1 Uterine Microvascular Sensitivity to Nanomaterial Inhalation: an *In Vivo* Assessment

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ABSTRACT

With the tremendous number and diverse applications of engineered nanomaterials incorporated in daily human activity, exposure can no longer be solely confined to occupational exposures of healthy male models. Cardiovascular and endothelial cell dysfunction have been established using *in vitro* and *in situ* preparations, but the translation to intact *in vivo* models is limited. Intravital microscopy has been used extensively to understand microvascular physiology while maintaining *in vivo* neurogenic, humoral, and myogenic control. However, a tissue specific model to assess the influences of nanomaterial exposure on female reproductive health has not been fully elucidated. Female Sprague Dawley (SD) rats were exposed to nano-TiO₂ aerosols (171 ± 6 nm, 10.1 ± 0.39 mg/m³, 5 hours) 24-hours prior to experimentation, leading to a calculated deposition of 42.0 ± 1.65 µg. After verifying estrus status, vital signs were monitored and the right horn of the uterus was exteriorized, gently secured over an optical pedestal, and enclosed in a warmed tissue bath using intravital microscopy techniques. After equilibration, significantly higher leukocyte-endothelium interactions were recorded in the exposed group. Arteriolar responsiveness was assessed using ionophoretically applied agents: muscarinic agonist acetylcholine (0.025 M; ACh; 20, 40, 100, and 200 nA), and nitric oxide donor sodium nitroprusside (0.05 M; SNP; 20, 40, and 100 nA), or adrenergic agonist phenylephrine (0.05 M; PE; 20, 40, and 100 nA) using glass micropipettes. Passive diameter was established by tissue superfusion with 10^{-4} M adenosine. Similar to male counterparts, female SD rats present systemic microvascular dysfunction; however the ramifications associated with female health and reproduction have yet to be elucidated.

INTRODUCTION

Microvascular reactivity is adversely altered in many female reproductive complications, including: preeclampsia (Vedernikov *et al.*, 1999; Brennan *et al.*, 2014; Osol *et al.*, 2014a) and intra-uterine growth restriction (Osol *et al.*, 2014b). Further, implantation and healthy gestation require precise and rapid maternal hemodynamic adaptations including uterine growth and microvascular remodeling for successful pregnancy.

Recently, we and others have investigated the influence of nanomaterial inhalation exposure in rodents during gestation and development, and have reported global vascular dysfunction within the macro- and microcirculation of the uterus in pregnant dams (Vidanapathirana *et al.*, 2014; Stapleton *et al.*, 2013). These vascular consequence are not limited to just the pregnant dam during gestation, but fetal pups exposed during gestation also display an impaired arterial dilation (Stapleton *et al.*, 2013). Further, neurobehavioral alterations within the offspring (Hougaard *et al.*, 2010), hepatotoxicity in the females in the F1 generation (Jackson *et al.*, 2013), and endothelium-dependent vascular dysfunction within the coronary and uterine circulations of the F1 generation (Stapleton *et al.*, 2014a). In addition, delays in litter delivery (Hougaard *et al.*, 2013), placental abnormalities (Yamashita *et al.*, 2011), increased fetal resorption and low birth weights (Jo *et al.*, 2013; Pietroiusti *et al.*, 2011), and nanomaterial translocation to the pups **via gestational connection** (Jo *et al.*, 2013; Yamashita *et al.*, 2011) **or lactation** (Snyder *et al.*, 2015; Sumner *et al.*, 2010) have been reported. These studies have also lead to broadened protocols of maternal exposure, with respect to anesthetic practices and maternal pulmonary exposures (Jackson *et al.*, 2011).

Female exposures to ENM during childbearing years may have long term consequences affecting successful implantation, placental, and fetal development associated with nanoparticle

translocation, pulmonary inflammation, and microvascular dysfunction. As human pregnancies are only clinically verified after implantation with the use of a blood test (Jovanovic *et al.*, 1987) and nanomaterial translocation to and deposition within the female reproductive organs has been identified (Sumner *et al.*, 2015) and systemic ENM excretion after exposure is low (Choi *et al.*, 2007) biological accumulation of nanoparticles pre-conception is highly plausible. However, the literature base and toxicological outcomes within these studies is low (Hougaard *et al.*, 2015).

While cardiovascular and endothelial cell dysfunction subsequent to engineered nanomaterial (ENM) exposure has been established using *in vitro* techniques, the study and identification of more complex physiological systems cannot be achieved. Therefore, systemic compensatory mechanisms (e.g. neural, endocrine) and their interactions remain largely unknown. Tissue-specific intravital microscopy may provide the translational step between *in vitro* observations and systemic responses *in vivo*. Intravital microscopy has been utilized extensively to understand microvascular physiology while maintaining *in vivo* neurogenic, humoral, and myogenic control; however it is not commonly employed in toxicological studies. In order to better understand the delicate nature of developmental physiology and specifically gestational nanotoxicology we must first fully evaluate normal maternal physiology in effort to identify microvascular development from virginity through pregnancy (Stapleton *et al.*, 2014b).

The uterine and endometrial vasculature have been shown to have differing local control mechanisms; most notably, the spiral arterioles are anatomically situated to develop into the uteroplacental arteries after implantation and are highly hormonally responsive (Robertson, 1976). Significant vascular remodeling of the spiral arterioles occurs during the luteal phase in response to the secretion of estrogen and progesterone (Deligdisch, 2000); however these vessels constrict as progesterone decreases in a non-pregnant state (Kelly *et al.*, 2001). Analysis of

individual spiral arterioles is notoriously difficult, due to their tortuous anatomy, spacing, and possible ischemia-reperfusion (Gannon *et al.*, 1997). Due to these unique anatomical differences within the endometrial vasculature, general vascular agonist delivery (dissolved in the superfusate) (Leonard *et al.*, 2013; Sweeney *et al.*, 2007; Sweeney *et al.*, 1999; Saha *et al.*, 1998), may not lead to a distinct evaluation of specific local mechanisms, but rather, complex reactivity in tandem or conducted in series. Whereas, iontophoresis locally applies agonists more discretely to assess microvascular reactivity within the immediate environment, with all systemic controls intact (Hungerford *et al.*, 2000).

As technological advancements continue to provide more complex models of pathophysiology, it is imperative to first evaluate normal physiology in an effort to fully characterize alterations from baseline (Hungerford *et al.*, 2000). In this case, the evaluation of the virgin uterine microvascular reactivity within a single phase of estrous after a single inhalation exposure (Figure 1A). Additionally, while the current study focuses on ENM exposures associated with our laboratory paradigm, the intravital methodology can be applied to a variety of conditions (e.g. diabetes, obesity, preeclampsia, fibroids, polycystic ovarian syndrome (PCOS), or surgical intervention). For consistency with these initial studies, we selected the basal or straight arterioles (Figure 1B), which serve to feed the endometrial layer of the uterus and are not immediately connected to the development of the uteroplacental vasculature. In contrast, adaptations in the pre-placental spiral arterioles, show significant remodeling during pregnancy, are more distensible and are highly responsive to hormonal fluctuation throughout the estrous cycle (Osol *et al.*, 2009; Robertson, 1976). While the majority of reports detailing the rate of vascular resistance are within a pregnancy model, it has been established that the major pressure decrease occurs between the systemic conduit arteries and the preplacental arteries in pregnant

and non-pregnant states (Moll *et al.*, 1973). This decrease in vascular pressure, with significant hormonal influence, is also mirrored in the ovarian circulation (Sweeney *et al.*, 2007). Given the possibility that hormonal fluctuations related to the reproduction cycle may influence vascular reactivity and inflammatory conditions, it remains crucial to confirm estrous status prior to microvascular assessments or xenobiotic exposure (Goldman *et al.*, 2007).

Therefore, the purpose of this study was to evaluate *in vivo* female arteriolar dysfunction and venular leukocyte adhesion and rolling associated with nano-TiO₂ inhalation during diestrus. The specific goals of the studies were to: (1) enhance the intravital microscopy preparation of the rat uterus for more sensitive toxicological assessments, (2) identify baseline uterine venular leukocyte activity and arteriolar function, and (3) quantify uterine venular leukocyte activity and arteriolar microvascular dysfunction 24-hours after nano-TiO₂ inhalation.

METHODOLOGY

Animal Model

Virgin female Sprague-Dawley (SD) rats were purchased from Hilltop Laboratories (Scottsdale, PA), provided food and water *ad libitum*, randomly assigned to either the control or exposure groups, and acclimated for 72 hours prior to use. Estrous cycle and verification of diestrus was determined by microscopic evaluation of vaginal smears (Goldman *et al.*, 2007; Stapleton *et al.*, 2013; Paccola *et al.*, 2013). In order to limit inflammatory and hormonal influences all experiments were performed in diestrus (Alsip *et al.*, 1996). All procedures were approved by the Institutional Animal Care and Use Committee of West Virginia University.

Particle Characterization

Nano- TiO₂ P25 powder, obtained from Evonik (Aeroxide TiO₂, Parsippany, NJ), has previously been shown to be a mixture composed primarily of anatase (80%) and rutile (20%) TiO₂, with a primary particle size of 21 nm and a surface area of 48.08 m²/g (Nurkiewicz *et al.*, 2008; Sager *et al.*, 2008; Sager *et al.*, 2009). As previously described, the nano- TiO₂ was prepared with care prior to aerosolization by drying, sieving and storing the powder (Knuckles *et al.*, 2012; Nurkiewicz *et al.*, 2008).

Inhalation Exposure

Our nanoparticle aerosol generator was developed, designed and tested specifically for rodent nanoparticle inhalation exposures (US patent #8,881,997, Yi *et al.*, 2014; Yi *et al.*, 2013). Briefly, it consists of a vibrating fluidized bed cylinder with a baffle, a vibrating Venturi disperser, and a cyclone separator. The nano-TiO₂ aerosols, generated from the bulk dry powder, were diluted and delivered to the animal inhalation exposure chamber at 90 LPM. The test atmospheres were monitored in real time and adjusted manually to assure a consistent and known exposure for each experimental animal with an Electrical Low Pressure Impactor (ELPI; Dekati, Tampere, Finland) and Scanning Mobility Particle Sizer (SMPS; TSI Inc, St. Paul, MN). During the inhalation exposure, the actual aerosol concentrations were measured using gravimetric methods to calculate inhaled dose. The particles were collected on 47 mm PTFE membrane filters and an XP2U microbalance (Mettler Toledo, Switzerland) was used to weigh the filters.

Rats were placed in the exposure chamber for a single 6-hour (1-hour to initiate exposure, 4-hours at the plateau concentration, and 1-hour complete exposure and bring aerosols concentrations back to baseline) nano-TiO₂ inhalation exposure 24-hours prior to microvascular

assessments. Once a steady-state aerosol concentration was achieved (final mass concentration of $10.1 \pm 0.39 \text{ mg/m}^3$ for nano- TiO_2), exposure duration was adjusted to achieve a single calculated deposition of $41.6 \pm 2 \text{ } \mu\text{g}$ nano- TiO_2 (exposed) or $0 \text{ } \mu\text{g}$ (control). Tremendous care was taken to achieve the same final mass concentration for each exposure; our ability to reliably and repeatedly characterize the aerosols in real time using the ELPI and SMPS enables us to achieve this day-to-day or exposure-to-exposure consistency (Yi *et al.*, 2013).

As previously described, total deposition was calculated based on mouse methodology previously reported (Porter *et al.*, 2013) and normalized based on rat weight and to rat minute ventilation (Stapleton *et al.*, 2013; Stapleton *et al.*, 2012a) using the following equation: $D = F \cdot V \cdot C \cdot T$, where F is the deposition fraction (10%), V is the minute ventilation based on body weight, C equals the steady state mass concentration (mg/m^3), and T equals the exposure duration (minutes) (Nurkiewicz *et al.*, 2008). Representative aerosol characterizations are presented in Figure 2.

Intravital Microscopy and Microiontophoresis

24-hours post-exposure, rats were anesthetized with Inactin (100 mg/mL i.p.) and placed on a heating pad to maintain a 37°C rectal temperature. With the animal supine, the trachea and carotid artery were cannulated, respectively, to ensure a patent airway and monitor mean arterial pressure and heart rate. After a 10-minute equilibration, an incision was made on the right iliac region of the abdomen and the right horn of the uterus was identified. The animal was then placed on its right side at a 45° angle to oppose development of pulmonary edema during the experiment. Leaving all innervations and feed vessels intact and uninjured, the right horn of the uterus was surgically exteriorized, gently secured using 5-0 suture over a uniquely designed

optical pedestal for uterine transillumination and observation, carefully maintaining the vasculature at its *in situ* length. Throughout the surgery and experimental period, the uterus was continuously superfused (3-4 mL/min) with a warmed (35 ° C) and equilibrated (95% N/5% CO₂) physiological salt solution (PSS; 119 mM NaCl, 25 mM NaHCO₃, 6 mM KCl, and 3.6 mM CaCl₂) as previously described (Nurkiewicz *et al.*, 2004b) with the addition of isoproterenol (10 mg/L; Sigma-Aldrich, St. Louis, MO) and phenytoin (20 mg/L; Sigma-Aldrich, St. Louis, MO) to reduce myometrial motility (Bohlen *et al.*, 1976; Nase *et al.*, 1996; Sweeney *et al.*, 1999; Leonard *et al.*, 2013). Previous use of isoproterenol and/or phenytoin at these concentrations in the mesentery had no effect on microvascular tone or reactivity (Bohlen *et al.*, 1976; Nase *et al.*, 1996; Sweeney *et al.*, 1999; Leonard *et al.*, 2013).

The animal preparation was then transferred to the stage of an intravital microscope coupled to a charge-coupled device video camera. Microvessels were visualized using a 20x water-immersion objective. Video images were viewed on a high-resolution color video monitor and digitally recorded for off-line analysis (Schneider *et al.*, 2012).

After a 20-minute equilibration period, leukocyte adhesion and rolling in separate second- or third-order venules (2 or 3 per rat) were counted for 1 minute in each venule studied using a 20x water immersion objective to characterize leukocyte trafficking after nano-TiO₂ exposure.

For assessment of basal arteriolar reactivity (1-3 per rat), micropipettes were pulled (Model P-97, Sutter Instruments, USA) from aluminosilicate glass capillary tubes (A100-64-10, Sutter Instruments) to an inner diameter of 2-4 µm and subsequently double-beveled, as previously described (Nurkiewicz *et al.*, 2004b). Micropipettes were backfilled with muscarinic agonist acetylcholine (0.025 M; ACh), and nitric oxide donor sodium nitroprusside (0.05 M; SNP), or

229 **adrenergic agonist phenylephrine (0.05 M; PE)** and positioned with the tip parallel to the vessel
230 wall to prevent accidental puncture associated with tissue movement using a micromanipulator
231 (Narishige, Tokyo, Japan) mounted on the stage platform (Gibraltar Industries, Buffalo, NY).
232 Microiontophoresis was conducted to evaluate endothelium-dependent dilation (EDD; ACh; 20,
233 40, 100, and 200 nA), -independent dilation (EID; SNP; 20, 40, and 100 nA), and vascular
234 smooth muscle responsiveness (VSM; PE; 20, 40, and 100 nA), by connecting the micropipettes
235 to a current programmer (model 260; World Precision Instruments, New Haven, CT) via an
236 Ag/AgCl wire. An additional Ag wire was submerged in the tissue bath to complete the circuit.
237 At the end of all experiments, passive maximum arteriolar diameter was established by
238 superfusing the tissue with 10^{-4} M adenosine.

239 The responses to ACh, SNP, and PE are presented as raw measurements (micrometers, μm)
240 and as calculated baseline active vascular tone via the equation $T = [D_M - D_{\text{BASE}} / D_M] * 100$, where
241 D_M is the maximum diameter measured during adenosine superfusion and D_{BASE} is vessel
242 diameter at baseline (Nurkiewicz *et al.*, 2004a). Since some diameters were significantly
243 different at baseline, we normalized reactivity to their maximum response, allowing for an
244 appropriate comparison of microvascular reactivity. This normalization used the following
245 equation: $[(D_{\text{SS}} - D_{\text{CON}}) / (D_M - D_{\text{CON}})] * 100$, where D_{SS} remains the steady-state diameter achieved
246 after each chemical bolus and D_{CON} is the baseline diameter measured immediately prior to the
247 microiontophoresis administration and D_M is the maximal response after adenosine
248 administration at the end of each experiment (LeBlanc *et al.*, 2009; Nurkiewicz *et al.*, 2004a;
249 Stapleton *et al.*, 2014a). All experimental periods were at least 2 minutes, all steady-state
250 diameters were collected for at least one minute, and all vessels recovered back to baseline for 2-
251 5 minutes after agonist and/or dosage administration.

Statistical Analysis

Data are expressed as mean $\pm$ standard error (SE), where "N" represents the number of rats studied and "n" represents the number of arterioles evaluated as previously described (Boegehold, 1998; Nurkiewicz *et al.*, 2008; Stapleton *et al.*, 2014a; Stapleton *et al.*, 2013). In these studies, the experimental unit was the arteriole, with one to three microvessels studied per rat; we consider these arterioles to be independent observations due to the considerable heterogeneity within any microvascular network (Frisbee *et al.*, 2011). Concentration-diameter plots were assessed by a two-way analysis of variance (ANOVA), with Student-Newman-Keuls post hoc test was used when appropriate and $p < 0.05$ was taken to reflect statistical significance.

RESULTS

Aerosol ENM Characterization

The count mean aerodynamic diameter was 171 ± 6 nm (ELPI; Figure 2A) and at steady-state this produced an aerosol concentration of 10.1 ± 0.39 mg/m³ (Figure 2B) and the count mean electrical mobility diameter was 165 ± 5 nm (SMPS; Figure 2C). These aerosol exposure parameters are consistent with previous studies in our research program (Stapleton *et al.*, 2014a; Stapleton *et al.*, 2013).

Animal Health and Vessel Characteristics

There were no significant differences between the experimental groups with respect to: age, weight, heart rate, or mean arterial pressure (Table 1). However, there was a significant difference between the sham and exposed baseline arteriolar diameters. The maximum diameters

were not significantly different between these groups, indicating at baseline, uterine arteriolar tone is significantly increased in exposed females by 12.8 ± 3.6 %. Interestingly, this phenomenon only occurred at baseline, after equilibration, prior to any functional assessments. As the functional assessments were randomized by dosage/amplitude and agonist, these differences in tone did not persist. Therefore, the tone was not significantly different at the initiation of each agonist assessment.

Leukocyte-Endothelium Activity

In diestrus, the average observed leukocyte-endothelium interactions in the 21 control venules was 8.48 ± 3.83 counts per minute, while the average observed interactions within the 24 observed venules of the exposed were significantly elevated (22.05 ± 4.21 counts per minute; 260% higher) within the uterine microcirculation (Figure 3), indicative of a pro-inflammatory state 24-hours after nano-TiO₂ inhalation.

Endothelium-Dependent Arteriolar Reactivity

There were significant differences between the exposed and control females with respect to the raw (Figure 4A) and normalized (Figure 4B) responses. As the uterine arterioles have been shown to be highly sensitive to and highly reliant on NO, the black line represents a normal dose-dependent dilation given increased concentrations of acetylcholine, with a near maximal response at 200 nA. The endothelium-dependent dysfunction, evident at lower dose of acetylcholine in the exposed group after nano-TiO₂ inhalation, may be attributed to an alteration in NO production, bioavailability, and/or smooth muscle signaling.

Endothelium-Independent Arteriolar Reactivity

There were no significant differences in the endothelium-independent responses to the NO-donor SNP in both the raw responses (Figure 5A) and the normalized reactivity (Figure 5B). While the raw responses may appear significantly different at baseline, they are not mathematically different ($p=0.11$). The control data is representative of a normal response to increased concentrations of SNP, with the highest dose recorded as a near maximal dilation. These findings are consistent with other responses 24-hours after nano-TiO₂ inhalation (Stapleton *et al.*, 2014a; Stapleton *et al.*, 2013), indicating appropriate smooth muscle responsiveness to bioavailable NO.

Vascular Smooth Muscle Contractility

The raw diameter measurements of vascular smooth muscle function were significantly altered at the highest dose of PE (100 nA) (Figure 6A). The control line for response is consistent with others, indicative of a controlled, step-wise constriction to increased concentrations of PE. When normalized for reactivity, there was a significant difference at the higher ejection currents (40 and 100 nA) (Figure 6B), with an attenuation of VSM contraction in the exposed group. These results indicate a reduced sensitivity to α -adrenergic stimulation.

DISCUSSION

The results of this study were gained by advancing an underutilized technique and applying it to a toxicological paradigm. This has provided valuable insight into the sensitive microvascular physiology and possible mechanisms of dysfunction within an intact female animal after ENM

exposure. This is also the first report of increased leukocyte activity, during diestrus, subsequent to nano-TiO₂ inhalation.

In addition to neurological and direct particle interaction, inflammation and/or inflammatory responses have long been the leading hypotheses connecting pulmonary exposure to dysfunctional microvascular outcomes. These inflammatory responses via local responses, systemic cascade, or in response to translocated particles may be visualized *in vivo* by an increase in leukocyte presence/activity and a plausible increase in vascular oxidative and/or nitrosative reactive species, which may act to reduce bioavailable NO through local scavenging (Stapleton *et al.*, 2012b). Increased leukocyte activity has been described in diestrus and associated with high progesterone levels (Paccola *et al.*, 2013); given this heightened activity exposure to ENM during this point in the estrus cycle may have an additive effect. Further, the development of the uterine intravital microscopy preparation will allow for future toxicological investigations of endocrine disruptors in the most relevant organ of interest to female reproduction.

While the use of suture to maintain position could influence local leukocyte activity, great care was taken to avoid vascular tissues when securing the uterine horn. Additionally, given the extensive use of identical surgical techniques within alternate tissue beds, including the mesentery (Nase *et al.*, 1996), spinotrapezius (Nurkiewicz *et al.*, 1998), and cremaster muscle (Hungerford *et al.*, 2000), the significant difference in leukocyte activity stems from nano-TiO₂ inhalation and correlates well with other findings from our group (Nurkiewicz *et al.*, 2004b). Moreover, the stage is set for future gender-based and tissue-based exposure assessments of leukocyte trafficking.

Previously, Rogers et al. (1992) demonstrated a reduction in leukocyte activity in the hours preceding implantation. It is unknown if leukocyte activity remains abnormally elevated through estrous after ENM exposure and if such an elevation may have deleterious effects on implantation. Therefore, due to the complexities associated with the uterine cycle and the demands placed during gestation, the reproductive ramifications of microvascular dysfunction and elevated leukocyte activity must be fully elucidated.

Despite the significant impact on future generations, our understanding of the uterine microcirculation in either pregnant (Rogers, 1992; Tawia *et al.*, 1992; Ruttner *et al.*, 2000) or non-pregnant states (Rogers *et al.*, 1990; Alsip *et al.*, 1996; Hill *et al.*, 1997; Sweeney *et al.*, 1999) remains limited (Osol and Moore, 2014b). While isolated microvascular preparations have provided crucial mechanistic data regarding vascular remodeling associated with pregnancy and gestational disease (e.g. preeclampsia) (Osol *et al.*, 2012; Cipolla *et al.*, 1997; Fuller *et al.*, 2011; Gokina *et al.*, 2009; Gokina *et al.*, 2003), these studies may not directly correlate to intact *in vivo* functional responses or specific toxicities. Initial *in vivo* studies of the endometrial microvascular anatomy, density, and function were based on methodology previously developed for mesenteric investigation and led to a cyclical understanding of endometrial and microvascular growth and degeneration (Bohlen *et al.*, 1976; Rogers *et al.*, 1990; Rogers *et al.*, 1993). More recently, intravital microscopy was elegantly conducted in a pregnancy model to determine if early gestational growth impacts microvascular reactivity (Leonard *et al.*, 2013). However, in each of these cases, the methodology required significant damage to the uterus (e.g. severing the ovarian ligament (Sweeney *et al.*, 2007; Sweeney *et al.*, 1999). Typically, the use of a microcautery and fine scissors are used to create a small opening in the uterine wall to insert a glass rod or fiber-optic probe into the uterine lumen (Rogers *et al.*, 1990; Alsip *et al.*, 1996). Alternatively, the

fetal implantation site can be excised to permit visualization (Leonard *et al.*, 2013). These surgical techniques may lead to unintentional tissue inflammation characterized by increased leukocyte-endothelial interactions and/or impaired uterine microvascular reactivity.

The previous point regarding artificial damage of microvascular beds is critical in xenobiotic toxicity studies because inflammation is one of the primary hypotheses associated with the microvascular dysfunction that follows pulmonary nanomaterial exposure (Stapleton *et al.*, 2012b). Increased leukocyte rolling and adherence (Nurkiewicz *et al.*, 2006), increased reactive species production (Nurkiewicz *et al.*, 2009; LeBlanc *et al.*, 2010) leading to reduced NO bioavailability (Nurkiewicz *et al.*, 2009; Minarchick *et al.*, 2015) that subsequently impairs normal vascular function has been well described in the cardiovascular toxicology literature, by our group and others (Channell *et al.*, 2012; Vedal *et al.*, 2013; Wingard *et al.*, 2011; Shannahan *et al.*, 2012). Because systemic dependence on NO signaling increases dramatically for numerous processes during pregnancy (Thompson *et al.*, 2001; Ni *et al.*, 1997; Mandala *et al.*, 2002), it is vital to identify any potential vascular sensitivities or dysfunction prior to conception to prevent possible health complications or pregnancy loss.

Uterine vascular anatomy of interest at this level includes arteriolar-venular pairing (Saha *et al.*, 1998), which allows for significant vascular crosstalk, matching of capillary blood in-flow and out-flow, and exchange of vasoactive factors (Hester *et al.*, 2002). These communications are frequently targeted during pathology. Specifically, local inflammation increases leukocyte activity, and amplified reactive species, can affect normal arteriolar reactivity and contribute to microvascular dysfunction (Hester *et al.*, 2002). The definitive association between increased inflammatory cell number and activity to microvascular dysfunction is unclear at this time. It may be that an increase in inflammatory cells, or inflamed environment may locally have higher

concentrations of free radicals (nitrosative and/or oxidative), as in other models of microvascular disease and dysfunction (Stapleton *et al.*, 2008).

This pro-oxidant environment may be responsible for a local reduction of bioavailable NO, shifting endothelium-dependent signaling from a predominantly NO signal to an arachidonic metabolite pathway, specifically through the cyclooxygenase (COX) enzyme. Production of COX metabolites may not be limited to prostaglandins, initiators of vasodilation, but also potent adrenergic vasoconstrictor thromboxane A₂. While this is the first systemic organ, similar findings have been described within skeletal muscle preparations (Knuckles *et al.*, 2012). This shift would account for the significant differences described within the ACh response, but the normal SNP reactivity as indicated within these studies. While others have reported an enhanced neurological (sympathetic) responsiveness within the microvasculature (Knuckles *et al.*, 2012), the normal *in vivo* environment, with neurological innervation intact may maintain for uniformity at baseline, but not be able to maintain functionality as the vasculature is challenged with a pharmacological agent, as evident in the ACh and PE results. Future studies, specifically associated with conception, may explore the vascular outcomes associated with ENM-initiated alterations to uterine innervation and neurological function (Gnanamanickam *et al.*, 2011).

These experiments fall under the inflammatory theory connecting pulmonary nanomaterial exposure to cardiovascular outcomes; however, there are a number of alternate mechanistic theories which need to be explored (Brook *et al.*, 2010). For example, the above speculation may be accurate; however, the possibility of direct particle interaction within the vasculature cannot be overlooked (Stapleton *et al.*, 2014c). In this instance nanomaterial translocation to the uterine vasculature, albeit a small fraction compared to injection or pulmonary deposition, (Sumner *et al.*, 2015; Stapleton *et al.*, 2012a) may act as a pro-oxidant, quenching local NO bioavailability

and initiating an inflammatory signaling cascade, leading to increased uterine venular leukocyte activity.

There were intentional restrictions placed on the experimental design of this work to enhance focus on novel methodology, reduce undue error, and highlight the potential of future studies, but limited the pharmacological output. To maintain consistency with our recent pregnancy studies, a single ENM selected for study, exposed at a single occupationally relevant high dosage and evaluated 24-hours after exposure (Stapleton *et al.*, 2014a; Stapleton *et al.*, 2013). Naïve nano-titanium dioxide may be considered inert compared to functionalized or highly reactive materials; therefore ENM with larger surface areas, altered physiochemical properties, or functionalization may lead to a greater inflammatory response and vascular dysfunction. We chose this exposure concentration and dosage, as an occupationally relevant dose which has led to uterine vascular dysfunction (Stapleton *et al.*, 2013). The 24-hour time point was selected for two main reasons: (1) in order to maintain a consistent estrous status and not interfere with cycle length alterations and (2) this time point has demonstrated the greatest microvascular dysfunction after ENM inhalation, with moderate recovery after 24 hours (Stapleton *et al.*, 2012a). Lastly, while exposure may take place at any point during the estrous there are significant alterations to cellular morphology during the estrous cycle, specifically associated with leukocyte infiltration and sensitivity to ACh during diestrus, therefore a single phase of the estrous cycle was selected for study (Dalle Lucca *et al.*, 2000; Goldman *et al.*, 2007; Westwood, 2008; Goldman *et al.*, 2007; Paccola *et al.*, 2013). This was preferred with the intent to highlight and distinguish inflammation associated with ENM exposure and limit error associated with normal leukocyte variability and arteriolar responsiveness during the full estrus cycle and is a consistent approach with previous uterine *in situ* approaches (Alsip *et al.*, 1996). This study was limited in its

approach to ensure that the vascular dysfunction and increased inflammation in the *in vivo* preparation was directly associated with the ENM exposure.

As these baseline measurements have now been identified after ENM exposure, others have indicated microvascular alterations associated with estrous status (Sweeney *et al.*, 2007; Rogers *et al.*, 1990); therefore, future studies should include a full timeline, which evaluate estrous and pregnancy in their entirety. These studies may aid in better understanding the mechanisms of normal uterine function to reduce health concerns associated with estrous cycle, conception, pregnancy, and menopause along with disease conditions associated with female reproductive physiology. As with any initial nanotoxicological pursuit, further evaluations including a dose-response curve, alternate exposure routes, and a range of ENM of varying material characteristics (e.g. shape, material, functional groups, etc.) need to be completed.

Overall, the estrous ovulatory cycle patterns 4-5 days (Goldman *et al.*, 2007) and previous studies have indicated microvascular dysfunction for at least 7 days post-exposure (Stapleton *et al.*, 2012a); therefore, female ENM exposure may interfere with implantation, placental formation, and fetal development as it does not provide an ideal environment for implantation or the initiation of a successful gestation. In conclusion, this study has adapted and improved uterine intravital microscopy allowing for microvascular toxicological assessments in regard to female reproductive health. These studies may also form the basis of analyses which evaluate female health in all stages of development and gestation.

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FIGURE LEDGENDS

Figure 1: Intravital Microscopy Schematic. (A) Identification of uterine vascular anatomy after adenosine superfusion. Arrows indicate: (1) uterine artery (thinner, lighter red), (2) arcuate artery (narrow red line above vein, encased in adipose), (3) radial artery (thin red line beyond point of the arrow), (4) basal/straight arterioles, and (5) spiral arterioles. (B) Representative Image of rat uterus exteriorization. Arrows indicate: (1) Tracheal intubation to aid with respiration, (2) carotid intubation to monitor vital signs during surgery, (3) vacuum line serving as a drain for the tissue bath, (4) exteriorized uterus, and (5) fiberglass pedestal. (C) Portrayal of intravital microscopy technique depicting: (1) real-time vital monitoring of heart rate, temperature, systolic, diastolic, and mean arterial pressures, (2) water-immersion microscope, (3) micromanipulator, (4) stage, platform, and optical pedestal, (5) heated physiological salt solution, (6) image acquisition for microvascular analysis, (7) glass iontophoretic pipette, and (8) representative basal arteriole.

Figure 2: Real Time Nano-TiO₂ Exposure Characteristics. A) Aerosol size distribution and characterization was determined using ELPI, which characterized the mass-based aerodynamic diameter as a mean of 171 ± 6 nm. B) Size-based aerodynamic diameter and mobility was characterized as 165 ± 5 nm. C) A representative image of the daily concentration distribution over the 5-hour exposure, portraying a maintained plateau at 10.1 ± 0.39 mg/m³. Values are means $\pm$ SE.

727 Figure 3: Leukocyte adhesion and rolling. Venular leukocyte trafficking within straight uterine
728 arterioles in diestrus is significantly increased after 24-hours after nano-titanium dioxide
729 inhalation .Values are means $\pm$ SE. * $p \leq 0.05$.

730 Figure 4: Endothelium-dependent arteriolar reactivity. Nano-titanium dioxide inhalation blunts
731 the raw (A) and normalized (B) uterine basal arteriole responsiveness to iontophoretically
732 applied acetylcholine. Values are means $\pm$ SE. * $p \leq 0.05$.

733 Figure 5: Endothelium-independent arteriolar reactivity. Raw (A) and normalized (B)
734 endothelium-independent reactivity within straight premyometrial uterine arterioles to the NO
735 donor spermine-NONOate remains unchanged after nano-titanium dioxide inhalation. Values
736 are means $\pm$ SE.

737 Figure 6: Vascular smooth muscle responsiveness. Nano-titanium dioxide exposure impairs α -
738 adrenergic responses at higher doses in raw (A) and normalized (B) responsiveness of uterine
739 basal arteriole to iontophoretically applied phenylephrine. Values are means $\pm$ SE. * $p \leq 0.05$.