Intravenous Administration of Multi-walled Carbon Nanotubes Affects the Formation of Atherosclerosis in Sprague-Dawley Rats

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Abstract: Intravenous Administration of Multi-walled Carbon Nanotubes Affects the Formation of Atherosclerosis in Sprague-Dawley Rats: Yu-Ying Xu, et al. Zhejiang University School of Medicine, China—Background: Carbon nanotubes (CNTs) have many potential applications, including as delivery systems for a variety of diagnostic or therapeutic agents. However, it has been suggested that exposure to carbon nano-materials may be a risk for the development of vascular diseases due to its impact on the vascular endothelium. Materials and Methods: Male Sprague-Dawley rats (180–200 g) were used to generate an atherosclerosis (AS) model, and the effect of intravenous administration of multi-walled carbon nanotubes (MWCNTs) on AS was studied. To further understand the underlying mechanisms, the effects of exposure of human umbilical vascular endothelial cells (HUVECs) to MWCNTs were examined. Results: Exposure to 200 μg/kg MWCNTs aggravated AS in this model. In addition, exposure to 50, 100 and 200 μg/kg MWCNTs increased the calcification of the aorta in the model. Short-term exposure also revealed that 200 μg/kg MWCNTs injured the endothelium in the aorta. MWCNTs disrupted the endothelial tight junction and induced endothelial cell death. Conclusion: The results demonstrated that MWCNTs could induce structural and functional changes in the endothelium, probably through vascular endothelialocyte injury, which eventually affected the development of AS in SD rats. (J Occup Health 2012; 54: 361–369)

Key words: Atherosclerosis, Calcification, Endothelial damage, Multi-walled carbon nanotubes

Data from abundance studies well indicate that air particulate pollution is directly linked to the adverse cardiovascular outcomes in the general population1). Epidemiological studies have indicated that individuals suffering from cardiovascular disease have increased susceptibility to particulate matter and other air pollutants2, 3). In addition, inhaled ultrafine particles exhibited considerably stronger toxicity when tested at equal mass dose with their fine counterparts4). Moreover, nano-sized particles, whose characterization is similar to ultrafine particles, can be rapidly translocated into the bloodstream in humans5), and there have been some reports showing the cardiovascular toxicity of nanoparticles6).

Carbon nanotubes (CNTs), such as single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs), have been widely used in different scientific fields, in particular as novel tools for biomedical and pharmaceutical applications due to their unique physicochemical properties7). A previous experimental study investigated their potential to be used to transport drug molecules to targeted tumor cells in an efficient and specific way8). Also, experiments carried out in small animals in vivo suggest the efficacy of CNTs as drug delivery vehicles9, 10). However, it has been suggested that after inhalation MWCNTs were translocated into the blood stream and can be found in remote organs11, 12). This finding suggests that MWCNTs could induce direct cardiovascular toxic effects independent of their passage through the lungs. With the rapid development of new MWCNT-based materials and technologies, there is a growing recognition that a fundamental understanding of the toxicological properties of MWCNTs is imperative.
Many in vivo and in vitro studies have shown that MWCNTs may induce oxidative stress and pulmonary inflammation\textsuperscript{31,44}. However, the toxicity of MWCNTs after they are introduced into the blood circulation is barely known\textsuperscript{45,46}. Furthermore, most i.v. exposure studies merely focused on the biodistribution and pharmacokinetics of carbon nanotubes\textsuperscript{17-19}. The potential cytotoxic or biological modulatory effects of carbon nanotubes on endothelial cells and the cardiovascular system still need to be investigated.

For this reason, we previously evaluated the direct toxic effects of MWCNTs on human umbilical vascular endothelial cells (HUVECs)\textsuperscript{20}. It was found that MWCNTs decreased cell survival, caused DNA damage and increased reactive oxygen species (ROS) in HUVECs. These results showed that MWCNTs could induce endothelial damage. Still, the influence of MWCNTs on the cardiovascular system, in particular, their effects on the progress of certain disease, such as atherosclerosis (AS), is not clear. AS is a major cardiovascular disease and the primary cause of heart disease and stroke. Therefore, if MWCNT exposure can influence the progress of AS, the broad range of applications of MWCNTs should be carefully evaluated. Thus, in the present study, we examined the effects of MWCNTs on AS using a Sprague Dawley (SD) rat model, which can be generated by feeding with high lipid chow after feeding vitamin D\textsubscript{3} (700,000 U/kg) via gavage in the first three days. In addition, we used a cell model in an effort to reveal the underlying mechanism.

Materials and Methods

Characterization of MWCNTs

MWCNTs were provided by Dr. F. Chen from Lawrence Berkeley National Laboratory. The characteristics of MWCNTs have been described by Guo et al.\textsuperscript{20}. Briefly, they were synthesized by using a chemical vapor deposition (CVD) method. Some of the physicochemical characteristics of the MWCNTs are as follows: purity ≥95%; average diameter, 30 nm; length ≥1 µm; and metal impurities, nickel 3.38%, yttrium 0.67%, and iron 0.13%. The MWCNTs were suspended in aqueous solution (1% Tween 80 or DMEM containing 10% fetal bovine serum) and then sonicated for 16 cycles of 5 seconds ultrasonication with a 6 seconds pause in an ice bath using an ultrasonic disruptor (200 W, JY 92-IIN, Scientz, Ningbo, China) each for the following exposures.

Establishment of an AS model and exposure

1) Model of AS

Establishment of an AS model was conducted based on the description by Pang et al.\textsuperscript{21}. Briefly, Sprague-Dawley rats were fed vitamin D\textsubscript{3} (700,000 U/kg) via gavage in the first three days, followed by feeding with high lipid chow for 4 months. The chow was a mixture of 2% cholesterol, 0.5% bile salt and 7% lard (all purchased from Sigma). The success of establishment of the model was determined by gross anatomy and histology 4 months after the beginning of feeding.

2) Animal administration and sampling

Sixty male Sprague Dawley (SD) rats weighting 180–200 g were randomly divided into 6 groups (N=10 for each group). Normal control rats received regular chow. For the AS model group, rats were orally fed vitamin D\textsubscript{3} and high lipid chow. For MWCNT exposure groups, rats were fed as a model group and received MWCNT injections (50, 100, 200 µg/kg bw, i.v., twice per week) after being fed vitamin D\textsubscript{3}. AS rats that received intravenous injections of 1% Tween 80 saline after being fed vitamin D\textsubscript{3} were used as a solvent control group. All rats were maintained on standard feed except for the above procedures. After 4 months, animals were anesthetized with pentobarbital (40 mg/kg i.p.), and blood samples were drawn from an artery. Aortas were harvested and fixed with 10% neutral formaldehyde. All animal experiments were conducted in compliance with the Standards of the Ethics Committee of Zhejiang Province for the Care and Use of Laboratory Animals.

3) Histological identification of AS

Aortas were dissected from the proximal ascending aorta to the bifurcation of the iliac artery, and the adventitial fat was removed. Then aortas were fixed in 10% neutral formaldehyde overnight. For en face analysis, aortas were rinsed in 70% alcohol for 30 seconds, stained with oil red O for 15 minutes, differentiated in 80% alcohol for 20 minutes and washed in running water for 1 hour. Aortas were then split longitudinally, pinned onto flat plates and photographed by a digital camera connected to a dissection microscope. The extent of AS was analyzed with Image-Pro Plus 5.0 (Media Cybernetics, Inc., Bethesda, MD, USA).

4) Analysis of calcification

For analyzing aortic calcification lesions, a paraffin-embedded tissue sample was sectioned at a thickness of 5 µm and stained with Alizarin red. Under a light microscope, Alizarin red-stained aorta tissues were evaluated as calcification lesions. Furthermore, the degree of calcification in each experimental group was calculated as the ratio of lesion area/original original luminal area (mm\textsuperscript{2}/mm\textsuperscript{2}).

5) Biochemical assay

Total serum cholesterol (CHO), total triglyceride (TG) and high-density cholesterol lipoprotein (HDL-c) were measured enzymatically using a Hitachi 7150 Autoanalyzer (Hitachi High-Technologies, Tokyo, Japan). The respective kits for the measurements
of CHO, TG, and HDL-c were all purchased from Beijing Applygen Technologies Inc., Beijing, China.

**Evaluation of endothelial damage in vivo**

Male SD rats (180–200 g) were fed a standard diet. The protocol was approved by the Ethics Committee of Zhejiang Province for the Care and Use of Laboratory Animals. The experimental animals were administered MWCNTs at a dose of 200 µg/kg for 7 days. Animals receiving 1% Tween 80 physiological saline for 7 days were used as a solvent control. Animals treated with bacterial LPS (Sigma Chemical, St. Louis, MO, USA) at a dose of 1 mg/kg for 12 hours before euthanasia were used as a positive control. The number of animals per group was 5.

1) **Immunohistochemistry**
   
   A small fragment of aorta was fixed in 10% buffered paraformaldehyde and subsequently embedded in paraffin blocks. Four-micrometer sections of tissue were used for immunohistochemistry. Endothelial cells were identified by the expression of factor VIII–von Willebrand factor (VWF) using a polyclonal antibody (dilution 1:1,000), dianaminobenzidine and a Vectastain Elite ABC and counterstained with hematoxylin. The images of aortic ring sections were obtained using a microscope with a × 40 objective.

2) **Plasma VWF detection**
   
   For the determination of plasma VWF, venous blood was drawn from the inferior caval vein, and citrated plasma was prepared by centrifugation at 3,000 rpm for 10 minutes. Plasma samples were stored at −80°C until analysis. Plasma VWF levels were determined by an ELISA method using commercial reagents (R&D Systems Inc., Minneapolis, MN, USA).

**Cell culture**

Human umbilical vein endothelial cell (HUVEC) lines were purchased from the American Type Culture Collection (ATCC) and cultured in DMEM medium containing 10% fetal bovine serum.

1) **Trypan blue exclusion assay**
   
   Cell viability was tested by using a trypan blue exclusion assay. Briefly, HUVECs were cultivated in six-well flat-bottom plates and treated with different concentrations of MWCNTs (0, 2, 5, 10, 20, and 50 µg/ml) for 24 hours, and then cell viability was measured under a microscope.

2) **Transendothelial electrical resistance (TEER)**
   
   HUVECs at passages 3–4 were seeded onto 6-well Transwell culture dishes and treated with an increasing concentration of MWCNTs (0, 2, 5, 10, 20, and 50 µg/ml). TEER was then measured by using a Millicell ERS-2 (Millipore) at 0, 6, 12, 24 or 48 hours after treatment. Each experiment was conducted in triplicate.

**Western blot analysis**

HUVECs were treated with different concentration of MWCNTs (0, 5, 20, and 50 µg/ml) for 24 hours, and then total proteins were isolated by homogenizing HUVECs with Radio Immunoprecipitation Assay (RIPA) lysis buffer (1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin). Protein concentration was determined by the bicinchoninic acid (BCA) method. Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Pall, Ann Arbor, MI, USA). After being blocked with 5% dried skimmed milk, membranes were incubated with different primary antibodies (VWF, Zonula Occludens-1 (ZO-1), p62 or atg8 antibodies) (1:1,000 dilution) overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibody (1:5,000) for 1 hour at room temperature. Membrane-bound antibodies were visualized using an enhanced chemiluminescence (ECL) system. Experiments were performed three or more times, and equal loading of protein was determined by measuring total β-actin expression.

**Statistical analysis**

Data are presented as means ± SD. Statistical analysis was performed using one-way ANOVA, the Student’s t-test and repeated measures in the general linear model. A probability level of *p*<0.05 was considered significant.

**Experimental Results**

**MWCNTs enhanced the formation of atheromatosis**

Atheromatosis lesions were examined with oil red staining. The animals in the model group had matured atheromatosis in the aorta, as shown by the presence of lipid streaks in the intima (Fig. 1A), whereas no lipid deposition was observed in the intima in the normal control (Fig. 1A) after 4 months. This demonstrated the successful establishment of the rat AS model. In rats exposed to 50 and 100 µg/kg MWCNTs, there was no significant difference in AS formation compared with the solvent group. On the other hand, exposure to 200 µg/kg MWCNTs significantly exacerbated the atheromatous lesion and lipid deposition in the aortic intima (Fig. 1A). The percentage of lesion area in the 200 µg/kg MWCNTs group (66.28%) was significantly higher than that in the solvent group (49.81%) (p<0.01) (Fig. 1B).

**MWCNTs increased aortic calcification**

Calcium precipitation may play a pathogenic role in AS. In this experiment, calcification mainly occurred at the medial intimal junction of the aortic wall.
Administration of 50, 100, and 200 µg/kg MWCNTs aggravated the deposition of calcium \( (p<0.01) \) (Fig. 2B).

**Serum lipids levels**

Blood samples were collected for measurement of total serum CHOL, TG and HDL-c. The results showed that there were significant differences between the model and normal group. However, exposure to 50, 100, and 200 µg/kg MWCNTs did not further influence the lipid levels (data not shown).

**MWCNTs induce endothelial cell damage in vivo**

As VWF release is increased when the endothelium is damaged, VWF levels have been proposed as a possible indicator of endothelial dysfunction\(^{23}\). In order to examine the direct effects of MWCNTs on the endothelium in the aorta, SD rats were treated with 200 µg/kg MWCNTs for 7 days. It was found that the endothelium was disrupted, as evidenced by the lack of continuity of the endothelial coating (Fig. 3A). Meanwhile, the VWF level was increased \( (p<0.05) \) (Fig. 3B) in serum after 7 days of exposure to 200 µg/kg MWCNTs.

**MWCNTs induce endothelial cell damage in vitro**

We further used a HUVEC cell culture model to elucidate the underlying mechanisms for MWCNT-induced endothelial damage. A trypan blue exclusion assay was used to measure the cell viability, and the results showed that MWCNTs decreased cell survival in a dose-dependent manner (Fig. 4A).

ZO-1 plays an important role in regulating cellular permeability\(^{24}\), while TEER is often used to evaluate the physical properties of endothelial intercellular junctions, which can indirectly influence cellular permeability\(^{22}\). To examine whether MWCNTs affected cellular permeability, ZO-1 protein expression and...
TEER were measured after treatment with MWCNTs. Also, we detected the level of VWF protein expression to observe the endothelial state. It was found that exposure to MWCNTs decreased TEER (Fig. 4B) in a dose- and time-dependent manner \((p<0.01)\) and that ZO-1 protein expression was decreased significantly \((p<0.01)\) (Fig. 4C–D), while VWF protein expression was increased significantly \((p<0.01)\) (Fig. 4E–F), thus indicating damage of endothelial cells and increased cellular permeability.

\textit{Autophagy is inhibited in MWCNT-induced endothelial cell damage in vitro}

Previously, we have shown that apoptosis was involved in the reduction of cell viability\(^{20}\). However, we were also interested to know whether other types of cell death, such as autophagy, might also contribute to the decreased cell viability. Therefore, cultured HUVECs were treated with MWCNTs \((0, 5, 20\) and \(50 \mu g/ml)\) for 24 hours. Two key proteins involved in autophagy, \(p62\) and \(atg8\) (including \(LC3-I\) and \(LC3-II\)), were observed using Western blot. It was found that exposure to MWCNTs increased \(p62\) protein and decreased \(LC3-I\) conversion to \(LC3-II\) (Fig. 5), thus indicating the inhibition of autophagy.

\textbf{Discussion}

In the present study, we evaluated the effects of MWCNTs on AS in a rat model. The findings in the present work are as follows: (1) MWCNTs exacerbate AS induced by Vitamin \(D_3\) and a high-cholesterol diet in rats, (2) MWCNTs induce damage in endothelial cells. The effects were manifested as distressed vessel homeostasis represented by lipid deposition and vascular injury.

The process required for SD rats to develop AS is relatively easy, e.g., feeding them a high lipid chow after feeding them vitamin \(D_3\) \((700,000 \text{ U/kg})\) via gavage in the first three days, although the duration is rather long (four months). Still, considering that laboratory rats have the advantages of moderate body type, easy breeding, more offspring and
low costs, using SD rats to develop AS is a simple and noninvasive method for generating an AS model. Epidemiological studies have shown that an important relationship exists between AS and exposure to particulate air pollution\(^1\,\,25\,\,26\)). Moreover, Araujo et al. reported that ultrafine particle (diameter<100 nm) exposures have a higher proatherogenic potential than fine particle exposures\(^27\). In our study, considering the potential biomedical application of MWCNTs as drug carriers, and since most of i.v. exposure studies have merely focused on the biodistribution and pharmacokinetics of carbon nanotubes\(^17\,\,19\)), we chose i.v. exposure to evaluate their vascular toxicity. Our data are the first to show that MWCNTs exacerbate AS in the SD rats model. Our results are in accordance with previous findings on other carbon nano-materials, despite differences in dosages, particle size, length of the experimental period, method of exposure and animal model. For example, a previous study had evaluated the effects of exposure to pulmonary SWCNTs on AS development in ApoE\(^{-/-}\) mice and found that exposure to SWCNTs was associated with AS acceleration\(^28\). Similarly, exposure of nano-sized carbon black by pulmonary instillation exacerbated the atherosclerotic lesions in the aorta of mice susceptible to the development of AS\(^29\).

A number of possible mechanisms have been proposed to explain the adverse health effects of MWCNTs, including their abilities to induce oxidative stress\(^15\,\,30\) and inflammation\(^31\), or induce platelet aggregation and vascular thrombosis\(^32\). Additionally, turbulences in blood vessels, especially in the endo-

**Fig. 4.** Multi-walled carbon nanotubes (MWCNTs) induced endothelial cell damage *in vitro*. (A) MWCNTs caused a decrease in cell viability in a dose-dependent manner for 24 hours. (B) HUVECs were cultured to confluence on 6-well Transwell\(^\text{TM}\) inserts. TEER was decreased in a dose-dependent manner for 6, 12, 24 and 48 hours. (C–F) The proteins ZO-1 (191 kDa) and VWF (220 kDa) were decreased after MWCNTs exposure for 24 hours. Data are presented as means \(\pm\) SD (n=3) (*\(p<0.05\) vs. solvent control; **\(p<0.01\) vs. solvent control). TEER: Transendothelial electrical resistance, ZO-1: Zonula Occludens-1, VWF: factor VIII-von Willebrand.
endothelium, could contribute significantly to the pathogenesis of AS. The endothelium, a monolayer covering the inner surface of blood vessels, plays a pivotal role in maintaining normal vascular homeostasis. The initial stage of atherogenesis involves endothelial dysfunction, and there is increasing evidence suggesting an association between impaired endothelial function and cardiovascular events\(^{(30)}\). Previously, we have shown that MWCNTs have a direct influence on cultured endothelial cells, as indicated by cytotoxicity and genotoxicity\(^{(20)}\). Our studies further indicated that MWCNTs decreased the expression of tight junction protein ZO-1 and increased vascular permeability. In addition, our \textit{in vivo} study demonstrated that the effects of MWCNTs were manifested as endothelial cell damage represented by increased expression of VWF in endothelial cells, as well as an increased serum VWF level. An increased plasma VWF level is considered as indication of endothelial dysfunction or activation\(^{(23, 34)}\).

Interaction of CNTs with endothelial cells triggers dose-dependent damage to cell function and viability\(^{(35)}\). Our results also showed a similar pattern. It is well known that apoptosis and autophagy are genetically regulated processes that could regulate cell fate. In some cellular settings, autophagy can lead to cell survival, either in collaboration with apoptosis or as a back-up mechanism when apoptosis is defective\(^{(36)}\). When mammalian cells are deficient in autophagy, the level of P62 is increased\(^{(37, 38)}\); during autophagy, LC3-I is conjugated to phosphatidylethanolamine to localize the resultant LC3-II to the autophagosomes, and a portion of the LC3, which is located in the inner membrane of the autophagosome, is degraded in autolysosomes\(^{(39)}\). In this study, the level of P62 was increased in a dose-dependent manner by treatment with MWCNTs. On the other hand, the LC3 level was increased by treatment with 5 and 20 \(\mu\)g/ml MWCNTs, and the LC3-II/LC3-I ratio was decreased upon treatment with 50 \(\mu\)g/ml MWCNTs. Taken together, such information indicated that MWCNTs inhibited autophagy in endothelial cells. These mechanisms of MWCNTs in endothelial cells were different from the mechanisms of MWCNTs in macrophages. MWCNTs probably trigger cytotoxic effects in phagocytic cells by reacting with macrophage receptor with collagenous structure (MARCO) on the plasma membrane and rupturing the plasma membrane\(^{(40)}\).

Taken together, the present study showed using an SD rat model that exposure to MWCNTs resulted in acceleration of development of AS. These results indicate a possible link between nanoparticles and AS.

**Acknowledgments:** We thank Prof. Fan-Qing Chen (Life Science Division, Ernest Orlando Lawrence Berkeley National Laboratory, Berkeley, CA, USA) for kindly providing the MWCNTs. This work was supported by grants from the National Natural Science Foundation of China (Nos. 30771824 and 30800923), Science Foundation of Chinese University (No. 2009QNA7021), Natural Science Foundation of Zhejiang Province (Nos. Y206537, R2100555 and Y2100353), Department of Science and Technology, Zhejiang Province (No. 2009C11122), and Ministry of Science and Technology, China (2009DFB30390).

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