]) and endothelial-dependent (acetylcholine [ACh]) and independent (sodium nitroprusside [SNP]) vasodilator responses of small mesenteric arteries, from male Wistar rats, before and after acute exposure to the SiNPs.
3. SiNPs were rapidly taken up by ECs lining mesenteric arteries. SiNPs attenuated Phe contractile responses, but did not alter the responses to KPSS or papaverine *ex vivo*, suggesting that the contractile machinery is unaffected by SiNP uptake.
4. Exposure of mesenteric arteries to SiNPs, *ex vivo*, under static conditions, led to an attenuation in endothelium dependent (ACh) dilator responses (at 5.32 $x10^{11}$ NP/mL), however, at a lower SiNP dose (1.01 x 10^{11} NPs/mL) there was no overall effect on ACh responses. The endothelium-independent (SNP) vasodilator responses were unaffected by incubation in SiNPs.
5. The compromise in dilator responses shown after *ex vivo* exposure at the higher SiNP dosage, may be attributed to SiNP effects on the EDHF-related pathway as demonstrated by our inhibition studies.

6. When injected intravenously *in vivo*, SiNPs only had a detrimental effect on constrictor (Phe) and endothelial dependent (ACh) dilator responses, at the lower agonist concentrations, similar to SiNP effects when infused under flow conditions.
7. A significant reduction in SNP-induced dilator responses was seen after SiNPs were injected *in vivo*. This may have clinical implications on the use of SNP drugs for patients with cardiovascular disease.
8. The dosage dependent influence of SiNPs on arterial function will help identify strategies for their safe clinical administration in the future.
9. Our findings highlight the importance of assessing the biosafety of nanoparticles for use in imaging diagnostics and medical interventions in order to minimise their toxicological influence on vessel contractility and function.

Figure legends

Graphical Abstract. A schematic illustration summarising the effect of SiNPs on the vasodilator function of ECs and VSMCs of the mesenteric artery and the potential associated mechanisms involved in mediating their behaviour *ex vivo*. AA; arachidonic acid, ACh; acetylcholine, BK_{Ca}; large-conductance calcium-activated potassium channels, Ca^{2+}; calcium ion, cAMP; cyclic AMP, cGMP; cyclic guanosine monophosphate, COX-2; cyclooxygenase-2*,* CREB; response element-binding protein, Cx 40; connexion 40, CYP; cytochrome P450, EDHF; endothelial-derived hyperpolarising factor (EDHF), EET; epoxyeicosatrienoic acid, eNOS3; endothelial-derived NO synthase 3 isotype, ERK; Extracellular-signal-regulated kinase, GC; guanylyl cyclase, Gp; G protein, GTP; guanosine-5‘ triphosphate, IK_{Ca}; intermediate-conductance calcium-activated potassium channels, IL6; interleukin 6, IL8; interleukin 8, K^+; potassium ion, K_v; voltage-gated potassium channels, M3; muscarinic receptor type 3, MKP-1; mitogen-activated protein (MAP) kinase phosphatase-1, NF-κβ; nuclear factor kappa beta, NO; nitric oxide, PKA; protein kinase A, PKG; protein kinase G, PLA_2; phospholipase A2, PLC; phospholipase C, R; receptor, SiNPs; silica nanoparticles, SK_{Ca}; small-conductance calcium-activated potassium channels, SOD; superoxide dismutase, TNFα; tumor necrosis factor alpha, TRP; transient receptor potential.

Figure 1. The Zeta potential output of the silica nanoparticles [SiNPs] (in water; A), a scanning electron micrograph of SiNPs with 97.85 ± 2.26 nm diameter (B; scale bar =100 nm) and fluorescence spectroscopy of the RBITC dye encapsulated SiNPs (C).

Figure 2. Transmission electron micrographs (A, B, C and D; scale bars = 1 μm, 100 nm, 0.5 μm and 200 nm, respectively) illustrating the internalisation of SiNPs into the vascular endothelium of the mesenteric arteries after incubation, *ex vivo* (A and B) and *in vivo* (C and D), respectively. The SiNPs are represented by black spherical structures inside ECs (arrows).

Figure 3. The influence of SiNPs injected *ex vivo* vs. *in vivo*, on the KPSS- (A) and Phe- (B) induced contraction. 'n' is number of vessels. Multi-variate repeated measures ANOVA. *p<0.05, ***p<0.001 and ****p<0.0001, error bars=SEM. P values correspond to comparisons between SiNPs incubated either *ex vivo* or *in vivo* vs. control Phe responses of mesenteric arteries.

Figure 4. The influence of SiNPs injected *ex vivo* vs. *in vivo*, on the ACh-induced relaxation in Phe pre-constricted vessels. 'n' is number of vessels. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, error bars=SEM. Multi-variate repeated measures ANOVA. P values correspond to comparisons between SiNPs incubated either *ex vivo* or *in vivo* vs. control responses of mesenteric arteries.

Figure 5. The influence of SiNPs injected *ex vivo* vs. *in vivo*, on the SNP- (A) and PAPA- (B) induced relaxation in Phe pre-constricted vessels. 'n' is number of vessel. ***p<0.001, error bars=SEM. Multi-variate repeated measures ANOVA. P values correspond to comparisons between SiNPs incubated either *ex vivo* or *in vivo* vs. control responses of mesenteric arteries.

Figure 6. The mean normalised percentage of Silica concentration present in *in vivo* tissues, expressed as a mean percentage of the actual dose injected (100%).

Figure 7. The influence of the inhibitor cocktail of L-NNA, apamin and TRAM-34 as well as indomethacin (A) and the co-incubation in SiNPs and L-NNA or SiNPs and SOD (B) on the ACh-induced relaxation in Phe pre-constricted vessels. 'n' is number of vessels. ***p<0.001 and ****p<0.0001, error bars=SEM. Multi-variate repeated measures ANOVA. P values correspond to comparisons between inhibitor-incubated vs. control ACh responses of mesenteric arteries.

Table 1. The influence of SiNPs and inhibitor incubation on vasoconstrictor and vasodilator responses of rat mesenteric arteries. E_{max} indicates maximal percentage response (contraction by Phe or relaxation by ACh or SNP respectively); pD2 values represent the –log (EC_{50}), concentration that produced half-maximal responses. Values are mean ± SEM. 'n' is number of vessels.

REFERENCES

AKBAR, N., MOHAMED, T., WHITEHEAD, D. & AZZAWI, M. 2011. Biocompatibility of amorphous silica nanoparticles: Size and charge effect on vascular function, in vitro. *Biotechnol Appl Biochem,* 58**,** 353-62.

ANG, J. C., HENDERSON, M. J., CAMPBELL, R. A., LIN, J. M., YARON, P. N., NELSON, A., FAUNCE, T. & WHITE, J. W. 2014. Human serum albumin binding to silica nanoparticles - effect of protein fatty acid ligand. *Phys Chem Chem Phys*.

BARBÉ, C., BARTLETT, J., KONG, L., FINNIE, K., LIN, H. Q., LARKIN, M., CALLEJA, S., BUSH, A. & CALLEJA, G. 2004. Silica Particles: A Novel Drug-Delivery System. *Advanced Materials,* 16**,** 1959-1966.

BORAK, B., BIERNAT, P., PRESCHA, A., BASZCZUK, A. & PLUTA, J. 2012. In Vivo Study on the Biodistribution of Silica Particles in the Bodies of Rats. *Advances in Clinical and Experimental Medicine,* 21**,** 13-18.

BRESLOW, N. E. & CLAYTON, D. G. 1993. Approximate Inference in Generalized Linear Mixed Models. *Journal of the American Statistical Association,* 88**,** 9-25.

CHAYTOR, A. T., TAYLOR, H. J. & GRIFFITH, T. M. 2002. Gap junction-dependent and -independent EDHF-type relaxations may involve smooth muscle cAMP accumulation. *American Journal of Physiology-Heart and Circulatory Physiology,* 282**,** H1548-H1555.

CORBALAN, J. J., MEDINA, C., JACOBY, A., MALINSKI, T. & RADOMSKI, M. W. 2011. Amorphous silica nanoparticles trigger nitric oxide/peroxynitrite imbalance in human endothelial cells: inflammatory and cytotoxic effects. *Int J Nanomedicine,* 6**,** 2821-35.

CORBALAN, J. J., MEDINA, C., JACOBY, A., MALINSKI, T. & RADOMSKI, M. W. 2012. Amorphous silica nanoparticles aggregate human platelets: potential implications for vascular homeostasis. *Int J Nanomedicine,* 7**,** 631-9.

DE WIT, C. & GRIFFITH, T. M. 2010. Connexins and gap junctions in the EDHF phenomenon and conducted vasomotor responses. *Pflugers Archiv-European Journal of Physiology,* 459**,** 897-914.

DIACONU, M., TACHE, A., EREMIA, S. A.-M. V., GATEA, F., LITESCU, SIMONA. & RADU, G. L. 2010. STRUCTURAL CHARACTERIZATION OF CHITOSAN COATED SILICON NANOPARTICLES –A FT-IR APPROACH. *U.P.B. Sci. Bull., Series B,* 72.

DUAN, J. C., YU, Y. B., YU, Y., LI, Y., HUANG, P. L., ZHOU, X. Q., PENG, S. Q. & SUN, Z. W. 2014. Silica nanoparticles enhance autophagic activity, disturb endothelial cell homeostasis and impair angiogenesis. *Particle and Fibre Toxicology,* 11.

EDWARDS, G., DORA, K. A., GARDENER, M. J., GARLAND, C. J. & WESTON, A. H. 1998. K+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature,* 396**,** 269-272.

FAROOQ, A., WHITEHEAD, D. & AZZAWI, M. 2013. Attenuation of endothelial-dependent vasodilator responses, induced by dye-encapsulated silica nanoparticles, in aortic vessels. *Nanomedicine (Lond)*.

FREESE, C., SCHREINER, D., ANSPACH, L., BANTZ, C., MASKOS, M., UNGER, R. E. & KIRKPATRICK, C. 2014. In vitro investigation of silica nanoparticle uptake into human endothelial cells under physiological cyclic stretch. *Part Fibre Toxicol,* 11**,** 1.

GAMUCCI, O., BERTERO, A., GAGLIARDI, M. & BARDI, G. 2014. Biomedical Nanoparticles: Overview of Their Surface Immune-Compatibility. *Coatings,* 4**,** 139-159.

HALD, B. O., JACOBSEN, J. C., BRAUNSTEIN, T. H., INOUE, R., ITO, Y., SORENSEN, P. G., HOLSTEIN-RATHLOU, N. H. & JENSEN, L. J. 2012. BKCa and KV channels limit conducted vasomotor responses in rat mesenteric terminal arterioles. *Pflugers Arch,* 463**,** 279-95.

HILGERS, R. H., TODD, J., JR. & WEBB, R. C. 2006. Regional heterogeneity in acetylcholine-induced relaxation in rat vascular bed: role of calcium-activated K+ channels. *Am J Physiol Heart Circ Physiol,* 291**,** H216-22.

HILGERS, R. H. & WEBB, R. C. 2007. Reduced expression of SKCa and IKCa channel proteins in rat small mesenteric arteries during angiotensin II-induced hypertension. *Am J Physiol Heart Circ Physiol,* 292**,** H2275-84.

HINTON, J. M. & LANGTON, P. D. 2003. Inhibition of EDHF by two new combinations of K+-channel inhibitors in rat isolated mesenteric arteries. *Br J Pharmacol,* 138**,** 1031-5.

HONING, M. L., SMITS, P., MORRISON, P. J. & RABELINK, T. J. 2000. Bradykinin-induced vasodilation of human forearm resistance vessels is primarily mediated by endothelium-dependent hyperpolarization. *Hypertension,* 35**,** 1314-8.

HU, J. & LIU, Y. 2015. Cyclic Strain Enhances Cellular Uptake of Nanoparticles. *Journal of Nanomaterials,* 2015**,** 8.

HWA, J. J., GHIBAUDI, L., WILLIAMS, P. & CHATTERJEE, M. 1994. Comparison of acetylcholine-dependent relaxation in large and small arteries of rat mesenteric vascular bed. *American Journal of Physiology - Heart and Circulatory Physiology,* 266**,** H952-H958.

ISMAIL, A. M. I., A.A.F., Z. & MOHAMED, A. S. 2010. Preparation of spherical silica nanoparticles: Stober silica. *Journal of American Science,* 6**,** 985-989.

ITOH, T., SEKI, N., SUZUKI, S., ITO, S., KAJIKURI, J. & KURIYAMA, H. 1992. Membrane hyperpolarization inhibits agonist-induced synthesis of inositol 1,4,5-trisphosphate in rabbit mesenteric artery. *The Journal of Physiology,* 451**,** 307-328.

JOKERST, J. V., KHADEMI, C. & GAMBHIR, S. S. 2013. Intracellular Aggregation of Multimodal Silica Nanoparticles for Ultrasound-Guided Stem Cell Implantation. *Science Translational Medicine,* 5.

JOSHI, A. & WOODMAN, O. L. 2012. Increased nitric oxide activity compensates for increased oxidative stress to maintain endothelial function in rat aorta in early type 1 diabetes. *Naunyn-Schmiedebergs Archives of Pharmacology,* 385**,** 1083-1094.

KASPER, J., HERMANNS, M. I., BANTZ, C., KOSHKINA, O., LANG, T., MASKOS, M., POHL, C., UNGER, R. E. & KIRKPATRICK, C. J. 2013. Interactions of silica nanoparticles with lung epithelial cells and the association to flotillins. *Arch Toxicol,* 87**,** 1053-65.

KIM, I. Y., JOACHIM, E., CHOI, H. & KIM, K. 2015. Toxicity of silica nanoparticles depends on size, dose, and cell type. *Nanomedicine,* 11**,** 1407-16.

KIM, J., CAO, L., SHVARTSMAN, D., SILVA, E. A. & MOONEY, D. J. 2011. Targeted delivery of nanoparticles to ischemic muscle for imaging and therapeutic angiogenesis. *Nano Lett,* 11**,** 694-700.

KITAZAWA, T. & KITAZAWA, K. 2012. Size-dependent heterogeneity of contractile Ca2+ sensitization in rat arterial smooth muscle. *J Physiol,* 590**,** 5401-23.

LEBLANC, A. J., CUMPSTON, J. L., CHEN, B. T., FRAZER, D., CASTRANOVA, V. & NURKIEWICZ, T. R. 2009. Nanoparticle inhalation impairs endothelium-dependent vasodilation in subepicardial arterioles. *J Toxicol Environ Health A,* 72**,** 1576-84.

LIBERMAN, A., MENDEZ, N., TROGLER, W. C. & KUMMEL, A. C. 2014. Synthesis and surface functionalization of silica nanoparticles for nanomedicine. *Surface Science Reports,* 69**,** 132-158.

LITTLE, T. L., XIA, J. & DULING, B. R. 1995. Dye Tracers Define Differential Endothelial and Smooth-Muscle Coupling Patterns within the Arteriolar Wall. *Circulation Research,* 76**,** 498-504.

LIU, X. & SUN, J. A. 2010. Endothelial cells dysfunction induced by silica nanoparticles through oxidative stress via JNK/P53 and NF-kappa B pathways. *Biomaterials,* 31**,** 8198-8209.

MOTWANI, M. S., RAFIEI, Y., TZIFA, A. & SEIFALIAN, A. M. 2011. In situ endothelialization of intravascular stents from progenitor stem cells coated with nanocomposite and functionalized biomolecules. *Biotechnol Appl Biochem,* 58**,** 2-13.

NEMMAR, A., ALBARWANI, S., BEEGAM, S., YUVARAJU, P., YASIN, J., ATTOUB, S. & ALI, B. H. 2014. Amorphous silica nanoparticles impair vascular homeostasis and induce systemic inflammation. *Int J Nanomedicine,* 9**,** 2779-89.

NURKIEWICZ, T. R., PORTER, D. W., HUBBS, A. F., STONE, S., CHEN, B. T., FRAZER, D. G., BOEGEHOLD, M. A. & CASTRANOVA, V. 2009. Pulmonary Nanoparticle Exposure Disrupts Systemic Microvascular Nitric Oxide Signaling. *Toxicological Sciences,* 110**,** 191-203.

PASSAGNE, I., MORILLE, M., ROUSSET, M., PUJALTE, I. & L'AZOU, B. 2012. Implication of oxidative stress in size-dependent toxicity of silica nanoparticles in kidney cells. *Toxicology,* 299**,** 112-124.

SANDOW, S. L., TARE, M., COLEMAN, H. A., HILL, C. E. & PARKINGTON, H. C. 2002. Involvement of myoendothelial gap junctions in the actions of endothelium-derived hyperpolarizing factor. *Circulation Research,* 90**,** 1108-1113.

SHUKUR, A., RIZVI, S. B., WHITEHEAD, D., SEIFALIAN, A. & AZZAWI, M. 2013. Altered sensitivity to nitric oxide donors, induced by intravascular infusion of quantum dots, in murine mesenteric arteries. *Nanomedicine,* 9**,** 532-9.

STÖBER, W., FINK, A. & BOHN, E. 1968. Controlled growth of monodisperse silica spheres in the micron size range. *Journal of Colloid and Interface Science,* 26**,** 62-69.

TENZER, S., DOCTER, D., ROSFA, S., WLODARSKI, A., KUHAREV, J., REKIK, A., KNAUER, S. K., BANTZ, C., NAWROTH, T., BIER, C., SIRIRATTANAPAN, J., MANN, W., TREUEL, L., ZELLNER, R., MASKOS, M., SCHILD, H. & STAUBER, R. H. 2011. Nanoparticle Size Is a Critical Physicochemical Determinant of the Human Blood Plasma Corona: A Comprehensive Quantitative Proteomic Analysis. *Acs Nano,* 5**,** 7155-7167.

ZHAO, L., WANG, Y., MA, X. & DENG, X. 2013. Oxidative stress impairs IKCa- and SKCa-mediated vasodilatation in mesenteric arteries from diabetic rats. *Nan fang yi ke da xue xue bao = Journal of Southern Medical University,* 33**,** 939-44.