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Originally published at:
http://doi.org/10.4172/2157-7439.1000201
Vascular Tissue Contractility Changes Following Late Gestational Exposure to Multi-Walled Carbon Nanotubes or their Dispersing Vehicle in Sprague Dawley Rats

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Abstract

Multi-walled carbon nanotubes (MWCNTs) are increasingly used in industry and in nanomedicine raising safety concerns, especially during unique life-stages such as pregnancy. We hypothesized that MWCNT exposure during pregnancy will increase vascular tissue contractile responses by increasing Rho kinase signaling. Pregnant (17–19 gestational days) and non-pregnant Sprague Dawley rats were exposed to 100 µg/kg of MWCNTs by intratracheal instillation or intravenous administration. Vasoactive responses of uterine, mesenteric, aortic and umbilical vessels were studied 24 hours post-exposure by wire myography. The contractile responses of the vessel segments were different between the pregnant and non-pregnant rats, following MWCNT exposure. Maximum stress generation in the uterine artery segments from the pregnant rats following pulmonary MWCNT exposure was increased in response to angiotensin II by 4.9 mN/mm² (+118%), as compared to the naïve response and by 2.6 mN/mm² (+40.7%) as compared to the vehicle exposed group. Following MWCNT exposure, serotonin induced approximately 4 mN/mm² increase in stress generation of the mesenteric artery from both pregnant and non-pregnant rats as compared to the vehicle response. A significant contribution of the dispersion medium was identified as inducing changes in the contractile properties following both pulmonary and intravenous exposure to MWCNTs. Wire myographic studies in the presence of a Rho kinase inhibitor and RhoA and Rho kinase mRNA/protein expression of rat aortic endothelial cells were unaltered following exposure to MWCNTs, suggesting absent/minimal contribution of Rho kinase to the enhanced contractile responses following MWCNT exposure. The reactivity of the umbilical vein was reduced with dispersion media and MWCNT exposure by both routes. These results suggest a susceptibility of the vasculature during gestation to MWCNT and their dispersion media-induced vasconstriction, predisposing reduced fetal growth during pregnancy.

Keywords: Vascular tissue contractility; Pregnancy; Uterine artery; Umbilical vein; Nanotoxicology; MWCNTs

Abbreviations: 5HT: Serotonin; Ach: Acetylcholine; ANG II: Angiotensin II; ANOVA: Analysis of Variance; BAL: Bronchoalveolar Lavage; (D)-MWCNTs: MWCNT suspended in DPPC and RSA based medium; DPPC1: 2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPC/ RSA DPPC: RSA and Phosphate Buffered Saline-based Medium; GD: Gestational Day; EC50: Half-maximal Effective Concentration; Enos: Endothelial Nitric Oxide Synthase; IL1β: Interleukin 1 Beta; IL6: Interleukin 6; IFNγ: Interferon, gamma; IT: Intratracheal Instillation; IV: Intravenous administration; MCP1: Monocyte Chemoattractant Protein-1; MWCNT Multi-walled Carbon Nanotube; NP: Non-pregnant; P: Pregnant; PAI1: Plasminogen Activator Inhibitor-1; PE: Phenylephrine; PSS: Physiological Saline Solution; RAEC: Rat Aortic Endothelial Cells; (S)-MWCNTs: Multi-walled Carbon Nanotubes dispersed in 10% surfactant; TNFα: Tumor Necrosis Factor, alpha; VEGF: Vascular Endothelial Growth Factor

Introduction

An increasing number of single- and multi-walled carbon nanotubes (SWCNTs and MWCNTs) are being designed and produced for various industrial and biomedical applications such as tracers of malignant cells, immunomodulators, contrast agents and as scaffolds in tissue engineering [1,2]. Pulmonary exposure to MWCNTs are reported to be associated with adverse effects similar to asbestos exposure [3] involving impairment in pulmonary functions [4] and activation of inflammatory responses in mesothelial cells [5]. MWCNTs are known to be taken-up by bronchial epithelial cells, increase pro-inflammatory cytokine production and induce cytotoxicity in in vitro studies [6,7].

When considering their bio-distribution, MWCNTs translocate to the lymph nodes following intratracheal instillation [8,9] and potentially to other extra-pulmonary organs including the liver, kidney and heart and contributing to various toxico-pathologies [10]. The extra-pulmonary effects of MWCNT exposure is reported to be associated with impairment of endothelial dependent relaxation in coronary arterioles [11] and increased coronary vascular tone enhancing indices of ischemia reperfusion injury [12]. The adverse pulmonary effects following occupational exposure to carbon nanotubes have been studied extensively in non-pregnant animal models [9,13]. The consequences of MWCNT exposure on the peripheral vascular system are yet to be studied adequately, particularly in the unique physiological stage of pregnancy.

In general, exposure to MWCNTs occurs by inhalation during occupational exposures in industry or in research laboratories [13-15].

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Received March 25, 2014; Accepted April 15, 2014; Published April 20, 2014


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Potential biomedical applications could also expose an individual to MWCNTs primarily by the intravenous route [16]. An animal model study on MWCNT exposure during pregnancy reported minimal effects on fetal development and maternal well-being following oral exposure to 8-1000 mg/kg/day of MWCNTs [17]. The expansive vascular remodeling that takes place during pregnancy [18,19] may predispose the maternal and fetal vasculature to be sensitive to nanomaterial exposures by various routes (i.e. pulmonary and intravenous) where increased concentrations of MWCNTs may directly reach the circulation. The consequence of any changes in vascular reactivity can potentially negatively influence the placental blood supply, impacting fetal growth and development. Following acute intravenous exposure, pristine carbon nanotubes are redistributed to the reticulo-endothelial system [16,20] with a significant proportion remaining in blood [21]. This is in contrast to functionalized forms, which are reported to be excreted unchanged via the kidney [22,23]. It can be assumed that these nanotubes come in direct contact with the vascular endothelium during their circulation and these interactions can potentially induce changes in vascular reactivity during pregnancy by various mechanisms.

Multiple vasoconstrictor agents including phenylephrine, endothelin 1, angiotensin II and serotonin act through Gq protein coupled receptors to regulate smooth muscle contraction in the vasculature. Downstream of this receptor, the RhoA/Rho kinase pathway plays a critical role in mediating contractile response in vascular smooth muscle cells. The active form of RhoA promotes activation of the Rho kinase (ROCK) that inhibits MLC phosphatase (MLCP) activity, the dephosphorylation of myosin and subsequent relaxation [24]. Alterations in the RhoA/Rho kinase pathway is reported to be involved in endothelial dysfunction, inflammation [25,26] and with exposure to particulate matter [27].

We hypothesized that MWCNT exposure during pregnancy would increase the contractile responses in uterine and placenta derived blood vessels by increasing the RhoA/Rho kinase activity. We also hypothesized that there will be differential effects on the contractile responses dependent on the route of exposure and the vascular bed location. Intratracheal instillation and intravenous administration were used as the two routes of exposure to identify these differential effects within thoracic aorta, mesenteric and uterine arterial segments.

Methods and Materials

MWCNT suspensions for exposure

Multi-walled carbon nanotubes (MWCNTs) were a generous gift from NanoTechLabs Inc. (Yadkinville, NC, USA) and the dry powder form was previously characterized [4]. The commercial grade, non-functionalized, hydrophobic carbon based nanotubes were suspended in non-polar solvents/dispersion media prior to in vivo exposure. MWCNTs for intratracheal instillation was suspended at 10% clinical grade surfactant (Infasurf®, ONY, Inc., Amherst, NY, USA), to mimic protein-lipid coating from lung surfactant, in sterile 0.9% saline (0.9% NaCl, B. Braun Medical Inc., CA, USA) as previously described [4] to a concentration of 150 µg/ml and the mixture was cup-horn sonicated for 2 minutes at 65% amplitude for a total energy of 10,817 Joules, using a Misonix ultrasonic liquid processor -1510R-MTH (Branson Ultrasonics Corp. Danbury, CT, USA). This suspension will be referred to as “(D)-MWCNTs” and had been previously characterized by Wang et al. [29].

In phosphate buffered saline (Sigma D5652 1X) and sonicated using the probe sonicator at 40% amplitude for 15 seconds. This dispersion medium will be referred to as “DPPC/RSA”. A MWCNT suspension of 150 µg/ml was made with DPPC/RSA and the mixture was cup-horn sonicated using a Misonix ultrasonic liquid processor -1510R-MTH (Branson Ultrasonics Corp. Danbury, CT, USA) at 65% amplitude for 2 minutes. This intravenous suspension will be referred to as “(D)-MWCNTs” and was previously described by Wang et al. [29].

Sprague Dawley rats

Pregnant and non-pregnant female, 10-12 week old Sprague Dawley rats were purchased from Charles River Laboratories (USA). All rats were acclimated for one week in East Carolina University (ECU) Department of Comparative Medicine's animal facility, housed under 12 hour light/dark cycles with standard rat chow and water provided ad libitum. The pregnant rat arrived in the facility between 9-12 days of gestation and the body weight was monitored once in every three days to assess the progression of pregnancy. All animal handling and exposure procedures were approved by the ECU Institutional Animal Care and Use Committee.

MWCNT exposure and dosing

Each pregnant and non-pregnant female rat was randomly assigned to either the MWCNT exposure or dispersion medium control group for each route of exposure to include a minimum of six animals in a group. Matched gestational day pregnancies were used to compare vehicle vs. MWCNT effects. The pregnant rats were exposed between 17-19 days of gestation, compatible with the third trimester of human pregnancy (i.e. late gestational stage). Rats were anesthetized using 2-3% isofluorane (Webster Veterinary, USA) dispersed in oxygen for exposure procedures. The 150 µg/ml MWCNT suspension was administered as a mass based dose of 100 µg/kg by weighing each rat just before the exposure. (S)-MWCNTs suspension or 10% surfactant was instilled intratracheally (IT) as previously described [4,12] for pulmonary exposure. A group of non-pregnant female rats was exposed to IT (S)-MWCNTs or 10% surfactant to evaluate any effect of life stage (pregnant vs. non-pregnant) on vascular tissue contractility. Intravenous (IV) administration of 100 µg/kg (D)-MWCNTs or DPPC/RSA was done in the pregnant rats through the tail vein using a 25G needle. Ten to twelve weeks old, pregnant (GD 17-19) and non-pregnant female rats were used as naïve controls.

Tissue and sample collection

All rats were anesthetized in a transparent sealed receptacle containing gauze soaked with 70% isofluorane (Webster Veterinary, USA) in propylene glycol (Amersco, OH, USA), separated from the animal by a desiccator plate/grid prior to euthanasia. Twenty-four hours following administration of the MWCNTs or vehicle, the rats were subjected to a midline incision and euthanized by pneumothorax. Whole blood (~1 ml) was withdrawn directly from the maternal right ventricle. A pooled fetal blood sample was collected from three fetuses in each pregnant dam (blood from these three fetuses were considered as one sample). Maternal and fetal whole blood samples were centrifuged (20,400 x g for 20 minutes), and serum supernatant was stored at -80°C for cytokine analysis.

Isolation of vessel segments

Three arterial beds and the umbilical vein were selected for pharmacological myographic studies. The uterine and mesenteric vascular beds were selected as they manifest both structural and
functional changes during pregnancy [30,31] with the uterine vasculature undergoing significant remodeling [32]. The thoracic aorta was included as proximal conduit vessel not anticipated to undergo significant remodeling, but still may express changes in pharmacological responses. Both uterine horns with the vascular loops, small intestinal loop with superior mesenteric artery and thoracic aorta were carefully excised and placed in ice cold physiological saline solution (PSS; mM) 140 NaCl, 5.0 KCl, 1.6 CaCl₂, 1.2 MgSO₄, 1.2 MOPS (3-[N-morpholino]-propane sulfonic acid), 6 d-glucose, 0.02 EDTA, and a pH of 7.4). Arterial segments with a length of 0.5 – 2.0 mm were isolated from the mid region of the main uterine artery (diameter 150-300 μm), first order mesenteric artery (diameter 150-250 μm), and thoracic aorta (diameter 2.3-3 mm). Two umbilical vein segments (diameter 400-550 μm) from umbilical cords of different fetuses implanted in the mid-uterine region were isolated from each dam.

Maternal and fetal serum cytokine analysis

The targets for maternal and fetal serum cytokine analysis were selected based on the reported cytokine targets in previous MWCNT exposure studies [4,33,34]. The selected serum cytokines and chemokines (IL6, IL10, TNFα, MCP1, VEGF, INFγ, and IL1β) were assessed using Milliplex MAP Cytokine/Chemokine Panel and Immunoassay (EMD Millipore MA, USA) according to the manufacturer's directions. Assays were run using Luminex 100/200 (Luminex, Austin, TX) and results reported using Luminex xPONENT® software versions 2.3/3.1.

Bronchoalveolar lavage cytology

Twenty-four hours following exposure to MWCNTs or dispersion media, a bronchoalveolar lavage (BAL) was performed on adult female rats as described previously [4]. Briefly, the right lung was lavaged in situ three times with repeated flushes of 26.25 mL/kg body weight of ice-cold Hanks balanced salt solution. The BAL fluids were centrifuged and the total number of cells was calculated using an automated cell counter (Cellometer, Nexcelom Bioscience, and Lawerence, MA, USA). A sample of 20,000 cells was centrifuged using a CytoSpin IV (Shandon Scientific Ltd., Cheshire, UK) and stained with a three-step hematology stain (Richard Allan Scientific, Kalamazoo, MI, USA). The differential cell count was determined by morphology, evaluating 300 cells per slide using light microscopy and each cell count is reported as a percentage of 20,000 cells.

Wire myographic studies

The dissected vessel segments were mounted into a DMT 610M multi-channel wire myograph system (Danish Myo Technology, Aarhus N, Denmark) using 40 μm wires or pins. All vessel segments were bathed in PSS at 37°C, bubbled with medical grade air during the entire myographic studies. The optimal resting tension for each arterial media, a bronchoalveolar lavage (BAL) was performed on adult female rats as described previously [4]. Briefly, the right lung was lavaged in situ three times with repeated flushes of 26.25 mL/kg body weight of ice-cold Hanks balanced salt solution. The BAL fluids were centrifuged and the total number of cells was calculated using an automated cell counter (Cellometer, Nexcelom Bioscience, and Lawerence, MA, USA). A sample of 20,000 cells was centrifuged using a CytoSpin IV (Shandon Scientific Ltd., Cheshire, UK) and stained with a three-step hematology stain (Richard Allan Scientific, Kalamazoo, MI, USA). The differential cell count was determined by morphology, evaluating 300 cells per slide using light microscopy and each cell count is reported as a percentage of 20,000 cells.

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different agonists and the differences were considered statistically significant if $p<0.05$. In addition, each concentration-response curve was also compared across treatment groups using a regression analysis by examining the best-fit values [36]. $EC_{50}$ values for concentration-responses in myographic studies were determined using the Hill equation. A two tailed $t$ test was used compare mean $EC_{50}$ umbilical vein stress generation, and cytokine expression levels between different treatment and control groups. One way ANOVA and Turkey post-hoc test was used for the analysis of fetal and placental weight on each day of gestation.

**Results**

**Characterization of MWCNT suspensions**

The MWCNT suspension in 10% surfactant in saline [(S)-MWCNTs] has been previously characterized by Wang et al. [4]. MWCNTs used in this study had a mean diameter of 22.5 ± 1.3 nm with a bimodal distribution with peaks at 12.5 and 25 nm and a length range of 10-100 µm and a surface area of 113.1 m²/g). The zeta potential of the particles in (S)-MWCNTs suspension was -57.3 mV with a mean hydrodynamic size of 915 nm. MWCNTs suspended in the DPPC, serum albumin and sterile phosphate buffered saline medium [(D)-MWCNTs] was characterized previously by Wang et al. [29] with a zeta potential of -20.8 mV with a mean hydrodynamic size of 793 nm.

**Maternal serum cytokine analysis**

The mean values serum cytokine levels of pregnant and non-pregnant rats 24 hours following exposure to MWCNTs or dispersion media for each route of exposure are reported in Table 1. The baseline cytokine levels in the naïve rats were relatively higher in the non-pregnant group compared to the pregnant group for all cytokines assessed except VEGF. In general, the cytokine profiles for the rats exposed to vehicle or MWCNT were lower in the non-pregnant group 24 hours following exposure to MWCNTs or dispersion media for each route of exposure when compared to the naïve group. TNFα levels were increased by five fold in the pregnant group (when compared to the naïve) following IV DPPC/RSA. TNFα levels were increased more than six fold in the serum following exposure to both dispersion media (10% surfactant group (Figure 1A). The response to angiotensin II following IT exposure to (S)-MWCNT during pregnancy was contributed to by the dispersal media as opposed to minimal changes in observed in the non-pregnant group.

**Maternal Bronchoalveolar Lavage (BAL) cell counts**

The percentages of differential cell counts in the bronchoalveolar lavage fluid are graphed in Supplementary Figure 1 and MWCNT induced changes in these cell counts were observed only in the pregnant group. The mean percentage of macrophages was 4.2% lower in the naïve pregnant group compared to the naïve non-pregnant group, and increased during pregnancy following exposure to both vehicles (by 4.8% with 10% surfactant and by 5.0% with DPPC/RSA) and intravenous (D)-MWCNT exposure (by 4.3%) compared to the naïve. In contrast, the mean epithelial cell percentage was 4% higher in the naïve pregnant group compared to the naïve non-pregnant group and was reduced by ~5% during pregnancy by following exposure to both vehicles and MWCNTs by both routes of exposure when compared to the naïve. The percentage of neutrophils was highest following exposure to (S)-MWCNTs via intratracheal instillation but was less than 1% of the total BAL cell counts. The percentages of eosinophils were not significantly different following exposure to MWCNTs during pregnancy.

**Responses of arterial segments 24 hours post-exposure to intratracheal (IT) instillation of (S)-MWCNTs or 10% surfactant in pregnant and non-pregnant female rats**

The contractile responses of the vessel segments from the pregnant and non-pregnant rats were different following IT exposure to (S)-MWCNTs. In general, the pregnant group manifested increased contractile responses in multiple vascular beds that were in part contributed to by the dispersal media as opposed to minimal changes in observed in the non-pregnant group.

**Main uterine artery**

The main uterine artery segments from pregnant and non-pregnant rats responded differently to the same dose of IT instilled (S)-MWCNTs. The maximum stress generation was increased in the pregnant group in response to phenylephrine by 2.6 mN/mm² (+37%) following IT exposure to (S)-MWCNTs when compared to the naïve, but was not significantly increased when compared to the responses from 10% surfactant group (Figure 1A). The response to angiotensin II following IT exposure to (S)-MWCNT during pregnancy was increased by 4.9 mN/mm² (+118%), as compared to the naïve and by 2.6 mN/mm² (+40.7%) as compared to the 10% surfactant exposed group (Figure 1B). In contrast, the stress generation in response to all 3 agonists was diminished in uterine artery segments from non-pregnant animals following (S)-MWCNTs exposure (Figure 1B, D and F). The relaxation responses to acetylcholine during 30 µM phenylephrine pre-contraction were not different in naïve, 10% surfactant and (S)-MWCNTs exposed pregnant groups (Figure 1G), but was diminished ~ 10% following (S)-MWCNTs exposure in the non-pregnant group.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>NP-naive</th>
<th>NP-IT 10% surfactant</th>
<th>NP-IT (S)-MWCNTs</th>
<th>P-naive</th>
<th>P-IT 10% surfactant</th>
<th>P-IT (S)-MWCNTs</th>
<th>P-IV DPPC/RSA</th>
<th>P-IV (D)-MWCNTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1ß (pg/ml)</td>
<td>56.0 ± 38.5</td>
<td>10.1 ± 3.8</td>
<td>7.3 ± 5.4</td>
<td>14.6 ± 7.1</td>
<td>42.1 ± 7.1</td>
<td>15.8 ± 5.7</td>
<td>70.2 ± 17.3</td>
<td>45.0 ± 12.5</td>
</tr>
<tr>
<td>IL6 (pg/ml)</td>
<td>1245.0 ± 826.0</td>
<td>640.0 ± 208.2</td>
<td>485.0 ± 209.0</td>
<td>77.9 ± 72.8</td>
<td>215.3 ± 62.6</td>
<td>183.0 ± 128.8</td>
<td>126.8 ± 92.1</td>
<td>229.4 ± 115.5</td>
</tr>
<tr>
<td>IL10 (pg/ml)</td>
<td>21.7 ± 9.0</td>
<td>13.4 ± 4.8</td>
<td>10.2 ± 6.4</td>
<td>5.8 ± 3.7</td>
<td>9.6 ± 4.4</td>
<td>7.3 ± 3.6</td>
<td>16.4 ± 6.0</td>
<td>9.8 ± 4.2</td>
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<tr>
<td>INFγ (pg/ml)</td>
<td>338.4 ± 135.3</td>
<td>189.6 ± 33.6</td>
<td>174.1 ± 57.3</td>
<td>198.5 ± 50.5</td>
<td>106.4 ± 10.8</td>
<td>131.0 ± 36.8</td>
<td>273.1 ± 102.2</td>
<td>242.0 ± 50.1</td>
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<tr>
<td>MCP1 (pg/ml)</td>
<td>823.2 ± 223.9</td>
<td>467.0 ± 106.6</td>
<td>540.2 ± 248.9</td>
<td>288.1 ± 96.0</td>
<td>492.9 ± 27.0</td>
<td>336.2 ± 96.5</td>
<td>510.6 ± 54.8</td>
<td>478.2 ± 36.2</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>53.7 ± 15.4</td>
<td>30.1 ± 4.3</td>
<td>29.4 ± 7.5</td>
<td>510.2 ± 111.9</td>
<td>377.4 ± 81.1</td>
<td>476.8 ± 42.2</td>
<td>421.8 ± 63.0</td>
<td>432.8 ± 56.6</td>
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<tr>
<td>TNFα (pg/ml)</td>
<td>40.9 ± 12.83</td>
<td>30.3 ± 4.6</td>
<td>27.0 ± 8.9</td>
<td>6.8 ± 3.9</td>
<td>45.3 ± 10.2</td>
<td>23.9 ± 9.9</td>
<td>43.7 ± 13.3</td>
<td>34.1 ± 10.4</td>
</tr>
</tbody>
</table>

IT: intratracheal instillation and IV: intravenous administration. P: pregnant and NP: non-pregnant. N/A: not available

**Table 1**: Cytokine levels in maternal serum 24 hours post-exposure to MWCNTs.

The maternal serum cytokines were evaluated using Milliplex MAP Cytokine/Chemokine Panel and Immunoassay (EMD Millipore MA, USA). The assays were run using Luminex 100/200 (Luminex, Austin, TX) and results reported using Luminex xPONENT® software versions 2.3/3.1. The mean and the SEM are reported for serum cytokines # indicates $p < 0.05$ when compared to naïve and P-IT 10% surfactant group.
Figure 1: Changes in the contractile responses of the main uterine artery following intratracheal instillation (IT) of MWCNTs. The changes in the contractile responses as assessed by wire myography of the main uterine artery from 17 - 19 days pregnant (A, C, E and G) and non-pregnant female (B, D, F and H) Sprague Dawley rats, 24 hours following intratracheal instillation of 100 µg/kg of (S)-MWCNTs or 10% surfactant. The stress generation in response to cumulative concentrations of phenylephrine (PE; A and B), angiotensin II (ANG II; C and D) and endothelin 1 (ET-1; E and F) are plotted. The percentage relaxation from a 30 µM phenylephrine pre-stimulation stress level in response to cumulative concentrations of acetylcholine (Ach; G and H) is graphed. * indicates $p < 0.05$ compared to 10% surfactant while # indicates $p < 0.05$ compared to naïve using repeated measures ANOVA ($n = 5 - 7$). The $p$ values were derived following the comparison of each concentration response curve across treatment groups using a regression analysis by examining the best-fit values.
(Figure 1H). The calculated EC₅₀ values for phenylephrine, angiotensin II, acetylcholine and HA-1077 were not different between the naive, 10% surfactant and (S)-MWCNTs treatment groups. Following (S)-MWCNTs exposure in pregnant rats, calculated EC₅₀ value for endothelin 1 (1.1 ± 0.3 nM) was significantly lower than the naive (3.4 ± 0.6 nM), but not different form the 10% surfactant exposed group (2.2 ± 1.0 nM, Supplementary Table 1).

First order mesenteric artery

The mesenteric artery segments from both pregnant and non-pregnant rats responded in a similar manner following IT (S)-MWCNT exposure. The stress generations in response to serotonin in the first order mesenteric artery segments were increased by ~ 4 mN/mm² following IT (S)-MWCNTs exposure compared to 10% surfactant exposed group (Figure 2E and F). The contractile responses

Figure 2: Changes in the contractile responses of the mesenteric artery following intratracheal instillation (IT) of MWCNTs

The changes in the contractile responses were assessed by wire myography of the first order mesenteric artery from 17 - 19 days pregnant (A, C, E and G) and non-pregnant female (B, D, F and H) Sprague Dawley rats, 24 hours following intratracheal instillation (IT) of 100 µg/kg of (S)-MWCNTs or 10% surfactant. The stress generation in response to cumulative concentrations of phenylephrine (PE; A and B), endothelin 1 (ET-1; C and D) and serotonin (5HT; E and F) are plotted. The percentage relaxation from a 30 µM phenylephrine pre-stimulation stress level in response to cumulative concentrations of acetylcholine (Ach; G and H) is graphed. * indicates p < 0.05 compared to 10% surfactant while # indicates p < 0.05 compared to naïve using repeated measures ANOVA (n = 4 - 7). The p values were derived following the comparison of each concentration response curve across treatment groups using a regression analysis by examining the best-fit values.
to phenylephrine or endothelin 1 and the relaxation response to acetylcholine were not changed following IT (S)-MWCNT exposure in the pregnant group (Figure 2A, C and G).

The mesenteric artery contractile responses to all 3 agonists were diminished in the non-pregnant rats exposed to 10% surfactant (Figure 2B, D and F), along with an impairment of acetylcholine dependent relaxation response (Figure 2H). Similar to the reported uterine vessels responses, the EC50 values of the mesenteric arteries responses following (S)-MWCNT exposure were not different except for endothelin 1 (Supplementary Table 2). The EC50 for endothelin 1-mediated responses was decreased in the 10% surfactant (1.4 ± 0.4 nM) group when compared to both naïve (5.4 ± 1.3 nM) and (S)-MWCNTs exposed group (5.0 ± 1.0 nM).

Thoracic aorta

The thoracic aortic segments from pregnant and non-pregnant rats responded differently to the IT exposure to (S)-MWCNTs. The contractile response to phenylephrine (0.001-10 µM) was reduced by 0.68 mN/mm² (25.4%) in the pregnant group following (S)-MWCNT exposure compared to the naïve, but was not different when compared to the 10% surfactant exposed group (Figure 3A). The contractile

Figure 3: Changes in the contractile responses of the thoracic aorta following intratracheal instillation (IT) of exposure to MWCNTs.

The changes in the contractile responses were assessed by wire myography of the thoracic aorta from 17 - 19 days pregnant (A, C and E) and non-pregnant female (B, D and F) Sprague Dawley rats, 24 hours following intratracheal instillation of 100 µg/kg of (S)-MWCNTs or 10% surfactant. The stress generation in response to cumulative concentrations of phenylephrine (PE; A and B) and endothelin 1 (ET-1; C and D) are plotted. The percentage relaxation form a 10 µM phenylephrine pre-stimulation stress level in response to cumulative concentrations of acetylcholine (Ach; E and F) is graphed. * indicates p < 0.05 compared to 10% surfactant while # indicates p < 0.05 compared to naïve using repeated measures ANOVA (n = 4 - 8). The p values were derived following the comparison of each concentration response curve across treatment groups using a regression analysis by examining the best-fit values.
response to endothelin 1 from the pregnant thoracic aorta segments was increased in both (S)-MWCNTs and 10% surfactant exposed groups when compared to the naïve. There was a noticeable relaxation response to highest concentration of endothelin 1 in the (S)-MWCNT exposed pregnant group (Figure 3C). In contrast, the contractile responses to phenylephrine and endothelin 1 were not affected by (S)-MWCNT or 10% surfactant exposure in the non-pregnant female rats (Figure 3B and D).

The acetylcholine (0.001-10 µM) mediated relaxation response was not different in the pregnant group (Figure 3E), but was increased in both (S)-MWCNTs and 10% surfactant exposed non-pregnant aortic segments when compared to the naïve (Figure 3F). The EC50 values were not different for the contractile and relaxation responses following (S)-MWCNT exposure (Supplementary Table 3).

Responses of arterial segments 24 hours post-exposure to intravenous (IV) administration (D)-MWCNTs or DPPC/RSA in pregnant rats

Twenty four hours following IV administration in pregnant rats, both (D)-MWCNTs and DPPC/RSA increased the maximal stress generation in the main uterine artery segments to a similar magnitude (3 - 4 mN/mm²) when compared to naive vessel segments with a similar concentration-response profile for the agonists: phenylephrine, endothelin 1 and angiotensin II (Figure 4A-C). The DPPC/RSA exposure elevated the baseline stress level of the uterine vessel segments while the (D)-MWCNT exposure had no additional effect. The relaxation responses of the main uterine artery to acetylcholine were not changed by IV (D)-MWCNT or DPPC/RSA exposure (Figure 4D). We did not proceed to do non-pregnant comparisons in this exposure group as the differences in the contractile responses were attributed sole to DPPC/RSA suspension and not to MWCNT exposure.

An increase in contractile response in the mesenteric artery segments was seen at higher concentrations of phenylephrine following (D)-MWCNT exposure (supplementary Figure 2A). All other contractile/relaxation responses of the mesenteric artery and aortic segments were not significantly different between the (D)-MWCNTs or DPPC/RSA exposure groups (supplementary Figures 2B-D and 3A-C). Unlike in the uterine artery, DPPC/RSA did not have a significant effect on the baseline stress level of the mesenteric artery or thoracic aorta. The EC50 values for all responses are reported in supplementary Tables 1-3 and were not different following MWCNT exposure except within the IV (D)-MWCNT exposure group during pregnancy (2.6 ± 0.2 µM compared to 1.8 ± 0.3 µM in the DPPC/RSA exposed group).

Contribution of Rho kinase activity on the vascular tissue contractility following exposure to MWCNTs

Maintenance of stress in the presence of Rho kinase inhibitor: Minor differences were observed in the relaxation responses to cumulative concentrations of the Rho kinase (ROCK) inhibitor, HA1077, during the stable phenylephrine pre-contraction for segments from all three vascular beds, regardless of the pregnancy state or route of exposure to the MWCNT (Figure 5 and Supplementary Figure 4). The EC50 values for the concentration responses are reported in Supplementary Tables 1-3 and were not significantly different following MWCNT exposure except within the IV (D)-MWCNT exposure group during pregnancy (2.6 ± 0.2 µM compared to 1.8 ± 0.3 µM in the DPPC/RSA exposed group).

**Figure 4:** Changes in the contractile responses of the main uterine artery following intravenous administration (IV) of MWCNTs.

The changes in the contractile responses were assessed by wire myography of the main uterine artery segments from 17 - 19 days pregnant (A, B, C and D) Sprague Dawley rats, 24 hours following intravenous administration of 100 µg/kg of (D)-MWCNTs or DPPC/RSA. The stress generation in response to cumulative concentrations of phenylephrine (PE; A), angiotensin II (ANG II; B) and endothelin 1 (ET-1; C) are plotted. The percentage relaxation from a 30 µM phenylephrine pre-stimulation stress level in response to cumulative concentrations of acetylcholine is graphed (Ach; D). * indicates p < 0.05 compared to naïve using repeated measures ANOVA (n = 5 - 8). The p values were derived following the comparison of each concentration response curve across treatment groups using a regression analysis by examining the best-fit values.
Figure 5: Changes in stress generation in the presence of a Rho kinase inhibitor following intratracheal instillation (IT) MWCNTs.

The reduction in stress generation is reported as the percentage relaxation from a phenylephrine (30 µM for uterine/mesenteric arteries and 10 µM for aorta) pre-stimulation stress level in response to cumulative additions of a Rho kinase inhibitor (HA1077). All responses were assessed by wire myography, 24 hours following intratracheal instillation of 100 µg/kg of (S)-MWCNTs or 10% surfactant from 17 - 19 days pregnant (A, C and E) and non-pregnant female (B, D and F) Sprague Dawley rats. Panels A and B: main uterine artery; C and D: first order mesenteric artery; E and F: thoracic aorta. * indicates p < 0.05 compared to naïve using repeated measures ANOVA (n = 4 - 6). The p values were derived following the comparison of each concentration response curve across treatment groups using a regression analysis by examining the best-fit values.
RhoA, ROCK and eNOS mRNA and protein expression in rat aortic endothelial cells: The mRNA expression of RhoA, ROCK1, ROCK2 and eNOS was not significantly changed in RAEC with 2-12 hour treatment with (S)-MWCNTs or (D)-MWCNTs when compared to untreated cells and vehicle controls (data not shown). Similarly, the protein expression of RhoA, ROCK and eNOS were not changed with 12 hours in vitro exposure to 10 µg/cm² of (S)-MWCNTs or (D)-MWCNTs as assessed by the In-cell Western Assay (Supplementary Figures 5 and 6).

Figure 6: Changes in contractile responses of the umbilical vein following maternal exposure to MWCNTs.

The changes stress generation in the umbilical vein segments were assessed by wire myography in response to 109 mM K⁺ depolarization (A and B) and 1 µM thromboxane agonist (U46619, B and D) 24 hours post-exposure to intratracheal instillation (IT) of 100 µg/kg of (S)-MWCNTs or 10% surfactant (A and C) or intravenous administration (IV) of (D)-MWCNTs or DPPC/RSA (B and D), from 17 - 19 days pregnant Sprague Dawley rats (n = 12). The percentage relaxation in response to sodium nitroprusside (SNP) following U46619 (1 µM) pre-contraction in the umbilical vein 24 hours post-exposure to intratracheal instillation of (S)-MWCNTs or 10% surfactant (E) or intravenous administration of (D)-MWCNT or DPPC/RSA is graphed (F) (n = 12).
Changes in the fetal components following MWCNT exposure

Changes in umbilical vein contractility: The reactivity of the umbilical vein (vessel from the placenta to the fetus) was assessed following both IT and IV administration. Stress generation during K+PSS and 1 µM of thromboxane mimetic (U46619) stimulations were not significantly different in umbilical vessel segments between MWCNT exposed (by either exposure route) and naïve animals (Figure 6A-D). The umbilical vein segments did not respond to acetylcholine and the relaxation response to 1µM sodium nitroprusside with a stable U46619 pre-contraction was not different following MWCNT exposure (Figure 6E and F).

Changes in fetal and placental weight: Mean weights of pregnant dams at the time of sacrifice were not significantly different between treatment groups (mean ± SEM): (S)-MWCNTs 298.2 ± 12.0 g (n=6), 10% surfactant 291.0 ± 10.8 g (n=6), (D)-MWCNTs 305.8 ± 7.8 g (n=6), DPPC/RSA 333.4 ± 24.9 g (n=6), and naïve 287.2 ± 10.9 g (n=10). The mean and range of litter size were also not different between the exposure groups: (S)-MWCNTs 10.7 (8-13), 10% surfactant 10.5 (10-11), (D)-MWCNTs 9.8 (9-11), DPPC/RSA 10.5 (9-12) and naïve 10.6 (8-13). Mean weights of the fetuses are reported in Figure 7A and B, after grouping them according to gestational day (GD). The mean fetal weight was reduced at GD 19 following MWCNT and dispersion media exposure by both routes and was evident across all gestational days studied following intravenous exposure. Gross external morphological abnormalities were not seen in the fetuses. An increase in the mean placental weight was observed following MWCNT exposure by both routes on GD 18 (Figure 7C and D).

Fetal serum cytokine analysis

The cytokines levels in the fetal serum were not significantly changed following exposure to MWCNT or dispersion media by either route of administration and are reported in Table 2.

Discussion

Twenty hours post exposure to MWCNT by either intratracheal or intravenous administration resulted in a limited alteration in isolated blood vessel segments’ responses to various pharmacological agents. The intratracheal instillation of 100 µg/kg of (S)-MWCNTs, was of note for we observed an increase in the contractile response to angiotensin II in the main uterine artery when compared to the response from the...
suspended in 10% surfactant, approximately 743,000 particles per rat. Employing these most recent data (not shown), a 100 µg MWCNT exposure mass translated to tidal volume in humans is 500 mL of air and average respiration rate weighing and moving dry materials, MWCNT were found to range which was calculated to correspond with a human alveolar deposition different facilities that handle carbon nanotubes in the United States, USA). These assays were run using Luminex 100/200 (Luminex, Austin, TX) and results reported using Luminex xPONENT.

Table 2: Cytokine levels in fetal serum 24 hours post-exposure to MWCNTs. The fetal serum cytokines (a pooled sample from three fetuses/dam) were evaluated using Milliplex MAP Cytokine/Chemokine Panel and Immunoassay (EMD Millipore MA, USA). These assays were run using LumineX 100/200 (LumineX, Austin, TX) and results reported using LumineX xPONENT software versions 2.3/3.1. The mean and the SEM are reported for serum cytokines from fetuses from 17-19 days pregnant Sprague Dawley rats (n = 4-6).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>naive</th>
<th>IT 10% surfactant</th>
<th>IV (S)-MWCNTs</th>
<th>IV DDPC/RSA</th>
<th>IV (D)-MWCNTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1β (pg/ml)</td>
<td>1455.0 ± 372.5</td>
<td>1597.0 ± 310.3</td>
<td>1657.0 ± 599.4</td>
<td>1932.0 ± 861.7</td>
<td>1500.0 ± 375.9</td>
</tr>
<tr>
<td>IL8 (pg/ml)</td>
<td>75.7 ± 16.8</td>
<td>277.0 ± 78.5*</td>
<td>64.7 ± 55.4</td>
<td>120.4 ± 86.5</td>
<td>58.7 ± 55.9</td>
</tr>
<tr>
<td>IL10 (pg/ml)</td>
<td>70.0 ± 17.0</td>
<td>76.0 ± 13.5</td>
<td>49.7 ± 6.1</td>
<td>117.2 ± 56.9</td>
<td>66.8 ± 12.2</td>
</tr>
<tr>
<td>INFγ (pg/ml)</td>
<td>283.3 ± 42.5</td>
<td>324.5 ± 96.4</td>
<td>278.7 ± 30.6</td>
<td>405.8 ± 139.0</td>
<td>355.9 ± 190.0</td>
</tr>
<tr>
<td>MCP1 (pg/ml)</td>
<td>903.4 ± 310.8</td>
<td>866.7 ± 137.7</td>
<td>936.0 ± 60.3</td>
<td>991.2 ± 184.6</td>
<td>813.2 ± 113.3</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>469.5 ± 83.0</td>
<td>483.8 ± 59.3</td>
<td>457.2 ± 52.2</td>
<td>620.2 ± 127.5</td>
<td>512.1 ± 47.0</td>
</tr>
<tr>
<td>TNFa (pg/ml)</td>
<td>5.9 ± 5.9</td>
<td>3.3 ± 3.3</td>
<td>11.5 ± 5.9</td>
<td>6.7 ± 4.2</td>
<td>3.1 ± 2.3</td>
</tr>
</tbody>
</table>

IT: intratracheal instillation and IV: intravenous administration, MWCNT: Multi-wall carbon nanotube, 10% surfactant: 10 % surfactant in saline, (S)-MWCNTs: MWCNT suspended in 10% surfactant, DDPC/RSA: vehicle used for IV MWCNT delivery and (D)-MWCNTs: MWCNT suspended in DPCC/RSA.

* indicates p < 0.05 when compared to naive.

Voluntary weight loss due to increased stress in the pregnant state. This increase in of stress generation of the uterine artery following MWCNT exposure was confined to the late gestational stage. Minimal changes in the contractile responses due to MWCNT exposure were seen in vessel segments from the other vascular beds studied in both pregnant and non-pregnant state. The enhanced contractile responses were not associated with comparable changes in relaxation responses with Rho kinase inhibition, suggesting that mechanisms other than RhoA/Rho kinase may underlie alterations in contractile responses. To our knowledge, this is one of the first attempts to identify pregnancy related changes in the contractile responses of several vascular tissues following exposure to MWCNTs by different routes.

The physiochemical characteristics of the suspensions of the MWCNTs are important in understanding any delivery characteristics. According to Henderson et al, the pulmonary responses are similar in response to particle exposure by either inhalation or instillation, provided similar lung burdens [37]. Intrapulmonary instillation has been suggested to deliver less well dispersed MWCNTs to the lung epithelium resulting in fewer adverse effects when compared to short term inhalational exposure [34,38]. On the other hand, instillation exposes the animal to an acute, higher concentration of nanoparticles compared to inhalational exposure over a long period [34], bypassing the nasal cavity. Accounting for these conditions, the results from our IT exposure might be used to speculate on the outcomes of long term inhalational exposure. Exposure levels have been identified in response to particle exposure by either inhalation or instillation, may be initiating the inflammatory cascade, contributing to changes in the contractile responses.

Several factors including the properties of MWCNT suspensions, route of exposure, pulmonary/systemic inflammatory response, pregnancy related physiological changes and sensitivity of the vascular bed may contribute to the differential contractile responses of vascular tissues that we have observed in this study. The reported zeta potentials of (S)-MWCNTs and (D)-MWCNTs suggest that both suspensions have a relatively good stability with minor agglomerate formation. The minor differences in the hydrodynamic size and zeta potential in different suspensions may not alone contribute significantly to modification of the vessel behavior via MWCNT exposure, as seen in different routes of exposure.

Previously reported distribution of MWCNTs following intravenous administration suggests that majority of the particles are distributed in the lungs following their first pass in circulation [23]. On the other hand, these particles are primarily distributed in the lungs following intratracheal instillation/ pulmonary exposure [44]. Considering these distribution patterns by both routes, we chose to study the immune mediated pulmonary responses by analyzing the cell counts in a broncoalveolar lavage. We report a pregnancy related increase in the BAL cell counts suggesting an inflammatory response, reported as higher percentages of macrophages with both dispersion media and (D)-MWCNTs and the increased neutrophils.
The alterations in contractile responses reported in this study (in response to angiotensin II and serotonin) are similar to the potentiation of stress generation reported with other cardiovascular pathologies, linked with elements of calcium sensitization and regulation of the contractions by RhoA/Rho kinase pathway [47,48]. As reported in Figure 5 and Supplementary Figure 4, there were only minor differences in the sensitivity to Rho kinase inhibition in all three vascular beds following exposure to MWCNTs or dispersion media by either route of exposure. Thus a response compatible with action of Rho kinase as being responsible for the augmented contractile responses was not evident with MWCNT exposure. Additionally, the lack of changes in Rho associated proteins and eNOS in rat aortic endothelial cells exposed to MWCNTs in vitro would suggest that this pathway is not mediating changes in the endothelial cell contribution to the augmented stress through regulation of eNOS as reviewed by Yao et al. [25] and Satoh et al. [48]. Thus in aggregate these data suggest that the rho kinase signaling was not a primary mechanism responsible for augmented stress generation observed following MWCNT exposure. Other mechanisms postulated to enhance force generation can include generation of reactive oxygen species [49], increased oxidative stress [50] and enhanced cyclooxygenase signaling [51]. SWCNTs have shown to increase oxidative stress and alter the mitochondrial signaling [50] and enhanced cyclooxygenase signaling [51]. SWCNTs have also been associated with increased TNFα levels [52] which can influence vascular tissues.

When trying to understand the IT exposed MWCNT induced changes, it is important to recognize that 10% surfactant used as a vehicle for suspending the MWCNTs also induces a notable increase in stress generation in response to agonist stimulation compared the contractile responses from naïve animal group. We suggest that (S)-MWCNTs may have a combined effect of both MWCNTs and surfactant changes, it is important to recognize that 10% surfactant used as a vehicle for suspending the MWCNTs also induces a notable increase in stress generation in response to agonist stimulation compared the contractile responses from naïve animal group. We suggest that (S)-MWCNTs may have a combined effect of both MWCNTs and surfactant and this effect is clearly demonstrated in response to angiotensin II in the main uterine artery segments during pregnancy. However, synthetic lung surfactant based suspensions are established for studying pulmonary exposure effects of MWCNTs [55] and we chose to use the same for our study and were surprised to see such a vascular response effect. The responses seen with intravenous exposure to MWCNTs appear to be due to properties of the dispersant medium rather than due to nanotubes as DPPC/RSA significantly increases the baseline stress generation. The different media for the two routes of exposure were selected to simulate the biological media that area related to the exposure routes and were speculated to have no/minimal effects on vascular contractile responses. The dispersant medium is known to affect the cellular uptake of the nanoparticles [56] and presumed to impact overall cellular function. The dispersant media are known to contribute to the composition of protein or lipid corona associated with the nanoparticles in the biological systems [57,58]. It may be likely that.
the corona on these MWCNT is different enough to mask a significant MWCNT effect.

Neither MWCNTs nor dispersion medium induced significant changes in contractile responses of umbilical vein segments, suggesting that these exposures may only be affecting the maternal side of the circulation. However, detrimental effects were seen in the mean fetal weight following MWCNT exposure via both routes along with a significant contribution by the dispersion media alone. The IV exposure to (D)-MWCNTs appeared to be more effective at reducing the fetal growth earlier in gestational exposure window studied, whereas the weight reduction following IT exposure is mainly attributed to dispersion media. The placental transfer of the nanoparticles is affected by multiple factors including the particle size, dispersion medium, and the stage of the pregnancy [59] which could contribute to effects on fetal growth. Additionally, fetal microvessel dysfunction following exposure to engineered nanomaterials which was recently proposed by Stapleton et al. [11] may be a possible underlying explanation for our observations of reduced fetal weight despite the absence of augmented contractile responses in umbilical circulation. Stapleton et al. [11] used the fetal tail artery as a representative vessel from the fetal microcirculation and reported decreased responses in both endothelium dependent and independent relaxation [60]. Their findings suggest the applicability of the Barker Hypothesis (i.e. the relationship between retarded growth in early life and risk of adult disease is due to long term effects on physiology and metabolism imposed by an adverse environment during critical periods of development) to explain the changes observed in the fetus following maternal nanoparticle exposure [60,61]. This hypothesis may also hold true for our MWCNT exposure scenario, suggesting that the differences in the fetal weight gain may be a reflection of limited blood supply due to increased contraction observed in the uterine vascular segments.

Conclusions

In conclusion, the observations in this study suggest that vascular contractility may change following MWCNT exposure depending on multiple factors, including life stage (pregnant or non-pregnant), route of exposure, MWCNT dispersion media and the target vascular bed. Multiple agonist-mediated responses are differentially affected between the pregnant and non-pregnant stages with a significant increase in the stress generation of the uterine artery in response to angiotensin II confined to the pregnant stage. These agonists alter the contractile mechanism through various signaling cascades and we assessed the contribution RhoA/Rho kinase pathway in mediating these responses. We were unable to demonstrate that the RhoA/Rho kinase signaling cascade was significantly altered in response to MWCNT exposure and could not account for the augmented contractile responses, suggesting that other pro-constrictor mechanisms are likely to be involved. Our comparisons with naïve rats revealed a significant influence of the dispersion media on vascular tissue contractility and fetal weight gain, suggesting MWCNT exposure in isolation has no/minimal effects under the exposure scenarios applied in this study.

Acknowledgements

We like to thank Dr. Walter Kline of ONY Inc. for the generous gift of Infasurf surfactant, Drs. Benjamin Harrison and Richard Czerw of Nanotech Labs Inc. for providing the multi-walled carbon nanotubes for this study. We would also like to thank Drs. Pu-Chun Ke and Apparao M. Rao for previous work on characterization of the dry powder form of the multi-walled carbon nanotubes and to Josh E. Pitzer for assistance in rat aortic endothelial cell culture and PCR. This project was funded by the National Institute of Environmental Health Sciences grant U19 ES019525 and an AHA Mid-Atlantic Affiliate pre-doctoral fellowship to AKV.

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Citation: Vidanapathirana AK, Thompson LC, Odom J, Holland NA, Sumner SJ, et al. (2014) Vascular Tissue Contractility Changes Following Late Gestational Exposure to Multi-Walled Carbon Nanotubes or their Dispersing Vehicle in Sprague Dawley Rats. J Nanomed Nanotechnol 5: 201. doi:10.4172/2157-7439.1000201