AIRBORNE CHALLENGES TO VOCAL FOLDS: STUDIES ON BARRIER FUNCTION AND INFLAMMATION

by

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ABSTRACT

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The vocal folds are located in the larynx and are responsible for voice production. The epithelium of the vocal folds is of the stratified squamous type. These epithelial cells along with the characteristic tight junctional complex create an active barrier, that protects the underlying connective tissue against air-borne xenobiotics. Damage to the epithelium can lead to penetration of xenobiotics and may cause inflammation in connective tissue. Particles and chemicals have different physical properties and have different capacities for penetrating epithelium and inducing inflammation in underlying tissues. In this study, we investigated the effects of one type of particle (single-walled carbon nanotubes) and one type of volatile chemical (acrolein) on vocal fold epithelium and connective tissue. The particle and the volatile chemical we choose represent common challenges in either the occupational environment or in daily life. We hypothesized that single-walled carbon nanotubes damage vocal fold epithelial barrier and promote fibrosis in connective tissue and that acrolein exposure impairs vocal fold epithelium and induces inflammation. In summary, the project (i) investigated the effects of nanoparticles on vocal fold epithelium and fibroblasts (Chapter 2), (ii) identified the damage from acrolein exposure on vocal fold epithelium and demonstrated the mechanism for this effect as lipid peroxidation induced cellular membrane damage (Chapter 3), (iii) identified the pathophysiological changes associated with sub-acute
acrolein exposure in rat laryngeal epithelium and lamina propria (Chapter 4). Overall, the results suggest that acrolein exposure damages vocal fold epithelium and induces a proinflammatory reaction. The nanoparticles do not affect vocal fold epithelium but may have a fibrotic effect on vocal fold fibroblasts.
CHAPTER 1. INTRODUCTION AND OVERVIEW OF THE PROJECT

1.1 Structure and function of vocal folds

It has been estimated that there are approximately 18 million adults suffering from voice problems each year[1]. About 10% of them seek treatment from healthcare professionals[1]. Among the patients who receive treatment, only about 29% of them report that the voice status improves[1]. The limited efficacy of treatments may be due to incomplete understanding of the pathophysiological mechanisms underlying these voice problems.

The larynx is located at the junction of the respiratory tract and the digestive tract. The vocal folds are housed within in the larynx. The vocal folds are a pair of membranous tissues. Each vocal fold consists of an epithelium, lamina propria, and muscle. The outermost surface of the vocal folds is called the epithelium, which is formed by stratified squamous cells and junctional complexes (Figure 1.1). In healthy human vocal folds, the stratified squamous epithelium runs between 5 to 10 cellular layers[2]. The tight junction complex between adjacent epithelia seals the intercellular space of the epithelia and prevents particles from passing through the intercellular spaces. This tight junctional structure provides an effective barrier to protect the underlying connective tissue from chemical and physical insults.

The tissue under the epithelia is the lamina propria. The lamina propria has three layers, superficial layer, intermediate layer and deep layer. The superficial lamina propria contains loose fibrous and elastic tissue and is also referred to as “Reinke’s space”. The epithelium and superficial lamina propria together form the main vibrating portion of
vocal folds[3]. Their vibration of vocal folds is flow-induced and essential for voice production. The pathophysiological changes to the vibratory portion disrupt phonation. The vibration of vocal folds produces voice and the abduction and adduction of the vocal folds is also essential for respiration and healthy swallowing respectively[4]. Besides being a barrier, the vocal fold epithelium also secretes mucins, transports ions, and is associated with water fluxes; and it is through these functions, that the epithelium actively regulates luminal surface composition. Moreover, since the laryngeal mucosa is exposed to a variety of xenobiotics, such as antigens, smoke, and environmental pollutants, the larynx is also considered as an important organ for decision-making on the type of immune response in the airway[5, 6].
Vocal folds epithelium is formed by stratified squamous epithelium with multiple cellular layers[2]. Below the epithelium is the lamina propria, which consists of superficial layer, intermediate layer and deep layer. The superficial lamina propria contains loose fibrous and elastic tissue.

Figure 1.1 Structure of Rat Vocal Fold Epithelium, Lamina Propria and Muscle under a Light Microscope[2].
1.2 Structure, sources, and toxic effects of single-walled carbon nanotubes

Nanomaterials are substances at least one dimension in range of 1 to 100 nm. Because of the ultra-small size of each unit of the material, the surface-area-to-volume ratio of these particles is extremely large. This differentiates the physical and chemical properties of nanomaterials from larger size materials.

Carbon nanotubes (CNT) are a type of nanoparticle produced in myriad industrial applications. The shape of carbon nanotubes is akin to rolling sheets of graphite into cylinders to form tubes, with two ends of the tubes sealed by the graphite sheet (Figure 1.2). Single-walled carbon nanotubes are the tubes formed by one sheet of graphite with only one layer of wall of the tube. Multi-walled carbon nanotubes are tubes produced by multiple rolled sheets of graphite and with multiple layers of tube walls.
SWCNT looks like a long tube rolled by a sheet of graphite. The diameter of the tube is on the nanoscale. However, the lengths is on the scale of µm[7].

Figure 1.2 Structure of SWCNT. (Picture is sourced from A Graf, 2016[7])
Carbon nanotubes (CNTs) have several unique mechanical, thermal, and electrical properties. These properties have resulted in wide applications in industrial fields including coating and filming, microelectronics, energy storage, and biotechnology[8]. The production of CNTs has increased substantially since 2006 and it has been estimated that the annual production reached 4.5 kilotons by 2011[8]. About 375 workers were reported to be directly working with CNTs in 2008 in the United States, and the workforce size was estimated to increase by 15%–17% annually[9]. Extrapolating to industries using all kinds of nanomaterial, the number of workers exposed will reach 6 million worldwide by 2020[10]. Workers who handle nanomaterials are at risk of being exposed to those materials. Studies suggest that workers who handle the CNTs directly are potentially exposed to airborne particles though inhalation if no exposure controls are provided. Although data on exposure level of CNTs in the workplace are limited, some studies on airborne CNTs have indicated a high concentration of CNTs in the personal breathing zone in occupational settings[11-13].

CNTs have a unique fiber-like shape as mentioned above. This shape is like asbestos, though the size of CNTs is much smaller (at nanoscale) compared to asbestos. Epidemiological studies indicate that asbestos exposure is associated with lung fibrosis and cancers[14], chronic laryngitis, epithelial hyperplasia[15], and laryngeal cancers[16]. Moreover, there is a significant latency period between the negative health outcomes and the initial asbestos exposure. For instance, it is reported that the latency of mesothelioma extends more than 20 years after asbestos exposure[17]. This prolonged latency renders it difficult to recognize and prevent the toxicities in time. It is not surprising therefore, given the similarities in shapes for CNTs and asbestos, that CNTs are also found to
induce lung fibrosis[18] and cancer[19, 20] in animal studies. Though the toxic effects of CNTs lack epidemiological evidence, the long latency of a variety of severe diseases caused by the similar shaped particles (asbestos), along with the explosive development of this industrial field, and the increasing number of workers exposed to CNTs raises a significant public concern about the potential adverse effects on worker’s health secondary to CNT exposures in occupational settings. Thus, the study on CNTs toxicities bears a significant public health importance.

As a type of nanoparticle, CNTs are capable of inducing oxidative stress[21, 22]. CNTs are also found to cause fibrosis in studies using in vitro lung fibroblast cultures[23] and in vivo studies of the lungs[24]. The mechanism of CNT-induced fibrosis may involve both direct and indirect effects[25]. CNTs promote fibroblast conversion into myofibroblasts, and increase the production of collagen either by directly interacting with fibroblasts or indirectly regulating phenotype and function of fibroblasts through cytokines released by epithelia and macrophages.

Although there are many studies reporting lung lesions following CNT exposure, few studies have evaluated pathologic changes to the larynx. Rats exposed to 5 mg/m³ multiwalled CNTs for 13 weeks within an inhalation exposure chamber show significant epithelial lesions in the larynx[26]. However, the underlying mechanisms for epithelial pathologies and connective tissue changes has not yet been examined prior to the experiments described in Chapter 2.
1.3 Exposure to cigarette smoke and its components

Tobacco smoke contains more than 5000 chemicals[27]. There are two phases of components in cigarette smoke, i.e. the particle and the gas. Data presented in Figure 1.3 show several major toxicants in smoke which have high concentrations per cigarette, summarized from a study using a research cigarette.
Among thousands of components in cigarette smoke, nicotine and carbon monoxide are on the level of mg/cig, and acrolein and several other toxicants are on the level of hundred µg/cig[28].

Figure 1.3 Major Cigarette Toxicants with Concentration on Level of mg/cig (A) and Hundreds µg/cig (B). (Data in picture is made using the data sourced from O Geiss, 2007[28])

Among thousands of components in cigarette smoke, nicotine and carbon monoxide are on the level of mg/cig, and acrolein and several other toxicants are on the level of hundred µg/cig[28].
The toxicities of each individual component and the concentration of the component in cigarette smoke together influence the toxicity of cigarette smoke. To assess the toxic effects induced by each component, a parameter named risk index is used[29]. Risk index is calculated by the exposure level of certain components in the cigarette smoke in µg/person/day (assuming 1 cigarette per day) divided by reference exposure level of that component. The reference exposure levels (REL) are the benchmark concentrations or reference doses calculated in risk assessment studies. REL represents a threshold, under which the exposure will not cause damage to humans. The risk index can be divided into a cancer risk index and non-cancer index based on the type of damage. The risk index can also be detailed specifically to the organs affected by the toxicity. For instance, acrolein is considered the most significant contributor to non-cancer respiratory effects, and hydrogen cyanide is the most toxic component for non-cancer cardiovascular effects[29] (Figure 1.4).
Non-cancer risk indices (NCRI) of major toxic components in cigarette smoke show distinct toxic effects on either respiratory system or cardiovascular system. Acrolein has the highest NCRI for respiratory effects[29].

<table>
<thead>
<tr>
<th>Non-cancer Effects</th>
<th>Constituent</th>
<th>Non-cancer Risk Index (NCRI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory effects</td>
<td>Acrolein</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>Acetaldehyde</td>
<td>3.78</td>
</tr>
<tr>
<td></td>
<td>Formaldehyde</td>
<td>0.83</td>
</tr>
<tr>
<td>Cardiovascular effects</td>
<td>Cadmium</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Hydrogen Cyanide</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>Arsenic</td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td>O-Cresol</td>
<td>0.071</td>
</tr>
<tr>
<td></td>
<td>Carbon Monoxide</td>
<td>0.068</td>
</tr>
</tbody>
</table>

Acrolein is the most significant contributor to non-cancer respiratory effects from cigarette smoke.

Figure 1.4 Non-cancer Risk Indices of Major Toxicants in Cigarette Smoke on Respiratory System and Cardiovascular System. (Data in picture is sourced from J Fowles, 2003[29])
1.4 Exposure to acrolein and its toxic effects

Acrolein is a highly reactive, unsaturated aldehyde. The boiling point is 53 °C. At room temperature, it is liquid. However, acrolein is highly volatile. It can readily change into a vapor. It is soluble in variety of solvents including water, alcohol, benzene, etc.

Acrolein exists ubiquitously in the environment. It is found in high concentration not only in cigarette smoke, but also in industrial waste from combustion, mobile exhaust, forest fires, etc. It is reported to be at a concentration between 0.5 to 3.186 ppbv in the ambient air in the U.S.[30]. The concentration of acrolein generated by pyrolysis of polyethylene foam reaches 76-180 ppm[30]. Moreover, mainstream cigarette smoke can have acrolein levels as high as 50-70 ppm[31]. For its high reactivity, acrolein has an exposure limit of 0.1 ppm over an 8-hour time-weighted average (TWA), as is recommended by both National Institute for Occupational Safety and Health (NIOSH) and Occupational Safety and Health Administration (OSHA)[32]. Besides inhalation, people are also exposed to acrolein through their diet, specifically with overheated vegetables and animal fats. Notably, acrolein can also be generated endogenously in certain pathophysiological processes, such as lipid peroxidation and metabolism of drug (cyclophosphamide), etc.

Studies in literature have been conducted to explore the mechanisms of acrolein toxicity. It is known that the toxic effects of acrolein are diverse. Generally, acrolein is strongly electrophilic, thus it interacts with nucleophiles. Therefore, it induces oxidative stress[33]. Acrolein binds to -lysine, -histidine, and -cysteine residues in protein and forms acrolein-protein adducts[34]. Besides proteins, acrolein also reacts with
nucleobases to create the acrolein-DNA adduction[35]. Acrolein is thought to be both an initiator and product of lipid peroxidation[36, 37]. It also induces mitochondrial dysfunction and participates in inflammation[33, 38]. Acrolein causes cell death through both apoptosis and necrosis[39].

1.5  **Hypothesis**

The overarching purpose of the experiments described below is to investigate the pathophysiological effects of environmental challenges on vocal folds. The challenges represent common airborne challenges that can be inhaled in environment and occupational settings. The challenges include exposure to agents in the form of particles (SWCNTs) or volatile chemicals (acrolein).

Specifically, we hypothesized that acute exposure to single-walled carbon nanotubes impairs vocal fold epithelial barrier and induces a fibrotic reaction in fibroblasts. We further hypothesized that acrolein exposure damages the vocal fold epithelial barrier and induces vocal fold inflammation, by causing cell membrane leakage and increasing cytokine expression through oxidative stress and NF-κB activation.

1.6  **Specific Aims**

**Specific Aim 1:** To determine whether acute exposure to single-walled carbon nanotubes damages the vocal fold epithelial barrier and to investigate the effects of single-walled carbon nanotubes on vocal fold fibroblasts in the lamina propria. Experiments were performed to
1) Evaluate the metabolic activity of vocal fold epithelium and fibroblasts following acute SWCNTs exposure;

2) Assess changes in vocal fold epithelial barrier function following acute SWCNT exposure;

3) Detect the levels of tight junction proteins in vocal fold epithelium following exposure to SWCNT; and

4) Investigate the expression of genes encoding fibrotic proteins after SWCNT exposure

**Specific Aim 2:** To determine whether acute acrolein exposure damages the vocal fold epithelial barrier and to investigate the mechanism of this damage to barrier integrity in vitro. Experiments were performed to

1) Detect the dose relationship between acute acrolein exposure and vocal fold epithelial metabolic activity;

2) Evaluate epithelial barrier function following acute acrolein exposure;

3) Assess expression of tight junction proteins; and

4) Detect the epithelial cellular membrane integrity and its related mechanism

**Specific Aim 3:** To investigate whether subacute acrolein exposure induces vocal fold inflammation and to evaluate the role of NF-κB in vocal fold inflammation in rat, using a whole-body exposure system. Experiments were performed to

1) Identify the pathological changes following sub-acute acrolein exposure;
2) Evaluate the expression of genes encoding proteins participating in an immune response;

3) Investigate the activation of NF-κB pathway; and

4) Investigate T lymphocyte infiltration following the acrolein exposure.

1.7 Structure of dissertation

Located at critical real estate offered by the aerodigestive tract, the vocal folds can be exposed to a variety of challenges in the air through inhalation. Xenobiotics in the air include both particles and volatile chemicals. The different physical properties of xenobiotics make influence the penetration and toxic effects differentially. In this project, we choose two typical challenges: the single-walled carbon nanotubes as a particulate insult and acrolein as a gaseous insult, to investigate how xenobiotics interact with the structure and function of the vocal folds, leading to impaired voice production in the long-term. Chapter 1 introduces background information on the structure and function of the vocal folds; the structure, properties and sources of single-walled carbon nanotubes; the components and toxicities of cigarette smoke constituents; and the toxic effects of acrolein. Chapter 2 presents results from an investigation of the effects of SWCNTs on vocal folds. Chapter 3 reveals the influence of acrolein on vocal fold epithelial barrier function. Chapter 4 detects the pathophysiological changes, especially inflammation in rat vocal folds. Chapter 5 summarize the findings of those studies in this dissertation and discusses future studies that should be conducted.
CHAPTER 2. EFFECTS OF SINGLE-WALLED CARBON NANOTUBES ON VOCAL FOLDS, EX VIVO & IN VITRO


2.1 Abstract

Airway exposure to nanoparticles is common in occupational settings. Inhaled nanoparticles have toxic effects on respiratory tissues. Vocal folds are at direct risk from inhaled nanoparticles. This study was designed to investigate the effect of single-walled carbon nanotubes (SWCNT) on vocal fold epithelium and fibroblasts. These two cell types were selected for this study as the epithelia are the outer layer of the vocal folds and fibroblasts are the most common cell type in connective tissue underlying the epithelium. 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 determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The epithelial barrier integrity was assessed with transepithelial resistance (TEER) and sodium fluorescein (NaFl) permeability. The expression of epithelial tight junctional protein occludin was measured using Western blot. Gene expressions of the fibroblast-specific protein 1 (FSP-1), α-smooth muscle actin (α-SMA), and collagen III (Col-III) were quantified using quantitative polymerase chain reaction (qPCR). We found the transcriptional expression of genes encoding FSP-1 and Col-III increased significantly following SWCNT exposure. There were no significant
differences between control and SWCNT groups on any of the other measures. Taken together our data suggest that SWCNT exposure induces vocal fold fibroblasts to a fibrotic phenotype, but it does not affect the tight junctional protein in epithelia. These data help us understand vocal fold defense mechanisms and lay the groundwork for studying the physiological effects of nanoparticle exposure in vivo.

2.2 Introduction

Carbon nanotubes (CNTs) are nanoparticles produced in myriad industrial applications[40]. Individuals are at risk for inhaling CNTs from the ambient environment[11-13]. Therefore, the adverse effects of CNTs on the respiratory system have been investigated. Exposure to multiwalled CNTs reduces ciliated cells in rodent trachea[41]. 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[42]. Pharyngeal aspiration of SWCNTs can cause granulomas and interstitial fibrosis[43]. 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/m3 multiwalled CNTs for 13 weeks within an inhalation exposure chamber presented significant epithelial lesions in the larynx[26]. 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[44-47]. 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[48]. 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 phono trauma or radiation injury can alter vocal fold extracellular matrix composition by increasing transcription and secretion of collagen, leading to fibrosis[49, 50]. 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[51]. The mechanism of CNT-induced fibrosis may involve both direct and indirect effects[25]. 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[52]. 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 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 lay the foundation for studying the impact of CNTs on health and voice production for future risk assessment and control.

2.3 Materials and Method

2.3.1 Materials

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 was 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.

2.3.2 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[48]. 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 2.1). 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.
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.
2.3.3 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.

2.3.4 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 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).
2.3.5 Epithelial resistance

The transepithelial electric resistance (TEER) was determined using an Ussing chamber system (Model 15362, World Precision Instruments, 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 (approximately 1 hour). Only epithelia with a TEER value exceeding 300 $\Omega \cdot \text{cm}^2$ were included in subsequent experiments[53]. The TEER values were measured by DataTrax software (World Precision Instruments, Sarasota, FL, USA) immediately prior to and following 5 hours of exposure to SWCNT or control (no SWCNT).

2.3.6 Epithelial permeability

Permeability experiments were conducted in the Ussing system in 14 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 studies. The apical surface of vocal fold epithelium was exposed to 100 ng/mL SWCNTs along with 1 mg/mL NaFl in HBSS-FBS or 1 mg/mL NaFl with HBSS-FBS (control). An aliquot (200 $\mu$L) 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 $\mu$L) 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) 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.

2.3.7 Occludin expression

Total proteins from six vocal fold epithelia in SWCNT and control groups were extracted in a homogenization buffer (5 mM EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid), 20 mM Tris [pH 7.5], 0.1% SDS, 1% Triton X-100, Protease Inhibitor Cocktail [Calbiochem], 10 µL/mL PMSF (phenylmethylsulfonyl fluoride), and 15 mM 2-mercaptoethanol). Protein concentrations were determined by BSA Protein Assay (Thermo Scientific). Samples were boiled with 2x Laemmli sample buffer (Bio-Rad). Samples were loaded on an 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 a 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. Immunoblots of occludin were visualized using enhanced chemiluminescence reagent-Western Blotting Substrate and the Bio-Rad Molecular Imager. The membrane was washed with TBST three times, tripping buffer for 15 minutes, and again with TBST three 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 immunoblots 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 the *ImageJ* software (NIH, Bethesda, MD), and occludin values were normalized to β-actin.

### 2.3.8 VFF cell culture

Immortalized human VFFs were obtained from Dr. Susan Thibeault at the University of Wisconsin-Madison[54]. VFFs were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Sigma Aldrich) supplemented with 10% FBS (Corning, New York, NY, USA), 1% penicillin/streptomycin, 1% minimal essential medium nonessential amino acid solution, and 200 µg/mL Geneticin (G418; Teknova, Hollister, CA, USA). 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 µg/mL, and sonicated with a duty cycle of 30% and amplitude of 30% for 30 seconds. SWCNTs were autoclaved and added to the VFF media at a final concentration of 100 ng/mL and incubated for 24 hours. The cells in the control group were incubated within the same medium without any SWCNT.

### 2.3.9 Fibrosis gene expression at the mRNA level

The VFF cells were incubated with or without 100 ng/mL SWCNTs for 24 hours. The cells were harvested by washing three 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 Col-III were quantified using quantitative real-time polymerase chain reaction (PCR). The real-time PCR was conducted in the CFX Connect Real-Time PCR Detection System (Bio-Rad) with iTaq Universal SYBR Green Supermix kit (Bio-Rad). The qPCR was performed using a protocol with 3-min initial denaturation at 95°C, 40 cycles of denaturation at 95°C, then 10-second gradient 55.0°C–65.0°C, and at last 30-second extension at 72°C. The qPCR samples were loaded in duplicate. The amplification efficiencies of target and reference genes were examined on the same load plate. The relative mRNA expressions of the target genes between SWCNT treated group and control group were assessed using ΔΔCt, 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 (Glyceraldehyde 3-phosphate dehydrogenase) in the same sample.

The forward and reverse primers for genes encoding FSP-1 (S100A4), α-SMA (ACTA2), Col-III (COL3A1) and GAPDH were designed using Primer Express 3.0 software (Applied Biosystems, Carlsbad, CA, USA). The primer sequence for human FSP-1 (S100A4) used in this study was (F) 5'-CTTCC CCTCT CTACA ACCCT CT-3', (R) 5'-GAGCT TGAAC TTGTC ACCCT CT-3'; for α-SMA (ACTA2) forwarded primer was 5'-CCTCC CTTGA GAAGA GTTAC GA-3', reverse primer was 5'-TGATG CTGTT GTAGG TGGTT TC-3'; for Col-III (COL3A1) forwarded 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 GGAAGAG-3, (R) 5'-ACTGG TTGAG CACAG GGTAC TT-3.'
2.3.10 VFF cell viability assay

To assess cell proliferation, the VFF cells were seeded at a concentration of 35,000 cells/cm² on a 96-well plate and left to attach overnight. VFFs were treated with or without 100 ng/mL SWCNTs for 24 hours. 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 540 nm.

2.3.11 Statistical analysis

Statistical analyses were completed in SPSS (version 20, IBM, Chicago, IL). Data that did not meet assumptions of normality were analyzed using nonparametric statistics. Group differences were assessed with median test (tissue viability), Wilcoxon signed-rank test (occludin expression), unpaired \( t \) tests (permeability, fibroblast viability, FSP-1, \( \alpha \)-SMA, and Col-III expression), and mixed analysis of variance (TEER). The differences between the means were considered significant if \( P \) values were equal to or less than 0.05.

2.4 Results

2.4.1 Epithelial viability

The epithelial viability remained largely unchanged after the tissues were exposed to 100 ng/mL SWCNTs in the incubation medium for 5 hours at 37°C (Figure 2.2), although a significant challenge effect among groups was detected (\( \chi^2 = 6.3, P = 0.043 \)). The Mann-Whitney posthoc analyses revealed a significant decrease in epithelial
viability between the positive control (boiled samples) and control, but no significant
difference was seen in the SWCNT group as compared with the control (N = 3, Z =
−1.528, \( P = 0.127 \), Figure 2.2).
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. SWCNT, single-walled carbon nanotubes. Data represent mean ± SD, N = 3. *: $p < 0.05$ as compared with controls.

Figure 2.2 Tissue Viability following 100 ng/mL SWCNT Exposure for 5 Hours.
2.4.2 Epithelial resistance

Epithelial resistance was maintained in vocal folds exposed to SWCNTs (N = 6, $F = 3.74$, $P = 0.08$, Figure 2.3). There were no significant differences between groups exposed to SWCNT and the control (N = 6, $F = 0.19$, $P = 0.672$, Figure 2.3), indicating that the integrity of the epithelial layer remained unchanged following SWCNTs exposure.
Figure 2.3 TEER Data following 100 ng/mL SWCNT and Control Exposure for 5 Hours.

SWCNT, single-walled carbon nanotubes; TEER, transepithelial electric resistance. Data represent mean ± SD, N = 6.
2.4.3 Epithelial permeability

There were no significant differences in permeability of SWCNT and control groups (N = 7, \( t = 1.462, P = 0.169 \), Figure 2.4), suggesting that SWCNTs exposure did not directly affect the epithelial barrier function.
No differences in permeability of epithelial tissue were observed in SWCNT (single-walled carbon nanotubes) and control groups. Data represent Mean ± SD, N = 7.
2.4.4 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, \( P = 0.285 \), Figure 2.5).
No significant differences were observed for occludin expression in SWCNT (single-walled carbon nanotubes) and control groups. Data represent Mean ± SD, N = 3.

Figure 2.5. Occludin Expression (Normalized by β-actin Levels).
2.4.5 VFF cell studies

When the immortalized human VFFs were incubated with or without 100 ng/mL SWCNTs for 24 hours, 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, Figure 2.6). Interestingly, among the three genes related with fibrosis, two of them exhibited significant increases in their expression following SWCNT exposure, that is, FSP-1 about 1.6-fold increase (N = 6, t = −3.573, P = 0.005, Figure 2.7) and Col-III about 1.5-fold increase (N = 6, t = −2.794, P = 0.019, Figure 2.7), whereas the mRNA level also showed a trend of increase, but not statistically significant, in the α-SMA expression (N = 6, t = −2.022 P = 0.071, Figure 2.7).
The viability was normalized to viability of control. SWCNT, single-walled carbon nanotubes. Data represent Mean ± SD, N = 6.

Figure 2.6 Fibroblast Viability following SWCNT Exposure.
The relative expressions of the target genes were normalized by the values of the control group. α-SMA, α-smooth muscle actin (ACTA2); Col-III, collagen III (COL3A1); FSP-1, fibroblast-specific protein 1 (S100A4). Data represent Mean ± SD, N = 6. *: p < 0.05 as compared with controls.

Figure 2.7. mRNA Level of Genes Encoding FSP-1, α-SMA, and Col-III.
2.5 Discussion

The unique properties of CNTs including the high elastic modulus and thermal and electrical conductivity have led to their widespread use in modern industry[55]. It has been estimated that several thousand tons of CNTs are produced annually around the world[8] in products including cables, wires, textiles, sensors, drug delivery systems, medical implants, electronics, coating, etc[8, 56]. About 375 workers were reported to be directly working with CNTs in 2008 in the United States, and the workforce size was estimated to increase by 15%–17% annually[9]. As an estimation of the whole nanotechnological field, there will be around 6 million workers worldwide, with 2 million of them in the United States alone by 2020[10]. Workers who handle the CNTs directly are potentially exposed to the airborne particles if no exposure controls are provided. Although data on exposure level of CNTs in the workplace are limited, some studies on airborne CNTs have indicated high concentration of CNTs in the personal breathing zone in occupational settings[11-13]. The National Institute for Occupational Safety and Health recommends a 1 µg/m³ exposure limit by considering both the health risks and limit of quantitation method[57]. CNTs are highly suspected to have toxic properties similar to asbestos due to similarities in their fiber-like shape[58]. Asbestos exposure is associated with lung fibrosis and cancers[14], chronic laryngitis, epithelial hyperplasia[15], and laryngeal cancers[16]. CNT exposures in animal studies reveal lung fibrosis[43] and cancer[19, 20], 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 interstitium[59] and reduce epithelial resistance in a tracheobronchial epithelial cell line model[48]. 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 the 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[48], 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 nonsignificant 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[60].

The current study exposed vocal fold epithelia to SWCNT concentrations reported in the literature. Therefore, the nonsignificant effects of SWCNT on vocal fold epithelial barrier function deserve further discussion. One potential reason for this nonsignificant 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\cite{44, 61}. 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 VFFs. We were interested in these effects since fibroblasts secrete ECM constituents which dictate the biomechanics of vocal fold tissue. Viable VFFs 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\cite{62}. 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, indicates a profibrotic response to continued SWCNT exposure.

### 2.6 Conclusion

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. VFFs 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.
2.7 Acknowledgments

We acknowledge the assistance of Elizabeth DiRenzo, PhD, in sharing the MTT protocol, and Abigail Durkes, DVM, for assistance in obtaining porcine larynges. James McMasters and Alyssa Panitch, PhD, assisted with the Zetasizer, and Sherleen Adamson, PhD, assisted with the Western blot procedure. Porcine larynges were obtained from Beutler’s Meat Processing, Lafayette, IN, and Monon Meat Packing, Monon, IN.
CHAPTER 3. ACROLEIN EXPOSURE IMPAIRS BARRIER FUNCTION OF VOCAL FOLD EPITHELIUM, EX VIVO


3.1 Abstract

Acrolein is a ubiquitous pollutant abundant in cigarette smoke, mobile exhaust, and industrial waste. There is limited literature on the effects of acrolein on vocal fold tissue, although there are clinical reports of voice changes after pollutant exposures. Vocal folds are responsible for voice production. The overall objective of this study was to investigate the effects of acrolein exposure on viable, excised vocal fold epithelial tissue and to characterize the mechanism underlying acrolein toxicity. Vocal fold epithelia were studied because they form the outermost layer of the vocal folds and are a primary recipient of inhaled pollutants. Porcine vocal fold epithelia were exposed to 0, 50, 100, 500, 900 or 1300 μM of acrolein for 3 hours; the metabolic activity, epithelial resistance, epithelial permeability, tight junction protein (occludin and claudin 3) expression, cell membrane integrity and lipid peroxidation were investigated. The data demonstrated that acrolein exposure at 500 μM significantly reduced vocal fold epithelial metabolic activity by 27.2% (p<0.001). Incubation with 100 μM acrolein caused a marked increase in epithelial permeability by 130.5% (p<0.05) and a reduction in transepithelial electrical resistance (TEER) by 180.0% (p<0.001). While the expression of tight junctional protein did not change in acrolein-treated samples, the cell membrane
integrity was significantly damaged with a 45.6\% increase of lipid peroxidation as compared to controls (p<0.05). Taken together, these data provide evidence that acute acrolein exposure impairs vocal fold epithelial barrier integrity. Lipid peroxidation-induced cell membrane damage may play an important role in reducing the barrier function of the epithelium.

3.2 Introduction

The vocal folds are paired, multi-layered, membranous tissues within the larynx. Vocal fold vibration is flow-induced and occurs between 100–300 times per second in conversational speech. Intact vocal fold abduction and adduction are also essential for respiration and healthy swallowing[4]. The outermost surface of vocal folds consists of 5 to 10 cell layers of squamous epithelial cells with tight junctions[2]. The epithelium forms a physical barrier to prevent inhaled xenobiotic penetration and protect underlying connective tissue and muscle. This stratified structure is unique compared to epithelia in other parts of respiratory system. Besides being a barrier, the vocal fold epithelium also secretes mucins, transports ions, and is associated with water fluxes to actively control surface composition[47, 63, 64].

The effects of exogenous insults, such as simulated gastric reflux, on the barrier function of vocal fold epithelium has been reported in the literature[65-67]. These noxious insults can compromise the epithelial barrier as measured by decreased epithelial resistance[67-69]. Tobacco smoke, for example, is an abundantly studied pollutant; 3-month exposure in rabbits causes hyperplasia with disturbed stratification on vocal fold
epithelium[70]. A reduction in desmosomes and enlargement of intercellular space has been observed in rats following 60-day tobacco exposure[45].

Acrolein, an unsaturated aldehyde with a high electrophilicity, is one of the major toxicants present in cigarettes (about 10–500 μg/cigarette)[71]. It is also formed by the combustion of fossil fuels, woods, plastics, and heating of animal fat[30, 31, 72, 73]. At room temperature, acrolein is present as a liquid, but is highly volatile. It also exists in the environment as a gas; this gas can contact the airway epithelium when inhaled. The current literature suggests that the mechanisms by which acrolein causes toxicity pertain to interaction with nucleophiles in a variety of local cellular structures[33-35, 38, 71], induction of oxidative stress[34, 74] with ensuing lipid peroxidation, and covalent binding with proteins to form adducts. Studies also show that acrolein acts as a mutagen, leading to damaged DNA and inhibited DNA repair in lung cells[71]. Moreover, it interferes with the immune response in the respiratory tract[75-80]. Whether and how acrolein directly affects the apical vocal fold epithelia, the first line of defense to foreign insults, is not known.

Voice problems including hoarseness and lowered fundamental frequency are commonly seen among smokers[81]. Smoking is reportedly capable of increasing the permeability and damaging the cell membrane in type I pneumocyte in guinea pig[82]. The application of cigarette smoke condensate to ex vivo porcine tissue did not alter epithelial barrier function[83], but these negative findings could be attributed to the acute exposure duration and dosage selected for study. Another reason for the non-effect could be that the cigarette smoke condensate contains only smoke particulates. The effects of components such as acrolein, which are mainly contained in the gaseous phase were not
examined. Acrolein is almost entirely found in the gaseous phase of mainstream smoke\cite{84, 85} and may play a role in vocal fold damage. Sub-chronic exposure of rats to acrolein for 13 weeks induces inflammation and hyperplasia in the respiratory tract including the larynx\cite{77}. Another acute study on vocal fold epithelium shows a reduction of sodium ion transport after 1-hour exposure with acrolein\cite{47}. Nonetheless, the mechanism whereby acrolein affects the vocal fold epithelial barrier remains elusive.

This study was designed to investigate the effects of acrolein on vocal fold epithelial barrier integrity. We hypothesized that acute exposure to acrolein may result in a dose dependent reduction in vocal fold epithelial viability. We also hypothesized that acrolein exposure may impair epithelial barrier integrity as indicated by a decreased epithelial resistance and an increased epithelial permeability. We further hypothesized that the impairment of barrier integrity may be caused by altered tight junction protein expression and/or cell membrane damage. The findings from this work will provide the groundwork to understand the effects of acrolein on vocal fold epithelia pathophysiology and its health impact on the human voice.

3.3 Materials and Methods

This study is exempt from the Institutional Animal Care and Use Committee (IACUC) of Purdue University, because the tissues were obtained from the slaughterhouse after sacrifice of pigs. Details are provided below.
3.3.1 Materials

Protease Inhibitor Cocktail was purchased from Calbiochem (San Diego, CA); bovine serum albumin (BSA) standards and Pierce LDH cytotoxicity assay kit from Thermo Scientific (Rockford, IL); Tris, sodium dodecyl sulfate (SDS), cDNA synthesis kit, iTaq Universal SYBR Green Supermix, 2xLaemmlie sample buffer and PVDF membrane from Bio-Rad (Hercules, CA). Primary rabbit anti-occludin antibody (ab31721) and primary mouse anti-4 HNE antibody (ab48506) were purchased from Abcam (Cambridge, MA); anti-rabbit IgG-HRP (goat) and anti-mouse IgG-HRP (goat) from Santa Cruz Biotechnology (sc-2004, sc-2005, Dallas, TX); enhanced chemiluminescence reagent (ECL) from Pierce Endogen (Rockford, IL); Alexa Fluor 488 goat anti-rabbit IgG (H+L) antibody (A-11034) from Thermo Scientific (Rockford, IL); and cyanine Cy™3 Goat Anti-Mouse IgG (H+L) (115-165-166) from Jackson ImmunoResearch Inc (West Grove, PA). Primers for qPCR analysis were obtained from Integrated DNA Technologies (Coralville, Iowa). Acrolein (99%), primary mouse anti-β-actin antibody and other chemicals were purchased from Sigma Aldrich (St Louis, Missouri). All reagents were of analytical grade, HPLC grade, or the best available pharmaceutical grade.

3.3.2 Vocal fold preparation

Seventy-two fresh male and female porcine larynges were obtained from two local, Indiana state-inspected and approved abattoirs and transported in cold phosphate buffered saline (PBS) to the lab. The larynges were dissected following protocols utilized in previous published studies[47, 65]. In brief, the larynges were bisected along the
midsagittal plane. The epithelium, basal lamina, and superficial lamina propria (referred as vocal fold epithelia below) were dissected from the larynges, and challenged with control (Hanks Balanced Salt Solution; HBSS), or acrolein in HBSS. The duration of challenge was 3 hours for all experiments. This duration was selected to represent an acute exposure model.

3.3.3 Epithelial metabolic activity assay

Vocal fold epithelial metabolic activity was assessed using a modified MTT assay following a published protocol[86]. Epithelia samples were dissected from 7 larynges (fourteen vocal folds), punched (4 mm in diameter), and weighed. Samples were incubated in oxygenated (95% O₂ and 5% CO₂) medium for 3 hr at 37°C in 7 groups: positive control (tissue boiled), control (HBSS), 50 μM acrolein, 100 μM acrolein, 500 μM acrolein, 900 μM acrolein and 1300 μM acrolein. Boiled tissues were used as positive controls since they are non-viable. Samples were then incubated in the MTT solution with 100 rpm rotation for 2 hr and rinsed with PBS. Finally, an aliquot (4 mL) of DMSO was added to each well to extract formazan while the tissues were completely minced. The absorbance of formazan was detected using an ELISA scanner (SpectraMax M2e, Molecular Devices, Sunnyvale, CA) at 570 nm. The viability index was calculated as the ratio of absorbance to the tissue weight (abs/mg). The viability index was normalized by the mean of that in control group to the percentage. Since the MTT revealed reduction of metabolic activity at acrolein concentrations ≥ 500 μM, we chose 100 μM, which
did not reduce the metabolic activity of the epithelial tissue, as the concentration of acrolein in the treated group in the ensuing experiments. This enabled us to avoid any interference from reduced metabolic activity in the experiments below.

3.3.4 Determination of transepithelial electrical resistance (TEER)

An Ussing chamber system (model 15362, World Precision Instruments, WPI, Sarasota, FL) was used for assessment of TEER values. Fourteen vocal fold epithelia were mounted on Lucite chambers that were filled with oxygenated HBSS, warmed to 37°C. Tissues were maintained in their entirety as a single specimen as they were dissected, approximately 1.5 cm long, 1 cm wide and 1 mm thick. Epithelia with TEER values \( < 300 \, \Omega \cdot \text{cm}^2 \) were considered viable and were used for this study[53]. This threshold for vocal fold epithelial viability is based on published literature[47, 65, 67].

The apical side of the epithelia was exposed to either 100 \( \mu \text{M} \) acrolein or control (without acrolein). The TEER values were determined using a voltage clamp (model DVC-1000, WPI, Sarasota FL) and DataTrax (WPI, Sarasota, FL). These techniques are routinely used in studies of airway epithelial physiology[87, 88]. An instant 2mV potential from voltage clamping equipment was presented to the tissue every two minutes, and the instant change of current was recorded by the software. The changed current was normalized by the area of the chamber where the tissue contacts with the solution (1.13 cm\(^2\)) and the resistance (TEER value) was calculated using the format \( R = \frac{V}{I} \), where \( R \) stands for resistance, \( V \) represents potential and \( I \) means the normalized current. The change of TEER value were calculated using the TEER value prior to and following 3 hours after acrolein exposure. The change of the TEER value in the two groups were
compared and percentage of change between the two groups were calculated. Seven vocal fold samples per group met the criteria for viability for a total of 14 vocal fold samples for this methodology.

3.3.5 Assessment of epithelial permeability

Epithelial permeability was measured in epithelia mounted on Ussing chambers (above). Fourteen epithelial tissues were exposed to 100 μM acrolein or control without acrolein. The experiments were conducted at the same time as the TEER experiments described above. Immediately following the treatment, an aliquot of 1 mg/mL sodium fluorescein (NaFl), a permeability marker, was added to the apical chamber. Samples (200 μL) of the medium on the basolateral side of the tissues were collected prior to and following 3 hours of acrolein/control exposure.

Fluorescence intensities were detected in duplicate using an ELISA scanner (SpectraMax M2e, Molecular Devices, Sunnyvale, CA) with the wavelength setting at 480 nm for excitation and 525 nm for emission. The permeability index represents the percentage of the fluorescein marker passing through the epithelial tissue. The permeability index was calculated by the change of the fluorescein intensity on the basolateral side divided by the fluorescein intensity on the apical side right after the sodium fluorescein was added and mixed.

3.3.6 Determination of levels of mRNAs encoding tight junctional proteins

The levels of mRNA encoding claudin3 and occludin were quantified using quantitative realtime polymerase chain reaction. Twelve vocal fold epithelial samples
were incubated with or without 100 μM acrolein. The total RNA was isolated from the epithelia and purified using NucleoSpin RNA isolation & purification kit following the manufacturer’s direction. RNA was then reverse transcribed into cDNA using a Bio-Rad iScript cDNA synthesis kit. The real-time PCR was conducted in the CFX Connect Real-Time PCR Detection system (Bio-Rad, CA). The qPCR was performed using a protocol with an initial 3-min denaturation at 95°C; then 40 cycles of 30-sec denaturation at 95°C, 10-sec gradient 55.0°C–65.0°C and 30-sec extension at 72°C. Each of the qPCR samples were run in duplicate using the iTaq Universal SYBR Green Supermix (Bio-Rad) kit. The amplification efficiencies of target and reference genes were examined by the amplification of a series of dilutions of control templates. ΔΔCt, the threshold cycle time value, was used for the evaluation of relative mRNA expression of the target genes between control and acrolein treated groups. The target genes Ct values were normalized with that of β-actin (ACTB) in the same sample. The relative expressions of target genes expression in acrolein compared to control were calculated by setting the control as 100%.

The forward and reverse primers for Claudin3 (CLDN3) and Occludin (OCLN) genes were designed using Primer Express 3.0 software. The primer sequences for porcine claudin3 (CLDN3) used in this study were: forward primer 5'-CCTAC GACCG CAAGG ACTAC -3 and reverse primer 5'-CATCT GGGTG GACTG GTCTC-3 (GenBank Accession No. NM_001160075). The primer sequences for occludin (OCLN) were: forward primer 5'-GGGGC TATAC AGATC CACGA -3 and reverse primer 5'-ATCAC CAATG CAGCA ATGAA -3 (GenBank Accession No. NM_001163647). The primer sequences for β-actin (ACTB) were: forward primer 5'- TGCGG CATCC ACGAA
ACTAC -3 and reverse primer 5-AGGGCCGTGATCTCCTTCTG-3 (GenBank Accession No. XM_003124280).

3.3.7 Western blot analysis of tight junctional proteins

Occludin protein expression was analyzed by Western blot. Total protein was extracted in a homogenization buffer (20mM Tris (pH 7.5), 5mM EGTA, 0.1% SDS, 10 μL/mL PMSF, 1% Triton X-100, protease inhibitor cocktail (Calbiochem, CA), and 15mM 2-mercaptoethanol) from 12 vocal fold epithelial samples challenged with 100 μM acrolein or control for 3 hours. A BSA assay kit was used to quantify the protein concentration. Isolated samples were boiled in the 2xLaemmli sample buffer (Bio-Rad) for 5 min. Samples were loaded onto the SDS-polyacrylamide gel and electrophoresed. The proteins were then transferred onto a PVDF membrane from the gel. The PVDF membrane was blocked in the 5% milk for 1 hr and incubated in the primary anti-occludin (1:1000) antibody at 4°C overnight. The membrane was then rinsed with mixture of Tris-Buffered Saline and Tween 20 (TBST) and incubated with the HRP linked secondary antibody (Goat anti-rabbit IgG-HRP, 1:3000) for 1 hr. ECL-Western Blotting Substrate was used for membrane immuno-blots, which was visualized and imaged using the Bio-Rad Molecular Imager. After imaging, the membrane was washed with tripping buffer for 15 min, and rinsed with TBST three times. Next, the membrane was incubated with primary anti- β-actin (1:10000) antibody overnight at 4°C, followed by incubation with the secondary antibody (Goat anti-mouse IgG-HRP, 1:5000) for 1 hr. The image of immune-blots for β-actin was obtained in the same procedure as described above. The intensity of bands for occludin (63 kDa) and β-actin (42 kDa) were quantified
using ImageJ software (NIH, Bethesda, Maryland). The relative expression of occludin was normalized to β-actin.

3.3.8 Cell membrane integrity assessment

The cell membrane integrity was analyzed using a lactate dehydrogenase (LDH) assay. Twelve vocal fold epithelial tissues were punched (6 mm in diameter) and incubated in oxygenated HBSS media at 37°C for 1 hour to fully release the LDH from the damaged cells. The tissues were placed in wells filled with either oxygenated control media or media with 100 μM acrolein. The media were then collected and diluted 10 times prior to assay. To avoid the interference caused by acrolein-protein adducts in the media containing LDH, 3% bovine albumin was added to the diluted media and incubated for 2 hr. The concentration of LDH in the sample media were detected using Pierce LDH Cytotoxicity Assay kit following manufacturer’s direction. An aliquot (50 μL) of diluted sample medium was mixed with 50 μL LDH reaction mixture in each well in a 96 wells plate. The absorbance was measured at 490 nm with the subtraction of absorbance at 680 nm. The absorbance was normalized by the weight of each tissue sample. The percentage of LDH release was normalized by the average of the control group.

3.3.9 Determination of lipid peroxidation

The product of lipid peroxidation, 4-HNE, was detected using immunohistochemistry. Eight vocal fold epithelial samples (6 mm in diameter) were incubated with or without 100 μM acrolein. The samples were fixed in 4% prophenol
aldehyde for 1 week and dehydrated in 30% sucrose for 1 week at 4°C. Tissues were frozen in liquid nitrogen before being sliced in microtome. Tissues were sectioned from apical side to basolateral side in the thickness of 35 μm. The sections were stored in cryoprotectant solution at -20°C (30% sucrose, 30% ethylene glycol, 0.05M PBS) before staining.

Four sections from each vocal fold sample were taken and washed in PBS followed by blocking in 5% normal donkey serum (NDS) with 0.3% Triton X-100, rotating for 90 min. The sections were then treated with primary anti-occludin antibody (1:1000) and anti-4 HNE antibody (1:100) at 4°C, overnight, followed by incubation with Alexa Fluor488 goat anti-rabbit IgG antibody (1:500) and cyanine goat anti-mouse IgG (1:500) at room temperature (RT) for 1.5 hr. Sections were rinsed with PBS between incubations of different antibodies, and mounted to slides with ProLong Gold anti-fade reagent and dried at room temperature overnight. Sections were imaged using a confocal microscope (C1-plus, Nikon). Images were analyzed using NIS Elements BR software. Confocal images (2~3 images covering entire epithelial area for each section) were used to quantify the intensities of 4-HNE and occludin in epithelia. The intensity quantification data from 4 sections of each vocal fold sample were collected. In one sample exposed to acrolein, two sections were damaged, leaving only 2 sections for further analysis. The average intensity of each vocal fold sample was calculated and processed to examine differences between control and acrolein exposed groups. Fifteen circles with diameter of 30 μm were applied systematically and covered almost the entire area of the epithelium in each image. The average intensities of each circle and average intensities of all fifteen circles in each image were calculated and shown by the software.
The average intensity of the target signal of each section was calculated by using the average intensity of each image. The average intensity of each vocal fold epithelium and the mean of the intensity of all four vocal folds in each group were further calculated based on the average intensity of each section.

3.3.10 Statistical analysis

All data are presented as mean ± SE. Statistical analyses were conducted using IBM SPSS for Windows (version 20, Chicago, Illinois). Analysis for tissue metabolic activity was performed using one-way ANOVA followed by Dunnett’s multiple comparison tests. Analyses of the differences between control and acrolein-exposed groups for TEER values and permeability were carried out by independent t-tests. All other analyses were completed with paired t-test. Differences between two means were considered significant if p values were equal or less than 0.05.

3.4 Results

3.4.1 Decreased epithelial metabolic activity following acrolein exposure

Following incubation with acrolein at the concentration from 50 to 1300 μM, the cell metabolic activity of the vocal fold epithelia showed a dose-dependent decline (Figure 3.1). The significance became evident when acrolein concentrations equaled or exceeded 500 μM (N = 7, F = 19.413, p<0.001). At concentrations of 500 μM, there was a 27.2% (p = 0.001) reduction in metabolic activity as compared to controls.
Epithelial metabolic activity was determined by the MTT assay. Ex vivo tissues were incubated with acrolein as the concentration indicated for 3 hours. Data represent Mean ± SE, n = 7, *: p < 0.05, §: p < 0.001 as compared to controls.

Figure 3.1 The Metabolic Activities of Vocal Fold Tissue following Acrolein Exposure.
3.4.2 Impaired barrier function following acrolein exposure

The values of TEER reflect the tightness of the cellular monolayer formed between adjacent cells. In control tissues without acrolein, the TEER values increased by 55.8 Ω*cm² after 3 hr incubation, while the acrolein-treated tissues showed a 44.7 Ω*cm² decrease in the TEER value, a reduction of 180.0% compared to the controls (N = 7, t = 5.023, p < 0.001, Figure 3.2 A), suggesting a reduced tightness of this vocal fold epithelial monolayer.

Further analysis of the barrier integrity by examining the leakage of a large molecular weight compound NaFl demonstrated that the concentration of NaFl in the basolateral chamber was increased by more than 2 folds (N = 7, t = -2.224, p = 0.045) (Figure 3.2 B). Thus, both the TEER values and NaFl assay provided unequivocal evidence suggesting a damaged vocal fold epithelial barrier after acrolein exposure.
Change of TEER (Ω cm$^2$)

Control 100 μM Acrolein

(A)
Acrolein impairs vocal fold epithelial barrier function with decreased TEER value (A) and increased permeability (B). Tissues were incubated with acrolein as the concentration indicated for 3 hours. (A). The TEER values were assessed by Ussing chamber system and associated voltage clamp. (B). The epithelial permeability was measured by fluorescent marker NaFl. Index of permeability represents the percentage of fluorescent marker passing through epithelium. Data represent Mean ±SE, n = 7, *: p < 0.05 as compared to controls.

Figure 3.2 Barrier Permeability Assay with or without Acrolein Exposure.
3.4.3 Acrolein exposure and expression of tight junctional proteins

Maintaining a tight barrier relies partly on the intact expression of tight junctional proteins. Thus, to understand the mechanism whereby acrolein altered the barrier permeability, we set out to examine if acrolein suppressed the expression of two key tight junctional proteins, i.e., occludin and caludin3, in the vocal fold, leading to openness of the barrier. The qPCR data in Figure 3.3 demonstrated that acrolein exposure did not significantly alter the level of mRNA encoding occludin (N = 6, t = -1.273, p = 0.259), nor did it affect the mRNA level of claudin3 (N = 6, t = -0.631, p = 0.556).

To verify the qPCR results, we directly examined the protein expression of occludin. Western blot analysis revealed that acrolein treatment did not affect the protein expression of occluding (data on occludin shown in Figure 3.4) as compared to controls (N = 6, t = -1.062, p = 0.337).

Thus, both qPCR and Western blot data established that the effect of acrolein on the tightness of the barrier seemed unlikely to be caused by its action on gene expression of barrier proteins.
No significant difference for expression of tight junction proteins was observed between two groups. Tissues were incubated with acrolein as the concentration indicated for 3 hours. The mRNA levels of occludin (OCLN) (A) and claudin3 (CLDN3) (B) were determined by qPCR. Data represent Mean ± SE, n = 6 (p = 0.259 for occludin and p = 0.556 for claudin3) as compared to controls.

Figure 3.3 Expression Levels of mRNAs Encoding Typical Tight Junctional Proteins.
The protein levels of occludin remained unchanged after acrolein exposure. Data represent Mean ± SE, n = 6, p = 0.337 as compared to controls. “C” represents control group and “A” represents acrolein treated group.

Figure 3.4 Western Blot Analysis of Occludin Protein with or without Acrolein Exposure.
3.4.4 Leakage of cell membrane and increased lipid peroxidation

LDH is an intracellular protein and usually present inside healthy cells. A significant increase of LDH in the culture medium is an indicator of cell membrane damage. With the assay, there was a 23.7% (N = 6, t = -4.807, p = 0.005) increase in LDH activity in the extracellular medium in acrolein-exposed groups as compared to controls (Figure 3.5). The data suggested that acrolein treatment caused cell membrane damage, which may contribute to acrolein-induced epithelial barrier impairment.
LDH release increased significantly in acrolein exposure group as compared to control group. Data represents LDH release in extracellular medium normalized by the average in the control group. Data represent Mean ± SE, n = 6. #: p<0.01 as compared to controls by paired t-test.

Figure 3.5 Cell Membrane Integrity Assessed by LDH Leakage.
The above findings on cell membrane damage led us to question whether
crolein toxicity was associated with the lipid peroxidation, since the latter is often the
cause of cell membrane damage. Our confocal data with immunohistochemistry
demonstrated that the expression of 4-HNE, a metabolic product of lipid peroxidation, in
the vocal fold epithelia was co-localized with occludin (Figure 3.6A). Acrolein exposure
significantly increased 4-HNE fluorescent intensity (N = 4, t = -3.440, p = 0.041, Fig
3.6B) as compared to controls. Further, the ratio of 4-HNE intensity to occludin was
greater by 45.6% after acrolein exposure (N = 4, t = -3.767, p = 0.033; Figure 3.6B).
These results indicate that the cell membrane damage may be due to the lipid
peroxidation caused by acrolein exposure, which underlies acrolein-induced barrier
permeability.
(A)
(B)
Figure 3.6 Immunohistochemical Study of Lipid Peroxidation Marker with or without Acrolein Exposure.

Signal intensity of 4-HNE adducts increased significantly following acrolein exposure. (A). A typical confocal image of vocal fold epithelia. 4-HNE was stained in red on the left panel; occludin was stained in green on the middle panel; and the merged signals in yellow was present on the right panel. (B). Quantification of signal intensities of 4-HNE and occludin. Data represent Mean ± SE, n = 4, *: p < 0.05 as compared to controls.
3.5 Discussion

The data from this study demonstrate that acrolein exposure disrupted vocal fold epithelial barrier function. The impairment in barrier function occurred at acrolein concentrations that preserved epithelial metabolic activity. The impaired epithelial barrier was due to lipid peroxidation-induced cell membrane damage. This study, for the first time in the literature, reveals that acrolein, a major toxicant of cigarette smoke, may damage vocal fold barrier integrity, leading to inflammation and subsequent voice disorders.

Acrolein is a ubiquitous pollutant in the environment. The concentration in the ambient air in the USA is about 0.5 to 3.186 ppbv[30]. Per recommendations from the National Institute for Occupational Safety and Health (NIOSH) and Occupational Safety and Health Administration (OSHA), the exposure limit to this chemical is 0.1 ppm over an 8-hour time-weighted average (TWA)[32]. However, under some circumstances the concentrations of acrolein are much higher than this recommended level. The concentration of acrolein in cigarette smoke can be as high as 70 ppm[31]. Acrolein concentrations in the smoke after combustion of wood, cotton, and pyrolysis of polyethylene foam range from 50 ppm to 180 ppm (0.41 mg/L) according to various reports[30]. In the current study, we used a 100-μM acrolein solution concentration which is equivalent to 5.6 mg/L. High concentrations have been used in other studies of vocal fold physiology[47] and it is noteworthy that this study involved a very limited exposure duration due to the ex vivo model. Thus, the toxic effects are likely to be even more extensive in vivo albeit at lower doses. This assumption, however, needs further experimental study.
The toxic effect of acrolein has implications for public health. It is estimated that 40.0 million adults (16.8%) in the USA are currently smokers[89]. Besides existing in the ambient environment, acrolein also can be generated endogenously though lipid peroxidation[36] and cancer drug (e.g. cyclophosphamide) metabolism[90]. Lipid peroxidation is a common mechanism in many diseases and pathological conditions. It has also been reported in the vocal fold wound healing literature[91]. Therefore, our study on the effect of acrolein on vocal folds also lays the groundwork for mechanistic investigations of pathological conditions involving lipid peroxidation of the vocal folds.

Upon exposure, acrolein caused a dose-dependent reduction of tissue metabolic activity, and the reduction only became significant when the concentration of acrolein reached at or above 500 μM. When a 100-μM acrolein concentration was chosen for LDH assay, we found significant cell membrane damage. It is possible that cell membrane damage may precede a reduction in metabolic activity following acrolein exposure. The membrane damage could be reversible or irreversible[92]. When the damage is too severe and becomes irreversible, necrosis may occur and metabolic activity will also be reduced. One reason for the different timelines in metabolic activity reduction and cell membrane damage following exposure may be that the cell membrane injury was in the early reversible stage and as such, did not cause mitochondrial damage or metabolic dysfunction[92]. The other possible reason may be that the MTT assay is not as sensitive as the LDH assay to acrolein toxicity when cell death is induced by cell membrane injury.

Acrolein at the concentration of 100 μM adversely affected vocal fold epithelial barrier integrity as measured by a reduction in epithelial resistance and an increase in
epithelial permeability. Similar findings have been reported in the literature. Primary culture of monolayer tracheal epithelium showed a reduction in epithelial potential difference and increased permeability following one-hour acrolein exposure[93]. Acrolein literature also reports changes in tight junction protein expression in lung epithelia[94]. Acrolein may increase tight junctional protein claudin5 expression at a relatively low dose (30 nM) but has been shown to increase expression at a relatively high dose (300 nM) in human lung endothelial cells following 4-hour exposure[94]. After 24-hour exposure to 10 ppm acrolein, claudin5 expression was upregulated in the mouse lung[94]. Though the effects of acrolein have been quantified on cuboidal airway epithelia, the effects of acrolein exposure on stratified squamous epithelia of the vocal folds has not been investigated. Given the routine mechanical stresses the vocal folds may be exposed to during speaking, it is important to investigate if acrolein exposure has similar effects on the stratified squamous epithelium of the vocal folds.

Xenobiotics can penetrate the epithelium via the paracellular and/or transcellular pathways. In healthy vocal fold epithelium, the tight junction complex seals neighboring epithelial cells by connecting adjacent cell membranes together restricting particle movement. In this study, we did not find any significant change in the expression of occludin and claudin3, two transmembrane proteins that occlude adjacent epithelial cells[95-98] and are expressed in larynx[69, 99]. This indicates that the barrier function reduction is unlikely to be associated with altered gene expression of tight junction proteins. The reason for no change in tight junction protein expression could be attributed to the short exposure duration (3 hours) or a lack of specific effect on the tight junction
complex in the vocal folds. It is possible that the functionality of the tight junction proteins may have changed by modifications of protein structure, but this was not investigated in the current study and should be the focus of future work.

Besides the paracellular pathway, epithelial barrier function can also be compromised through the transcellular pathway. Acrolein exposure caused leakage of cell membrane and increased lipid peroxidation. This is expected since acrolein is a highly electrophilic chemical with a great tendency to induce oxidative stress and lipid peroxidation[36, 74, 100]. While the reaction is on the membrane, the consequence is increased barrier permeability, which may allow xenobiotics to gain access to the deep layer of vocal folds. Thus, our data provide new insight into mechanisms of acrolein toxicity on larynx. There are some limitations to the study that must also be discussed. Acrolein concentrations used in the current study were higher than that found in routine environments. Using a high concentration enabled the investigation of changes to a variety of underlying mechanisms that regulate barrier function, and future studies will look at more physiologically relevant levels. A higher concentration was also needed as we were studying an acute exposure rather than documenting the chronic effects.

Future studies will also study the effects of removing acrolein via reversal and measuring specific xenobiotic penetration. In vivo studies using environmental chambers will also help us understand the effects of chronic exposure and cumulative effects of acrolein on vocal folds and airway tissue.

3.6 Conclusion

Acute acrolein exposure impairs vocal fold epithelial barrier integrity. The reduced barrier function is associated with lipid peroxidation damage on cell membrane.
The damaged barrier may lead to the invasion of xenobiotics to the deep layer of the vocal folds. These data lay the groundwork for a future mechanistic study of inflammation on vocal folds caused by acrolein exposure in vivo.
3.7 Acknowledgments

We thank Dr. Sherleen Adamson for her assistance us with the confocal microscopy procedure. We also acknowledge Dr. Elizabeth Erickson DiRenzo for sharing the protocol for the modified MTT assay. Porcine larynges were obtained from Indiana state-inspected and approved slaughterhouses, Beutler Meat Processing, Lafayette, IN and Monon Meat Packing, Monon, IN.
CHAPTER 4. ACROLEIN EXPOSURE INDUCES LARYNGEAL INFLAMMATION, IN VIVO

4.1 Abstract

Acrolein is an abundant component of cigarette smoke. It is considered to contribute to the most non-cancer effects on respiratory system among all cigarette smoke components. Cigarette smokers frequently complain about voice changes. However, the mechanism of the voice changes induced by smoke remains elusive. A previous study demonstrated impairment of vocal fold epithelia barrier function following acute acrolein exposure, and further that the damage was likely caused by lipid peroxidation of cell membrane. This current study was designed to investigate the pathophysiological changes especially inflammatory reactions in rat larynges following subacute acrolein exposure by inhalation. Male Sprague Dawley rats (4 months old) were exposed to either 3 ppm acrolein (acrolein exposure group) or filtered air (control group) in a whole-body exposure chamber for 5 hours/day, 5 days/week, and the treatment lasted for 4 weeks in total. Rats were sacrificed 4 hours after the last exposure and tissues were collected immediately after the sacrifice. The presence of typical pathological changes was evaluated using hematoxylin and eosin staining of paraffin embedded larynges by a blinded, board-certified pathologist (N=4). Expression of proinflammatory cytokines was assessed using quantitative PCR (N=6), and the phosphorylation of NF-kB was detected using Western blot (N=3). Histological images revealed edema and epithelial cell death and sloughing of the vocal folds. The qPCR analysis showed that the expressions of genes encoding interferon regulatory factor (Irf-5), and chitinase-3-like protein 3 (Chl3l3) were up-regulated by 75.1% and 59.6% respectively (p<0.01 for both genes). However,
no significant differences were found between the two groups for the gene expression of
tumor necrosis factor alpha (Tnf), interleukin 1 alpha (Il1α), interleukin 1 beta (Il1β),
interleukin 6 (Il6), interleukin 10 (Il10), interleukin 12B (Il12B), interferon gamma
(Ifng), granulocyte-macrophage colony-stimulating factor (Csf2, arginase (Arg1),
resistin-like molecule alpha (Retnla). Western blot revealed a 76.8% increase in
phosphorylation of NF-kB P65 on the site of Ser536 (p<0.05). No difference for T
lymphocytes infiltration in vocal folds was found between two groups. These findings
suggest that exposure to acrolein induces edema and a mild proinflammatory reaction in
larynx. The inflammatory response appears to involve NF-kB regulation and alteration of
gene expressions of proinflammatory cytokines. This study lays the groundwork for
further mechanistic investigation of acrolein-induced inflammatory reaction.

4.2 Introduction

It has been reported that there are about 40 million adults who are cigarette
smokers in the U.S[89]. Cigarette smoking is not only the most important risk factor of
chronic obstructive airway diseases[101], but is also considered to cause pathological
changes to larynx and dysphonia[81]. Cigarette smokers frequently report hoarseness and
lowered fundamental frequency of voice[81, 102]. However, the mechanism for
dysphonia is unclear.

The vocal folds are a pair of membranous tissues in the larynx, which produce
voice through vibration. Smoking is reported to increase the thickness of epithelium in
the supraglottic region and vocal fords[103]. Smoking is also the main risk factor for
Reinke’s edema reported by several epidemiological studies[104, 105]. The manifestation
of the histological change is associated with the dose of the smoking and the duration of the smoking[105]. Since Reinke’s edema changes the mechanical properties of vocal folds, it induces hoarseness[106]. It is important to understand the mechanism of edema induced by smoking, so that the treatment can be effective to improve the voice quality of patients[6]. So far, the underlying mechanism of edema affect cigarette smoke exposure is not well known.

Animal studies in literature have investigated the effects of cigarette smoke on inflammation in larynx. Exposure of rabbit to cigarette smoke for three months (10 minutes each time, twice per day) showed hyperplasia with disturbed stratification in epithelium in vocal folds[70]. Another study of passive smoking of rats for 75 days reported similar histological changes including hyperplasia in epithelium. However, no inflammation was found[46]. To study the effect of passive smoking, rats were exposed to passive smoke for 120 days (20 minutes each time, four times per day) on the 12th day after birth. The mucosa of the subglottic area exhibited moderate and focal inflammation in the exposed group as compared to the control group[107]. An inflammatory reaction was also suggested by a study administering cigarette smoke solution to rat larynges for 4 weeks. Increase expression of TNFα and IL6, along with Muc5ac were detected following the exposure. The up-regulation of inflammatory cytokines was recovered after a 90-day cessation period[108].

Tobacco smoke is a mixture of more than 5000 chemicals[27]. Acrolein is considered the most significant contributor to non-cancer effects on respiratory system among all the components in cigarette smoke[29, 109]. The concentration of acrolein in mainstream tobacco smoke frequently reaches 50-70 ppm[31]. Besides tobacco smoke,
acrolein also exists abundantly and ubiquitously in the environment, including in mobile exhaust, combustion waster from industry, forest fires, overheated animal fats and vegetables[38].

As a type of highly reactive unsaturated aldehyde, acrolein induces a variety of toxicities on the respiratory system, cardiovascular system, and neural system. The effects on the respiratory system have been studied extensively. Exposure of the Dahl hypertension-sensitive (DS) rat lines to 4 ppm acrolein (6 hours/day, 5 days/week) induced death in 2 weeks and the histological evaluation revealed an epithelial necrosis with edema and hemorrhage in lung[110]. Similarly, exposure to acrolein for 62 days using Fischer-344 rats showed bronchiolar epithelial necrosis and edema with increased macrophages in lung[111]. Nasal pathological alterations were noted following exposure to 3 ppm acrolein for 3 weeks in Sprague-Dawley rats. Significant changes include hyperplasia, squamous metaplasia of epithelium covering nasal cavity and infiltration of neutrophils[112]. Besides hyperplasia and metaplasia of epithelium, effects of inflammation in the respiratory system were also demonstrated following exposure to 4.9 ppm acrolein for 13 weeks in hamsters, rats, and rabbits. The infiltration of neutrophils was found in nasal cavity in rats and hamsters. Accumulation of alveolar macrophages was shown in rat lungs. The larynx was also reported to change with hyperplasia in epithelium of vocal folds[113]. The inflammation in respiratory system induced by acrolein exposure was confirmed by another study with Fischer-344 rats following exposure to 1.8 ppm acrolein for 13 weeks. Notably, inflammation in the larynx was also detected in that study, along with hyperplasia and metaplasia of epithelium[77]. Another study following the intranasal administration of acrolein also demonstrated inflammation
with increased activated macrophages in mouse lung following one week and four weeks exposures. Moreover, inflammation was promoted via activation of NF-κB pathway[76]. However, whether the histological changes in the larynx, especially the inflammation induced by acrolein are similar as those changes induced by cigarette smoke is not known. To understand the histological changes and the mechanism of these changes in the larynx, we conducted this study with sub-acute exposure of rats to acrolein. We hypothesized that acrolein exposure damages the vocal fold epithelium and induces vocal fold inflammation. We further hypothesized that the inflammation was caused by increased cytokine expression through NF-κB activation.

Very few studies have investigated the type of immune response, as characterized by the cell type of infiltrated leukocytes, during inflammation induced by cigarette smoke and acrolein exposure. In cigarette smoke induced chronic obstructive pulmonary disease (COPD), pathogenic T lymphocytes are considered to play a critical role in promoting inflammation and epithelial cell death[114]. Transferring CD3+ T cells from the lungs of mice which were exposed to cigarette smoke to Rag2−/− mice induces accumulation of macrophages and neutrophils[114]. Meanwhile, study of exposure to acrolein (instillation of 5 μmol/kg acrolein) for 4 weeks led to increased activated macrophages in lung in mice[76]. Increased CD8+ T lymphocytes were identified in the mouse lung following exposure to acrolein for 12 weeks[115] as well. The epidemiological study revealed the increase of CD4+ T cells in both epithelium and lamina propria in larynx in cigarette smokers[116]. To identify whether T cells play an important role in initiating the accumulation of other leukocytes and inducing inflammation in vocal folds following acrolein exposure as shown in lung with exposure
to cigarette smoke, we further hypothesized that the T cells would increase in larynx following the sub-acute acrolein exposure.

This study lays the foundation for further mechanistic investigation of edema and inflammation induced by acrolein in the larynx.

4.3 Materials and Methods

4.3.1 Animals

Male Sprague-Dawley rats, 4-month of age at purchase, were obtained from Envigo RMS, Inc (Indianapolis, IN). At the time the exposure started, the rats were 320-420g. All rats were maintained in a temperature and humidity controlled room with a 12-h light/12-h dark cycle and allowed to acclimate for 6 days prior to treatment in the animal facility at Purdue University. All rats had access to filtered water and standard rat chow ad libitum throughout the study. The study was approved by the Institutional Animal Care and Use Committee at Purdue University.

4.3.2 Chemicals and reagents

Chemicals and reagents were purchased from the following sources. Primary antibodies included rabbit anti-rat phospho-NF-κB p65 (Ser 536) antibody, mouse anti-rat NF-κB p65 antibody (3033, 6956; Cell Signaling, Danvers, MA); rabbit anti-human CD3 antibody (A0452; Agilent, Santa Clara, CA); secondary antibodies included: anti-rabbit IgG-HRP (goat) and anti-mouse IgG-HRP (goat) (sc-2004, sc-2005, Santa Cruz Biotechnology, Dallas, TX). Protease Inhibitor Cocktail was from Calbiochem (San Diego, CA); bovine serum albumin (BSA) standards and Pierce LDH cytotoxicity assay
kit was purchased from Thermo Scientific (Rockford, IL); sodium dodecyl sulfate (SDS),
Tris base, glycine, Triton X-100, 2xLaemmli sample buffer, clarity Western ECL
substrate, Polyvinylidene Difluoride (PVDF) membrane, cDNA synthesis kit and iTaq
Universal SYBR Green Supermix were from Bio-Rad (Hercules, CA). Ethylene glycol-
bis (β-aminoethyl ether)-N, N, N', N'-tetracetic acid (EGTA), Phenylmethylsulfonyl
fluoride (PMSF), 2-mercaptoethanol were purchased from Sigma Chemicals (St Louis,
MO); enhanced chemiluminescence reagent (ECL) was from Pierce Endogen (Rockford,
IL). Primers for qPCR analysis were obtained from Integrated DNA Technologies
(Coralville, Iowa). Acrolein gas cylinders were ordered from Praxair (Danbury, CT).
Refresh charcoal filters were from World Precision Instruments (Sarasota, FL). All
reagents were of analytical grade, HPLC grade, or the best available pharmaceutical
grade.

4.3.3 Acrolein administration

A whole-body 50L exposure chamber (47.6cm x 32.4cm x 32.4cm) (EZ-179,
World Precision Instruments, Sarasota, FL) was used for acrolein exposures. A gas
cylinder containing 350 ppm acrolein in nitrogen (Praxair, CT) was used to generate the
exposure atmosphere. For the acrolein exposure group, the exposure atmosphere was
produced by mixing the acrolein in nitrogen from the gas cylinder with filtered air. The
flow rates of highly concentrated acrolein (350 ppm) gas and filtered air were monitored
and controlled using two mass flow controllers separately. The two air flows were then
mixed in a “T” tube to achieve a final concentration of 3 ppm of acrolein with a total flow
of 8 L/min. The final flow then went into the chamber through the inlet on one side of the
chamber. The expired air was passed through the outlet on the other side of the chamber and through a charcoal filter (World Precision Instruments, FL) into a chemical ventilation hood (Figure 4.1). The actual chamber concentration was monitored by an VOC detector (Mocon Baseline, CO). The detector was calibrated using a 10-ppm acrolein gas cylinder and the accuracy was checked with another VOC detector (MiniRAE 3000, RAE system, CA) once every month. The acrolein concentration of the whole exposure system was tested to be stable for 5 hours before the experiment. Concentrations reported by VOC detector at one hour after exposure everyday were used for calculating the average acrolein concentration through the whole study. Rats were placed in the chamber and were exposed to 3ppm acrolein for 5 hours every day, 5 days per week for four weeks. For the control group, the rats were exposed to filtered air in the chamber. All other conditions were identical for the acrolein exposed group and control group. All rats were given filtered water and standard rat chow ad libitum during the exposure.
Acrolein concentration was controlled by a flow meter & a controller to modulate the flow rate of highly concentrated acrolein (350 ppm) and filtered air to 3 ppm. Two air flows were mixed at the “T” tube and connect to the inlet of the chamber. The outlet of the chamber connected to the charcoal filter in the fume hood from where it was vented.

Figure 4.1 Structure of Concentration Controlled Whole-body Exposure System.
4.3.4 Euthanasia and histopathology

Rats were euthanized by I.V. administration of overdosed sodium pentobarbital (Beuthanasia®) at 100 mg/kg. Exsanguination was administrated after cessation of heartbeat. Larynges were dissected immediately after rat death. Laryngeal tissues were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. For histologic evaluation (N=4), the blocks of larynges were sliced into 4-μm coronal sections. One section from every 6 sections in the mid-membranous region was chosen for hematoxylin and eosin staining. All microscopic slides underwent histological evaluation based on standard histopathological morphology by a veterinary pathologist who was blinded to the exposure conditions. The histology changes on vocal folds were assessed on six aspects: 1) epithelial hyperplasia; 2) epithelial sloughing; 3) epithelial cell death; 4) mononuclear cells infiltration; 5) polymorphonuclear leukocytes (PMN) infiltration; and 6) edema. The infiltration of mononuclear cells and PMNs were graded on four levels: 0, normal; 1, increased infiltrated cellular number in lamina propria; 2, increased cellular number in epithelium; and 3, increased infiltrated cellular number in muscles. All the other items were evaluated as either normal or alteration present.

For Western blot, the whole larynges were dissected, snap-frozen and stored in -80 °C (N=3). For quantitative polymerase chain reaction (qPCR), the larynges of rats were dissected and placed under an operating microscope for microdissection (N=6). The tissues from the luminal surface deep to cartilages (not included) in larynges (from the level of top at the thyroid cartilage to the level of first tracheal ring) were collected and snap-frozen in liquid nitrogen and stored in -80 °C for further uses.
4.3.5  Quantitative Polymerase Chain Reaction (qPCR) detection for gene expression at mRNA level

The mRNA level of genes encoding tumor necrosis factor alpha (Tnf), interleukin 1 alpha (Il1α), interleukin 1 beta (Il1β), interleukin 6 (Il6), interleukin 10 (Il10), interleukin 12B (Il12B), interferon gamma (Ifng), granulocyte-macrophage colony-stimulating factor (Csf2), interferon regulatory factor (Irf5), arginase (Arg1), resistin-like molecule alpha (Retnla), chitinase-3-like protein 3 (Chi3l3) were quantified using qPCR. Total RNA was isolated from the tissues collected from larynges and purified using TRIzol reagent (Life Technologies). The RNA was reverse transcribed to cDNA using the Bio-Rad iScript cDNA Synthesis Kit according to manufacturer’s instructions. The qPCR analyses were performed in duplicate using CFX connect Real-Time PCR Detection System (Bio-Rad). iTaq Universal SYBR Green Supermix kit (Bio-Rad) was used for the qPCR detection. The qPCR was performed using a protocol described previously[44]. The amplification efficiencies of both target genes and reference genes were tested on the same plate with samples. Relative expressions of the target genes were assessed using ΔΔCt, which were calculated using the threshold cycle time values (Ct values) in qPCR. The Ct values in the target genes were normalized with the ones of β-actin in the same sample. The forward and reverse primers for genes being tested in qPCR were designed using Primer3[117]. The sequence of each primer is listed below (Table 4.1):
Table 4.1 Primer Design for Quantitative PCR Analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence 1</th>
<th>Gene</th>
<th>Primer Sequence 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>CTCTGACCCCCATTACTCTGAC</td>
<td>IFN-γ</td>
<td>GCACAAAGCTGTCAATGAAACTC</td>
</tr>
<tr>
<td>(Tnf)</td>
<td>TACTTCAGCGTCTCGTGTGTTC</td>
<td>(Ifng)</td>
<td>CTCTCTACCCCAGAATCAGC</td>
</tr>
<tr>
<td>IL-1α</td>
<td>TCCTAAATCCTCTGAGCTTG</td>
<td>GM-CSF</td>
<td>GAGAACGAAAGAGAAACGAGACG</td>
</tr>
<tr>
<td>(Il1a)</td>
<td>ACAGATTTGGTATCATGACTGC</td>
<td>(Csf2)</td>
<td>CCCGTAAGACCTGCTTTGTATAG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AGTGTGGATCCCCAAACAATACC</td>
<td>Irf-5</td>
<td>GGAGAAGAGGAGGAAGAGGAAG</td>
</tr>
<tr>
<td>(Il1b)</td>
<td>AACTGTGCAGACTCAAACCTCA</td>
<td>(Irf5)</td>
<td>TGGGTAAGAGAATAGGGTGCTA</td>
</tr>
<tr>
<td>IL-6</td>
<td>GTCAACTCCATCTGCCCTTCAG</td>
<td>Arg1</td>
<td>AGTATGGCAATTGGAAGCATCT</td>
</tr>
<tr>
<td>(IL6)</td>
<td>GGCAGTGGCTGTCAACAACAT</td>
<td>(Arg1)</td>
<td>GGGAACTTTTCTTTTCAGTTTCT</td>
</tr>
<tr>
<td>IL-10</td>
<td>CAACTGCATAGAAGCTACGTG</td>
<td>RetnlA</td>
<td>GCCAAGCTGTCAATAAGGAGG</td>
</tr>
<tr>
<td>(Il10)</td>
<td>GGTACAAACGAGGTTTTTCCAAG</td>
<td>(RetnlA)</td>
<td>ATACATCAAATGCGCATGAGTC</td>
</tr>
<tr>
<td>IL-12B</td>
<td>AGAACTCTCAGGTGGAGGTCAG</td>
<td>Chi3L3</td>
<td>TATCAATCTCCATCCGACACTG</td>
</tr>
<tr>
<td>(Il12b)</td>
<td>TCCTTCGCTTTTTTCTTTTGC</td>
<td>(Chi3L3)</td>
<td>TGTAGAGGGTCACTCAGAGAT</td>
</tr>
<tr>
<td>β-actin</td>
<td>GGCACCACACTTTTCTACAATGA</td>
<td>β-actin</td>
<td>CATGATCTGGGTCACTTTTCA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence 1</th>
<th>Gene</th>
<th>Primer Sequence 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Actb)</td>
<td>CATGATCTGGGTCACTTTTCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3.6 Western blot analysis of phosphorylation of NF-κB

Activation of the NF-κB pathway was analyzed by Western blot. The phosphorylation of NF-κB were determined by the intensities of bands of phosphorylated NF-κB normalized to the intensities of bands of total NF-κB. Total protein of larynges was extracted using a homogenization buffer (20 mM Tris buffer with pH at 7.5, 1% Triton X-100, protease inhibitor cocktail, 5 mM EGTA, 10 µL/mL PMSF, 0.1% SDS, and 2-mercaptoethanol) from each sample. The concentration of total protein of each sample was quantified using a BSA assay kit. Isolated protein samples were mixed with 2xLeammlie at the ratio of 1:1 and boiled for 5 min. Samples were concentrated using a CentriVap Concentrator (Labconco, MO) for 30 min. The samples were loaded to the SDS-polyacrylamide gel. The proteins were electrophoresed and separated by molecular weights. A PVDF membrane was used to transfer the proteins and blocked by 5% milk for 1 hour. The membrane was then moved to primary anti-rat phospho-NF-κB p65 antibody (1: 500) at 4 °C overnight. The mixture of tris-buffered saline and Tween 20 (TBST) was used to rinse the membrane. The membrane was then incubated in the secondary antibody (Goat anti-rabbit IgG-HRP, 1:3000) for 1 hour. ECL-Western Blotting Substrate was used for membrane incubation of 5 min. The bands of protein were visualized and imaged in the Bio-Rad Molecular Imager. The membrane was washed using the ECL stripping buffer and rinsed using TBST after imaging. The membrane was then incubated in the primary anti-rat NF-κB p65 antibody (1:1000) at 4 °C overnight, followed by the secondary antibody (Goat anti-mouse IgG-HRP, 1:5000) incubation. The image of bands was taken in the Bio-Rad Molecular Imager as described above. The intensities of bands for phospho- NF-κB and total NF-κB were quantified
using the software of ImageJ (NIH, Bethesda, Maryland). Relative phosphorylation of NF-κB was normalized to the total NF-κB.

4.3.7. Immunohistochemistry for T lymphocytes

Laryngeal tissues (N=5) were fixed in 10% neutral buffered formalin for 48 hours after dissection. Fixed tissues were then dehydrated and embedded in paraffin. The blocks were sliced into 5 μm coronal sections. One section out of every seven sections was chosen from all the sections for staining of immunohistochemistry. Those slides were incubated in 60 °C for two hours and deparaffinized. The slides were then rehydrated to water through a series of alcohols. Antigen was retrieved in Roden Decloaker solution (Biocare Medical) in the steamer at 95 C for 20 minutes. After retrieval, slides were kept at room temperature for 20 more minutes to cool down and were then rinsed with Tris buffer. A circle was drawn on the slide using a hydrophobic pen to keep reagents inside. Slides were put in a Dako Cytomation IHC autostainer. Sections were blocked using 3% hydrogen peroxide (Fisher) for 5 minutes and 2.5% Goat Serum (Vector) for 20 minutes. Then the sections were incubated in 1:200 primary rabbit anti human CD3 antibody (Dako) for 30 minutes. Sections were rinsed and incubated in ImmPRESS HRP goat anti-rabbit secondary antibody for 30 minutes. ImmPACT DAB kit (Vector) was used for the final staining. After DAB incubation, slides were rinsed and counterstained by hematoxylin in a Leica H&E autostainer. Sections were covered by cover slides and dried. A positive and negative rat microarray control was included in the staining.
All slides were scanned using Aperio digital pathology slide scanner (Leica Biosystems, IL) with the 20x objective. Areas of lamina propria and epithelium of vocal folds were drawn using annotation tools in the software of Aperio eSlide Manager. The upper margin and lower margin of vocal folds were identified as the narrowest part of lamina propria and epithelium of vocal folds.

The threshold of the intensity restricted to strong positive staining was determined by an experienced board-certified veterinarian pathologist. The area of strong positive staining and total region of interest were calculated by the Aperio software and exported as raw data. The ratio of area of strong positive staining to the area of the total region was calculated and analyzed as the percentage of T cell staining infiltration by area.

4.3.8. Statistical analyses

All data are presented as mean ± SD. Analysis of histological assessment was performed using Fisher exact test. Comparisons of acrolein exposure group and control group for gene expression (qPCR), phosphorylation of NF-κB (Western blot), and T lymphocytes infiltration (Immunohistochemistry) were carried out by independent t-tests. Differences were considered significant when p values were equal or less than 0.05. All statistical analyses were performed using IBM SPSS (version 20, Chicago, Illinois).

4.4 Results

4.4.1 Concentration of acrolein in the exposure chamber

The stability of the whole-body exposure system was monitored and confirmed before conducting exposure studies. The actual chamber concentrations monitored by the
VOC detector (Mocon Baseline, CO) was 3.1 ± 0.9 ppm for the target concentration of 3 ppm.

4.4.2 Change in body weight

No animal death occurred during challenge exposure. The average body weight of rats in the control group before exposure (to filtered air) was 361.6g ± 26.2g. The average body weight of rats in the acrolein exposure group before the exposure was 387.2g ± 41.6g. The average weight of rats increased by 5.1% in control group while it decreased by 6.2% in the acrolein exposure group. The body weight changes were significantly different between two groups (N = 18, p < 0.001) (Figure 4.2 A. B)
Rat body weight decreased significantly following acrolein exposure. A. Body weights in two groups over time. B. Percentage of body weight change in two groups. Data represent Mean ± SD, n = 18 (each group), #: p < 0.01 as compared to controls.

Figure 4.2 Percentage of Changes in Rat Body Weight following Exposure to Acrolein or Filtered Air (Control).
4.4.3  Histological evaluation

Blinded evaluation of vocal folds revealed mild edema in the lamina propria of the acrolein group as compared to the control group (N = 4, \( p = 0.029 \), Table 4.1, Figure 4.3). Mild edema was identified histologically as non-staining material expanding the lamina propria of affected vocal folds.

Besides edema, three out of four vocal folds from acrolein exposure group showed epithelial sloughing and epithelial cell death. The desquamated epithelial cells were shrunken with pyknotic nuclei, which was suspected as potential apoptotic bodies. Conversely, none of the vocal folds from control group showed any change in epithelial sloughing or epithelial cell death. However, the differences in incidence of epithelial sloughing and epithelial cell death did not reach statistical significance on a Fisher exact test (N = 4, \( p = 0.143 \); \( p = 0.143 \), Table 4.2, Figure 4.3).

No epithelial hyperplasia was observed in vocal folds in either group. One out of four vocal folds in the acrolein exposure group showed increased number of mononuclear cells, suggesting infiltration of either macrophages and/or lymphocytes. However, this change was also found in one of the vocal folds in control group. No infiltration of polymorphonuclear leukocytes (PMNs) was observed in any vocal folds in acrolein exposure group. There were no significant differences in epithelial hyperplasia, mononuclear cells infiltration, or PMN infiltration between acrolein exposure group and control group. (Table 4.1, Figure 4.3A, B)
Table 4.2 Blinded Histology Evaluation Scores of Vocal Folds Following Acrolein Exposure or Control (Filtered Air) Exposure

<table>
<thead>
<tr>
<th></th>
<th>Epithelial Hyperplasia</th>
<th>Epithelial Sloughing</th>
<th>Epithelial Cell Death</th>
<th>Mononuclear Infiltrate</th>
<th>PMN Infiltrate</th>
<th>Edema</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Control3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Control4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Acrolein1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Acrolein2</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Acrolein3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Acrolein4</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: N=4. Each row represents a different animal. The infiltration of mononuclear cells and PMNs were graded into four levels: 0, normal; 1, increased infiltrated cellular number in lamina propria; 2, increased cellular number in epithelium; 3, increased infiltrated cellular number in muscles. All the other items were evaluated as either normal or alteration present.
Control  4 weeks acrolein

(A)
Images of H-E staining of vocal folds in low magnification (A, x4 objective) and high magnification (B, x10, x20 objectives). Vocal folds in acrolein exposure group showed edema and epithelial desquamation with apoptotic body (rectangular frame).

Figure 4.3 Representative Images of H-E Staining of Vocal Folds following Acrolein or Filtered Air (Control) Exposure.
4.4.4 Gene expression of proteins participating in immune response

Expressions of genes encoding Irf5 and Chi3l3 increased significantly at the mRNA level in the acrolein exposure group as compared to the control group (N = 6, \( p = 0.002 \), \( p = 0.009 \) respectively). The expressions of all the other genes were not significantly different between two groups. (Figure 4.4)
Gene expression evaluated using quantitative PCR. Expressions of Irf-5 and Chi3l3 increased following acrolein exposure. Data represent Mean ± SD, n = 6, #: p < 0.01 as compared to controls.

Figure 4.4 Expressions of mRNA Encoding Proteins in Proinflammatory Reaction.
4.4.5 Activation of NF-κB pathway

Phosphorylation of NF-κB increased significantly in the acrolein treated group (N=3, \( p = 0.042 \)), suggesting activation of the NF-κB pathway following 4-weeks of acrolein exposure (Figure 4.5).
Phosphorylation of NF-κB p65 was detected using Western blot. Protein levels of phosphor-NF-κB were normalized by the protein levels of total NF-κB. Data represent Mean ± SD, n = 3, *: p < 0.05 as compared to controls.

Figure 4.5 Increased Phosphorylation of NF-κB p65 following Acrolein Exposure Assessed by Western Blot.
4.4.6 CD3+ cells infiltration

Generally, CD3+ cells were found to be present very rarely in vocal folds in both groups. There was no significant difference between acrolein exposure group and the control group for the percent of area stained as strong positive to the total area of vocal folds (N = 5, p = 0.309). This finding suggests no increased infiltration of T lymphocytes into vocal folds following acrolein exposure (Figure 4.6, (A) control group; (B) acrolein exposure group).
Vocal folds were stained by DAB and hematoxylin. DAB staining represents CD3+ cells (T lymphocytes). Both vocal folds in control group and acrolein exposure group revealed very few CD3+ cells (pointed by black arrow). There was no significant difference in the percentage of CD3+ staining area to total vocal fold area between two groups.

Figure 4.6 Images of CD3+ Staining on Vocal Folds following Acrolein or Filtered Air (Control) Exposure.

Vocal folds were stained by DAB and hematoxylin. DAB staining represents CD3+ cells (T lymphocytes). Both vocal folds in control group and acrolein exposure group revealed very few CD3+ cells (pointed by black arrow). There was no significant difference in the percentage of CD3+ staining area to total vocal fold area between two groups.
4.5 Discussion

The results from this study demonstrated that sub-acute acrolein exposure induced vocal folds edema and probably epithelial sloughing and cell death. Epidemiological data in the literature suggest that cigarette smoke is the main risk factor of Reinke’s edema[105]. Since acrolein is a major toxicant in cigarette smoke, which has the highest non-cancer risk index on respiratory effects[29], it is worth understanding if acrolein exposure induces edema. The edema on vocal folds shown in our study suggests the possibility of such a hypothesis. Though the edema in our study was mild and not limited to Reinke’s space, the underlying mechanism of the edema following acrolein exposure may also apply to Reinke’s edema in cigarette smoking. It is found that Reinke’s edema may be caused by the unusual blood vessel networks with loops and branching[118]. Whether acrolein-induced edema accompanies alteration of blood vessels awaits further study.

In our study, we also found epithelial sloughing and cell death in three pairs of vocal folds out of four in the acrolein exposure group. (None in the control group.) Though the statistical analysis did not reveal significance; the non-significance could be due to the small sample size in the current study. Acrolein exposure is found to induce pulmonary epithelial cell death in a previous mouse study[76]. Our previous in-vitro study with porcine epithelial tissue also reveals lipid-peroxidation induced cell membrane damage in epithelium following acute acrolein exposure. Moreover, with an increase of acrolein dose, the epithelium metabolic activity declined dramatically[44]. In the histologic evaluation, cell death was identified in the squamous epithelium and characterized by shrunken cells with contracted nuclei.
The quantitative PCR results demonstrated increase of interferon regulatory factor (Irf5) and chitinase-3-like protein 3 (Chi3l3) expression. However, no difference in expression of other cytokines was observed. Irf5 is a transcription factor expressed in macrophages and neutrophils. It mediates macrophage activation during inflammation. Macrophages play a major role in innate immune system to clear the antigens. They are also important for adaptive immune system as a type of antigen-presenting cells. Activated macrophages have two main groups, i.e., M1 and M2. M1 macrophages function to promote inflammation, while M2 macrophages function to repair tissues and limit inflammation[119]. Irf-5 is highly expressed in M1 macrophages as compared to M2 macrophages and it promotes proinflammatory reactions. Irf-5 up-regulates the genes encoding proinflammatory cytokines such as IL12B and IL23A and represses expression of genes encoding anti-inflammatory cytokines such as IL-10[120]. Chi3l3 is a type of lectin. It is a marker of M2 macrophages in mouse and has anti-inflammatory effect[121]. However, the exact mechanism of its effect on macrophages is not understood yet.

Although the histology evaluation did not reveal any differences in inflammation besides edema between two groups, the upregulation of Irf-5 and Chi3l3 suggests activation of both M1 and M2 macrophages, indicating the existence of mild proinflammatory reactions in larynx. Our Western blot result further proved the proinflammatory reaction by showing the activation of NF-κB pathway which is considered to play key role in regulating immune response during inflammation[122]. NF-κB is a heterodimer, which functions as a transcription factor regulating the genes encoding proinflammatory cytokines. At the inactivated status, the dimers exist in cytoplasm binding proteins called inhibitors of κB (IκB). Once the NF-κB dimer leaves
the IκB, it is released to enter the nucleus and promote expression of some genes. NF-κB pathway can be activated in several steps. Most of the studies revealed the mechanism of its activation via ubiquitination of IκB[122]. The signals such as cytokines and ROS can activate IκB-kinase (IKK) complex. This IKK complex phosphorylates IκB and makes it go into a process called ubiquitination[123]. IκB is then degraded. Thus, the NF-κB dimers are set free to get into the nucleus. Another important mechanism to activate NF-κB is to phosphorylate the dimer itself[123]. The phosphorylation of certain domains in NF-κB is proved to enhance its function of transactivation[124, 125]. A previous study on cigarette smoke found inflammation in the rat lung with activation of NF-κB pathway without degradation of IκB[126], suggesting that the ubiquitination of IκB may not the mechanism for NF-κB activation following cigarette smoke exposure. Moreover, the acrolein exposure study from mice proved the phosphorylation of NF-κB p65 at the site of Ser536[76]. Thus, we chose the mechanism of phosphorylation of NF-κB as the hypothesis for the activation of NF-κB pathway. The result of increased phosphorylation confirmed our hypothesis and is consistent with previous research on lung[76].

Finally, we investigated T cell (CD3+) infiltration in vocal folds following acrolein exposure. As a result, we did not find any significant change in T cell infiltration in acrolein exposure group. This result is in contrast with a previous study showing increased CD 8+ T cells in mouse lung[115]. The non-significant result in vocal folds when compared to pulmonary tissues may be due to the difference in nature of these tissues. Vocal folds have a unique histological structure with very few capillaries, which makes it difficult to induce infiltration of leukocytes.
Our study is the first study to investigate the proinflammatory reaction of acrolein on the vocal folds at the level of gene expressions of proteins participating in the immune response, activation of a related regulating pathway, and the infiltration of T cells. We confirmed edema in vocal folds after acrolein exposure. We also identified the mild inflammatory reaction with upregulation of genes related with macrophages activation. The phosphorylation of NF-κB was also detected.

This study enhanced our understanding of the mechanism of cigarette smoke induced laryngitis and edema. It lays the groundwork for future mechanistic studies along several directions, including the mechanism of edema on vocal folds and studying the infiltration and activation of macrophages in vocal folds following acrolein exposure. Future studies will also evaluate the effects on vocal folds following co-exposure of acrolein and other components in cigarette smoke.

4.6 Conclusion

Subacute acrolein exposure induces vocal fold edema and probably epithelial sloughing and cell death. The exposure promotes mild inflammatory reactions with increased gene expression of Irf-5 and Chi3l3, suggesting activation of macrophages. The proinflammatory reaction may be due partly to activation of NF-κB via the phosphorylation of NF-κB p65. The inflammatory changes are not likely due to the participation of T lymphocytes.
4.7 Acknowledgments

We thank Dr. Jae Hong Park and David McMillan for assisting us with the whole-body exposure system development. We also acknowledge Ruth Anderson and Nathan Garrison for assisting us with the rats’ exposure. We thank Dr. Jason Cannon, John Wise, Rachel Foguth, and Victor A. Bernal-Crespo for the assistance with Immunohistochemical examination. We thank NIH R01DC011759 (National Institutes of Health/ National Institute on Deafness and Other Communication Disorders) for sponsoring this study.
CHAPTER 5. SUMMARY AND FUTURE DIRECTION

5.1 Summary

In summary, the research question guiding these data was to determine the effects of common environmental pollutants in the form of particles and volatile chemicals on vocal fold pathobiology. A variety of methodologies and paradigms were used across rodent and swine animal models. Below, I summarize specific findings. Section 5.1.1 summaries the findings of vocal folds exposure to SWCNTs. Section 5.1.2 summaries the findings of vocal folds exposure to acrolein.

5.1.1 SWCNT exposure on vocal fold physiology

Viable excised vocal fold epithelium is robust to acute challenges from SWCNT. There was no change in epithelial metabolic activity, barrier function or tight junction protein level following acute SWCNT exposure at a relatively high concentration when compared to its concentration in occupational environment.

However, the exposure of viable vocal fold fibroblasts to SWCNTs at the same concentration increased the expression of profibrotic genes, though no reduction of metabolic activity was observed at that concentration.

These results suggest that the vocal fold epithelium may function as a robust barrier to prevent the invasion of SWCNTs from the occupational environment. However, if the epithelium is damaged, and SWCNTs get access to the underlying connective tissue, the fibroblasts in lamina propria may undergo the pathophysiological process of fibrosis (Figure 5.1).
Figure 5.1 Mechanistic Figure of Change to Vocal Folds following Exposure to SWCNTs.
5.1.2 Acrolein exposure on vocal fold physiology

Vocal folds epithelium is susceptible to damage from acrolein, which is a highly reactive, soluble chemical. The metabolic activity of epithelium was reduced in a dose-dependent pattern in response to acute acrolein exposure. The epithelial barrier function and cellular membrane integrity were significantly impaired by acute acrolein treatment at relatively high concentrations (more than 10 times higher than possible exposure levels of acrolein in environment). The cellular membrane damage was primarily related to acrolein induced lipid peroxidation (Figure 5.2).

Our data also suggest that subacute acrolein exposure at a relatively low concentration induces edema in rat vocal folds. The epithelium may undergo cell death with apoptosis. Furthermore, acrolein exposure also promotes a mild inflammatory reaction in larynges with increased expression of proteins involving activation of macrophages. The NF-κB activation may play an important role in the inflammatory reaction. As a major toxicant in cigarette smoke, acrolein-induced changes present clinically with a profile similar to that in cigarette smoke (Figure 5.2).
Figure 5.2 Mechanistic Figure of Change to Vocal Folds following Exposure to Acrolein.
5.2 Future Directions

The effects of particle exposures on vocal folds should be further investigated using an in-vivo exposure system. In our ex vivo study with excised porcine vocal fold epithelial tissue, we did not expose vocal fold epithelial tissues to acrolein for long durations because the tissue could only survive for up to 6 hours outside of the body. To investigate the effects induced by particle exposures at low concentrations (environmentally-related) but at longer durations, an in vivo animal exposure model should be used. The aerosolization of particles could be applied to the in vivo study to mimic physiologically-realistic occupational exposure environments. Moreover, with the in-vivo exposure system, systematic reactions such as the immune response could be studied as well.

The in vitro study revealed epithelial barrier damage following acute acrolein exposure. However, the concentration was higher than that typical in the environment. The effects of acrolein exposure at environmental concentrations on vocal folds epithelial barrier functions should be assessed with the in vivo exposure system. The reversal effects on epithelial barrier function and inflammation in lamina propria after acrolein cessation also awaits future studies.

We will further study the mechanism of edema on the vocal folds, the infiltration and activation of macrophages in vocal folds following acrolein exposure. To investigate the effects of NF-κB activation on edema and macrophage infiltration, specific inhibitors to NF-κB can be applied, such as transcriptional activity inhibitor JSH23.
Acrolein exists with many other toxicants in cigarette smoke. The effects of co-exposure of acrolein with other chemicals such as acetaldehyde and hydrogen cyanide may be complicated. Future studies will evaluate the effects on vocal folds following co-exposure of components in cigarette smoke.

Our findings from SWCNTs study and acrolein study raise an interesting question of whether vocal folds are more sensitive to challenges of volatile chemicals (e.g. acrolein) than particles (e.g. SWCNTs). However, our current data cannot make a conclusion on that question because of the different methodologies used in the SWCNTs and acrolein studies. To further investigate the differences in sensitivity of vocal folds to different challenges, these challenges should be examined using the same research design.
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126. Marwick JA, Kirkham PA, Stevenson CS, Danahay H, Giddings J, Butler K.