Published in final edited form as:

J Voice. 2017 November ; 31(6): 662–668. doi:10.1016/j.jvoice.2017.03.014.

Acute nanoparticle exposure to vocal folds: a laboratory study

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Abstract

Objective—Airway exposure to nanoparticles is common in occupational settings. Inhaled nanoparticles have toxic effects on respiratory tissue. Vocal folds are also at direct risk from inhaled nanoparticles. This study investigated the effects of single-walled carbon nanotubes (SWCNT), a type of nanoparticle, on vocal fold epithelium and fibroblasts. These cell types were selected for study since the epithelium is the outer layer of the vocal folds and fibroblasts are the most common cell type in connective tissue underlying the epithelium.

Methods—Native porcine vocal fold epithelium and cultured human vocal fold fibroblasts were exposed to SWCNTs (100 ng/mL) and control (no SWCNT), in vitro. Epithelial and fibroblast viability were measured using MTT assay. Epithelial barrier integrity was assessed with transepithelial resistance and sodium fluorescein permeability. Epithelial tight junctional protein occludin expression was measured with the Western blot. Gene expressions of the fibroblast specific protein 1 (FSP-1), α -smooth muscle actin (α -SMA), and collagen III (Col-III) were assessed using quantitative polymerase chain reaction.

Results—Transcriptional expression of genes encoding FSP-1 and Col-III were increased significantly following SWCNT exposure. There were no significant differences between control and SWCNT groups on any of the other measures.

Conclusions—SWCNT exposure induces vocal fold fibroblasts to a fibrotic phenotype. These data help us understand vocal fold defense mechanisms and lay the groundwork for studying the physiological effects of nanoparticle exposure in vivo.

Keywords

vocal fold epithelium; carbon nanotubes; fibroblasts; fibrosis

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Conflict of Interest: None

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Introduction

Carbon nanotubes (CNTs) are nanoparticles produced in myriad industrial applications.¹ Individuals are at risk for inhaling CNTs from the ambient environment.²⁻⁴ Therefore the adverse effects of CNTs on the respiratory system have been investigated. Exposure to multi-walled carbon nanotubes reduces ciliated cells in rodent trachea.⁵ Tracheal instillation of single-walled carbon nanotubes (SWCNTs, 10 mg/mL) in mice for 7 days induces epithelial granulomas and interstitial inflammation. Longer exposures (90 days) result in extended inflammation and necrosis of lung tissue.⁶ Pharyngeal aspiration of SWCNTs can cause granulomas and interstitial fibrosis.⁷ Though there are many studies reporting lung lesions following CNT exposure, very few studies have evaluated pathological changes to the larynx. Rats exposed to 5 mg/m³ multi-walled carbon nanotubes for 13 weeks within an inhalation exposure chamber presented significant epithelial lesions in the larynx⁸. However, the underlying mechanisms for epithelial pathologies and connective tissue changes have not been studied.

The vocal fold epithelium consists of stratified squamous cells and junctional complexes. This structural specificity provides an active barrier to xenobiotics and protects the underlying connective tissue against chemical and physical insults. This epithelial barrier can be adversely affected by pollutants found in cigarettes.⁹⁻¹² Though no study has investigated the effect of CNTs on vocal fold epithelium, CNTs are reported to reduce epithelial resistance in a tracheobronchial epithelial cell line.¹³ Fibroblasts are the most common cell type in the vocal folds and are important for synthesizing extracellular matrix (ECM) components such as collagen and elastin. However, stress resulting from phonotrauma or radiation injury can alter vocal fold ECM composition by increasing transcription and secretion of collagen, leading to fibrosis.^{14,15} The effects of CNT on vocal fold fibroblasts have not been investigated although there is literature from lung tissue. For example, mice exposed to 5 mg/m³ SWCNT in a whole body chamber for one year showed more than two-folds increase in lung collagen¹⁶. The mechanism of CNT induced fibrosis may involve both direct and indirect effects¹⁷. CNTs promote fibroblast conversion into myofibroblasts, and increase production of collagen either by directly interacting with fibroblasts or indirectly regulating phenotype and function of fibroblasts through cytokines released by epithelia and macrophages.

The objective of this study was to determine whether one type of CNT (single walled; SWCNTs) would have detrimental effects on vocal fold epithelia and fibroblasts. SWCNTs have a single layer of graphene cylinder, are small in diameter, and have a fiber-like shape.¹⁸ We investigated the effects of SWCNT exposure on epithelial viability, resistance, and permeability. We also quantified the effects of SWCNT on occludin expression, which is a tight junction protein. Finally, we assessed the effects of SWCNT exposure on fibroblast viability and gene expression of fibroblast specific protein 1 (FSP-1), α -smooth muscle actin (α -SMA), and Collagen III (col-III). These genes are biomarkers of fibrosis. The current study is the first step towards our understanding of the effects of CNTs on vocal folds. As CNTs become more ubiquitous, quantifying the underlying pathophysiological changes in response to acute exposures is needed as a pre-requisite to more chronic studies. Eventually,

these data will lay the foundation for studying the impact of CNTs on health and voice production for future risk assessment and control.

Materials and Methods

Reagents

SWCNTs (#900-1301, long, purified, outer diameter <2nm, length: 5–15µm, purity: >90% CNT~ >50% SWNT*, ash < 2% wt., Amorphous carbon: <5% wt.) were purchased from SES Research (Houston, TX). Protease Inhibitor Cocktail was purchased from Calbiochem (San Diego, CA). Sodium dodecyl sulfate (SDS), Tris, PVDF membrane and 2xLaemmli sample buffer were purchased from Bio-Rad (Hercules, CA). Bovine Serum Albumin (BSA) standards were purchased from Thermo Scientific (Rockford, IL). Primary rabbit anti-occludin antibody was purchased from Abcam (Cambridge, MA). Primary mouse anti-β-actin antibody was purchased from Sigma Aldrich (St Louis, Missouri). Goat anti-rabbit IgG-HRP and Goat anti-mouse IgG-HRP were from Santa Cruz Biotechnology (Dallas, TX). Enhanced chemiluminescence reagent (ECL) was obtained from Pierce Endogen (Rockford, IL). TRIzol reagent and Vybrant MTT cell proliferation assay kit were purchased from Thermo Fisher Scientific (Waltham, MA) iTaq Universal SYBR Green Supermix kit was obtained from Bio-rad (Hercules, CA). All other chemicals were obtained from Sigma Aldrich (St Louis, Missouri).

SWCNT, Control, and Positive Control

SWCNTs were prepared using FBS at a concentration of 1 mg/mL and sonicated (Fisher Scientific Sonic Dismembrator Model 500) with a duty cycle of 30%, amplitude of 30% for 30 seconds. The SWCNT-FBS media mixture was diluted to final concentration of 100 ng/mL of SWCNT and 15% FBS (10% FBS for cell culture) using HBSS and then sonicated. This concentration was selected from published literature.¹³ A Nano Zetasizer ZS90 (Malvern Instruments, Worcestershire, UK) was used to show that at least some SWCNTs were still dispersed in the media at 5 hours as compared to control media (Fig 1). The control media was HBSS with 15% FBS (10 % FBS for cell culture) and did not contain any SWCNTs. A positive control (boiled tissue) was used for epithelial viability.

Vocal fold epithelial dissection

Porcine larynges were obtained from local slaughterhouses. Larynges were transported to the laboratory in cold phosphate buffered saline (PBS). Larynges were hemisected along the mid-sagittal plane and the epithelium, basal lamina, and superficial lamina propria (referred as vocal fold epithelia hereafter) were separated from the connective tissue and muscle and then moistened with Hanks' Balanced Salt Solution (HBSS). Vocal fold epithelia were exposed to one of three conditions: SWCNT; control (no SWCNT); positive control (boiled) for 5 hours.

Epithelial viability

Nine epithelial samples (6 mm diameter) were obtained via biopsy punch, weighed, and incubated in oxygenated (95% O₂ and 5% CO₂) SWCNT, or control (no SWCNT) media for 5 hours at 37 °C. Boiled vocal fold epithelia served as positive control. Samples were

incubated in MTT solution in a twelve-well culture plate while rotating at 100 rpm for 2 hours and then rinsed with PBS for 1 minute. Finally, formazan was extracted using 4mL DMSO from minced tissues. The absorbance of formazan was measured at 570 nm with an ELISA scanner (SpectraMax M2e, Molecular Devices, Sunnyvale, CA) with DMSO as blank. The viability index for each tissue was calculated by the ratio of the absorbance to the tissue weight (abs/mg).

Epithelial Resistance

Transepithelial electric resistance (TEER) was measured using an Ussing chamber system (model 15362, World Precision Instruments, WPI, Sarasota, FL) coupled with an associated voltage clamp (model DVC-1000). Twelve vocal fold epithelia were mounted on the chambers of a calibrated Ussing system. Chambers on both sides of the tissue were filled with 5 mL HBSS-FBS which was oxygenated and warmed to 37 °C. TEER was measured once the short-circuit current reached stable baseline (approximate 1 hour). Only epithelia with TEER exceeding 300 $\Omega \cdot \text{cm}^2$ were included in subsequent experiments.¹⁹ The TEER values were measured by DataTrax software immediately prior to and following 5 hours of exposure to SWCNT or control (no SWCNT).

Epithelial Permeability

Permeability experiments were conducted in the Ussing system in fourteen vocal fold epithelial tissues. Sodium fluorescein (NaFl) was selected as the permeability marker. Only those vocal folds with TEER greater than 300 $\Omega \cdot \text{cm}^2$ were selected for these studies¹⁹. The apical surface of vocal fold epithelium was exposed to 100 ng/mL SWCNTs along with 1 mg/mL sodium fluorescein (NaFl) in HBSS-FBS or 1 mg/mL NaFl with HBSS-FBS (control). An aliquot (200 μL) of media from the luminal side was also collected right after NaFl was mixed to serve as the original concentration of fluorescence. After 5 hours of exposure, another aliquot (200 μL) of media was obtained from the basolateral chamber of the Ussing system. The fluorescence was measured in duplicate using an ELISA scanner (SpectraMax M2e, Molecular Devices, Sunnyvale, CA) with excitation wavelength of 480 nm and emission wavelength of 525 nm. The permeability index was calculated as the ratios of intensity of NaFl on basolateral side to the intensity of NaFl on the luminal side.

Occludin expression

Total protein from six vocal fold epithelia in SWCNT and control groups was extracted in a homogenization buffer (5mM EGTA, 20mM Tris (pH 7.5), 0.1% SDS, 1% Triton X-100, protease inhibitor cocktail (Calbiochem, CA), 10 $\mu\text{L}/\text{mL}$ PMSF, and 15mM 2-mercaptoethanol). Protein concentrations were measured by BSA protein assay (Thermo Scientific). Protein samples were boiled with 2xLaemmli sample buffer (Bio-Rad). Samples were loaded on a SDS-polyacrylamide gel, electrophoresed, and transferred onto a PVDF membrane. The membrane was blocked for 1 hour at room temperature, subsequently incubated with primary rabbit anti-occludin (1:1000) antibody overnight at 4°C. The membrane was washed with mixture of Tris-Buffered Saline and Tween 20 (TBST) and incubated with the Goat anti-rabbit IgG-HRP (1:3000) at room temperature for 1 hour. Immuno-blots of occludin were visualized using ECL-Western Blotting Substrate and the Bio-Rad Molecular Imager. The membrane was washed with TBST 3 times, tripping buffer

for 15 minutes, and again with TBST for 3 times. Next, the membrane was incubated with primary mouse anti- β -actin (1:10000) antibody at 4 °C overnight. The membrane was washed and incubated with Goat anti-mouse IgG-HRP (1:5000) at room temperature for 1 hour. The immuno-blots of β -actin were visualized with the same method mentioned above. The band intensities of occludin (63 kDa) and β -actin (42 kDa) were quantified using ImageJ software (NIH, Bethesda, Maryland) and occludin values were normalized to β -actin.

Vocal fold fibroblast cell culture

Immortalized human vocal fold fibroblasts (VFF) were obtained from Dr. Susan Thibeault at the University of Wisconsin-Madison.²⁰ VFF were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS; Corning), 1% penicillin/streptomycin, 1% minimal essential medium (MEM) nonessential amino acid solution, and 200 μ g/mL Geneticin (G418; Teknova). Cells were used at passages 8–12 for all experiments and maintained at 37°C and 5% CO₂.

The VFF cells were seeded at a density of 25,000 cells/cm² in a 24 well plate and allowed to attach overnight. SWCNTs were dissolved in FBS at stock concentrations of 5 μ g/mL and sonicated with a duty cycle of 30%, amplitude of 30% for 30 seconds. SWCNTs were autoclaved and added to the VFF media at final concentrations of 100 ng/mL and incubated for 24 hours. The cells in the control group were incubated within the same medium without any SWCNT.

Fibrosis gene expression on mRNA level

The VFF cells were incubated with or without 100 ng/mL SWCNTs for 24 hr. The cells were harvested by washing 3 times with D-PBS to clear the nanoparticles and then by adding TRIzol to the wells and collecting the lysate. The total RNA was isolated and purified using TRIzol reagent. The RNA was reverse transcribed to cDNA (Bio-rad iScript cDNA synthesis kit). The levels of mRNA encoding FSP-1, α -SMA and collagen 3 were quantified using quantitative real-time polymerase chain reaction. The real-time PCR was conducted in the CFX Connect Real-Time PCR Detection system (Bio-Rad, CA) with iTaq Universal SYBR Green Supermix (Bio-Rad) kit. The qPCR was performed using a protocol with 3-min initial denaturation at 95 °C; 40 cycles of denaturation at 95°C, then 10-sec gradient 55.0°C – 65.0°C and at last 30-sec extension at 72°C. The qPCR samples were loaded in duplicate. The amplification efficiencies of target and reference genes were examined in the same load plate. The relative mRNA expressions of the target genes between SWCNTs treated group and control group were assessed using 2^{-Ct} which were calculated with the threshold cycle time values (Ct values) obtained in qPCR detection system. The target genes Ct values were normalized with that of GAPDH in the same sample.

The forward and reverse primers for genes encoding FSP-1, α -SMA, collagen 3 and GAPDH were designed using Primer Express 3.0 software. The primer sequences for human FSP-1 used in this study were: (F) 5-CTTCC CCTCT CTACA ACCCT CT-3, (R) 5-GAGCT TGAAC TTGTC ACCCT CT-3; for α -SMA forwarded primer was 5-CCTCC CTTGA

GAAGA GTTAC GA-3, reverse primer was 5-TGATG CTGTT GTAGG TGGTT TC-3; for collagen 3 forward primer was 5-AATCA GGTAG ACCCG GACGA-3, reverse primer was 5-TTCGT CCATC GAAGC CTCTG-3; and for GAPDH primers were (F) 5-AGCAA GAGCA CAAGA GGAAG AG-3; (R) 5-ACTGG TTGAG CACAG GGTAC TT-3.

VFF cell viability assay

To assess cell proliferation, the VFF cells were seeded at a concentration of 35000 cells/cm² in a 96 well plate and left to attach overnight. VFF were treated with or without 100 ng/mL SWCNTs for 24 hr. MTT assay was performed according to manufacturer's protocol. Briefly, fresh phenol red free medium was added to the wells along with MTT solution and incubated at 37 °C for 4 hours. Majority of the medium with MTT was removed, leaving only 25 µL in each well. An aliquot of 70 µL DMSO was then added into each well to dissolve the formazan. The absorbance was read at 540nm.

Statistical analysis

Statistical analyses were completed in SPSS (version 20, Chicago, Illinois). Data that did not meet assumptions of normality were analyzed using non-parametric statistics. Group differences were assessed with Median test (tissue viability), Wilcoxon signed-rank test (occludin expression), unpaired t-tests (permeability, fibroblast viability, FSP-1, α-SMA and collagen 3 expression), and mixed-ANOVA (TEER). The differences between the means were considered significant if p values were equal to or less than 0.05.

Results

Epithelial viability

The epithelial viability remained largely unchanged after the tissues were exposed to 100 ng/mL SWCNTs in the incubation medium for 5 hr at 37°C (Fig. 2), although a significant challenge effect among groups was detected ($\chi^2 = 6.3$, $p=0.043$). The Mann-Whitney post-hoc analyses revealed a significant decrease in epithelial viability between the positive control (boiled samples) and control; but no significant difference was seen in SWCNT group as compared to control ($N=3$, $Z = -1.528$, $p=0.127$, Fig. 2).

Epithelial resistance

Epithelial resistance was maintained in vocal folds exposed to SWCNTs ($N = 6$, $F = 3.74$, $p = 0.08$, Figure 3). There were no significant differences between groups exposed to SWCNT or control ($N=6$, $F = 0.19$, $p = 0.672$, Figure 3).

Epithelial permeability

There were no significant differences in permeability of SWCNT and control groups ($N = 7$, $t = 1.462$, $p=0.169$, Fig. 4).

Occludin expression

No significant differences were observed for occludin expression after SWCNT or control exposure suggesting that SWCNTs did not change the expression of occludin ($N = 3$, $Z = -1.069$, $p = 0.285$, Fig. 5).

VFF cell studies

When the immortalized human vocal fold fibroblasts (VFF) were incubated with or without 100 ng/mL SWCNTs for 24 hr, the cell viability as determined by MTT assay did not change significantly between SWCNT group and controls ($N = 6$, $t = 1.417$, $p = 0.187$, Fig. 6). Interestingly, among three genes related with fibrosis, two of them exhibited significant increases in their expression following SWCNT exposure, i.e., FSP-1 about 1.6 fold increase ($N = 6$, $t = -3.573$, $p = 0.005$, Fig 7) and collagen-3 about 1.5 fold increase ($N = 6$, $t = -2.794$, $p = 0.019$, Fig. 7), while the mRNA level also showed a trend of increase, but not statistically significant, in α -SMA expression ($N = 6$, $t = -2.022$ $p = 0.071$, Fig. 7).

Discussion

The unique properties of carbon nanotubes (CNTs) including high elastic modulus and thermal and electrical conductivity have led to widespread use in modern industry.²¹ It has been estimated that several thousand tons of CNTs are produced annually around the world²² in products including cables, wires, textiles, sensors, drug delivery systems, medical implants, electronics, coating, etc.^{22,23} About 375 workers were reported to be directly working with CNTs in 2008 in the US, and the workforce size was estimated to increase by 15%–17% annually.²⁴ As an estimation of the whole nanotechnological field, there will be around 6 million workers worldwide with 2 million of them in the US alone by 2020.²⁵ Workers who handle the CNTs directly are potentially exposed to the airborne particles if no exposure controls are provided. While data on exposure level of CNTs in the work place are limited, some studies on airborne CNTs have indicated high concentration of CNTs in the personal breathing zone in occupational settings.^{2–4} The National Institute for Occupational Safety and Health (NIOSH) recommends a $1\mu\text{g}/\text{m}^3$ exposure limit by considering both the health risks and limit of quantitation method.²⁶ CNTs are highly suspected to have toxic properties similar to asbestos due to similarities in their fiber-like shape.²⁷ Asbestos exposure is associated with lung fibrosis and cancers,²⁸ chronic laryngitis, epithelial hyperplasia²⁹ and laryngeal cancers.³⁰ CNT exposures in animal studies reveal lung fibrosis⁷ and cancer,^{31,32} but relevant toxic effects of CNTs on the larynx are unknown. This is important because the vocal folds are essential for voice production and airway protection.

The vocal fold epithelium protects underlying connective tissue from environmental insults. Impairment of the epithelial barrier may allow the penetration of xenobiotics and induce inflammation. Previous studies have demonstrated that SWCNTs are deposited in alveolar interstitium³³ and reduce epithelial resistance in a tracheobronchial epithelial cell line model.¹³ However, the effects of SWCNTs on vocal fold epithelia may be quite different from those in the lung due to epithelial differences in these organs. As the first defense line against xenobiotic insults in larynx, the stratified squamous cell type in vocal folds may be more effective in withstanding structural and functional damage.

To understand the vulnerability of the vocal folds to SWCNTs, we quantified changes in viability and barrier function after SWCNT exposure. A concentration of SWCNT (100 ng/mL), which was shown to decrease resistance in a tracheobronchial epithelial cell line,¹³ was used in this study. The MTT assay result indicated that epithelial cells were still metabolically active after 5 hours of SWCNT exposure. The epithelial resistance and permeability did not change after SWCNT exposure, suggesting that barrier integrity was preserved. These data are consistent with the non-significant changes in occludin expression levels. Occludin is a transmembrane protein and a major component of the tight junction strand, which seals the intercellular space; its normal expression is associated with the barrier function of epithelia.³⁴

The current study exposed vocal fold epithelia to SWCNT concentrations reported in the literature. Therefore, the non-significant effects of SWCNT on vocal fold epithelial barrier function deserve further discussion. One potential reason for this non-significant effect is the short exposure duration. The acute exposure duration of 5 hours was selected because the vocal folds were viable over this time period. Previous data have shown that short duration exposure to acidic challenges and pollutants reduce the vocal fold epithelia resistance.^{9,35} However, epithelial barrier integrity was preserved for 5 hours after SWCNT exposure, suggesting that the vocal folds may be able to effectively defend against nanoparticles in the short-term.

We further investigate the direct effects of SWCNTs on vocal fold fibroblasts. We were interested in these effects since fibroblasts. Viable vocal fold fibroblasts were incubated with SWCNTs directly for 24 hours and fibrosis was evaluated with the transcription level of genes encoding FSP-1, α -SMA and Col-III. FSP-1 is a calcium binding protein that identifies fibroblasts, which increase during pulmonary fibrosis.³⁶ α -SMA is expressed in the differentiated myofibroblasts. Col-III levels also increase with fibrosis. Increase in levels of FSP-1 and Col-III gene expression, along with the trend of increase in α -SMA indicate a profibrotic response to continued SWCNT exposure.

Conclusions

Vocal fold epithelia and fibroblasts were exposed to low concentrations of SWCNT. There were no significant changes in epithelial viability, resistance, permeability, and occludin expression. Vocal fold epithelia show resistance to nanotoxicity induced by SWCNT during an acute exposure. Vocal fold fibroblasts showed a profibrotic response when directly exposed to SWCNT for 24 hours. The current data lay the groundwork for further investigation of the effects of inhaled nanoparticles on vocal fold tissue using in vivo models to fully understand the pathophysiological changes following pollutant exposures.

Acknowledgments

Funding: Funding was provided from R01DC011759 (National Institutes of Health/National Institute on Deafness and other Communication Disorders).

We acknowledge the assistance of Elizabeth DiRenzo, Ph.D., in sharing the MTT protocol and Abigail Durkes, DVM for assistance in obtaining porcine larynges. James McMasters and Alyssa Panitch, Ph.D., assisted with the Zetasizer and Xue Fu, Ph.D., assisted with the Western blot procedure. Porcine larynges were obtained from

Beutler's Meat Processing, Lafayette, IN and Monon Meat Packing, Monon, IN. The study was funded by R01DC011759 (National Institutes of Health/National Institute on Deafness and other Communication Disorders).

References

1. Thostenson ET, Ren Z, Chou TW. Advances in the science and technology of carbon nanotubes and their composites: a review. *Compos Sci Technol*. 2001; 61:1899–1912.
2. Maynard AD, Baron PA, Foley M, Shvedova AA, Kisin ER, Castranova V. Exposure to carbon nanotube material: Aerosol release during the handling of unrefined single-walled carbon nanotube material. *J Toxicol Env Health Pt A*. 2004; 67:87–107.
3. Han JH, Lee EJ, Lee JH, et al. Monitoring multiwalled carbon nanotube exposure in carbon nanotube research facility. *Inhal Toxicol*. 2008; 20:741–749. [PubMed: 18569096]
4. Bello D, Wardle B, Yamamoto N, et al. Exposure to nanoscale particles and fibers during machining of hybrid advanced composites containing carbon nanotubes. *J Nanopart Res*. 2009; 11:231–249.
5. Ohba T, Xu J, Alexander DB, et al. MWCNT causes extensive damage to the ciliated epithelium of the trachea of rodents. *J Toxicol Sci*. 2014; 39:499–505. [PubMed: 24849684]
6. Lam CW, James JT, McCluskey R, Hunter RL. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. *Toxicol Sci*. 2004; 77:126–134. [PubMed: 14514958]
7. Shvedova AA, Kisin ER, Mercer R, et al. Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. *Am J Physiol-Lung Cell Mol Physiol*. 2005; 289:L698–L708. [PubMed: 15951334]
8. Kasai T, Umeda Y, Ohnishi M, et al. Thirteen-week study of toxicity of fiber-like multi-walled carbon nanotubes with whole-body inhalation exposure in rats. *Nanotoxicology*. 2015; 9:413–422. [PubMed: 25030099]
9. Liu X, Zheng W, Sivasankar MP. Acute Acrolein Exposure Induces Impairment of Vocal Fold Epithelial Barrier Function. *PLoS One*. 2016; 11:e0163237. [PubMed: 27643990]
10. Isik AC, Kalender Y, Yardimci S, Ergun A. Environmental tobacco smoke in rats. *J Otolaryngol*. 2004; 33:382–386. [PubMed: 15971655]
11. Duarte JL, de Faria FA, Ceolin DS, Cestari TM, de Assis GF. Effects of passive smoke inhalation on the vocal cords of rats. *Braz J Otorhinolaryngol*. 2006; 72:210–216. [PubMed: 16951854]
12. Levendoski EE, Sivasankar MP. Vocal fold ion transport and mucin expression following acrolein exposure. *J Membr Biol*. 2014; 247:441–450. [PubMed: 24648011]
13. Banga A, Witzmann FA, Petrache HI, Blazer-Yost BL. Functional effects of nanoparticle exposure on Calu-3 airway epithelial cells. *Cell Physiol Biochem*. 2012; 29:197–212. [PubMed: 22415089]
14. Allen J. Cause of vocal fold scar. *Curr Opin Otolaryngol Head Neck Surg*. 2010; 18:475–480. [PubMed: 20861796]
15. Johns MM, Vasantha K, Eric B, et al. Radiation Fibrosis of the Vocal Fold: From Man to Mouse. *Laryngoscope*. 2012; 122:S107–S125. [PubMed: 23242839]
16. Shvedova AA, Yanamala N, Kisin ER, et al. Long-term effects of carbon containing engineered nanomaterials and asbestos in the lung: one year postexposure comparisons. *Am J Physiol Lung Cell Mol Physiol*. 2014; 306:L170–182. [PubMed: 24213921]
17. Vietti G, Lison D, van den Brule S. Mechanisms of lung fibrosis induced by carbon nanotubes: towards an Adverse Outcome Pathway (AOP). *Part Fibre Toxicol*. 2016; 13:11.doi: 10.1186/s12989-016-0123-y [PubMed: 26926090]
18. Murray A, Kisin E, Tkach A, et al. Factoring-in agglomeration of carbon nanotubes and nanofibers for better prediction of their toxicity versus asbestos. *Part Fibre Toxicol*. 2012; 9:10. [PubMed: 22490147]
19. Phillips JE, Wong LB, Yeates DB. Bidirectional transepithelial water transport: measurement and governing mechanisms. *Biophys J*. 1999; 76:869–877. [PubMed: 9929488]
20. Chen X, Thibeault SL. Novel isolation and biochemical characterization of immortalized fibroblasts for tissue engineering vocal fold lamina propria. *Tissue Eng Part C Methods*. 2009; 15:201–212. [PubMed: 19108681]

21. Sinnott SB, Andrews R. Carbon Nanotubes: Synthesis, Properties, and Applications. *Crit Rev Solid State Mat Sci*. 2001; 26:145–249.
22. De Volder MFL, Tawfick SH, Baughman RH, Hart AJ. Carbon Nanotubes: Present and Future Commercial Applications. *Science*. 2013; 339:35.
23. Zhang Q, Huang JQ, Qian WZ, Zhang YY, Wei F. The road for nanomaterials industry: a review of carbon nanotube production, post-treatment, and bulk applications for composites and energy storage. *Small*. 2013; 9:1237–65. [PubMed: 23580370]
24. Schubauer-Berigan MK, Dahm MM, Yencken MS. Engineered carbonaceous nanomaterials manufacturers in the United States: workforce size, characteristics, and feasibility of epidemiologic studies. *J Occup Environ Med*. 2011; 53:S62–7. [PubMed: 21654420]
25. Roco MC. The long view of nanotechnology development: the National Nanotechnology Initiative at 10 years. *J Nanopart Res*. 2011; 13:427–445.
26. Current Intelligence Bulletin 65: Occupational exposure to carbon nanotubes and nanofibers. Centers for Disease Control and Prevention web site; Apr. 2013 No. 2013–145. Available at: <http://www.cdc.gov/niosh/review/peer/HISA/nano-pr.html>
27. Donaldson K, Aitken R, Tran L, Stone V, Duffin R, Forrest G, Alexander A. Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety. *Toxicol Sci*. 2006; 92:5–22. [PubMed: 16484287]
28. Loomis D, Dement JM, Wolf SH, Richardson DB. Lung cancer mortality and fibre exposures among North Carolina asbestos textile workers. *Occup Environ Med*. 2009; 66(8):535–542. [PubMed: 19282317]
29. Kambic V, Radsel Z, Gale N. Alteration in the laryngeal mucosa after exposure to asbestos. *Br J Ind Med*. 1989; 46:717–723. [PubMed: 2489023]
30. Offermans NSM, Vermeulen R, Burdorf A, et al. Occupational asbestos exposure and risk of pleural mesothelioma, lung cancer, and laryngeal cancer in the prospective Netherlands cohort study. *J Occup Environ Med*. 2014; 56:6–19. [PubMed: 24351898]
31. Grosse Y, Loomis D, Guyton KZ, et al. Carcinogenicity of fluoro-edenite, silicon carbide fibres and whiskers, and carbon nanotubes. *Lancet Oncol*. 2014; 15:1427–1428. [PubMed: 25499275]
32. Sargent L, Porter D, Staska L, et al. Promotion of lung adenocarcinoma following inhalation exposure to multi-walled carbon nanotubes. *Part Fibre Toxicol*. 2014; 11:3. [PubMed: 24405760]
33. Mercer RR, Scabilloni J, Wang L, et al. Alteration of deposition pattern and pulmonary response as a result of improved dispersion of aspirated single-walled carbon nanotubes in a mouse model. *Am J Physiol Lung Cell Mol Physiol*. 2008; 294:L87–97. [PubMed: 18024722]
34. Wong V, Gumbiner BM. A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. *J Cell Biol*. 1997; 136:399–409. [PubMed: 9015310]
35. Erickson E, Sivasankar M. Simulated reflux decreases vocal fold epithelial barrier resistance. *Laryngoscope*. 2010; 120:1569–75. [PubMed: 20564752]
36. Lawson WE, Polosukhin VV, Zoia O, et al. Characterization of Fibroblast-specific Protein 1 in Pulmonary Fibrosis. *Am J Respir Crit Care Med*. 2005; 171:899–907. [PubMed: 15618458]

Figure 1.

Particle size distribution of control and SWCNT media at 0 and 5 hours. The shift of peaks in the media over time suggest particle aggregation and agglomeration at 5 hours. The peak around 10nm in SWCNT media demonstrates that some particles were still dispersed at 5 hours.

Figure 2. Tissue viability following 100 ng/ml SWCNT exposure for 5 hours. The viability index was calculated by the ratio of the absorbance to the tissue weight (abs/mg). Boiled vocal folds were used as positive control.

Figure 3.
TEER data following 100 ng/ml SWCNT and control exposure for 5 hours.

Figure 4.
Permeability index following SWCNT exposure.

Figure 5.
Occludin expression (normalized by β -actin levels).

Figure 6. Fibroblast viability following SWCNT exposure. The viability was normalized to viability of control.

Figure 7. mRNA level of genes encoding FSP-1, α -SMA, and collagen 3. The relative expressions of the target genes were normalized by the values of the control group.