Acute Nanoparticle Exposure to Vocal Folds: A Laboratory Study

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Summary: Objectives. 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 as 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 was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Epithelial barrier integrity was assessed with transepithelial resistance and sodium fluorescein permeability. Epithelial tight junctional protein occludin expression was measured with 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 was 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*.

Key Words: Vocal folds–Epithelium–Carbon nanotubes–Fibroblasts–Fibrosis.

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 multiwalled CNTs 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] Although there are many studies reporting lung lesions following CNT exposure, very few studies have evaluated pathologic changes to the larynx. Rats exposed to 5 mg/m^3 multiwalled CNTs for 13 weeks within an inhalation exposure chamber presented significant epithelial lesions in the larynx.[8] 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] Although 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 components such as collagen and elastin. However, stress resulting from phonotrauma or radiation injury can alter vocal fold extracellular matrix composition by increasing transcription and secretion of collagen, leading to fibrosis.[14,15] The effects of CNT on vocal fold fibroblasts (VFF) have not been investigated, although there is literature on its effects on lung tissue. For example, mice exposed to 5 mg/m^3 SWCNT in a whole-body chamber for 1 year showed more than twofold increase in lung collagen.[16] The mechanism of CNT-induced fibrosis may involve both direct and indirect effects.[17] 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

Accepted for publication March 23, 2017.

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

Conflict of interest: None.

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Journal of Voice, Vol. ■■, No. ■■, pp. ■■-■■

0892-1997

http://dx.doi.org/10.1016/j.jvoice.2017.03.014

of fibrosis. The current study is the first step toward 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 prerequisite 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: <2 nm, length: 5–15 μm, purity: > >90% CNT~ >50% SWCNT, 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, Polyvinylidene Difluoride (PVDF) membrane, and 2x Laemmli 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, MO). Goat Anti-Rabbit IgG-HRP and Goat Anti-Mouse IgG-HRP were from Santa Cruz Biotechnology (Dallas, TX). Enhanced chemiluminescence reagent was obtained from Pierce Endogen (Rockford, IL). TRIzol reagent and Vybrant MTT cell proliferation assay kit were purchased from Thermo Fisher Scientific (Waltham, MA), and iTaq Universal SYBR Green Supermix kit was obtained from Bio-Rad. All other chemicals were obtained from Sigma Aldrich.

SWCNT, control, and positive control

SWCNTs were prepared using fetal bovine serum (FBS) at a concentration of 1 mg/mL, and sonicated (Fisher Scientific Sonic Dismembrator Model 500) with a duty cycle of 30% and amplitude of 30% for 30 seconds. The SWCNT-FBS media mixture was diluted to a final concentration of 100 ng/mL of SWCNT and 15% FBS (10% FBS for cell culture) using Hank's Balanced Salt Solution (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 with the control media (Figure 1). The control medium 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 midsagittal plane, and the epithelium, basal lamina, and superficial lamina propria (referred to as vocal fold epithelia hereafter) were separated from the connective tissue and muscle and then moistened with HBSS. Vocal fold epithelia were exposed to one of three conditions: SWCNT, control (no SWCNT), and 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% O_2 and 5% CO_2) SWCNT, or control (no SWCNT) media for 5 hours at 37°C. Boiled vocal fold epithelia served as positive control. Samples were incubated in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution in a 12-well culture plate while rotating at 100 rpm for 2 hours and then rinsed with PBS for 1 minute. Finally, formazan was extracted using 4-mL 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 In-

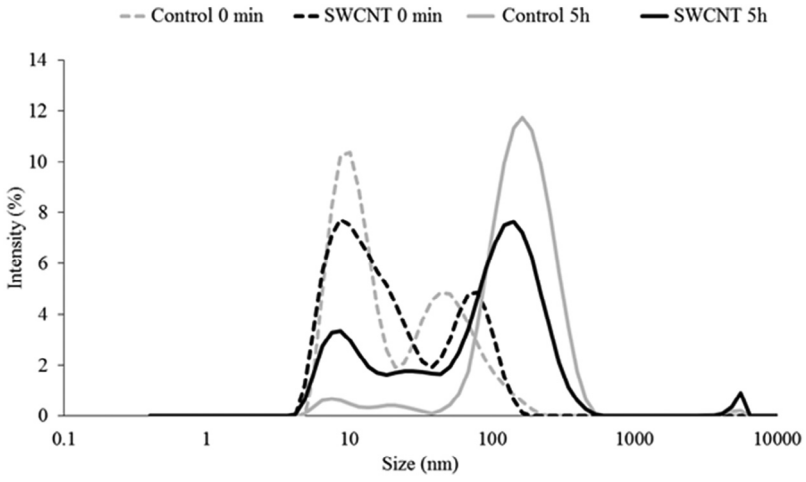

FIGURE 1. Particle size distribution of control and SWCNT media at 0 and 5 hours. The shift of peaks in the media over time suggests particle aggregation and agglomeration at 5 hours. The peak around 10 nm in SWCNT media demonstrates that some particles were still dispersed at 5 hours. SWCNT, single-walled carbon nanotubes.