MODELING THE EFFECTS OF ARSENIC INHALATION EXPOSURE ON APICAL BRONCHIAL EPITHELIAL BARRIER FUNCTION

By

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I. Acknowledgements:

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Modeling the Effects of Arsenic Inhalation Exposure on Apical Bronchial Epithelial Barrier Function

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II. Abstract

This thesis is a research-based approach to determining an experimental model that accurately mimics inhalation of environmentally relevant concentrations of arsenic. The model will be established by using an immortalized human bronchial cell line seeded on filters that allow access to the apical and basolateral surfaces of the upper respiratory epithelium. Further, this model will be used to understand how inhalatory arsenic exposure affects the upper airway epithelial barrier function via tight junction proteins and subsequently contributes to respiratory disease.

III. Introduction:

Arsenic is a heavy metalloid toxicant found in nature as a combination with other elements resulting in either organic or non-organic arsenic compounds [1]. It is found primarily in soil and rock, although it may be present in air and/or water through natural or anthropogenic processes such as volcanic eruptions, melting of metal ores, mining or incinerators. In addition to be a naturally occurring element, arsenic is also used industrially for a variety of purposes, including pesticides, animal feed additives, metal alloys, lead-acid car batteries, and semiconductors [2].

The most common exposure to arsenic is through the ingestion of contaminated food sources, however areas with high industrial activity, hazardous waste sites or urban areas can have significant water and air arsenic pollution as well. Despite being a large component of the problem, arsenic exposure is not limited as an occupational hazard. The World Health Organization (WHO) estimates that more than 200 million people worldwide are chronically exposed to arsenic levels above the safety threshold of 10 ppb [3]. Generally, the average exposure is minimal, but negative physiological impacts from chronic, low-dose arsenic exposure have been documented [2].

Numerous studies have examined the health impacts of arsenic exposure through an ingestion route that commonly occurs through consumption of contaminated drinking water. The findings from these studies indicate that arsenic exposure can lead to increased risk of infection, decreased lung function, respiratory effects, and/or obstructive lung disorders [4-7]. Additionally, the incidence of lung cancer is strongly correlated with chronic arsenic exposure via either ingestion or inhalation routes [8]. While providing

context to the larger problem, these connections have been established primarily through the use of epidemiological studies. In order to clarify our understanding of these findings, it becomes critically important to understand the physiological mechanism(s) of the detrimental respiratory effects seen in these studies.

In order to evaluate the impact of arsenic on the lung epithelium, it is necessary to have a general understanding of lung anatomy and physiology from both a system and cellular perspective. The respiratory system can be divided into three subsections: the upper airway, the conducting airway and the respiratory airway. The conducting airway, which will be the main focus of this research project, functions to moisten, warm and clean air. As air moves from the upper areas of the lung to the respiratory airway, gas exchange and blood oxygenation occurs [9].

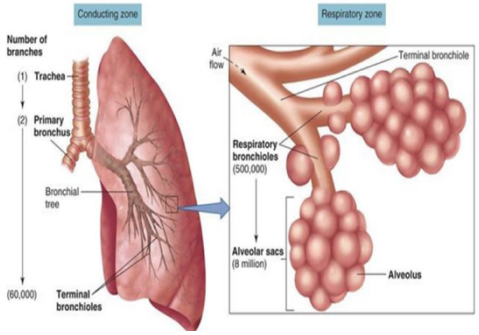

Figure 1: Anatomy of the respiratory system. The respiratory system is divided into the conducting zone and respiratory zone. Anatomically, the conducting zone is comprised of the nasal structures, trachea, the primary bronchi, and bronchioles. The respiratory zone includes the respiratory bronchioles and alveoli [18].

The lumen of the conducting airway is lined by a continuous barrier composed of epithelial cells. This, combined with the physiological function of the conducting airway to remove damaging inhalants, demonstrates the necessity of maintaining proper barrier function and homeostasis of the tissue [9]. On a molecular level, tight junction proteins are critical in maintaining the barrier function of the epithelium [10]. Ultimately, the presence of tight junction proteins within the epithelial barrier contributes to regulation of paracellular ion and molecule diffusion, cell polarity, and innate immunity [10].

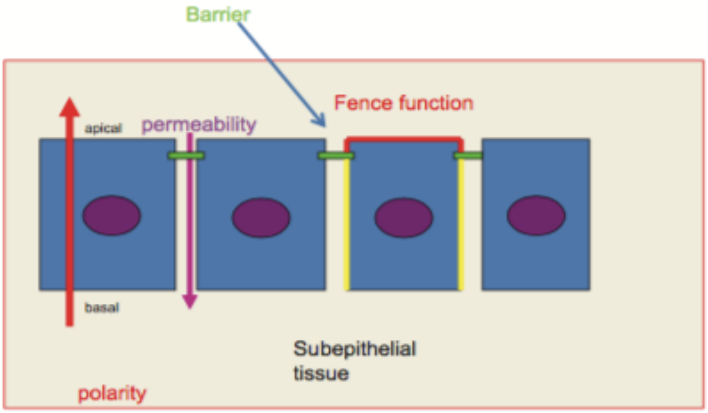

Figure 2: The epithelial cell layer contains tight junctions (green) that regulate the permeability of solute and ions through the paracellular space (downward purple arrows). The tight junctions create a barrier to the paracellular space, prevent pathogens from reaching the subepithelial tissue, and determine cell polarity. Cell polarity separates the apical and basolateral surface of the cell membrane and prevents mixing of membrane proteins [11]

Damage to the conducting airway epithelial barrier through via changes in tight junction proteins can compromise the ability of the respiratory epithelium to function as an effective physical barrier and prevent infiltration of pathogens. Alterations to the conducting airway tight junction proteins have been observed in various respiratory illness such as chronic infection, asthma, and COPD [10,11]. Other noxious inhalants, including environmental pollution and particulates, can have negative physiological effects through the internalization of occludin, one of the molecular components of tight junctions [11].

Arsenic exposure is known to have negative effects to lung function but the full context of the physiological reasoning behind this has not been fully determined. Previous experiments have attempted to clarify the underlying mechanisms by creating a model of the conducting airway epithelium and using laboratory techniques to assess measurements of barrier function [10].

Following basolateral exposure of mouse tracheal epithelial (MTE) cells to environmentally relevant concentrations of arsenic, the study noted a decrease in TER compared to control, suggesting the epithelial barrier had in some way been compromised. Further analysis of immortalized human bronchial epithelial cells (16HBE14o-) reveals molecular changes including disturbances in the localization patterns of tight junction proteins claudin and occludin and alterations to the level of expression and posttranslational modifications [10].

This experiment focuses the basolateral region of the epithelium, which functions to model arsenic exposure through an ingestion route. Conversely, addition of arsenic from the apical side of the epithelial barrier mimics the inhalation route of exposure. While it is known that inhalation and ingestion pose similar risk to developing adverse respiratory events from arsenic exposure [8], there is less understanding of the physiological changes that occur at a cellular level through apical arsenic exposure.

Recreating inhalation of arsenic can be complicated due to the nature of the apical respiratory epithelium. This project will attempt to solidify this model through the use of an immortalized human bronchial epithelial cell line (16HBE14o-). Ultimately, this model will be used to quantitatively assess barrier function of the conducting airway epithelial barrier through dose response concentrations of arsenic exposed apically, as compared to how the barrier responds to basolateral exposure [10].

Given the implications that were determined from the previous experiments of basolateral arsenic exposure, we hypothesized that there would similar changes in the localization of tight junction proteins. However, the results of this initial study do not indicate a significant difference in transepithelial resistance (TER). This suggests that arsenic exposure through an inhalatory route does not incur the same changes to the respiratory epithelial physiology as exposure via ingestion.

V. Methods

Tissue culture methods

Description: 16HBE14o- cells are grown and maintained through tissue culture methods. The two main protocols using tissue culture were 1) maintenance of the cell line through tissue culture flasks and 2) seeding 16HBE14o- cells on filters for TER experiments,

1) Immortalized human bronchial epithelial cells (16HBE14o-) were grown on a collagen, fibronectin, Bovine Serum Albumin (CFB) matrix-coated tissue flasks in a controlled growth medium (CGM) of Minimal Essential Medium with Earle's salts supplemented with 10% FBS, 2mM glutamax, penicillin, and streptomycin. The cells were grown at 37°C in a 5% CO_2 atmosphere. The CGM was replaced every other day until confluence. At confluence, the cells were split on CFB-coated semipermeable filters (6.5 mm diameter and 3 μm pore size) and cultured at 37°C in a 5% CO_2 atmosphere.

2) When seeding the 16HBE14o- cells on Transwell semipermeable filters, the splitting dilution resulted in 200,000 cells/filter. The cells were grown in MEM supplemented with 10% fetal bovine serum (FBS) 2mM glutamax, non-essential amino acids (NEAA), penicillin, and streptomycin. Upon initial seeding, the media dose was 75 μL apical and 600 μL basolateral. Each subsequent cell feed prior to air-liquid interface transition received a media dose of 100 μL apical and 600 μL basolateral.

Air liquid interface transition refers to the addition of 100 μL apical HBSS instead of supplemented MEM. This mimics the small volume of liquid found near the apical surface of airway epithelia [14].

Transepithelial resistance (TER) measurement methods

Barrier function can be measured quantitatively by understanding how tight junctions create electrical resistance between the apical and basolateral domains.

Corrected resistance is calculated by subtracting the background resistance from the measured resistance of each filter and multiplying by the effective surface area (0.33 cm^2). Background resistance was found by measuring a CFB coated filter free of cells. The final resistance measurement is reported in $\Omega \cdot cm^2$. A decrease in TER compared to control is suggestive of a decrease in integrity of the epithelial barrier.

The TER was measured using an EVOM epithelial ohmmeter and an EndOhm 6 tissue resistance measurement chamber. Readings were collected according to the manufacturer's protocol [13].

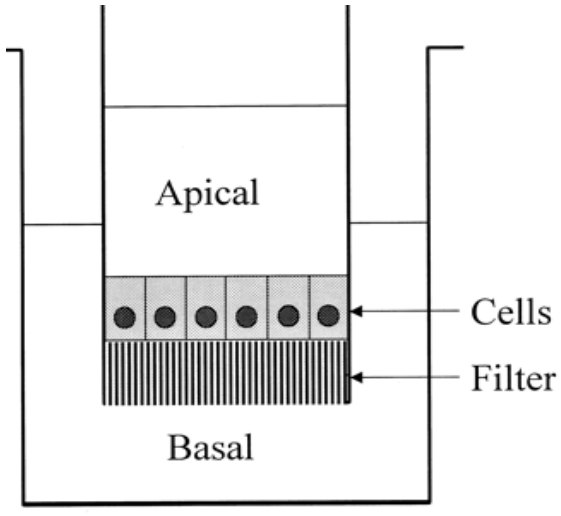

Figure 3: Diagram of Transwell semipermeable filters. This shows how the plating of cells allows distinct access to basolateral and apical sides of the established epithelium [12].

Statistics

The data collected in this thesis was analyzed using GraphPad prism (version 5.0d), ANOVA, and t-tests when appropriate. A $p < 0.05$ was considered significant.

Transepithelial resistance (TER) experiment methods

Acute arsenic exposure:

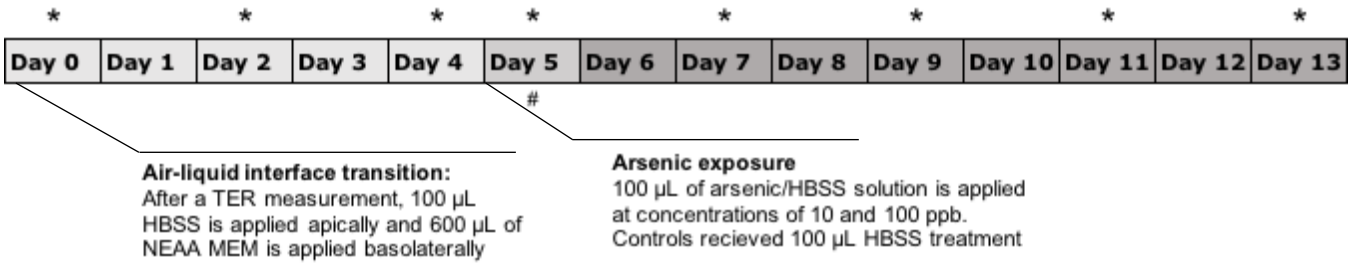

Figure 4: Timeline for acute apical arsenic exposure experiments. Arsenic/HBSS solution was applied apically to the filters initially at Day 5 (0, 10, or 100 ppb dose) once a sufficient corrected TER (≤ 200 Ω·cm2) was established at air-liquid interface. Each successive measurement received an arsenic-free HBSS dose. "*" indicates a TER reading. "#" indicates an arsenic treatment.

Chronic arsenic exposure:

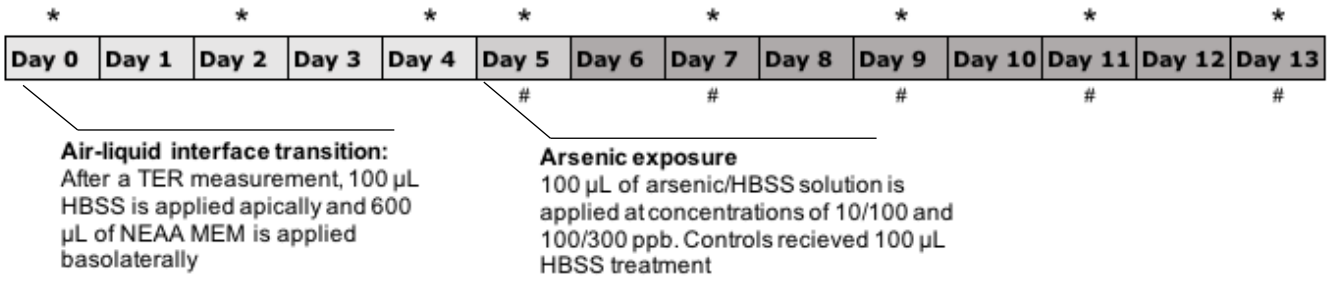

Figure 5: Timeline for chronic apical arsenic exposure experiments. Arsenic/HBSS solution was applied apically to the filters initially at Day 5 (0, 10, or 100 ppb dose) once a sufficient corrected TER (≤ 200 Ω·cm2) was established at air-liquid interface. Each successive measurement received the same HBSS/arsenic or HBSS dose (control). "*" indicates a TER reading. "#" indicates an arsenic treatment.

VI(a) Results: Acute exposure

We first began to evaluate the acute apical exposure of arsenic onto 16HBE14o- cells with a previously established air-liquid interface. We monitored the changes in transepithelial resistance (TER) over three days. On day 1, the highest exposure concentration of arsenic (100 ppb) resulted in a slight, but insignificant loss of TER when compared to the control (arsenic-free) or 10 ppb arsenic containing media.

Experiment 1: 0, 10, 100 ppb dose response

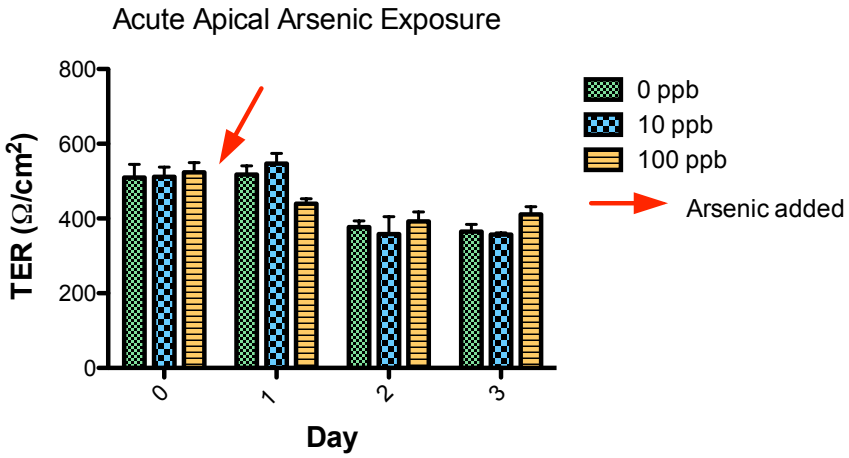

Figure 6: Acute dose response of arsenic applied to the apical domain 16HBE14o- cell monolayers with established TER ($\leq 200\ \Omega \cdot cm^2$) were exposed to arsenic-free (0 ppb) or arsenic supplemented (10 and 100 ppb) HBSS solution at Day 0. The red arrow signifies when arsenic was applied to the filters assigned a 10 or 100 ppb dose.

The next experiment we did was to evaluate a chronic apical exposure of arsenic on 16HBE14o- cells with a previously established air-liquid interface. We monitored the changes in transepithelial resistance (TER) over three days. While there were slight decreases in TER, none of the changes in either concentration were significant when compared to each other or to the control (arsenic-free).

VI (b). Results: Chronic exposure

Exposure 1: 0, 10, 100 ppb dose response

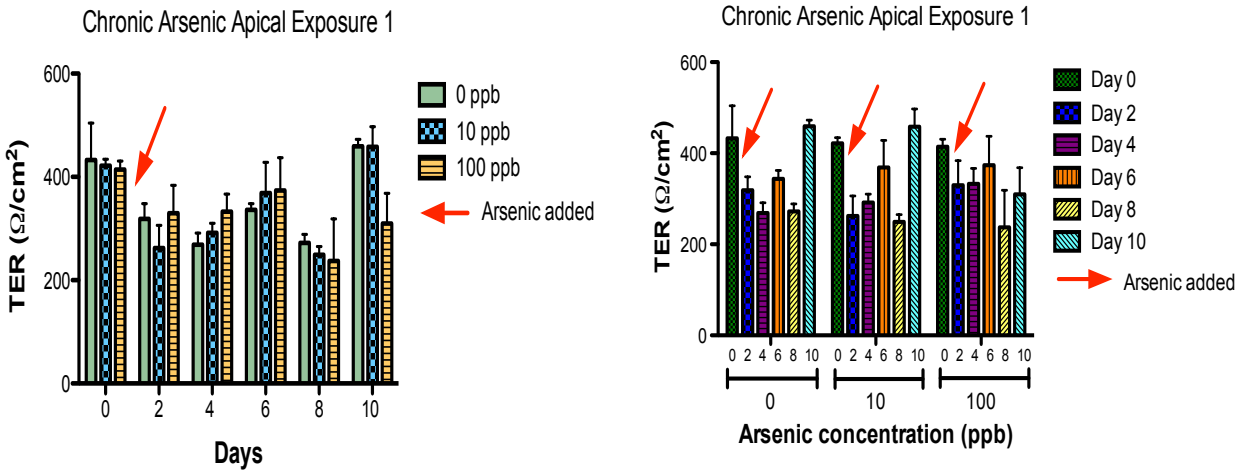

Figure 8: Chronic dose response of arsenic applied to the apical domain (experiment 1). 16HBE14o- cell monolayers with established TER ($\leq 200\ \Omega{\cdot}cm^2$) were exposed to arsenic-free (0 ppb) or arsenic supplemented (10 and 100 ppb) HBSS solution at first at Day 0 and every other day after. The red arrow signifies when arsenic was applied to the 10 and 100 ppb designed filters.

VII. DISCUSSION

Arsenic is an environmental toxicant and has known physiological impacts [2]. The purpose of the experiments described in this thesis project is to build on previous knowledge and more fully understand the underlying mechanism of arsenic toxicity in the conducting airway.

It is thought that through an ingestion route of exposure, negative health outcomes are mediated in part by arsenic's effect on tight junction protein localization and post-translational modification. These effects ultimately result in changes to epithelial barrier function of the respiratory epithelium. Since epithelial barrier health plays a critical role in innate immunity, altered function is at least one mechanism for increased risk of respiratory infection seen with arsenic exposure [10]. Understanding arsenic's impact on lung physiology has critical relevance considering a United Nation's report states arsenic toxicity is the second most important hazard related to drinking water and human health [15]. While ingestion of arsenic via contaminated food and water sources is a large public health issue, inhalation exposure of arsenic is also relevant and contributes to our overall

understanding of arsenic's effects of human physiology.

Inhalation exposure of arsenic can occur in occupational settings or downwind from contaminated mining sites and is thought to have similar detrimental effects on respiratory function [8]. The mechanism for this, however, has yet to be clarified. The experiments in this report work toward determining a suitable model to mimic arsenic inhalation and determine how the respiratory epithelial barrier function is impacted. Specifically, whether these changes are similar to the previous experiments that modeled arsenic ingestion. The quantitative assessment of barrier function is conducted by experimentally measuring transepithelial resistance (TER) in control and dose response arsenic exposed cells. TER has been determined to be an adequate indicator of barrier function and thus, is an important tool in clarifying whether arsenic has compromised the respiratory epithelium. Through an understanding of epithelium physiology, we know that the polarity of the cell monolayer is mediated by tight junction proteins. The presence of tight junctions allows for separation of apical and basolateral surfaces. The ingestion route experiments mentioned previously expose the cells to arsenic from this basolateral surface considering the close proximity to circulatory structures [16]. In the upper respiratory tract, the apical surface of the epithelial monolayer would be directly exposed to inhaled air entering the lungs. The tight junctions provide separation of these two domains and have distinct membrane proteins and cell functions [16]. Thus, while the previous experiments provide insight on a potential impact of inhalation exposure of arsenic, it is important to more clearly understand if the arsenic-induced changes in barrier function are the same compared to basolateral exposure.

The overall trend gathered from data in this thesis suggests that apical arsenic exposure does not incur the same decrease in transepithelial resistance that was found in basolateral exposure. At both dose responses in chronic and acute exposure patterns, there were no significant alterations in TER readings when compared to control. These findings are important in providing a general inclination regarding the effects of arsenic inhalation. However, a continuation of this experiment would be useful to more clearly define the impacts of arsenic on the respiratory epithelium. Potential experiments could model those completed in the Sherwood et. al. paper [10] and include genetic and immunocytochemistry studies. One foundational component of this research is to identify an experimental model which most accurate represents the physiology of the upper airway epithelium. Modifications to the model used could prove useful in achieving this.

In general, it is worth discussing why there were noticeable difference between the basolateral and apical arsenic exposure experiments. We speculate that the lack of response to apical arsenic exposure, as evidenced by no significant changes in TER, may be due to the transport of arsenic into the cell. If arsenic is not entering the cell, there wouldn't be the same epithelial damage. Differences in the transporters that are present on the apical and basolateral membrane could serve as a mechanism for why this occurs.

Combined, the insights gained from both the inhalation and ingestion route experiments will create a more representative picture of how arsenic changes lung physiology at a molecular level. Arsenic exposure in occupational and residential settings is becoming an increasingly relevant area of study. While there has been a previous focus on arsenic

contamination of watersheds, other sources of exposure such as dust, air, and soil have become more prevalent in toxicological research [17]. This has particular applicability in areas in close proximity to former or current mining sites, smelting sites, industrial plants, and designated Superfund areas [17]. Ultimately, a combination of research from multiple disciplines will be most advantageous in determining the overall risk that arsenic poses to the public health. This includes studies of sediment migration, arsenic bioavailable, and systems toxicology. The work conducted in thesis project contribute this body of knowledge by providing a detailed representation of the cellular changes that occur to the upper airway when exposed to environmentally significant arsenic concentrations. Altogether, this research informs preventative and remedial action for public health and policy officials to address the severity of arsenic contaminated sites and implementation of safety measures to avoid occupational risk.

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