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Environmental Factors Influencing Oxidative Stress In Respiratory Cells

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Biographical Sketch

Carresse L. Gerald was born December 18, 1985, in Winston-Salem, North Carolina. She received the Bachelor of Science degree in Animal Science from North Carolina Agricultural and Technical State University in 2008. She received her Master of Science degree in Animal Health Science in 2010 from North Carolina Agricultural and Technical State University. She is a candidate for a doctorate in Energy and Environmental Systems.

Dedication

I would like to thank God for giving me the opportunity to grow and develop as a young woman. This dissertation is dedicated to my daughter, Cana, and my son, Carter. I want to thank my mother Cynthia Cecil, my father Bruce Gerald, my grandmother Mary Cecil, grandfather Cloys Cecil, and my great-grandma Maggie Shepherd. If it wasn't for their continued support, I would not have had this honor.

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Abstract

Agriculture workers inhale a variety of dusts, gases, microbes and compounds on a daily basis. Several respiratory diseases display inflammation and oxidative stress as key factors of pathogenesis. To better understand the effects of agricultural dusts on the airway there first needs to be characterization of dust components. Thus, we hypothesized that confinement facilities contain high levels of respirable particles, bacteria, and elements of respiratory importance. We further hypothesized that SCF dust alone or in combination with diepoxybutane (DEB) will result in oxidative stress and phytonutrients from the sorrel plant will reduce these effects. Several bacterial species were identified via amplification of the 16S ribosomal DNA gene and or biochemical selection, and include Escherichia coli, Listeria sp., Bacillus sp., Staphylococcus and Clostridium species. Each dust extract showed pH altering effects (p-value < 0.05) and resulted in acidic changes with the exception of small ruminant dust (alkaline). Respirable particles (< 10 μm) were found in dairy and poultry dusts. More inhalable particles were found in swine, small ruminant and equine dust samples. Exposure to swine dust extract increased intracellular hydrogen peroxide, 8-isoprostane and nitric oxide levels in NHBE cells in vitro and pretreatment with sorrel prevented such increases (p-value<0.001, p-value<0.001 and p-value<0.05 respectively). Elucidating the mechanism of agricultural dust mediated oxidative stress, 'dust+DEB' mediated oxidative stress and agriculture-related inflammatory airway diseases will provide insight for better understanding of respiratory diseases caused by chronic exposure to CAFO-like facilities and development of improved animal management practices to ultimately decrease the incidences of respiratory disease.

CHAPTER 1

Introduction

Agriculture workers are prone to develop respiratory illnesses associated with acute and or chronic inhalation of dusts and are similar to chronic obstructive pulmonary diseases (COPD). However, many of the symptoms do not easily fit within a particular disease category. Example respiratory diseases include asthma-like syndrome, chronic bronchitis and farmer's lung. These diseases are associated with a high concentration of free radicals in tissues such as asthma. Our lab has established that oxidative stress occurs in airway cells exposed to swine confinement facility dust in vitro (Gerald et al, 2010). In addition to dust exposures, a high prevalence of agricultural workers smoke, (do Pico, 1996) and tobacco smoke increases the free radical concentration within the airway (Borgerding & Klus, 2005). Butadiene is a byproduct of petroleum factories and is present in tobacco smoke (Yadavilli & Muganda, 2004; Brunneman, Kagan, Cox & Hoffman, 1990). Published reports state that there is oxidative stress and DNA damage in lymphocytes exposed to a butadiene metabolite, diepoxybutane (DEB) (Yadavilli et al, 2007). However, to our knowledge no studies have examined the impact of DEB on the airway epithelia in combination with dusts. Many plants have been said to have increased antioxidants and thus have the ability to protect against free radicals and reduce the effects of oxidative damage. Antioxidants bind to free radicals preventing them from causing damage by binding to cellular components. *Hibiscus sabdariffa* (sorrel) is a plant that exhibits the ability to ward off oxidative stress. Recent studies in our lab have supported this claim (Gerald et al, 2013). Agriculture workers are exposed to many variants of dusts which can potentiate airway inflammation. These symptoms are similar to common airway diseases such as asthma and chronic bronchitis but also vary. Smoking is also a key contributor to respiratory diseases by the

addition of free radicals leading to oxidative stress. The goal of this study is to fill in gaps in the knowledge base/published literature on oxidative stress mediated respiratory disease and to evaluate novel approaches for preventing agriculture-related respiratory diseases.

The central hypothesis is that agricultural workers exhibit respiratory complications due to oxidative damage mediated by environmental factors (i.e. agricultural dusts and cigarette smoke chemicals) and that sorrel extracts can relieve these symptoms. The rationale for this study is based on preliminary studies in our lab that show environmental factors may mediate oxidative stress in airway epithelia and sorrel plant extract can decrease oxidative stress in this tissue. Through *in vitro* studies with respiratory cells (and lymphocytes) we have shown that exposure to agricultural dusts from animal husbandry units modulate genes and proteins responsible for controlling oxidative stress responses and pretreatment with sorrel extracts reduce these effects. While the significance of these changes in respiratory cell dynamics is not know at this time, the work summarized here is expected to provide insight for understanding the exposures experienced by agricultural workers and the mechanisms governing associated cellular and molecular processes.

CHAPTER 2

Literature Review

Various agricultural rearing strategies are dependent on type of production and animal species, but are usually broken up into three main categories: confinement, semi-confinement and pasture based. The dust that accumulates over time in each type of facility is complex due to the rearing strategy, animal species and many other various factors. The prevalence of respiratory illness is in part due to the variations between management systems. However, there is difficulty in distinguishing between symptoms, which make the disease harder to treat. To further complicate the respiratory nature of agriculture workers is the fact that more than half are reported as cigarette smokers (do Pico, 1996). Cigarette smoke's complexity can also exacerbate previous conditions which can perplex medical professionals on how to diagnose and treat respiratory complications. Our interest is in investigating a more holistic approach to prevention, that includes natural ways to combat oxidative stress which is common to both agriculture dust mediated airway disease and cigarette smoke laden airways.

2.1 Animal Housing and Rearing Strategies

Agriculture is a main staple to the world. Meat production (beef and veal, pork, and chicken) for the world was 236,537 (thousands of metric tons) and meat consumption was 234,624 in 2010 (Census, 2012). North Carolina is the second largest hog producer with 25% (of gross farm receipts) and poultry producing state in America with 36.9 % (turkeys and broilers) of US value respectively (United States Department of Agriculture Census of Agricultural, 2007). Rearing styles vary depending on species and production (small-scale or grand-scale). Due to the demand of swine and poultry, a confinement system is usually more efficient. Confinement systems allow animals to be grown faster, stocked densely and protected

from many environmental factors such as diseases and weather extremes. Semi-confinement facilities that house ovine and caprine species (small ruminant), cattle (dairy), and horses are less constrained compared to the confinement practices. Semi-confinement animals have the option of having an inside and outside component. The air quality is less concentrated compared to the confinement facilities which are often faced with poor ventilation and an accumulation of dust. However, many outside factors can influence prevalence of disease (parasite infection) and weight gain.

Despite successes garnered from concentrated animal feeding operations (CAFOs) they typically have a bad reputation and may have a negative impact on the environment because the waste can be a burden on surrounding neighborhoods. CAFOs are operations where animals are kept and raised in confinement facilities on a relatively small amount of land. In CAFOs, feed is brought to animals as opposed to eating on free range pastures. These types of operations are mostly utilized in food-animal companies in order to produce more animals and associated commodities in a relatively short amount of time. The limited amount of space aids in higher production and higher weight gain in CAFO-maintained animals. In the U.S., the most common food animals maintained CAFOs are swine and poultry. Methane and ammonia are common gases that plague both indoor and outdoor animal rearing environments; however, the levels of such gases are often intensified by confinement housing approaches. For Swine, manure pits sit underneath animals and the waste the animals produced is collected there. Gases that are formed in the pits include hydrogen sulfide, ammonia, methane and carbon dioxide (Spurzem, Romberger,Von Essen, 2002). In the U.S. the amount of animal waste (i.e., urine, manure and carcasses) produced by CAFOs is 130 times more than human waste (United States Senate 1997).

The environmental impact exerted by animals reared in pasture-based operations is not much better. Pasture raised dairy and beef cattle are fed high fiber diets which leads to an increase of methane emissions compared to feed practices in confinement facilities which results in less methane generation (Siegford, Power & Grimes-Casey, 2008). Pasture-based rearing strategies are known to support the normal behavior of the animals but animals can be exposed to pathogens and the environment can take harsh assaults. However, disadvantages to this management practice include increase of parasitic infections (Roepstorff and Murrell, 1997), soil erosion due to animal behaviors such as rooting and little to no control over environmental conditions (e.g. temperature and weather).

In the study conducted by Gustafsson (1999), three piggeries for growing and finishing pigs were accessed for the settling rate of dust, ventilation rate and dust suppression. From this work, it was noted pig number and weight have an impact on dust generation and dust concentrations are usually higher in the daytime than at night as expected. However, it was stated that ventilation rates were highly limited because the concentration of the dust affects the settling rate. The dust levels were decreased dramatically in the piggery defined as cold climate, uninsulated, with straw bedding and natural ventilation when compared to the climate control insulated piggery. Also noted from this study, was the ability to reduce dust loads by spraying water or a water-oil combination. There are so many varying factors that can influence the dust accumulation in a livestock or poultry facility. In conjunction, there are several practices animal husbandry facilities can utilize to reduce the risks of dust inhalation.

2.2 Agricultural Dusts

Agricultural dusts tend to be a very complex mixture of grain/feed particles, microbial products (endotoxins), hair and skin cells (animals and humans), feces, a variety of gases, metals

and other components. Odorous emissions from agriculture waste have raised concerns regarding CAFOs for many years (Esteban, 1997). In a study done by Schiffman and colleagues (2001) the gaseous compounds from swine facilities were analyzed via GC/MS, and they found 167 different compounds including but not excluding alcohols, acids, aromatics, ethers, nitrogen-related compounds and phenols. It was also reported that odors can be smelled past 1000 ft of swine houses. During a typical day's work, farmers that work in animal confinement facilities may be exposed to inorganic substances, food grains, organic dusts (containing bioaerosols such as fungi, bacteria or their components) as well as gases (e.g. ammonia) and chemicals (e.g. pesticides, disinfectants). Many airborne pathogenic microorganisms, including viruses and bacteria, can be transmitted great distances through the airflow (Jones & Harrison, 2004; Brown & Hovmoller, 2002; Shinn, Bing-Canar, Cailas, Peneff & Binns, 2000).

In Roy and colleagues (2003), bacterial DNA was found more in farm barns and farm houses than urban houses. Bacteria can potentiate respiratory symptoms and illnesses in human workers. *Bacillus anthracis* is a gram positive rod-shaped bacterium that is also a spore former. When the conditions are not favorable to the bacteria to grow they become dormant and commence into a spore. These spores can be inhaled, ingested and absorbed through lesions in the skin. Most of the time, anthrax infections will resolve themselves, however there have been occasions where anthrax can be fatal. One study has shown that the endospore of *B. anthracis* interacts with some free radicals to maintain in hostile environments in its normal environment. In Baillie et al, 2005, it is reported the *B. anthracis* endospore possesses its own antioxidants SOD and catalase, to combat free radicals such as hydrogen peroxide and superoxide. Furthermore, this study also states that the endospore shows no negative side effects once exposed to superoxide. These key factors also contribute to virulence that can target

macrophages because endospores need to be phagocytized before they can germinate (Ireland & Hanna, 2002; Weiner & Hanna, 2003). The gram positive bacteria *Listeria monocytogenes* can thrive in a variety of environmental conditions (Gandhi & Chikindas, 2007). Listeriosis is a rare food borne illness but can be quite severe causing central nervous systems problems such as meningitis, septicemia and abortion (Carpentier & Cerf, 2011). It is usually found in dairy products like soft cheeses, in meat, poultry and in raw milk (CDC, 2013). Salmonella is of importance to the pork industry because it is the second leading foodborne illness. Salmonellosis costs 80.3 million dollars worldwide annually. It is also the leading cause of hospitalization and death (Majowicz et al, 2010; Scallan et al, 2011). Swine (and other mammals) and avian sources are known carriers of Salmonella. *Escherichia coli* are common gram negative bacteria found in the intestines of mammals. Cattle are reservoirs of the strain *E. coli* O157:H7 (Pruimboom-Brees, Morgan et al, 2000). This particular strain is known to have been the causative agent in sporadic cases and outbreaks since the 1980s, with the source of the bacterium linked back to cattle (Laegreid, Hoffman et al, 1998). Clostridium species such as *C. botulinum* have the potential to create foodborne illness as well. These gram positive bacteria normally dwell in soil and are spore-formers similar to the bacillus group. Interestingly, *Clostridium* botulinum possesses the deadliest toxin in the world (Schantz and Sugiyama, 1974).

In addition to the bacteria themselves, toxic bacterial components can also be found in agriculture dusts. Commonly found components are endotoxins, which are part of the outer membrane of gram negative bacteria (Schierl et al, 2007). Lipopolysaccharide is the infectious portion or endotoxin of the membrane that induces a cellular response or causes disease. In animal houses, the major contributors to endotoxin-contaminated organic dusts are animal feces and bacteria-contaminated plant materials (Schierl et al, 2007). Endotoxins are potent inducers

of neutrophilic airway inflammation and are thought to be a major risk factor for adverse health effects of the airways among farmers (Mathiesen, Von Essen, Wyatt & Romberger, 2004; Schierl et al, 2007). In particular, microorganisms, or their fragments, can be passively or actively released into the air in response to the changes of the hygienic situation of animal houses and meteorological conditions (Charavaryamath & Singh, 2006). The majority of dust-borne fungi in agricultural settings are of the *Aspergillus* species. Typical amounts of aerosols inhaled are 10^4 to 10^7 CFU/ m^3 of bacteria and 10^3 to 10^6 CFU/m^3 of fungi (Kirkhorn, 2002).

Although there are numerous epidemiological reports summarizing the respiratory effects associated with inhalation of agricultural dusts by farmers, fewer, if any, reports explore the molecular effects on the airway epithelium. Thus, it is intriguing to characterize agricultural dusts from animal husbandry units and evaluate which components have a negative impact on the airway epithelium.

2.3 Respiratory Pathophysiology

The respiratory system consists of two parts: the conducting portion and respiratory portion. The conducting portion is comprised of nasal cavities, nasopharynx/oropharynx, larynx, trachea and two primary bronchi. The above mentioned passages lead to the gas exchange region within the lung. Inside the lung space, the primary bronchi are divided into smaller branches called bronchioles. This is also known as the bronchial tree. The bronchioles are at the last remnants of the conducting portion. The respiratory portion is the location of gas exchange and is one of the respiratory system's main functions, along with air conduction and air filtration. The respiratory portion consists of respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli.

Conditioning must occur before air reaches the gas exchange region. This happens in the conducting areas via warming, moistening and removal of debris. Any particulates not stopped by the nasal cavity will be trapped in mucous in the conducting portion of the airway. The mucous blanket that covers the trachea, bronchi and other parts of the conducting passages also serves as a protectant against dehydration from the inhaled air. Cilia are specialized hair-like projections that undulate to coordinate a sweeping motion known as mucociliary clearance. This allows for expelling the mucus by moving up the respiratory tract or expelling the mucus by moving it down the esophagus so that it can be neutralized by the acids in the stomach. Mucociliary clearance is regulated by the free radical, nitric oxide (Jain, Rubinstein et al, 1993).

The trachea is a short flexible extension of the larynx and its walls condition the inspired air. There are four layers of the trachea: mucosa (ciliated pseudostratified epithelium and elastic fiber-rich laminia propia), submucosa (denser connective tissue than the lamnia propia), cartilage (C-shaped hyaline cartilages), and adventitia (connective tissue). The morphology of the trachea is contributed largely to the C-shaped hyaline cartilages that are stacked on top of one another; providing support and protection against tracheal collapse.

There are several types of cells in the ciliated pseudostratified columnar epithelium of the conducting system: goblet, ciliated, basal, brush and small granule cells. The main cell types in tracheal epithelium are the first three types mentioned. Goblet cells synthesize and secrete mucus and function as progenitor cells that can differentiate into simple columnar cells when exposed to external toxins. Mucous cell numbers increase during chronic irritation of the airway. Increasing amounts of toxins over time can lead to a condition known as goblet cell metaplasia which is known to be a precursor to cancer. Ciliated cells are the most prominent cell type in the trachea. Cells are equipped with cilia, short hair-like structures on the apical surface. Goblet

cells and ciliated cells work in concert to produce the mucociliary escalator that removes inhaled particles from reaching the lungs. Basal cells are progenitors of other cell types. These small rounded cells lie close to the basal lamina. They do not possess many organelles, which is typical of stem cells.

The two branches that follow the trachea are known as the main bronchi (right and left). Each bronchus enters the lung and separates out into lobar bronchi. These branches support a lobe of the right (three lobes= three branches) and left lung (two lobes = two branches). Further branching extends into eight bronchopulmonary segments (left lung) and ten bronchopulmonary segments (right lung). This branching continues, coinciding to the number of segments for each lung.

The bronchi and trachea share the same histological structure until the bronchi enter the lung. The structure of the wall transforms from cartilage rings into an amorphic shape. The shape of the bronchi began to look circular which is a distinct difference from the tracheal morphology. The cartilage decreases as the bronchi are reduced in size. Smooth muscle develops to form a layer around the wall as cartilage disappears. Once the branches become 1 mm in diameter, the branch is known as a bronchiole. The bronchi can be divided into five layers: mucosa (pseudostratified epithelium), muscularis (continuous layer of smooth muscle), submucosa (relatively loose connective tissue), cartilage layer (discontinuous cartilage plates) and adventitia (moderately dense connective tissue).

Bronchioles measure less than 1 mm in diameter and continue to branch from the segmented bronchi to the terminal bronchioles to the respiratory bronchioles. Cartilage and glands are not absent in the bronchioles. Larger bronchioles have ciliated, pseudostratified columnar epithelium as well as goblet cells, but as they narrow, the cell type slowly develops

into a simple ciliated columnar epithelium and goblet cells disappear. A thick smooth muscle layer is found in the wall of the bronchioles. The terminal bronchioles are the smallest conducting bronchioles and histologically are lined with cuboidal epithelium. In the terminal bronchioles, clara cells are seen to increase in number as ciliated cells decrease. Clara cells do not possess cilia and appear to be rounded on the apical surface. These cells secrete proteins, clara cell secretory protein (CC16), and lipoproteins, however when lung injury is present the amount of proteins secreted decreases due to cell injury. These proteins help to protect the bronchioles. In a study by Elizur and colleagues (2007), the authors determined that clara cells (murine clara cell line, C22) impact macrophage recruiting cytokines TNF-α post-exposure to lipopolysaccharide (LPS) *in vitro* and *in vivo*. Other cell types found in the bronchioles are brush cells and small granule cells. Respiratory bronchioles are dual functioning, participating in air conducting and gas exchange (the first of the bronchioles). They are lined by cuboidal epithelium (ciliated and clara cells) and have narrow diameters.

Alveoli are the terminal sites of gas exchange between air and blood and are an expansion of the respiratory bronchioles. Microscopically, the alveoli appear to be scattered and thin-walled outpocketings. The alveoli are encompassed by capillaries that bring the blood close to the inhaled air inside the alveoli. Alveolar ducts are elongated airways that have hardly any walls and their periphery is ringed of smooth muscle. Alveolar sacs are usually found near the end of the alveolar duct. The epithelium present in the alveolar region consists of Type I and Type II alveolar cells. Type I cells are very thin squamous cells and line the majority of the alveoli. Type II cells are cuboidal secretory cells and are as plentiful as Type I cells; however, due to their cell morphology they only cover 5% compared to 90% (Type I cells). Type II cells can secrete an alveolar lining surface-active agent, surfactant. Following lung injury, Type II

cells are known to proliferate and can be classified as hyperplasia of Type II cells marking alveolar lung injury and repair. Surfactant can actively clear out foreign materials in the alveoli (Wright, 2003). Another common cell type found in the alveoli is alveolar macrophages. These immune cells reside within connective tissue and the air space of the alveolus and can peruse the surface to remove any inhale particulate matter such as dust and pollen. Autopsies performed on people dwelling in urban areas and on smokers manifest macrophages post-engulfment of carbon particles and silica particles. Our lab has also conducted *in vivo* murine dust exposure studies and observations from those studies showed that chronic inhalation exposure to swine confinement facility dust for eight weeks enhanced phagocytosis and vacuolation by lung macrophages (Pender et al, 2011).

Size of airborne particles can not only predict how many particles may enter into the respiratory system but also where it is deposited in the airway. Upper airways can harbor micro-sized particles and nanoparticles are transported via diffusion. Recent studies show that ultra-fine particles (<100 nm) can exhibit more severe inflammation than larger sized particles of the same material (Donaldson, Li,& MacNee, 1998; Frampton, 2001; Oberdörster, 2001;Oberdörster, Ferin, & Lehnert, 1994). Caveolae are found prominently on alveolar type I cells and pulmonary capillaries cell membranes. These cave-like structures have a main function in endocytosis and can transport nanoparticles from the lungs and blood (Oberdoster et al, 2005; Rejman et al, 2004). Deeper areas of the lung can also be targets for micro and nano-sized particles. Due to low surface tension of surfactant secretions, micro and nano sized particles can easily transfer to the liquid wall layer (Gehr, Schurch, Berthiaume, Im Hof, & Geiser, 1990).

2.4 Agriculture-Related Respiratory Diseases

Agriculture work can potentiate illness and injury. In Alterman et al (2008), it was reported that farm workers have high prevalence of the following symptoms: running nose/watery eyes (34.3%), hay fever and sinus problems (32.1%), shortness of breath (13.7%) and chronic bronchitis (3.8%). There are millions of agricultural workers at risk for pulmonary diseases, including veterinarians, managers, inspection and processing workers and many others (do Pico, 1996). They can work with a wide variety of chemicals and substances daily such as pesticides, grain, feces, bodily fluids (i.e. milk). Some of these substances contribute to agriculture lung diseases, include grain particles, fungi, bacteria, viruses, silicates, pesticides, nitric oxide and ammonia (do Pico, 1996).

As previously stated, agriculture workers manifest a complex overlapping group of respiratory disorders including acute bronchitis, chronic bronchitis, asthma, interstitial disease and acute lung injury (Spurzem et al, 2002). Wyatt and colleagues (2008) found that hog barn dust can impact the normal stimulation of cilia in bovine ciliated cells. This can lead to defective mucociliary clearance and particulates not being excreted out of the airway efficiently. In Mathisen et al (2004), it was observed that hog barn dust can facilitate lymphocyte adhesion to human airway epithelia in a time and concentration dependent manner *in vitro*. This knowledge supports the fact that swine dust can mediate an immune response via ICAM-1 (intercellular adhesion molecule) and PKC-α (protein kinase C) expression. Romberger et al, 2002 reported that hog barn dust stimulate cytokines IL-8 and IL-6 in human bronchial epithelial cells *in vitro*. This explains why some persons exposed to swine dust/agriculture dusts manifest neutrophilia (increased number of neutrophils), because IL-8 is a recruiter of neutrophils. IL-6 is a pro-inflammatory cytokine which has been shown to exacerbate blood and airway secretions (Wang

et al, 1997; Wang, Malmberg, ER, Larsson & Palmberg, 1999). Pro-inflammatory mediator COX-2 is expressed in human bronchial epithelial airways that have been exposed to particulate matter (Zhao et al, 2009). Zhao and colleagues also observed a release of IL-6 in this same study.

Farmer's lung disease (FLD) is a form of occupational respiratory disease that is common in agricultural workers due to chronic exposure to a high volume of grain particles, microorganisms and gaseous compounds (Gbaguidi-Haore, Roussel et al, 2009). Some signs and symptoms manifested in farmer's lung respiratory disease are dyspnea, fever/night sweats, tiredness, coughing, headaches and general sickness (Gbaguidi-Haore, Roussel et al, 2009). This disease is dependent upon climate; especially in areas of heavy rainfall and colder temperatures because wet hay and feed will be stored inside, increasing the likelihood of growth and inhalation of mold (Spurzem et al, 2002).

The most common occupational lung disorder is asthma in workers which spans a variety of industries and occupations (Chan-Yeung & Malo, 1995). This disease manifests as airflow limitation and bronchial hyper-responsiveness due to certain work environments. Occupational asthma may be caused by pollens and animal dander because they have high molecular weight antigens. Storage mites and yeasts are commonly found in agriculture housing since the agricultural environment is a fertile ground for these types of organisms. There is an array of potential irritants that could mediate asthma in an agriculture environment such as endotoxin and ammonia. Asthma-like syndrome does not necessarily need a predisposition to IgE-mediated inflammation. Healthy people are known to undergo bronchial hyper-responsiveness without prior exposure to stimulants. Vogelzang and colleagues (1998) conducted a study in which 171 swine workers were followed for 3 yrs. They noticed that an annual decline in FEV_1 was

significantly associated with endotoxin exposure. They also noted swine confinement workers have an increased risk of the bronchial hyper-responsiveness. According to a meta-analysis on health associations between asthma and lung cancer (Santillan et al, 2003), asthma may increase the risk of lung cancer by reducing the clearance of toxins and carcinogens in the bronchoalveolar epithelium.

Acute and chronic bronchitis are suggested to be high in prevalence in farmers, many of whom also have a decreased in lung function. The most common respiratory disease is chronic bronchitis in grain elevator workers and grain and animal production workers (Frank, Mcknight, Kirkhorn, Gunderson, 2004). About 3-30% of nonsmoking general farming population experience chronic bronchitis. Alterman et al (2008) reports 3.8% of farm workers suffer from bronchitis. Also from this study, workers exposed to grain dust, swine confinement facilities and poultry houses have a higher risk for developing chronic bronchitis. Furthermore, non-smoking, swine confinement facility workers had a prevalence of 25% compared to 12% in other farm workers. Also noted, swine and poultry producers are more likely to exhibit a decrease in lung function. Organic dusts have the ability to mediate bronchitis. Endotoxins, plant particles, soil particles and inorganic dusts may have a role in disease manifestation. Chronic bronchitis is also a risk factor for lung cancer among non-smokers (Santillan, Carmago & Colditz, 2003). Thus it is important to understand which environmental factors such as cigarette smoke and dust from agricultural settings are contributing to respiratory illnesses and disease.

2.5 Cigarette Smoke and Respiratory Disease

do Pico, 1996 reported that 71% of farm workers who reported respiratory illnesses were also smokers. Tobacco smoking is a common habit worldwide and contributes greatly to indoor inhalable particulates (Paoletti De Berardis, Arrizza, Granato, 2006). Tobacco smoke has three

distinct categories: mainstream, sidestream, and environment tobacco smoke (ETS). Mainstream smoke is the smoke from the mouth end of the cigarette during a puff, while side stream smoke is smoke from the lit end of the cigarette (aka second hand smoke). They both can diffuse into the air and become diluted via physical and chemical changes to create environmental tobacco smoke.

Cigarette smoke is a complicated mixture which has particulate and gaseous phases and is comprised of over known 4000 constituents. The particulate phase consists of carbonaceous particles, heavy metals and aromatic hydrocarbons. The most abundant gas is carbon monoxide, along with others such as benzene and formaldehyde (Paoletti et al, 2006). Butadiene, a byproduct of fossil fuel combustion, has also been found in tobacco smoke at 4 ng/puff (Thweatt, Harward Sr et al, 2007). Many of these components are also free radicals which will to be discussed in detail later. Thweatt and colleagues (2007) also noted 1 $X10^{16}$ alky- and alkyoxy-radicals have been found in the vapor phase of one cigarette, which is equivalent to 5 $x10^{14}$ radicals per puff. Normally these radicals would have an extremely short halflife of only about a fraction of a second but, in cigarette smoke; free radicals have been known to have half-lives of five minutes (Borgerding & Klus 2005).

Smoking can increase the risk of bronchitis six-fold and may result in potential synergistic effects with agriculture respiragen exposure (Melbostad & Eduard, Magnus, 1997). In the study conducted by Glader et al (2005), the authors noticed an increase of IL-8 and MUC5A in differentiated NHBE after being exposed to a cigarette smoke extract at 15 and 30%. IL-8 is a cytokine responsible for white blood cell recruitment. Sadowska and colleagues (2007) demonstrated the activation of cytokines and transcription factors such as IL-8 and NF-κB post-cigarette smoking and subsequent oxidative stress. MUC5A is responsible for producing mucins

and is known to be implicated in respiratory diseases via mucus hypersecretion. The cigarette smoke component, butadiene, can particularly contribute to toxicity.

2.6 Butadiene's Toxicity on Tissues

Butadiene (BD) is a known toxic chemical classified as a mutagen, a carcinogen and a major contributor to air pollution. This chemical is produced directly, or as a byproduct, by many synthetic rubber-producing factories for automobile tires, plastics and polymers (Melnick and Sills 2001). The population of BD exposed persons typically is comprised of workers in these industrial factories. However, BD can be found in automobile exhaust and cigarette smoke (Adam et al, 2006; Grant, Leopold et al, 2007). The National Toxicology Program has 1, 3-butadiene classified as a human carcinogen. The toxic effects of this chemical are known. However, the underlying molecular mechanisms are not fully understood and metabolites of this chemical are known to be the main contributors to butadiene's toxicity. Metabolites include 1,2-epoxy-3-butene (EB), 1,2-3,4 diepoxybutane (DEB) and 1,2-dihydroxy-3,4 epoxybutane (EBD) (Yadavilli, Martinez-Ceballos et al, 2007), with the most potent being DEB (Kligerman and Hu 2007). DEB has been shown to produce forms of reactive oxygen and nitrogen species (RONS), such as hydrogen peroxide, and to damage DNA (Erexson & Tindall, 2000). DEB is known to be cytotoxic but all the mechanisms in this pathway have yet to be uncovered.

The metabolite DEB has been shown to cause cellular death in TK6 lymphoblasts by means of apoptosis and not necrosis (Yadavilli and Muganda, 2004). Apoptosis, also known as programmed cell death, is the body's way of cleaning up nonfunctional cells without creating a disturbance. Studies have shown that oxidative damage can play a vital role in apoptosis (Curtin, Donovan et al, 2002). The mediators of the apoptosis pathway upon exposure to DEB in lymphoblasts were defined as being oxidative stress (Yadavilli, Martinez-Ceballos et al, 2007).

DNA damage/oxidative stress occur in lymphoblasts exposed to the butadiene metabolite DEB (Yadavilli, Martinez-Ceballos et al, 2007). Also noted was the ability of antioxidants Tiron and N-acetyl cysteine (NAC) to prevent DEB-induced apoptosis. This indicates that oxidative stress is involved in apoptosis because antioxidants have the ability to bind free radicals and therefore relieve the cells of those stressors. These findings show that DEB induced apoptosis was significantly reduced as compared to cells that were not exposed to the antioxidants, Tiron and NAC. These reports propose a correlation between oxidative stress and DEB-induced apoptosis. In the present study, DEB will be used at levels similar to those who smoke. We are linking the DEB exposure to similar effects as it would occur in agriculture workers who smoke. This could elucidate some of the mechanisms of respiratory diseases that occur in agricultural workers as determining if there are common disease elements such as oxidative stress. This work may provide a means to further characterize oxidative stress that occurs in lymphocytes as well as other cell types such as airway tissue like normal human bronchial epithelium and A549 lung cancer cell lines.

As stated previously, cigarette smoke is a habit worldwide and a large population of agricultural workers smokes cigarettes. Cigarette smoke can mediate lung diseases and lung cancer. Tobacco smoke can lead to chronic obstructive pulmonary disease (COPD) (Haswell, Hewitt et al, 2010). According to Ohta and colleagues (1998), IL-8 was decreased in alveolar macrophages exposed to cigarette smoke. IL-8 is a cytokine usually expressed in inflammation and functions to recruit other inflammatory cells. Without IL-8, negative conditions can be prolonged and increase the risk of disease. Cigarette smoke can also activate transcription factor NF-κB (Hasnis, Bar-Shai, Burbea & Reznick, 2007). do Pico and colleagues (1996) reported that 71% of farm workers who reported respiratory illnesses were also smokers. Chronic

bronchitis is not only common in farm workers but in cigarette smokers as well. Cigarette smoking has been noted as a major risk factor for lung cancer, with approximately 80% of lung cancer deaths being due to tobacco smoke *(*Cancer Facts & Figures 2012). Smoking also can contribute to the exacerbation of current respiratory diseases. With the vast collection of literature on the deleterious effects of cigarette smoke on the lungs available, we would like to investigate the impact of combinatorial effects of exposure to chemicals and or biological factors present in agricultural dust and cigarettes on the respiratory system, particularly the airway epithelium.

2.7 Oxidative Stress and Tissue Damage

Radicals of oxygen have important roles in physiological processes including cell signaling and pathogen killing as part of the immune system. RONS can have beneficial effects especially in low/moderate concentrations against infectious agents (Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006; Sugiura & Ichinose, 2008; Bowler & Crapo, 2002). Excessive levels of free radicals can exert pathological effects in the body at the molecular, cellular and tissue/organ levels. To protect against damage, the body must maintain a balance between oxidants and antioxidants. However, once the level of free radicals is higher than antioxidants, oxidant stress occurs. Oxygen- nitrogen derived free radicals are reactive molecules with well-established roles in cell injury. They are responsible for airway inflammation in asthma, airway hyper-responsiveness, and airway remodeling in animals and humans. These unstable chemicals cause damage by binding to nucleic acids, proteins and lipid membranes. These radicals are normally produced in cells during mitochondrial respiration, energy generation and apoptosis in phagocytes (endogenous). However, there are external sources that can contribute to free radical inside of the body (exogenously) such as particulate matter, microbes and tobacco smoke. Free

radicals are chemicals that do not have a full outer valence orbital. The presence of unpaired electrons gives the molecule the ability to readily bind to other substances so that they overcome their electron deficiency. Oxygen $(O_2)^-$ derived free radicals include: superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-). By losing electrons, these three species are ready for optimal binding (Day & Suzuki, 2005).

Nitrogen-derived reactive species are produced enzymatically from the oxidation of L-arginine into nitric oxide (NO) by nitrogen oxide synthases (NOS). Nitrogen species are also known to bind with oxygen free radicals to produce other free radicals. An example includes peroxynitrite ($ONOO^-$). This unstable anion is known to permeate cell membranes (Waterman & Adler, 2008). An important reactive nitrogen species studied in this work is inducible nitric oxide synthase (iNOS). It is one of three NOS isoforms (nNOS, eNOS and iNOS) found in mammalian systems and is the only inducible form. Nitric oxide has a dual role in oxidant stress and as a pro- and anti-inflammatory mediator. Inducible NOS is stimulated by inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL-1β) and interferon (IFN)-γ, all of which are regulated by the transcription factor NF-κB. Further, the promoter region of iNOS contains binding sites for NF-κB. In asthmatics, iNOS has been shown to be important in airway inflammation and remodeling (Prado et al, 2006). Physical loss of epithelial lining cells is considered one proximate cause of the airway hyper-responsiveness to inhaled mediators (Comhair et al, 2005). Post-stimulation iNOS generates NO for long periods of time in large quantities. However, normally these free radicals are removed rapidly before they cause dysfunction and necrosis (Comhair, Erzurum, & Serpil, 2002).

In the airways of animals and humans, antioxidants counterbalance the free radical concentration, preventing oxidant stress. Antioxidants are molecules that can prevent oxidant

stress by binding to free radicals and precluding them from interacting with lipids, proteins or DNA molecules. They degrade the free radicals down to less harmful compounds such as hydrogen peroxide, water and diatomic oxygen. Some of the major enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidase and catalase (Suguira & Ichinose, 2008; Bowler & Crapo, 2002). There are also non-enzymatic sources of antioxidants, such as vitamin C, vitamin E and glutathione which may be present in dietary supplements. When tissues have been depleted of antioxidants, or the individual is experiencing an infection (i.e., respiratory diseases), the levels of oxidants rise over the levels of antioxidants and can result in RONS damage to the tissues. There is evidence that shows that after exposure to inhaled toxins mammalian airways can alter antioxidant/oxidant homeostasis (Stringer, Freed et al, 2004).

The impact of RONS on airway tissue includes contraction (and relaxation) of airway smooth muscle, fibroblast activation, hypersecretion of mucus glands, cell injury and pro-inflammatory mediators production in epithelial cells (Sugiura & Ichinose, 2008). There are two main types of oxidants: endogenous and exogenous. Endogenous sources are from inside the individual and include phagocytic apoptosis which would be high in swine already infected with diseases such as PRRSV and PRCV. Exogenous sources come from the individual's external environment, for example LPS from excreted feces in swine confinement facilities. Evidence suggests airway inflammation in asthmatics is due to RONS levels that exceed antioxidants (Sugiura & Ichinose, 2008). Oxidative and nitrative stress leads to a loss of superoxide dismutase activity and to downstream events that are characteristic of asthma, including apoptosis, shedding of the airway epithelium and hyper responsiveness (Comhair et al, 2005). Studies in animal models have shown that an increase of oxidant levels correlates with an increase in the production of mucins (Adler & Li, 2001), the protein component of mucus. Airway epithelial

cells can undergo aberrations in membrane permeability and lead to malformations in DNA and protein.

Other key oxidant stress pathway members in this study include Heme oxygenase decycling 1 (Hmox1 gene or HO-1 protein) and Cyclooxygenase (COX; also called prostaglandin g/h synthase). These enzymes have two distinct functions in oxidant stress. Hmox1 is the gene responsible for encoding heme oxygenase (HO-1), a potent anti-inflammatory and anti-oxidant stress enzyme that facilitates heme catabolism (Rahman, Biswas, & Kode, 2006). There are three isoforms of heme oxygenase (HO-1, HO-2 and HO-3); the inducible isoform, HO-1, is investigated in this project because it has been shown to be induced in studies using *in vitro* and *in vivo* models of airway oxidant stress (Lim et al, 2000 ;Hisada, Salmon, Nasuhara & Chung, 2000; Almoiki et al, 2008). Cyclooxygenase is an enzyme responsible for converting arachidonic acid into several types of inflammatory mediators including prostaglandins and leukotrienes (Szczeklik & Sanak, 2002; Gylifors, 2007). Both of these classes of molecules have been associated with upper and lower respiratory diseases. There are two isoforms of COX: COX1 which is constitutively expressed and COX-2, an inducible type. It is active in promoting inflammatory reactions and is associated with various inflammatory diseases such as arthritis and asthma in humans (Waterman & Adler, 2008). Therefore these genes make good candidates for studying similar effects in porcine airway cells *in vitro*. These pathways may provide insight for understanding porcine airway diseases such as PRRS and PRCV.

It is well-established that oxidants are associated with airway diseases such as COPD, asthma and chronic bronchitis; we propose that free radicals mediate airway inflammation and stress which contribute to onset and exacerbation of such diseases. Certain respiratory diseases,

such as asthma, are associated with a high concentration of free radicals in tissues. Previous studies in our lab have shown that swine confinement facility dust activates NF-kB and up-regulates proinflammatory genes, such as iNOS and COX-2, in porcine airway cells (Gerald et al, 2010). Thus the impetus to explore the present work is to investigate oxidative stress in airway cells that have been exposed to dusts from various husbandry units and examine a possible holistic approach to prevent oxidative stress in respiratory disease.

2.8 Phytonutrients

Phytonutrients are active compounds from plant sources that can provide immune-boosting, and well as other health-promoting benefits. Research has been conducted on various foods and the efficacy in preventing cancer (Greenwald, Clifford et al, 2001). Some phytonutrients can function as precursors to antioxidants. Diets enriched with antioxidants can be beneficial in providing a protagonist effect to the free radicals. Sorrel (*Hibiscus sabdariffa*) is a plant that naturally grows in India, China, and the West Indies. The calyces of the plant have a plethora of phytonutrients, and, in this study, we will further investigate their properties. Phytonutrients include phenolic acids, flavonoids and lignans along with many others. According to Valerio (2001), flavonoids can increase the activity of endogenous antioxidant glutathione and other antioxidants as well as detoxifying agents. Sorrel use has been implicated in studies using *in vitro* models as well as in animal models to deter carcinogenic effects. Rats pre-exposed to sorrel and then exposed to carcinogens were protected against harmful effects (Essa, 2006). There is also evidence that sorrel can provide hepatoprotection (Amin, 2006), cardioprotection (Olaleye, 2007) and has antibacterial properties (Fullerton & Williams, 2010). Previous studies in our laboratory have shown that sorrel possesses antioxidant properties (unpublished results; personal communication with Dr. Christa Watson, Harvard University),

potentially through free radical scavenging and enhancement of endogenous antioxidant levels (i.e., glutathione, catalase and superoxide dismutase). Sorrel components may also provide anti-inflammatory effects. In Christian et al, 2006, red sorrel effectively decreased in COX-2 (pro-inflammatory protein) production.

The commercially available antioxidant, NAC, is known to have anti-apoptotic and growth-promoting properties. This thiol compound contains mucolytic properties and is a direct pre-cursor to reduced glutathione (Sadowska, Manuel-y-Keenoy, De Backer, 2007). This antioxidant can also be as efficient in neutralizing H_2O_2 as glutathione; however, it lacks in carrying capacity (Benrahmoune, Therand & Abedinzadeh, 2000; Gillissen, 1997). It has the potential to also inhibit respiratory bursts via post-engulfment neutrophils *in vitro* (Stolarek, Bialasiewicz, Nowark 2002) which could indicate anti-inflammatory roles of sorrel as well. In bronchial epithelia that were exposed to silica, NAC was able to inhibit activities of transcription factor NF-κB.

2.9 Specific Aims and Hypothesis

Natural antioxidants hold promise for reducing inflammation and oxidative stress, thus it is of interest to investigate the ability of plants such as sorrel to alleviate of symptoms caused by dusts and cigarette smoke. Therefore, the overall goal of this project is to characterize animal husbandry dusts, determine the potential impact of agricultural dusts on the respiratory epithelia and potential combinatorial effects of cigarette smoke components; and investigate how phytochemicals from sorrel attenuate these effects. The central hypothesis is that agricultural workers exhibit respiratory complications due to oxidative damage mediated by environmental factors (*i.e.*, agricultural dusts and cigarette smoke chemicals) and that sorrel extracts can relieve

these symptoms. The central hypothesis was investigated by exploring the objectives listed below.

The objectives are:

1) **Characterize physical and biochemical composition of agriculture dusts from the various husbandry units at NC A&T SU farm.**

   Working Hypothesis: Confinement facilities will harbor smaller particles, bacteria, and chemicals compared to semi-confinement facilities.

2) **Identify DEB induced oxidative stress mechanisms associated with early stages of apoptosis in human airway epithelial cells and lymphoblasts.**

   Working Hypothesis: Oxidative stress and apoptosis levels will be higher in cells that are exposed to DEB.

3) **Identify the mechanism of oxidative stress in airway cells that have been exposed to agricultural dusts and analyze oxidative stress reduction capacity of sorrel in respiratory cells and lymphocytes exposed to dusts.**

   Working Hypothesis: Pretreatment with sorrel-derived antioxidants will lower the oxidative effects in cells exposed to dusts.

CHAPTER 3

A Biophysiochemical Analysis of Settled Livestock and Poultry Housing Dusts

3.1 Introduction

Animal agriculture is a multi-billion dollar global industry and meat products supply an ever increasing demand; the No. 1 consumed meat in the world is pork (36%), followed by poultry (33%) and beef (24%) (FAO, 2012). Due to the high demand for meat, some livestock production operations shifted to efficient confinement systems that allow faster production in environmentally controlled buildings that safeguard against temperature extremes, predators and disease incidence. However, a consequence of confinement production is reduction in air quality associated with accumulation of dust and gases. Semi-confinement production facilities usually have indoor and outdoor components and thus, dust is typically less concentrated compared to the confinement facilities.

Animals, farmers and workers within animal confinement facilities may be exposed to higher levels of inorganic substances, feed grains, organic dusts, microbes and their products, gases and chemicals (e.g. pesticides, disinfectants) compared to outdoor operations. Grain dusts contribute heavily to agricultural dust composition among swine, dairy and poultry farms (Donham et al, 1986; Lee et al, 2007). For example, pioneering studies by Donham and coworkers reported respiratory dysfunction in swine facility workers chronically exposed to dust consisting of feed particles, bacterial endotoxin, gases and other components (Donham et al, 1986; 1995). Gaseous compounds from swine facilities contain at least 167 different compounds including alcohols, aromatics, and nitrogen-related compounds (Schiffman et al, 2001). Simultaneous exposure to poultry production dust particulates and ammonia resulted in a synergistic decline in pulmonary function in workers (Donham et al, 2002).

Although it is well accepted that exposure to animal housing dust is associated with respiratory symptoms in workers (Linaker & Smedley, 2002), reports summarizing the respiratory effects associated with inhalation of agricultural dusts linked to specific dust components are limited. Thus, it is intriguing to characterize dusts from animal husbandry units to gain a better understanding of inhalation exposures and risks. To begin assessing exposures, settled dust samples were collected from raised surfaces at the swine and poultry confinement units, and the dairy, small ruminant, and equine semi-confinement buildings at the North Carolina A&T State University (NCA&T) Farm, and five other farms across the state of North Carolina. The purposes for collecting the samples were to determine the chemical, physical and microbial composition of settled dusts that could affect respiratory health by inhalation.

3.2 Materials and Methods

Approach. The analyses were primarily conducted on dusts collected from the livestock units at the NCA&T Farm (Table 1). Samples were also collected from two small ruminant, two horse and one beef cattle farms across the state. Qualitative and quantitative evaluations were conducted to identify physical, chemical and microbial constituents of the dust.

Table 1.
North Carolina A&T State University Farm animal husbandry facilities.

	Facility Type	Year Built	Cleaning regimen[a]	Animals	Animal No.	Feed Type	Bedding
Poultry	C	2004	PW	Broilers, Layers, Heritage birds	4000, 400, 50	Pellet	Wood shavings
Swine	C	1983/2006	PW, SF	Yorkshire, Landrace	150	Pellet	Slatted floors
Equine	SC	2005	GPB	Arabian[c] Quarter Horse[c2]	3	Pasture, Alfalfa hay	Wood shavings
Sm. Rum.	SC	1998	GPB, broom	Boer goats, Sr. Croix sheep	50,20	Pasture, Pellet	Wood shavings
Dairy	SC	2006	PW[b]	Holstein, Jersey	45[d]	Pasture (80%), Pellet, Corn silage	Canvas mat & wood shavings

[a]Cleaning practices, generally on a monthly basis or as needed when changing animal groups.
[b]Twice daily, especially after milking.
[c] 1Mare, [c2]2 geldings
[d]Milking, dry, heifers, calves
C, Confinement
SC, Semi-confinement
PW, pressure washed
SF, slated floor
GPB, gas powered blower

The first analyses were designed to characterize physical components of the dust. This was accomplished by using scanning electron microscopy (SEM) and simple gravimetric analysis to ascertain particle size and morphology. The second set of analyses included the chemical analyses including element levels and pH determinations. Finally, microbiological characterization was performed utilizing endotoxin quantification, identification via selective media and DNA quantitation and gene analysis by polymerase chain reaction (PCR).

The aforementioned characterization strategy for dust samples was selected based on housing and species type, biological components of the dust (i.e., bacteria important to respiratory disease or foodborne illness) and chemicals used in the upkeep of the facilities. Therefore, it was imperative to observe the various dimensions of the dust to determine the size of the particles in relation to where they may deposit along the respiratory tract. Chemical analyses were conducted to characterize dust from different species/unit settings. Animals housed in semi-confinement facilities can track soil from the pasture inside and elements from soil can become aerosolized and contribute to dust composition. Finally, we conducted microbiological analyses to provide bacterial profiles for dust samples.

3.2.1 Dust sampling and mixture preparation. Settled dust was obtained from raised surfaces at the North Carolina Agricultural and Technical State University swine, poultry, equine, dairy and small ruminant (caprine and ovine) units. Samples were also collected from five other farms across North Carolina: 1-Equine, 2-Caprine, 3-Bovine (beef cattle) & Equine, 4-Bovine (beef cattle), 5-Caprine & Ovine. For sampling, approximately 10-15 grams of settled dust on fixtures was brushed into a ziptop plastic bag using a cosmetic brush and transported immediately to the laboratory for further processing as previously described with a few

modifications (Mathisen et al. 2004). Briefly, a 1:10 (w/v) dust mixture (DM) was prepared by combining 0.5 grams of dust with 5 mL of Hank's buffered saline solution (HBSS). The mixture was vortexed for one minute and left to stand at room temperature for one hour. The DM was used for pH readings and gravimetric analysis as detailed below. Dust samples were collected from the same locations within each building several times over a two year period (for NCA&T units only).

3.2.2 pH altering capacity. To analyze the pH of animal housing DM, pH meter readings were measured. Prior to reading pH, the DM samples were inverted and vortexed to mix particles.

3.2.3 Gravimetric analysis. To investigate dust particle size, gravimetric analysis was performed using a modified method of Lioy and colleagues (2002). The P2 and P5 filter papers (Thermo Fisher Scientific, Rockford, IL) were selected to study thoracic and respirable particles; P2 has particle retention of 1-5 µm and P5 has particle retention of 5-10 µm. Briefly, the DM samples were separately passed through P2 and P5 filters. Particle retention was estimated from average pre- and post-filter weights. Each dust sample was analyzed at least three times for all units.

3.2.4 Scaninng electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS). To analyze dust particulate size, procedures similar to Lioy and coworkers (2002) were used. For SEM analysis, portions of the dust were placed on conductive carbon tape and a Hitachi SU8000 Field Emission Scanning Electron Microscope (Hitachi High Technologies America, Dallas, TX) was used to capture images. Particle size estimation was completed using the Quartz X-One software. X-ray imaging was performed for elemental

analysis (energy dispersive technology) on each dust sample. Two or three SEM images were captured for each sample.

3.2.5 Trace element analysis. Samples underwent a nitric acid digestion prior to analysis via inductively coupled plasma-optical emission spectrometry (ICP-OES). Nitric acid (5 ml) was added to 0.25 grams of dust from each agricultural unit in a beaker (250 ml). To obtain a consistent reflux, a watch glass was used to cover the mouth of the beaker. Nitric acid (5 ml) was added continuously until the solid substance was dissolved; totaling approximately 20-30 ml of nitric acid depending on dust consistency. This continued until the liquid was a clear yellow color. Sample volumes were brought to 50 ml final volume with distilled water and filtered using #42 Whatman filter paper. For ICP-OES analysis, standards containing the following elements were used: aluminum, calcium, chromium, iron, potassium, manganese, magnesium, sodium, phosphorus, silicon, zinc, cadmium, copper, nickel and lead. Varian 710-ES ICP-OES was used to analyze samples using a procedure adapted from EPA Method 3050B Acid digestion of sediments, sludges and soils.

3.2.6 Endotoxin assay. To quantitate endotoxin levels in dust samples, the Pierce LAL Chromogenic Endotoxin Quantitation assay (Thermo Fisher Scientific, Rockford, IL) was used according to manufacturer's instructions. Readings were performed three times using a microplate reader at 410 nm.

3.2.7 Biochemical identification of dust microbiomes. Animal units at the NCA&T Farm were swabbed using a sterile cotton swab, and placed in 1% peptone water for transport. Swabs were streaked onto various selective agar plates (see Table 2) and cultured at 37°C overnight.

Table 2
Selective media for bacterial identification.

Medium Symbol	Medium Name	Bacterial Selection
Centrimide	Centrimide	Pseudomonas
MYP	Mannitol egg yolk polymixin	Bacillus
TSI	Triple Sugar Iron	E.coli, Pseudomonas, Salmonella
Oxford	Oxford	Listeria
SMAC	Sorbitol MacConkey	E. coli
XLD	Xylose Lactone Deoxycholate	Salmonella
RC	Reinforced Clostridial	Clostridium
BP	Baird Parker	Staphylococcus
TSA	Tryptic Soy agar	Most grow (universal)

3.2.8 Microbial DNA isolation and 16S ribosomal gene analysis. Evaluation of the 16S ribosomal DNA gene, the genomic sequence that encodes the 16S portion of prokaryotic ribosomes and is thus present in all bacterial species, is an established approach for detection and identification of bacterial species (Wilson et al. 1990). For bacterial detection and identification, DNA was isolated from dust samples (0.1-0.5 grams) using the PowerSoil DNA isolation kit (Mo Bio, Carlsbad, CA) according to manufacturer instructions. Following DNA quantitation, 16S rDNA gene amplification was performed via polymerase chain reaction (PCR) with primer sets obtained from published reports (Table 2). DNA (100 ng) was combined with forward and reverse primers (0.2 μM each), 2X GoTaq Green (Promega, Madison, WI) and nuclease-free

water was added to bring the reaction up to 25 µl. Samples were amplified using an iCycler thermal cycler (Bio-Rad, Hercules, CA) using a hot start (94°C, 1 minute), followed by 35 cycles of denaturation (94°C, 30 seconds), annealing (see Table 2 for annealing temperatures, Tm; 30 seconds) and elongation (72°C, 7 minutes). PCR products were visualized by agarose gel electrophoresis, ethidium bromide staining and photodocumentation.

3.2.9 Statistical analysis. Analyses were performed using Prism version 5.0. (GraphPad Software, Inc., San Diego, CA) using analysis of variance (ANOVA). Differences between means were considered statistically significant when P values were less than 0.05. Bonferroni or Dunnett's posttest corrections were utilized to evaluate differences among means. All experiments were performed at least three times unless otherwise noted and values are reported as means ± standard error (SE) or standard deviation (SD).

3.3 Results

Physically, all dusts were variations of brown in color. The poultry unit dust was fluffy and the lightest in color. NCA&T dairy dust was grainy/pebbled and darkest of the dusts. Swine dust was fine in consistency. Equine dusts were hard in texture and light in color. Small ruminant dusts are less fluffy than poultry unit dust. Overall, the cattle dusts are coarser than all other dusts.

3.3.1 Animal husbandry dust particle sizes. Through gravimetric analysis, smaller particles in the size range of 1-5 µm were detected for each NCA&T unit based on five-fold ($p<0.001$) higher retention levels on P2 filters compared to P5 filters (Figure 1). This indicates more particles ≤ 5 µm passed through the P5 filter. These data were consistent with particles

measurements determined via SEM (Figure 2). The largest particle sizes were detected in dairy building dust, for which the average particle size was 37.86 µm. The NCA&T dairy unit also had the greatest particle size range, 5.11 µm - 154.5 µm, while the poultry unit had the some of the smallest particles with an average size of 12.5 µm and a narrow particle size range of 5.23 - 26.93 µm (Figure 3, Table 3). By way of simple gravimetric analysis and SEM analysis it was determined that animal housing buildings contained higher levels of respirable and thoracic particles (1-5 µm) than large inhalable particles (<30 µm) based on the American Conference of Industrial Hygienists (ACGIH) classification (WHO 1999). Table 3 provides a summary of particle size averages and ranges for dusts collected from NCA&T Farm.

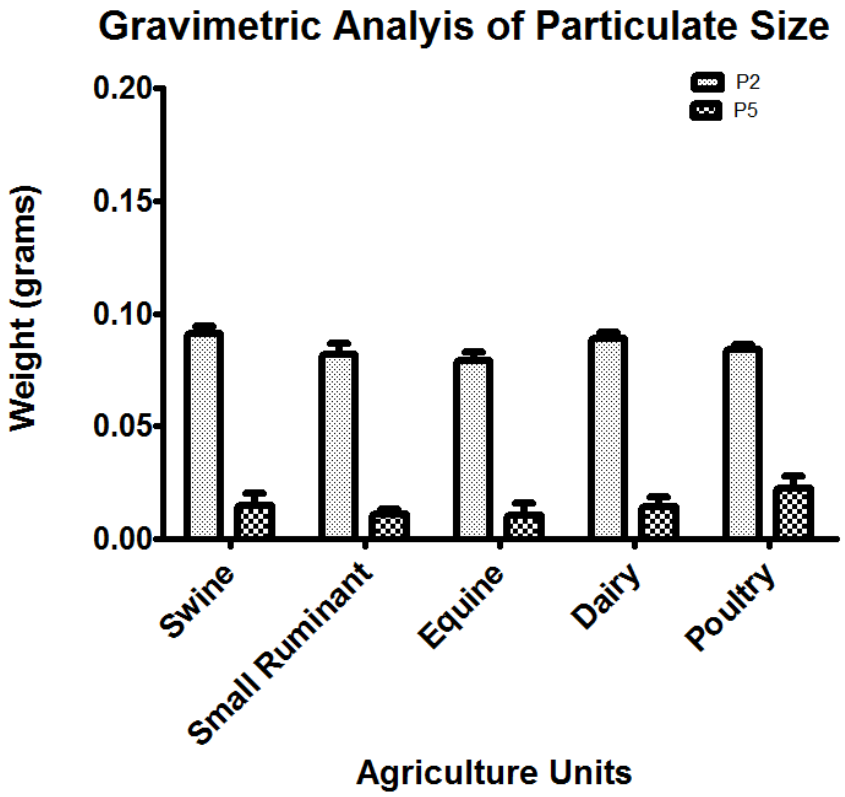


Figure 1. Gravimetric analysis of DM from agricultural units. Statistical analysis of agricultural units DM particles weights on P2(1-5 µm) and P5(5-10 µm) papers when filter types and agricultural unites were compared using a two way ANOVA (conducted at the 95% confidence interval) and a bonferroni posttest. Data are presented as Means ±SEM.

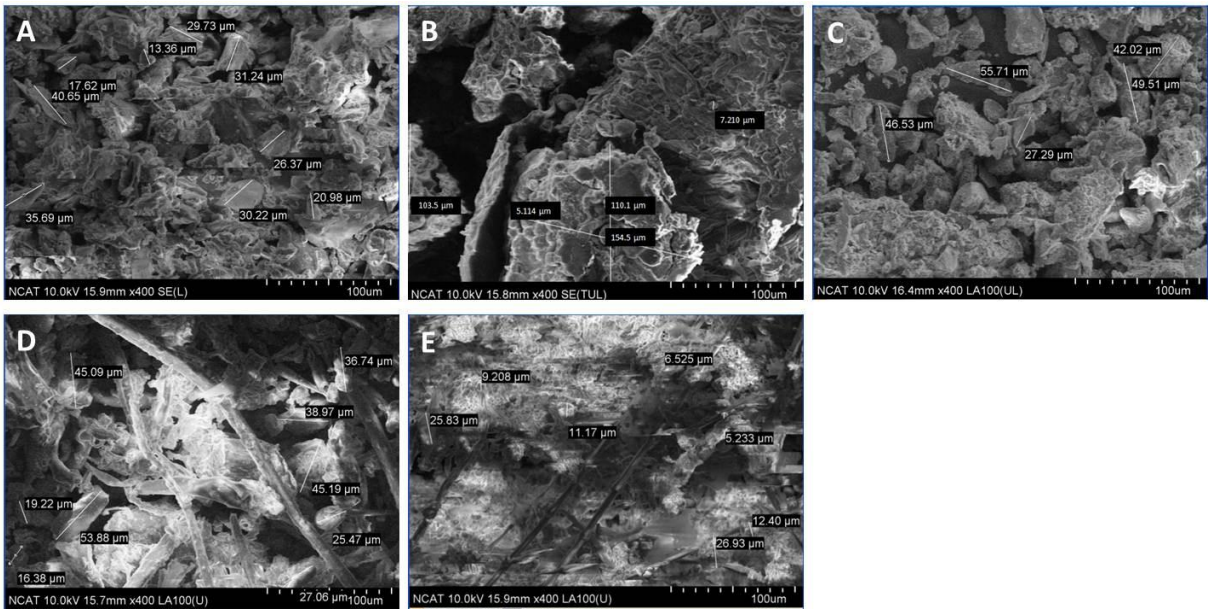


Figure 2. SEM analysis for classification of particles from dusts from husbandry units at NC A&T SU farm. A-Swine, B- dairy, C- equine D- poultry, E- small ruminant. All images are at 400X.

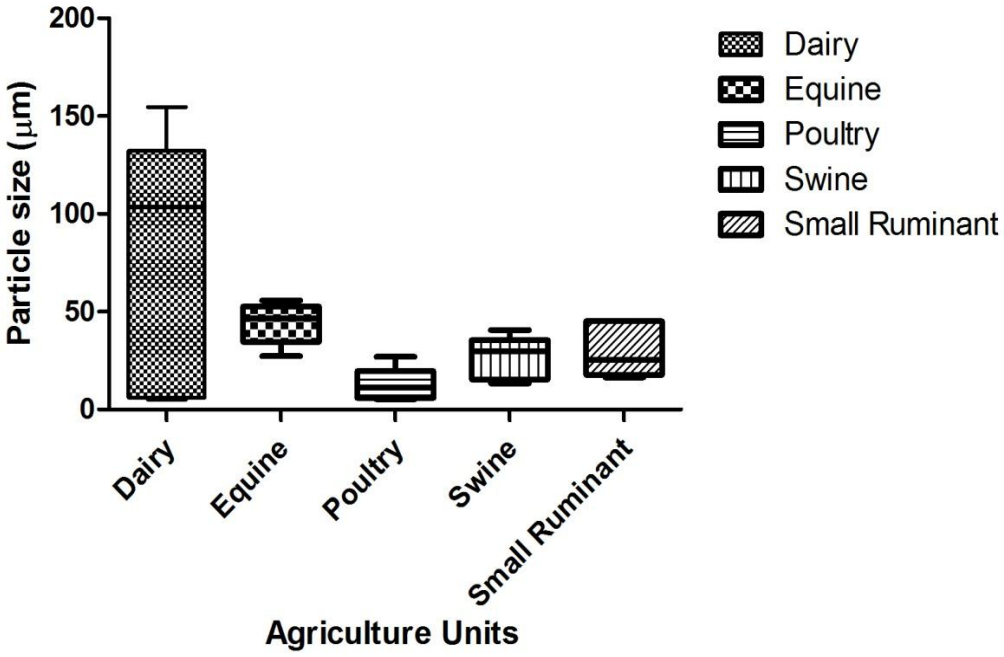


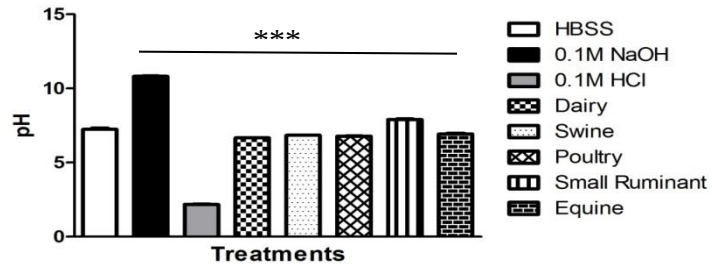
Figure 3. Particle sizes of the agriculture dusts by SEM. Particle sizes were quantified using the quartz program via SEM analysis. Data are presented as Means ±SEM, n=5.

Table 3.
Animal husbandry unit dust particle size estimation by SEM.

Agriculture Unit	Average Particle Size	Particle Size Range (μm)	Standard Deviation	Mean	Standard Error
Dairy	76.10	5.11-154.50	66.77	76.08	29.86
Equine	44.20	27.29-55.71	10.68	44.21	4.79
Poultry	12.50	5.23-26.93	8.63	12.45	3.86
Small Ruminant	30.27	16.38-45.19	13.96	30.27	6.24
Swine	26.30	13.36-40.65	10.90	26.31	4.87

3.3.2 Dust particles have an acidic pH. With the exception of the alkaline small ruminant dust suspension (pH of 7.9), the pH of aqueous suspensions of all dust samples were acidic (Dairy = 6.67, Swine = 6.84, Poultry = 6.78, Equine = 6.93) compared to the basic HBSS control (Figure 4).

A.



B.

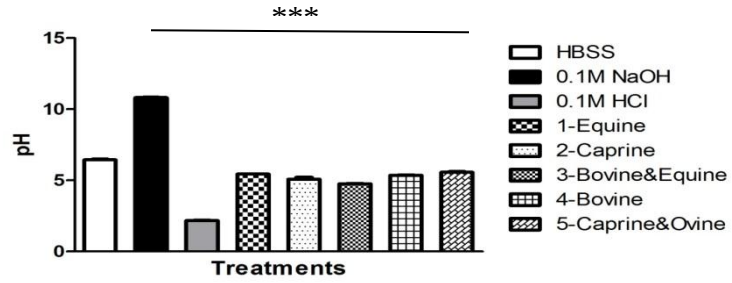


Figure 4. Analysis of agricultural dust-mediated pH changes from agricultural units. A, Dairy, Swine, Poultry, Small Ruminant and Equine dusts' from NC A&T SU farm were tested for pH changes. B, 1-Equine, 2-goat, 3-beef cattle & horses, 4-beef cattle, 5-sheep & goats farm dusts were tested for pH changes. Data are presented as Means ±SEM, n=3. *** p-value<0.001 when compared to HBSS.

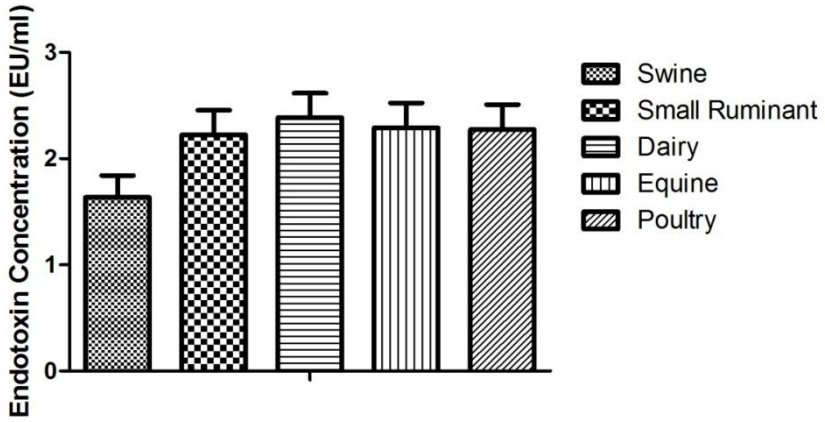
3.3.3 Element concentrations determined by SEM/EDS and ICP-OES. As showed in tables located in the Appendix, most of the elements detected were in the ppm (μg/g) concentration range. Phosphorus and sulfur levels were 5-10 fold higher in swine unit dust compared to other units this is likely due to low ventilation and higher levels of feces.

3.3.4 Endotoxin levels and bacterial presence. Dust endotoxin levels were quantified for all livestock units sampled. The levels ranged between 2.39 Endotoxin Units (EU/ml) (dairy) and 1.64 EU/ml (swine) for NCA&T animal units, and between 2.37 EU/ml (5-Caprine and Ovine) and 2.84 EU/ml (1-Equine) for other farms (see Figure 5). There were no differences in endotoxin levels among dusts tested.

To identify bacterial species present in NCA&T Farm animal housing dusts, selective growth media and PCR analysis were performed. Bacteria of interest included species common to agricultural settings known to be clinically important to food borne and respiratory illnesses. Most of the selective agars were effective at growing bacteria from the various dusts collected from animal husbandry units. As summarized in Table 2, positive bacterial growth corresponds to the detection of Bacillus (MYP); E. coli, Pseudomonas, Salmonella (TSI); Listeria (Oxford); Clostridium (RC); and Staphylococcus (BP). E. coli was also detected on Sorbitol MacConkey media from the swine unit only. Tryptic soy agar, a permissive growth medium, showed growth for all units.

Figure 5 depicts successful bacterial growth and amplification of the 16S ribosomal gene from NCA&T Farm dust DNA samples. Bacterial growth was observed for each of the selective agars with the exception of Centrimide, which exclusively detects Pseudomonas species and XLD, a selective medium for Salmonella species (see Table 2). Notably, 16S rDNA from more bacterial species were detected in the swine unit dust compared other units and include Listeria, Clostridium, Bacillus, Pseudomonas, Salmonella, E. coli, Staphylococcus, and Actinomycetes (Figure 5). However, Listeria 16S rDNA had the highest levels ($p<0.01$) was detected in dusts from all five of the NCA&T Farm animal housing buildings (Figure 5).

A.



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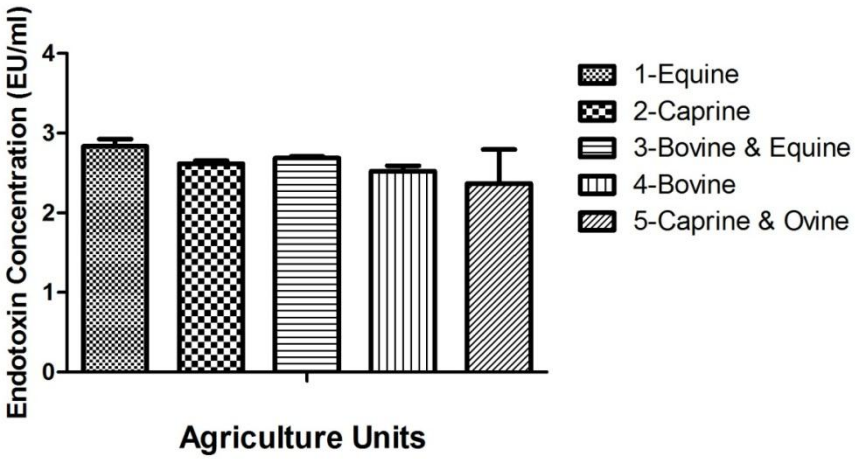


Figure 5. Endotoxin levels in the agriculture units. There were no differences between the endotoxin levels in the various units. Data is presented as endotoxin units per ml. Data are represented as Means ±SEM, n=3.

Figure 5 depict successful bacterial growth and amplification of the 16S ribosomal gene from dust DNA samples, respectively. Bacterial growth was observed for each of the selective agars with the exception of Centrimide, which exclusively detect Pseudomonas species and XLD, a selective medium for Salmonella species (Table 4). Notably, 16S rDNA from more

bacterial species were detected in the swine unit dust compared other units and include Listeria, Clostridium, Bacillus, Pseudomonas, Salmonella, E. coli, Staphylococcus, and Actinomycetes (Figure 5). However, Listeria 16S rDNA had the highest levels ($p<0.01$) was detected in dusts from all five of the NCA&T farm animal housing buildings (Figure 5).

Table 4.
Bacterial identification via media selection.

Species	Centrimide	MYP	TSI	Oxford	SMAC	XLD	RC	BP	TSA
Swine	-	+	+	+	-	-	+	+	+
Sm. Rum.	-	+	+	+	-	-	+	+	+
Dairy	-	+	+	+	-	-	+	+	+
Poultry	-	+	+	+	+	-	+	+	+
Equine	-	+	+	+	-	-	+	+	+

+, bacterial colony/lawn growth observed.

-, no bacterial colony/lawn growth observed.

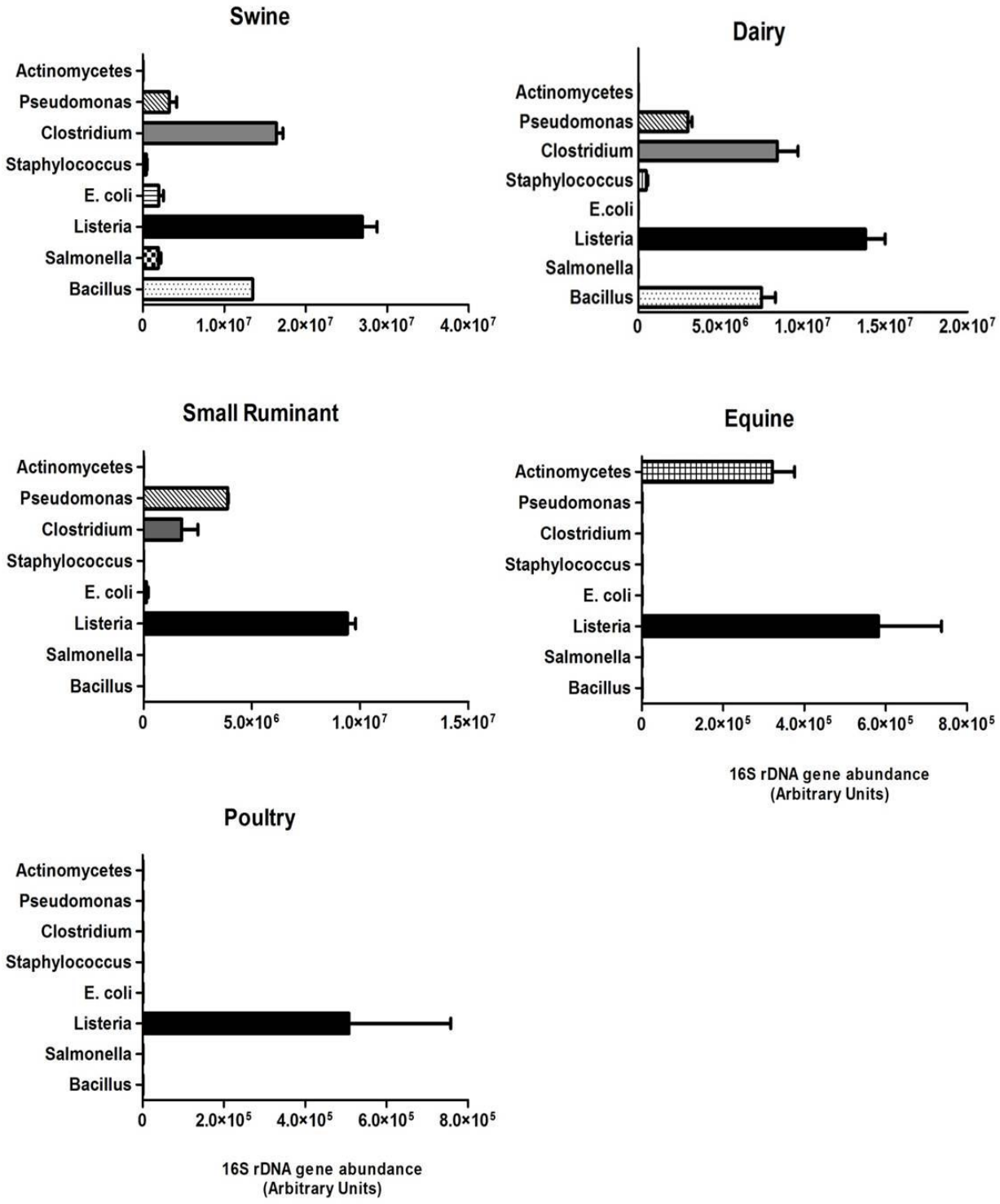


Figure 6. Detection of bacterial 16S ribosomal DNA gene in dust samples. Soil DNA extractions were prepared and primes specific for species specific bacterial 16S ribosomal DNA gene were used to amplify targets. A, Bacterial presence of DNA isolated from Agricultural Dusts via Polymerase Chain Reaction quantified by densitometry. √, 16S rDNA gene detected, ND, not detected. Data are presented as Means ±SEM, n=3.

3.4 Discussion

Dust collected from animal production facilities is extremely complex due to the nature of the facilities, species, feed, and cleaning practices. Cleaning regimens coupled with ventilation are key factors in controlling air quality and dust accumulation. Dusts from animal husbandry units tended to contain higher levels of respirable particles (<5 μm) regardless of housing or animal type. Inhalable dust particles are small enough to stay airborne. According to the International Organization for Standardization (ISO) and ACGIH, the single most important factor influencing deposition along the respiratory tract is the "aerodynamic diameter" of a particle (ISO, 1995; ACGIH, 1999). Larger particles (i.e., 30-100 μm) are inhalable and may deposit anywhere along the respiratory tract. Thoracic particles are smaller (<10 μm in diameter) and deposit with in tracheobronchial region, while respirable particles (<2-5 μm; very small < 0.5 μm) can travel to the gas exchange/alveolar region of the lungs (ACGIH, 1999). Respirable and inhalable dust levels within poultry houses can range from 0.02 to 81.33 mg/m3 and 0.01 to 6.5 mg/m3, respectively (Ellen et al, 2000). This study reports higher levels of respirable and thoracic particles than inhalable particles among all units tested.

Trace elements detected in this study were consistent with published reports for metals such as zinc, manganese and iron (Demmers et al, 2003). Higher levels of phosphorus and sulfur in the NCA&T swine unit compared to other units is likely due to presence of feces; swine manure contains phosphorus and sulfur.

Respirable particles can enter systemic circulation, distribute throughout the body and elicit an immunological response owing to the increased number of sites nanoparticles have to react on cell membranes and a greater capacity to absorb and transport toxic substances (Garnett & Kallinteri, 2006). Ultra-fine particles (< 100 nm) can elicit more severe inflammation than

larger sized particles of the same material (Oberdörster, 2001; 2005). Animal husbandry units can release a variety of particulates into the atmosphere. Airborne microorganisms, including viruses and bacteria, can be transmitted great distances through the airflow (Jones & Harrison, 2004). This study reported presumptive positives for bacteria based on PCR and growth detection. Roy (2003) reported that bacterial DNA was found more in farm barns and farm houses than urban houses. Bacteria and their components can potentiate respiratory symptoms and illnesses in agriculture workers. Listeria monocytogenes can cause foodborne illness in humans and disease in cattle, sheep and goats (Nightingale et al, 2004). Typical amounts of aerosols inhaled are 104 to 107/ m3 of bacterial CFUs and 103 to 106 fungal CFUs/m3 (Kirkhorn, 2002). The presence of select bacteria observed in the present study is consistent with reported levels. Endotoxins are potent inducers of neutrophilic airway inflammation and are a major risk factor for asthma among farmers (Charavaryamath and Singh, 2006), horses (Pirie et al, 2003) and sheep (Purdy et al, 2002). There were no differences in the endotoxin levels among farm dust samples tested in this study. However, levels were relatively low compared to other published works (Romberger, 2002). Variation in endotoxin levels is likely due to differences in animal species and numbers, feed types and production styles. For example, higher dust, bacterial diversity and endotoxin levels were detected in cage-housed than floor-housed poultry operations (Just et al, 2011).

In a study utilizing an environmentally controlled swine confinement facility designed or controlled dust exposure, researchers reported endotoxin levels at 32.5 µg g-1 as well as 7.0 $x10^7$ CFU and recurrent cases of PRRS, swine influenza, Actinobacillus pleuropneumonia, atrophic rhinitis and enzootic pneumonia in piglets (Demmers et al,

2003). Thus, inhalation of endotoxin-laden dust promotes susceptibility to respiratory dysfunction in animals and humans.

Reduced air quality in production facilities may contribute to respiratory dysfunction in farmers/farm workers, animals and perhaps farm visitors. Donham and Gustafson (1982) estimated 700,000 people in the United States are exposed to animal confinement units through their occupations. They also reported over 60% (survey of 2459 Iowa livestock workers) reported adverse effects such as cough, sore throat, runny nose and tightness of chest among others. On the molecular side, studies have shown that agriculture dusts can also alter normal physiological balances. Agriculture workers manifest a complex overlapping group of disorders including acute bronchitis, chronic bronchitis, asthma, interstitial disease and acute lung injury (Spurzem et al, 2002). Wyatt and colleagues (2008) showed that hog barn dust can impact the normal stimulation of cilia in bovine ciliated cells. This can lead to defective mucociliary clearance and particulates not being excreted out of the airway efficiently. Mathisen et al (2004), showed hog barn dust can facilitate lymphocyte adhesion to human airway epithelia in a time and dose dependent manner in vitro. Hog barn dust stimulates secretion of cytokines interleukin (IL) -8 and IL-6 by human bronchial epithelial cells in vitro (Romberger et al, 2002). This potentially explains why some persons exposed to agriculture dusts manifest neutrophilia; IL-8 is a recruiter of neutrophils.

Two important limitations of the present study are that analysis was performed on settled dust and a modest number of farms were sampled. It is possible that the results would differ for dusts collected using a different sampling method. Reports summarizing results of samples collected using air sampling devices tend to yield higher levels of ultrafine particles in the respirable or nanometer range (Demmers et al, 2003; Donham et al, 1986). However, an

understanding of settled dust particles would provide insight into the nature and interactions of larger dust constituents which are more likely to accumulate and persist within animal production housing. It is important to understand both dust categories, that is, larger and smaller dust particles.

With regard to the number of units sampled, husbandry dust characterization studies depend heavily on access to animal production facilities. With the exception of swine and poultry, two-three husbandry units per species were analyzed in the present study. Ultimately, there is a need for more studies of this nature to fully understand agricultural dust exposures.

3.5 Conclusions

Agricultural dust in animal production buildings consists of a complex mixture of grain/feed particles, microbial products (endotoxin), a variety of gases, metals and other components. Chronic inhalation of such animal production dust has been associated with occupational respiratory symptoms in farmers and workers; lesser is known about potential effects on animals. The present study found more respirable versus inhalable particles in all livestock unit dust samples and provides some preliminary evidence in possible differences for dust particle sizes and bacterial species among livestock units that need to be more fully explored.

CHAPTER 4

Apoptotic and Oxidative Effects of Diepoxybutane on Airway Cells and Lymphoblasts

4.1 Introduction

The environmental pollutant, 1,3-butadiene (BD) is important in the industrial world particularly in the manufacturing of synthetic rubbers and plastic products. It is also a component of cigarette smoke and automobile exhaust, thereby exposing not only factory workers but the general population as well. BD is classified as a carcinogen by the International Agency for the Research on Cancer (IARC) and is a known toxicant. Metabolically, BD is broken down into three primary active metabolites, 1,2-epoxy-3-butene (EB), 1,2:3,4-diepoxybutane (DEB) and 3,4-epoxy-1,2-butanediol (EBD). These three metabolites have the ability to be carcinogenic, mutagenic and genotoxic, inducing chromosomal aberrations, micronucleus formation and sister chromatids (Bond, 2001; Kligerman, 2007;Murg, 1999). Of these three metabolites 1,2:3,4-diepoxybutane (DEB) is the most potent metabolite and causes the highest amount of genotoxicity. The incurring of high levels of genotoxicity contributes to the induction of crosslinking DNA (inter-strand and intra-strand) (IARC, 2007) (Millaird et al, 2006). A study by Yadavilli et al (2009), DEB induced elevated cellular levels of tumor suppressor p53 in human B lymphoblastic TK6 cells. Diepoxybutane mediates DNA damage through generating free radicals and contributing to oxidative stress which mediates apoptosis in human lymphoblasts (Erexson & Tindall, 2000) (Yadavilli et al, 2007).

Because butadiene is normally inhaled we investigated the effect of DEB on normal human bronchial epithelial cells. There are a relatively small number of published studies on the oxidative stress in DEB- exposed airway cells. To examine the levels of oxidative stress, we quantified levels of 8-isoprostane, a biomarker for lipid peroxidation.

Apoptosis is a form of programmed cell death with physiologic and pathologic actions. For example, the cells between the digits in human embryos undergo apoptosis. Another purpose of apoptosis is to eliminate damaged, infected or possible cancerous cells. In some cases, such as cancer, there is too little apoptosis and far too much apoptosis in conditions such as Alzheimer's disease (Nagata, 1996). Characteristics of apoptosis are cell shrinkage, nuclear shrinkage and apoptotic bodies. It also occurs usually within single cells or small groups of cells.

Free radicals are chemically unstable molecules which have a high affinity for binding to DNA, protein and nucleic acids due to their' unfilled outer electron valence shell. Free radicals are produced as a result of mitochondrial respiration as well as other normal physiological processes. Free radicals can also be a result from environmental factors such as cigarette smoke. In most circumstances, antioxidants can bind free radicals, preventing the build up of high levels of free radicals which can cause tissue damage. However, the system can be overwhelmed and free radicals can cause tissue damage in a condition known as oxidative stress. We hypothesized that DEB will increase apoptosis and reactive oxygen species (ROS) production in human B lymphoblasts and decrease proliferation and increase oxidative stress in NHBE cells. Our objectives for this study were to analyze DEB-mediated reactive oxygen species production and apoptosis at acute time points in lymphoblasts and identify DEB-mediated oxidative stress in NHBE cells.

4.2 Materials and Methods

4.2.1 Chemicals. Diepoxybutane (11.267) and a antioxidant, N-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich Chemical Company. Acridine-orange (AO, 10 mg/ml) used for apoptosis staining and CM-H_2DCFDA (5-(and-6)-chloromethyl-2`,7-

dichlorodihyrofluorescein diacetate) stain for free radical detection were purchased from Molecular Probes. Psiva, another stain for apoptosis, was purchased from IMGENEX.

4.2.2 Exposure of cells to diepoxybutane. The human B lymphoblastic cell line TK6 (American Type Culture Collection) were cultured at 2 x 10^5 cells/ml in RPMI 1640 media, supplemented with 10% fetal bovine serum (FBS, Invitrogen Life Technologies) and 2 mM l-glutamine. Cells were passaged into fresh media at 24 hours prior to each experiment. Cells were washed and seeded into fresh media. For DEB exposures, DEB stock [11.27 M] was made in media to obtain a 1M solution and a 1:1 dilution of 1M DEB was added to the media of each individual treatment group for a final concentration of 10 mM. NAC (20 mM) treatments occurred one hour prior to DEB exposures. For NHBE exposures, cells were allowed to reach 90-95% confluency for cytotoxicity, nitric oxide and 8-isoprostane assays. For proliferation studies in NHBE cells, confluency of cells were no more than ~75%. Exposures times ranged from 7 to 24 hours for TK6 and NHBE cells. Concurrent experiments of dust extract (5% and 10%) and sorrel treatments (0.1% and 1%) were conducted in triplicate.

4.2.3 Assessing cell viability. Viability was determined via the trypan blue exclusion assay and was quantified on Beckman Coulter ViCell Cell counter prior to further experimentation. Cells were counted on 50 different planes; the viability and viable cells/ml were gleaned. This assessed the health of cells before conducting assays. For non-DEB exposed viabilities had to be at least above 95% and for DEB exposed cells viability had to be ~84-89%.

4.2.4 Quantitation of diepoxybutane-induced apoptosis. In order to characterize the cell death we analyzed the levels of apoptosis in Tk6 cells acridine orange and pSiva staining occurred. Acridine orange (AO) and pSIVA staining were used to analyze the levels of apoptosis in Tk6 cells. Samples were counted in five fields of brightfield and fluorescence

microscopy. Cells that produced green on the cell membrane (pSiva) and orange nuclei (acridine orange) were recorded as apoptosis positive. The percent of apoptotic cells was calculated by dividing the number of apoptotic cells divided by the total number of cells in brightfield (multiplied by 100). This was performed using acridine orange as previously described in Yadavilli and Muganda (2004) to identify late apoptosis. Cells (1×10^5) were used for both staining.

4.2.5 Measurement of reactive oxygen species. Lymphoblasts (TK6) cells were pre-incubated with 20mM NAC for 1 hour prior to DEB treatment of cells (0, 10 µM) for the 3, 8 and 19.5 hours. Cells were washed with PBS once and were then incubated with PBS/DCF (5 µM) for 20 minutes on ice. Cells were then washed again in PBS and resuspended in PBS and subjected to analysis via microplate reader format. To evaluate the total number of cells by nuclear staining and to distinguish apoptotic cells via nuclear morphology, Hoecsht 33342 (4 µg/ml final concentrations) was added after cells were read via microplate reader. For statistical purposes, ROS (green) positive cells were counted from five different fields and the percentage of ROS positive was obtained.

4.2.6 Measurement of proliferation. Cells (NHBE) were cultured in a tissue cultured treated clear 96 well plate using NHBE complete media. Subconfluent cell cultures (75%) were evaluated using the BrdU proliferation kit. Labeling and treatments were done simultaneously and the kit was used per manufacturer's instructions.

4.2.7 Measurement of 8-isoprostane. The 8-isoprostane EIA kit was obtained from Cayman Chemical Company. Supernatants were collected post-exposure and diluted either 1:10 or undiluted. Standards used were 500, 200, 80, 32, 12.8, 5.1, 2.0, 0.8 pg/ml in duplicate for each plate. The plate was loaded with appropriate samples, read at 405 nm using the

SpectroMax M5 microplate reader and analyzed with Softmax 5.0. The concentrations were quantified with the kit similar to Van Hoydonck et al (2004).

4.2.8 Lactate dehydrogenase release. To determine the cytotoxic effect of the agricultural dusts on the NHBE cells, the lactate dehydrogenase release (LDH) assay was utilized. After treating the cells, supernatant was collected and centrifuged at 4,000- 5,000 RPM. The resulting cell pellet which formed was not disturbed, and the supernatant was transferred to a new clean tube. The supernatant was subjected to the cytotoxicity kit obtained from Roche Diagnostics and used per manufacturer's instructions.

4.2.9 Nitric oxide production. Supernatants from NHBE cell cultures exposed to DEB were collected and analyzed using Griess reagent kits (Promega) to analyze levels of NO. Supernatants were collected and centrifuged for 5 minutes at 4,000-5,000 RPM. Without disturbing the cell pellet, the supernatant was collected in a new microfuge tube. Kit was used per manufacturer's instructions. Nitrite standards were used at the various concentrations: 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0 µM. Samples (50 µl) were mixed with sulfanialamide solution and kept at room temperature protected from light for 10 minutes in a clear 96 well plate. NED solution was added after the first 10 minute incubation and 10 minute incubation followed. The samples were subjected to the versamaxx plate reader and read at 520 nm.

4.2.10 Statistical analysis. All results were expressed as means of ±SEM of at least three experimental replicates. Statistical differences between the means were determined with a one way analysis of variance (ANOVA) using Graphpad Prism software was utilized significance reported as P value of ≤ 0.05 was considered significant.

4.3 Results

4.3.1 DEB impairs viability in human lymphoblasts. We utilized a dye exclusion method, trypan blue, to assess cell viability. Trypan blue is a dye that is taken up 5.2by cells whose cell membrane is compromised. Cell membranes are selectively permeable and only allow certain molecules to cross in and out. Therefore, dead cells, which have taken up the dye, will appear blue under the microscope, whereas live cells will be clear. This also allows us examine cellular health of the lymphocytes that have been exposed to DEB. As expected we observed a trend that suggests a decrease in viability over time in the lymphocytes exposed to DEB (Figure 7). This is similar to the findings of Yadavilli and Muganda 2004.

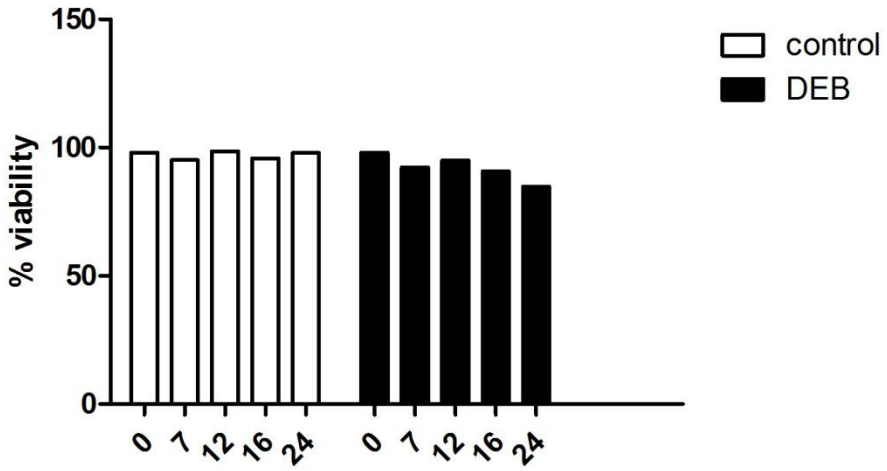
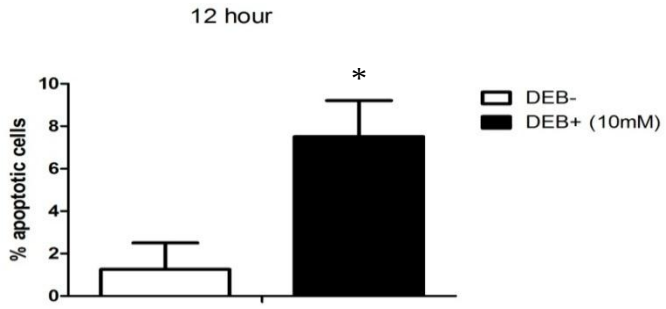


Figure 7. Viabilities of DEB exposed and non-DEB exposed TK6 lymphoblasts. Cells were counted on the Beckman Coulter ViCell cell counter; viabilities were calculated and recorded for the following times: 0, 7, 12, 16 and 24 hours.

4.3.2 Early apoptosis in lymphoblasts exposed to DEB. Acridine orange is a cell permeable dye which binds to nucleic acids and, when combined with ethidium bromide, can identify apoptotic cells via fluorescent microscopy. As previously stated, human lymphoblasts exposed to DEB undergo apoptosis. In Figure 8A we see a significant increase in the percent of

apoptotic cells exposed DEB for 12 hour via the Psiva fluorescent microscopy staining. As shown in figure 8B, the ROS scavenger NAC decreased apoptosis in lymphocytes after a 16 hour DEB exposure. One-way ANOVA evaluation of results revealed higher levels of apoptotic cells in DEB exposed cells at the 12 hour exposure (A) and that NAC significantly decreased the levels of apoptotic cells after a 16 hour DEB exposure (value<0.05) compared to control.

A.



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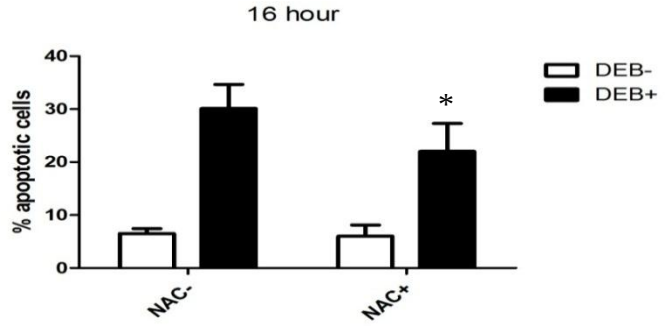


Figure 8. DEB mediated apoptosis in lymphocytes. Cells were exposed to DEB (10 mM) for 12 and 16 hours. A. Increase of apoptotic lymphocytes after 12 hour DEB exposure. AO staining occurred and cells were counted via fluorescent microscopy. B. Apoptosis was increased in lymphocytes post 16 hour DEB exposure. Cells were pretreated with NAC (20 mM) before the DEB exposure. pSiva staining was conducted and cells were counted via fluorescent microscopy. Data presented as mean ± SEM, n=4 *p-value<0.05, DEB+ compared to DEB+ pretreated with NAC.

4.3.3 DEB increased ROS production in human lymphocytes at 19.5 hours. Free radicals in large and uncontrolled levels may be responsible for tissue damage. Figure 9 depicts higher levels of ROS as determined by microplate reader (figure 9A) and the microscopy (figure 9B) methods. There was a significant increase in the fluorescence emitted which is indicative of hydrogen peroxide levels in DEB exposed lymphoblasts (p-value<0.05) when a t-test was conducted. The percent of ROS positive cells were increased significantly after a 19.5 hour DEB exposure under a one-way ANOVA (p-value<0.01) in B.

A. B.

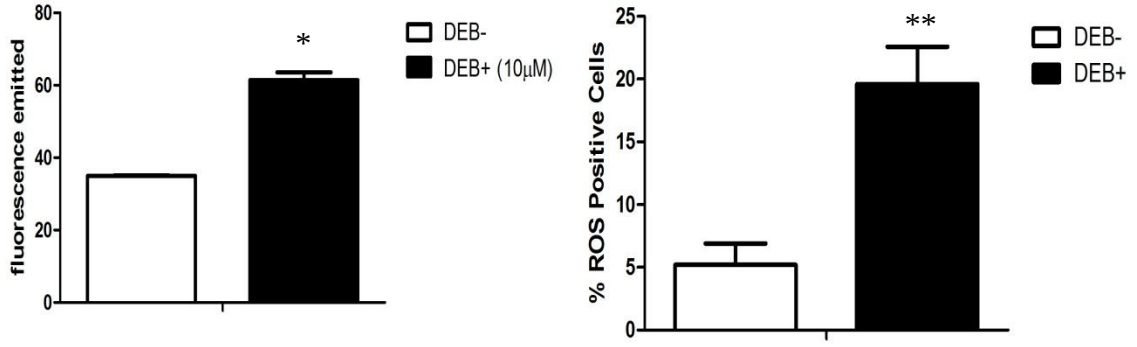


Figure 9. ROS production in TK6 lymphoblasts following 19.5 hour DEB exposure. Lymphoblasts were subjected to DCF staining after DEB exposure and two methods of characterizing ROS production: microplate reader (A) and fluorescent microscopy (B). Higher levels of ROS where present in cells exposed to the DEB after 19.5 hours. Data are presented as mean ±SEM, n=4. *-p-value<0.05, **-p-value<0.01.

4.3.4 ROS production in human lymphoblasts unaltered after acute DEB exposures. To investigate if the ROS are produced at earlier time points than 24 hours, TK6 lymphoblasts were exposed to DEB for 3 hours and incubated with DCF to determine oxidant levels. We also pretreated one group of cells with the antioxidant NAC to determine if it could potentially lower amounts of oxidants. Figure 10 represents the levels of oxidants at 3 hours post-treatment.

There was no increase in the amount oxidants between the non-exposed versus DEB groups and there were no differences between the NAC pretreated groups. No significant differences were found among the NAC pretreatmented cell cultures.

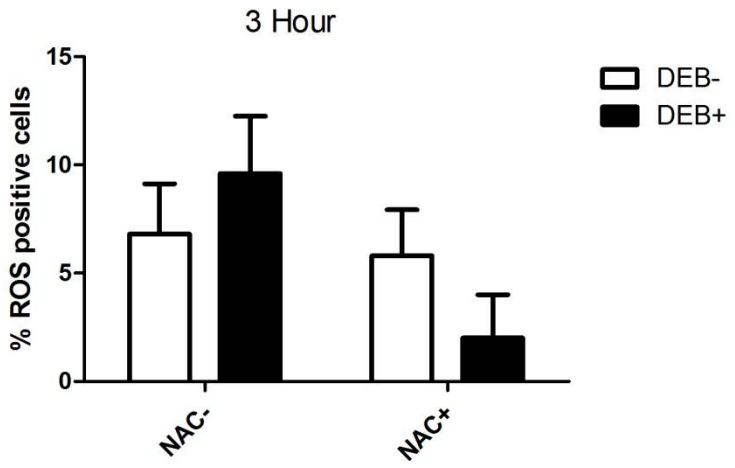


Figure 10. Acute exposure of DEB, 3 hours on TK6 human lymphocytes. Reactive oxygen species production in cells exposed to DEB for 3 hours, microscopy. Pretreatment of NAC (20mM) occurred one hour prior to DEB treatment. Levels measured via microscopic analysis. Data are presented as mean ±SEM.

4.3.5 Proliferation of NHBE cells exposed to DEB. Proliferation of lymphoblasts exposed to DEB did change. We expected to see the same results in the NHBE cells however, the proliferation was similar in the DEB exposed NHBE cells compared to the control (figure 11).

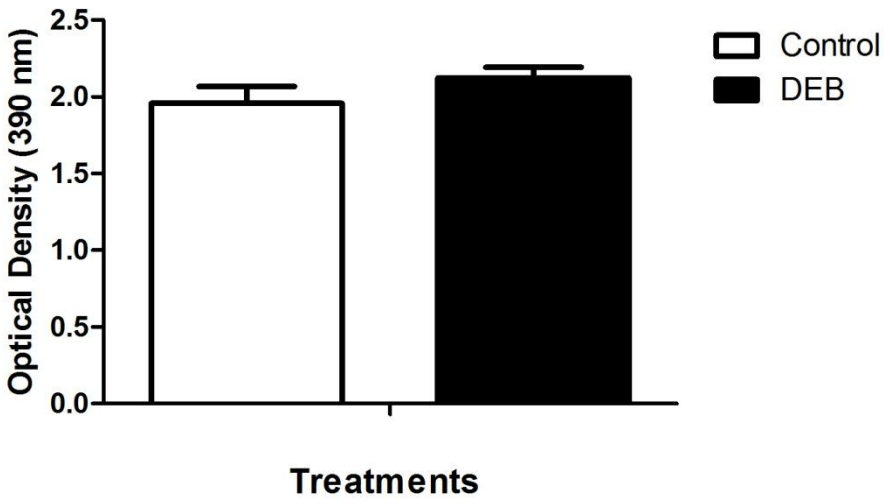
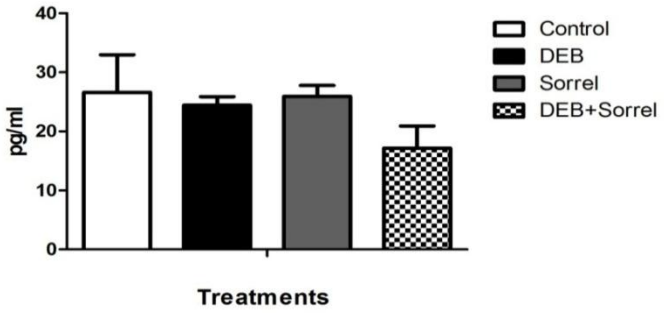


Figure 11. Proliferation of NHBE cells post 24 hour DEB exposure. Cells were exposed to 10 mM DEB for 24 hours. Data presented as mean ±SEM, n=12.

4.3.6 8-isoprostane levels were unaltered after DEB exposure. A common biomarker of lipid peroxidation and oxidative stress, especially in airway epithelium is 8-isoprostane. In this study we analyzed the effects of 8-isoprostane on NHBE cells that have been exposed to DEB for 8 hours and cells that were pretreated with NAC followed by a DEB exposure (also 8 hours). There were no significant differences between the treatments for levels of 8-isoprostane in this study.

A.



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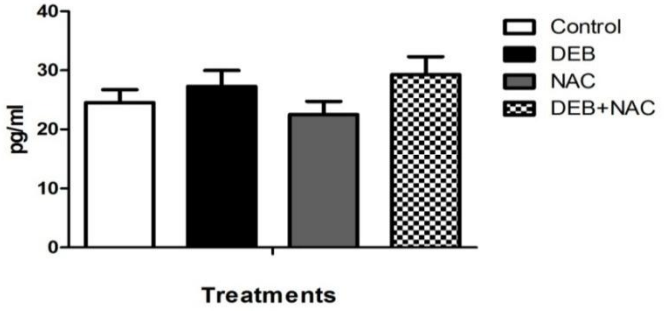


Figure 12. Levels of 8-isoprostane after DEB exposure in NHBE cells. A. NAC pretreated NHBE cells. B. Sorrel pretreated NHBE cells. Cells were exposed to 20 mM NAC (1 hour), sorrel (1%) 10mM DEB (8 hours) and DE (10%). Data are presented as mean ± SEM, n=3.

4.3.7 NHBE cells exhibited no cytotoxicity post-DEB exposure. Lactate dehydrogenase leakage is an indicator of cell membrane damage. Therefore, the more LDH released corresponds to the level of the cell membrane damage and to the level of cytotoxicity. We expected to see the DEB cause high levels of cytotoxicity due to the nature of the DEB in the lymphocytes. However there was no cytotoxicity found via the LDH release assay. These data may be invalid due to the low levels of cytotoxicity found in all treatments.

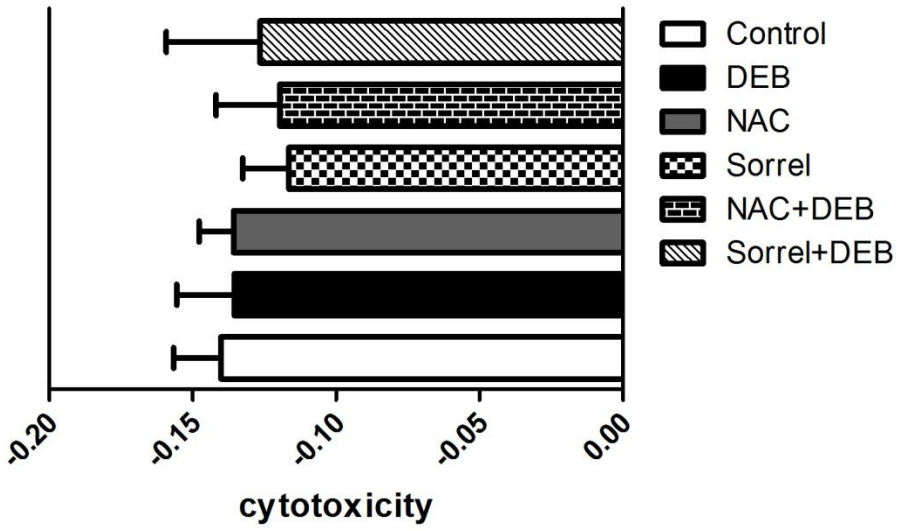


Figure 13. Cytotoxicity levels in NHBE cells pretreated to antioxidants and exposed to DEB for 7 hours. NAC (20 mM) and sorrel (1%) pretreatments for one hour prior to DEB (10mM) exposure (7 hours). Data are presented as mean ±SEM, n=6.

4.3.8 NO levels in NHBE after exposure to DEB. Nitric oxide is a well known free radical and functions in the regulation of pulmonary disease (Jorens PG 1993; Gaston B 1994). DEB did not cause an increase in nitric oxide levels; in fact, the levels of NO decreased in comparison to the control (figure 14). The decrease in NO levels could be due to the depletion of this signaling molecule. NO is known to have a role in pro-inflammatory signaling and DEB could have caused NO to increase but after seven hours, levels could have decreased NAC and sorrel were significantly different from the control as expected, and the pretreatment of NAC did not alter the nitric oxide levels before the 7 hour DEB exposure. However the sorrel pretreatment and corresponding DEB exposure did decrease NO levels significantly when compared to the control.

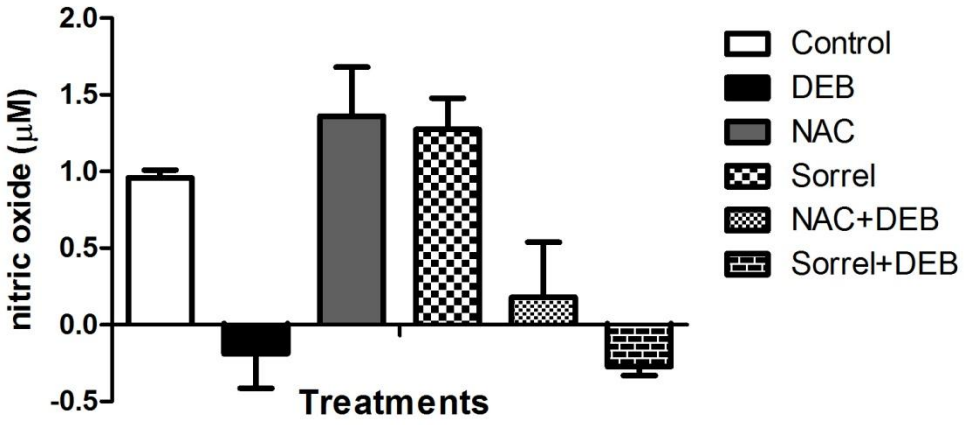


Figure 14. NO production post-DEB exposure in NHBE. Cells were exposed to pretreatments for one hour, DEB exposures occurred for 7 hours. Data are presented as mean ±SEM, n=3.

4.4 Discussion

Apoptosis can occur as a result of maintaining homeostasis, but can also be induced via environmental factors. Oxidative stress, a condition of imbalanced levels of oxidants compared to antioxidants, can also be induced by environmental factors and may play a role in the pathogenesis of apoptosis. Examples of some environmental factors that can cause apoptosis and oxidative stress are cigarette smoke and automobile exhaust. DEB, the most potent metabolite of the carcinogen BD (Yadavilli et al, 2007), has been shown to induce apoptosis, via oxidative stress in human B lymphoblasts.

In this study we investigated the apoptotic and oxidative stress inducing effects of DEB in lymphoblasts following exposures of less than 24 hours. As hypothesized, lymphoblast viability decreased and there were higher levels of apoptosis at 12 and 16 hours exposures in DEB exposed cells. The health of lymphoblasts were compromised post-DEB exposures. L-cysteine is a precursor to glutathione, a known antioxidant and known as NAC is commercially

available and currently used for a host of diseases. NAC effectively reduced apoptosis in lymphocytes at 16 hours but did not alter levels of ROS at 3 hours. These are confounding results due to studies stating NAC is known to function by increasing glutathione levels and DEB causes glutathione conjugates (mechanism of toxicity) (Boogaard et al, 1996). NAC was also shown to reduce ROS in lymphoblasts exposed to DEB at 24 hours in the study conducted by Yadavilli et al, 2007. We do observe higher levels of ROS in lymphoblasts exposed to DEB for 19.5 hours. It is anticipated that more in depth studies on acute versus chronic DEB exposures of lymphoblasts will illuminate more information on the DEB toxicity mechanisms in regards to apoptosis and oxidative stress.

We expected to observe decreased NHBE cell proliferation following DEB exposure *in vitro*; however, proliferation remained unaltered. When 8-isoprostane levels were quantified, there was no difference between control and DEB exposed cells. It is plausible that the dose of DEB administered to NHBE cells was not effective in eliciting a response in airway cells. In this study, lymphocytes had a tendency to undergo apoptosis more readily, while airway cells were less likely to possibly due to the fact that they encounter so many various pollutants on a daily basis. Similar findings were observed with NO evaluation compared to 8-isoprostane studies.

NO levels of the experimental groups were lower than the control groups. From this data it seems as if DEB decreases NO production. This observation needs to be further investigated. Due to NO's many origins it can be difficult to pinpoint what physiological activity it might have in the airway (Ricciardolo, 2003).

According to the LDH release results, none of the treatments were deemed cytotoxic and a dose response could not be established. Perhaps there was some interference with the reagents which resulted in low levels of "LDH release." Further investigations using additional

cytotoxicity detection assays should be considered to analyze the cytotoxicity of DEB in airway cells. The concentration of DEB (10mM) is optimal for lymphocytes which are known to undergo apoptosis rapidly however, potentially too low for airway cells which encounter onslaughts frequently.

4.5 Conclusion

In conclusion we observed a trend of decreasing viability in DEB exposed lymphoblasts over time. As expected, we also detected higher levels of apoptotic cells at 12 and 16 hours. NAC was able to decrease levels of apoptosis in DEB exposed lymphoblasts. We believe this is a result of NAC scavenging free radicals and therefore reducing apoptosis. ROS production was higher in DEB exposed cells compared to control cells. However, we did not detect a decrease in ROS at shorter DEB exposures, and NAC was not effective in reducing ROS levels. This could be due to a short exposure time which did not give enough time for ROS to build up. More studies should be conducted to monitor the apoptosis and ROS in lymphoblasts that are exposed to DEB in an acute manner.

More analysis should be conducted to validate DEB toxicity in NHBE cells. Perhaps an increase in the concentration of DEB for this cell type could result in an observable response. The effect of antioxidant pretreatments in decreasing oxidative stress could not be validated with NHBE cells under the conditions reported here. Future studies should established an effective doses of DEB and NAC to evaluated oxidative stress and antioxidative effects in NHBE cells.

CHAPTER 5

Pretreatment with Sorrel Reduces Biomarkers of Oxidative Stress in Airway Epithelial Cells Exposed to Agricultural Dusts *in vitro*

5.1 Introduction

Veterinarians, managers, inspection and processing workers and many others in the agriculture profession may be susceptible to illness and injury following agricultural environment exposures (do Pico, 1996). Acute bronchitis, chronic bronchitis, asthma, interstitial disease and acute lung injury are examples of pulmonary diseases that affect agriculture workers (Spurzem et al, 2002). Exposure to animal husbandry dusts, including dusts from barns that house sheep goats, horses, cattle, swine and poultry, is the leading cause of these manifestations. Many of the aforementioned respiratory diseases are mediated by cytokines including interleukin-6 (IL-6) and IL-8; pro-inflammatory cytokines whose levels are known to increase after dust exposure in airway cells following swine dust exposure (Romberger et al, 2002). IL-8 is also a potent chemokine and recruiter of neutrophils. It is well known that neutrophils, and other phagocytic cells, contribute to an increase of oxidative stress by releasing free radicals as a tool to control bacteria and other harmful substances as well as being released from neutrophils that have undergone a respiratory burst. We believe oxidants, whether from endogenous or exogenous sources, contribute to respiratory complications. Oxidants mediate injury by binding to important macromolecules such as proteins, lipids and DNA and inhibit their normal function. However, more studies are needed to fully understand the molecular events modulated by oxidants.

Recently, phytonutrients including flavonoids, stillbenes and lignans from functional foods (e.g., fruits and vegetables) have become of interest because these natural, active

compounds from plants have been studied for their antioxidant, disease prevention actions. Phytonutrients are known to strengthen immunity as well as other health-promoting benefits. Some phytonutrients can function as precursors to antioxidants (Fullerton et al, 2008). The commercially available antioxidant acetylcysteine, also known as N-acetylcysteine or N-acetyl-L-cysteine (NAC), is a thiol compound that has mucolytic potential and is a direct pre-cursor to reduced glutathione (Sadowska, Manuel-y-Keenoy, De Backer, 2007). NAC is also known to decrease respiratory bursts caused by neutrophils which can lead to a decrease in free radicals and ultimately oxidative stress (Stolarek, Bialasiewicz, Nowark 2002) and Nordgren and colleagues (2013) provided evidence that maresin-1 can decrease levels of IL-8 and IL-6 in bronchial epithelial cells. Maresin-1 can function as a lipid mediator which can be a form of oxidative stress. An antioxidant enriched diet can provide benefits such as decreasing free radical levels and supplying the body with free radical eliminators. *Hibiscus sabdariffa (*sorrel) is a plant native to the West Indies and China which has been studied recently for it antimicrobial and anti-imflammation capacity (Fullerton et al, 2008; 2011). Sorrel calyces contain potent phytonutrients that are rich in antioxidants including vitamin C (Fullerton et al, 2008). In Essa (2006), rats pre-exposed to sorrel were protected against the harmful effects of carcinogens. In our lab, there have been ongoing studies that suggest sorrel may have protective effects against dust mediated inflammation. It is believed that this occurs through free radical scavenging and enhancement of endogenous antioxidant levels (i.e., glutathione, catalase and superoxide dismutase).

In the present study we investigated the hypothesis that pretreatment with sorrel will reduce animal husbandry dust mediated oxidative stress through reduction of oxidative stress biomarkers (e.g., nitric oxide and 8-isoprostane) in airway epithelial cells. Respiratory cells that

were pretreated with sorrel and stimulated with extracts of husbandry dust showed a significant decrease in oxidative stress compared to control cells that were not pretreated with sorrel.

5.2 Materials and Methods

5.2.1 Cell culture. Normal human bronchial epithelium (NHBE; Lonza, Walkersville, MD) cells were maintained in incubators at 37°C and 5% CO_2 and cultured in NHBE complete media (1:1 mixture of BEBM /DMEM, and Lonza SingleQuots® and Nystatin. Cells were used at passage 2 and were seeded on rat tail collagen-coated tissue culture plates at a density of 1 x 10^4 cells/cm^2 or as noted until confluent. Prior to experimental investigations, cells were switched to a medium that did not contain epidermal growth factor (EGF) or bovine pituitary extract (BPE) overnight.

5.2.2 Sorrel extraction and isolation. Dried sorrel calyces were purchased from a local grocery store and freeze-dried. The freeze-dried calyces were grounded to powder and then stored at -20 °C until use. The phenolics in powdered freeze-dried calyces were extracted by the ultrasound-assisted method. Phenolics of the calyces were extracted from 10 g of ground, freeze-dried samples using 100mL of 80% aqueous methanol. The mixture of freeze-dried powder and 80% aqueous methanol was sonicated for 20 minutes with continual nitrogen gas purging. The mixture was filtered through Whatman (Maidstone, United Kingdom) #2 filter paper using a Buchner funnel and rinsed with 50mL of 100% methanol. Extraction of the residue was repeated using similar conditions. The two filtrates were combined and transferred into a 1-L evaporating flask with an additional 50 mL of 80% aqueous methanol. The solvent was evaporated using a rotary evaporator at 40°C. The remaining phenolic concentrate was dissolved in 50mL of 100% methanol and diluted to a final volume of 100 mL using distilled deionized water obtained with a NANOpure water system (Barnstead, Dubuque, IA, USA). The mixture

was centrifuged at refrigerated temperatures using a Sorvall (DuPont, Wilmington, DE, USA) RC-5B refrigerated superspeed centrifuge, at 10,000 g for 20 minutes and then stored at -4°C for future use.

5.2.3 Lactate dehydrogenase release. To determine the cytotoxic effect of the agricultural dusts on the NHBE cell, the lactate dehydrogenase (LDH) assay was used by Roche. The enzyme resides in the cytoplasm of mammalian cells. When the plasma membrane is damaged, LDH can be released and can be marker of cell viability. Post-treatment, 1.5 ml of supernatant was collected and centrifuged for 5 minutes at 4,000-5,000 RPM. The resulting cell pellet, which was formed, was not disturbed and the supernatant was transferred to a new clean tube. The supernatant was subjected to the cytotoxicity kit obtained from Roche Diagnostics and used per manufacturer's instructions.

5.2.4 Nitric oxide production. Supernatants from exposed cells were used to measure NO levels using Greiss reagent kit obtained from Promega. Supernatant was collected and centrifuged for 5 minutes at 4,000-5,000 RPM. Without disturbing the cell pellet, the supernatant was collected in a new microfuge tube. The kit was used per manufacturer's instructions.

5.2.5 DCF staining. Cells were grown in a 96-well black, clear bottom tissue culture plate format. Cultures (90-95% confluency) were washed with PBS and loaded with 10 μM dichlorofluroscein (DCF; Invitrogen) at 37°C for 50 minutes while wrapped loosely in foil to protect form light. DCF was aspirated off, surfaces were washed twice with PBS and cultures were either pre-treated or not with an antioxidant (NAC or Sorrel) and stimulated with dust extracts. Plates were read on a SpecrtoMax M5microplate reader according to manufacturer's instructions for 3.5 hours.

5.2.6 Proliferation assay. Cells were cultured in a clear 96 well plates until they reached 75% confluency. Cells were then pretreated with antioxidant, stimulated with dust extract and analyzed using the BrdU cell proliferation assay (Roche Diagnostics) as per manufacturer's instructions.

5.2.7 Prostaglandin production. To evaluate the levels of prostaglandin or PGE_2, an EIA Kit (Enzo Life Sciences, Plymouth Meeting, PA) was utilized according to manufacturer's instruction. Briefly, appropriate components were brought to room temperature before use. Standards were made from 2500 pg/ml to 39.1 pg/ml. 100 ml of standards or samples were added to each well, except for controls. (Several different controls were made, in accordance to instructions, for the blank, Bo, NSB and total activity. Control wells received different treatments, depending on desired end point.) Next, 50 ml of PGE_2 conjugate and then 50 ml of PGE_2 antibody was added to standard and sample wells. The plate was then incubated for two hours, at room temperature, while being shaken. All wells were then aspirated and washed three times in wash buffer. Next, 200 ml of pNpp substrate was added to all wells for 45 minutes. Finally, 50 ml of stop solution was added to all wells. The plate was read at 405 nm.

5.2.8 Measurement of 8-isoprostane. Following cell exposure to antioxidant pretreatment and dust extract stimulation, the 8-isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI) was used according to manufacturer's instructions to evaluate oxidative stress. Supernatants were collected post-exposure and diluted either 1:10 or left undiluted. The concentrations were quantified as previously reported (Van Hoydonck et al. 2004).

5.3 Results

5.3.1 Cytotoxicity levels of NHBE cells exposed to the various agriculture dusts. Lactate Dehydrogenase (LDH) is an enzyme that normally resides in the cytoplasm of the cell.

When it is released and leaks into the surrounding environment, suggesting cell membrane damage. The level of LDH in culture media is directly proportional to the level of cytotoxicity. In this study, we evaluated the cytotoxicity of the various semi-confinement facilities agriculture dusts: dairy, equine and small ruminant, as well as the swine and poultry confinement facilities shown in figure 15. The exposure to the equine and swine dust increased in a toxicity concentration dependent manner. The poultry dust toxicity levels were very similar across the concentrations. The small ruminant and dairy dust toxicity were inverted. In the dairy dust, extract exposed cells 1% and 10% showed low levels of toxicity while the 5 and 8% showed higher levels. Small ruminant dust extracts caused higher levels of toxicity in 5 and 10% whereas 1 and 8% were lower.

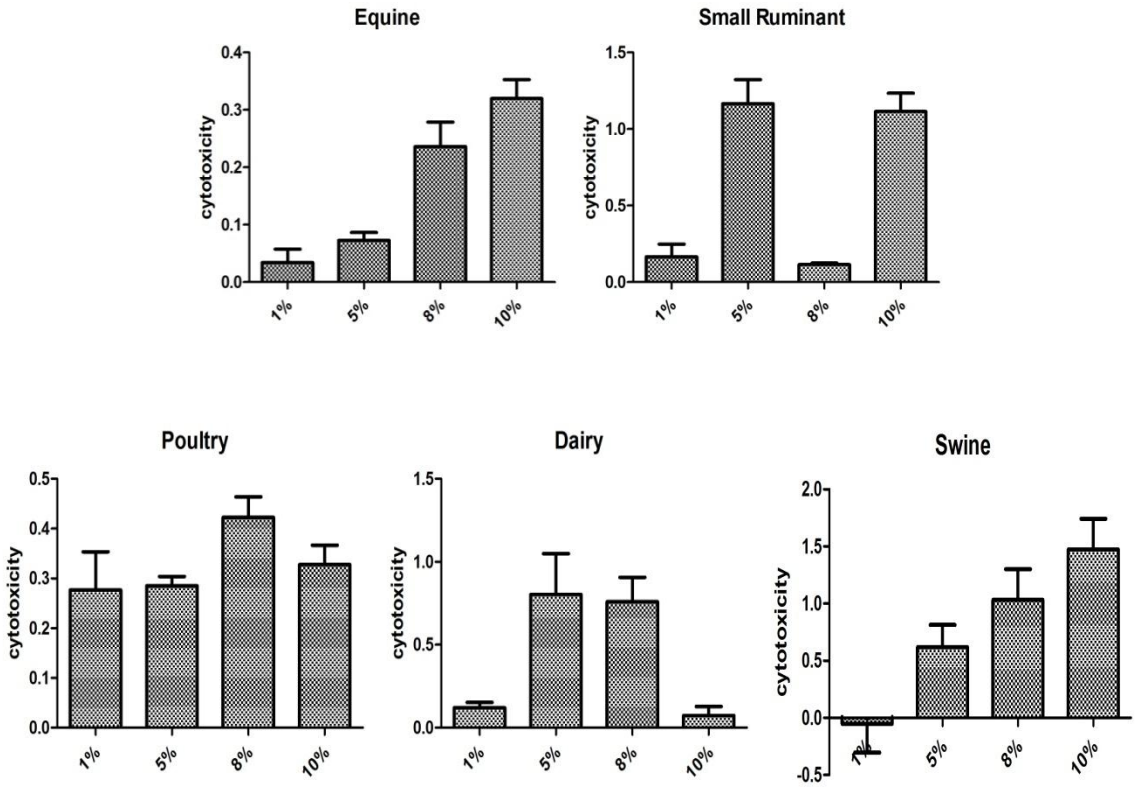
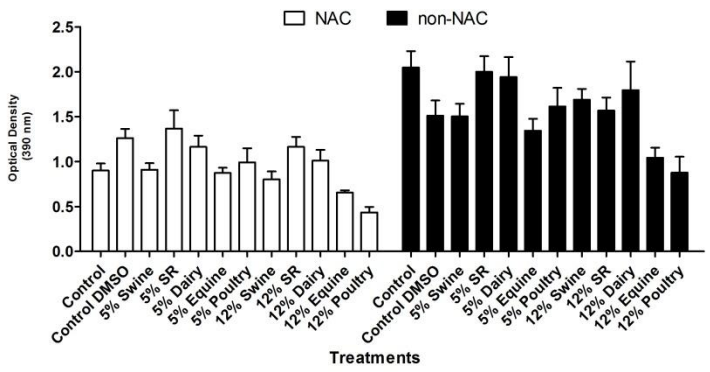


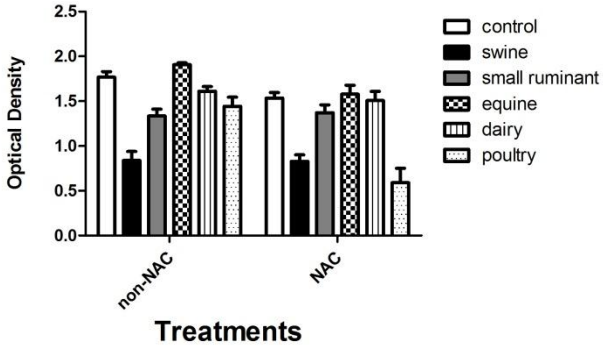
Figure 15. Cytotoxicity analysis of agricultural dusts on NHBE cells for 6 hours. Lactate dehydrogenase assessment was utilized to identify plasma membrane damage of NHBE cells exposed to swine, small ruminant, poultry, dairy, and equine husbandry dusts. Data are presented as mean ± SEM, n=3.

5.3.2 Proliferation of airway cells following agricultural dust exposure. Along with cytotoxicity data to show the health of the cells exposed to the different agricultural dusts, we also examined the proliferation in three different cell types. In figure 16A , NHBE cells were exposed to the various agricultural dusts at 5 and 12%, with one group of cells receiving a pretreatment of antioxidant scavenger, NAC, for one hour prior to dust exposure. Higher levels of proliferation in cells not exposed to NAC. In figure 16B, A549 cells were exposed to the various dusts (12%) for 24 hours. A similar affect was observed in the NAC treated cells, a marked decrease in proliferation in cells exposed to the antioxidant NAC. In figure 16C, PTBE cells were exposed to swine dust at 5% and pretreated with sorrel (0.1 and1%) for one hour before dust exposure. No differences were present in between treatments for proliferation conducted in the porcine cells. We expected to see a decrease in proliferation in cells exposed to dust extract. The proliferation of cells should be kept normal at a steady rate. Cells that increase or decrease proliferation can indicate a signaling problem. In this study, the proliferation was measured by staining the DNA (in particular thymidine) and the resulting color produced indicates how much DNA synthesis occurred.

A.



B.



C.

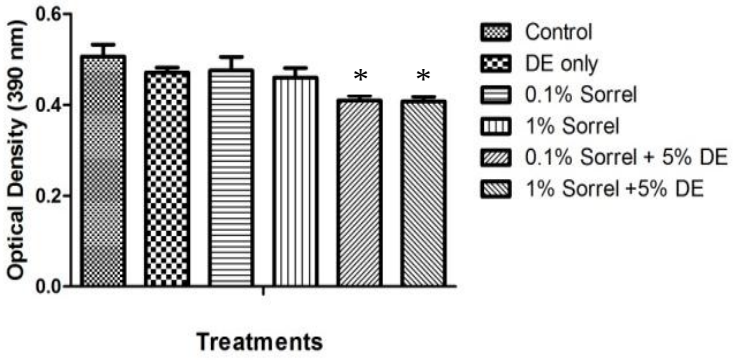
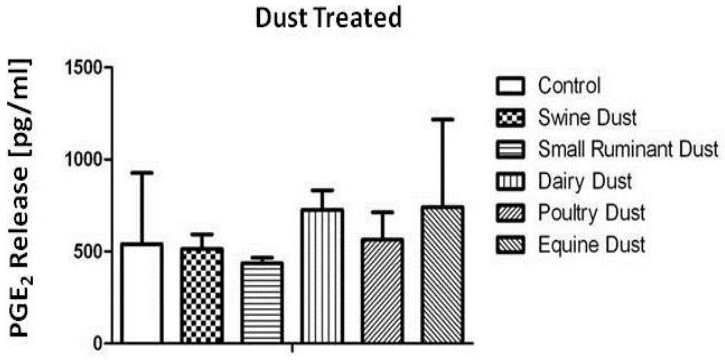


Figure 16. Proliferation of airway cells exposed to various agricultural dusts. A. Proliferation of NHBE cells exposed to the various agricultural dusts at 5% and 12% (swine, small ruminant, dairy, equine, and poultry) and NAC (10 mM) for 24 hours. B. Proliferation of A549 cell line exposed to the same dusts (12%) for 24 hours and pretreated with NAC (10mM). C. PTBE cells exposed to swine dust extract (5%) for 24 hours and pretreated with sorrel (1%) for one hour. Data represents the mean ± SEM, n=8. *-p-value < 0.05.

5.3.3 Effects of Sorrel pretreatment on prostaglandin production. Prostaglandin is a member of arachidonic pathway and is known to modulate inflammation. In this study, we analyzed the levels of prostaglandin via an ELISA assay on NHBE cell supernatant that had been exposed to the various agriculture dusts at 5% and also pretreated a group of cells prior to dust exposure with sorrel extract (1%). We did not see differences between the various treatments on the dust only treated cells (figure 17A). We also observed no differences when sorrel was utilized as an antioxidant pretreatment (figure 17B).

A.



B.

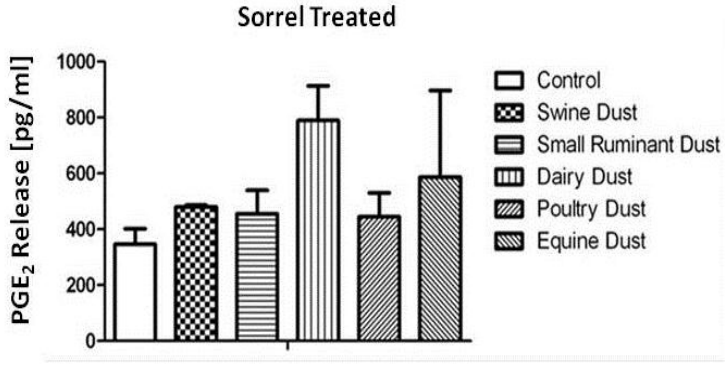
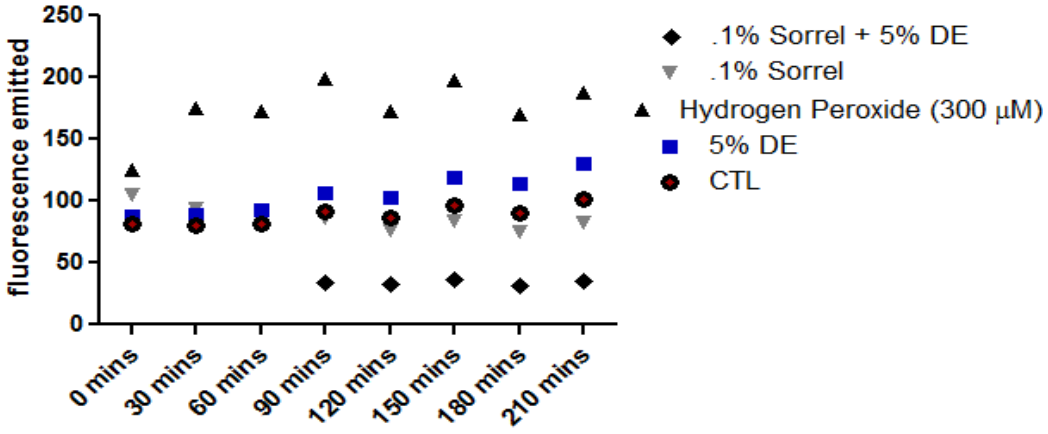


Figure 17. Prostaglandin production of NHBE after dust exposure and Sorrel pretreatment. A. NHBE cells exposed to the various agricultural dusts. B. NHBE cells were pretreated with sorrel (1%) for 1 hour before dust treatment (5%). Data represents the mean ± SEM, n= 3.

5.3.4 Oxidant levels in airway cells exposed to swine dusts. We examined the levels of free radicals that airway cells produce following antioxidant/dust exposure. In figure 18A, PTBE cells were exposed to 5% swine DE, a pretreatment of sorrel (.1%) and hydrogen peroxide (300 µm) for a positive control. Cells were exposed to the various treatments for 3.5 hours (210 minutes) and readings were taken every thirty minutes. The hydrogen peroxide significantly increased the oxidant levels when compared to the control. The cells treated with a sorrel pretreatment prior to dust exposure had significantly lower levels of oxidants compared to the sorrel only , control and dust only treatments. We expected to see higher levels of oxidants in cells exposed to dust only; however, it did not differ from the control. We also conducted a concentration gradient of swine confinement unit dust on NHBE cells over one hour (figure 18B). We observed an increase of hydrogen peroxide production in a time and dose-dependent manner the various dust treatments ($p<0.001$). The hydrogen peroxide as the high control did not increase the levels of oxidants as expected.

A.



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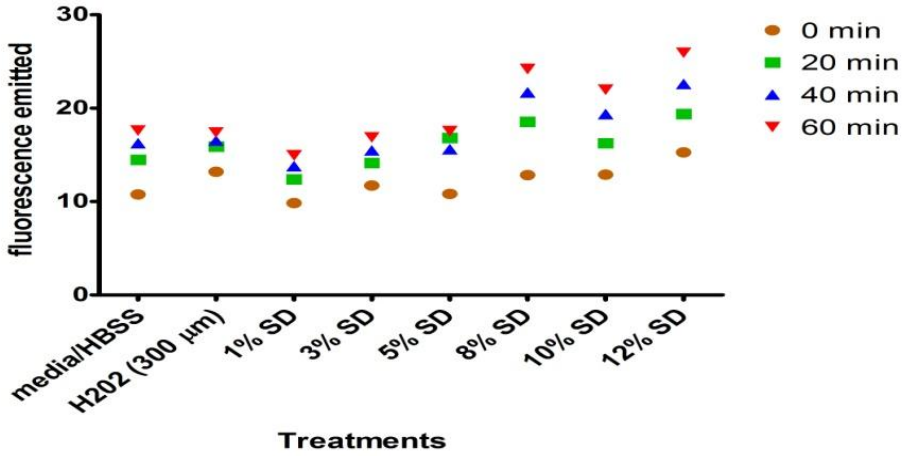
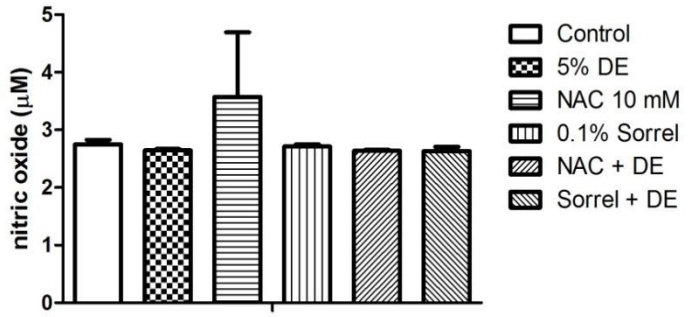


Figure 18. Hydrogen peroxide production in airway cells post swine dust extract exposure. A. Oxidant levels of PTBE cells exposed to 5% swine dust. PTBE cells were exposed to media (control), hydrogen peroxide (300 µM), NAC (10mM), and pretreated with 1% sorrel for one hour. Data represented as the mean ± SEM, n=8. B. Oxidant levels of NHBE cells exposed to various concentrations of swine dust over 60 minutes. Measurement of oxidants in NHBE cells exposed to Swine Dust (1,3,5,8,10 and 12 %) for 0, 20, 40, & 60 minutes. Oxidant production increased in time and concentration dependent manner. Data presented as mean ±SEM, n=16.

5.3.5 Levels of nitric oxide in airway cells pretreated with antioxidants and exposed swine dust. Nitric oxide is a free radical that is normally found as a signaling molecule and is

predominant in the respiratory tract. In this study, we analyzed the levels of the nitric oxide in cells exposed to swine dusts as well as an antioxidant pretreatment of NAC or sorrel. In figure 19A, PTBE cells were pretreated with sorrel (0.1%) or NAC (10 mM) for one hour prior to a four hour 5% DE exposure. We expected to see increased levels of nitric oxide in the DE exposed cells, however we did not. In fact, we did not see any differences in the various treatment groups. In figure 19B, we exposed NHBE cells to 10% swine DE for 7 hours as well as a one hour pretreatment prior to the DE exposure. The NAC and sorrel were increased to 20 mM and 1% respectively. The 10% DE increased the nitric oxide levels when compared to the control group. The antioxidant pretreatments had low levels of NO similar to the control. We expected to see a more dramatic decrease in NO levels in the combined treatments, NAC and sorrel (pretreatments) and the 7 hour DE exposed cells;however, the sorrel significantly decreased the NO levels when compared to the DE exposed group whereas NAC did not decrease the levels. NAC may be as efficient in neutralizing H_2O_2 as glutathione; however, it lacks in carrying capacity (Benrahmoune, Therand & Abedinzadeh, 2000; Gillissen, 1997).

A.



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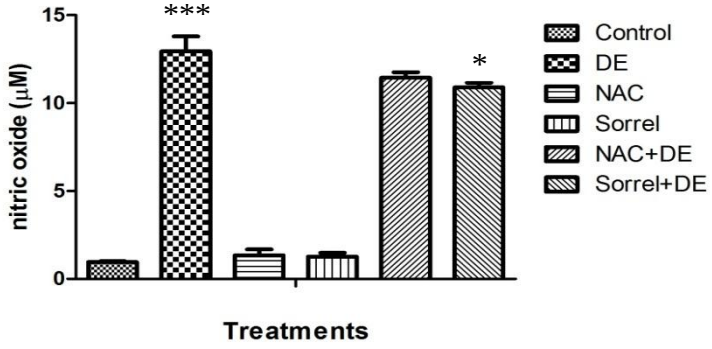


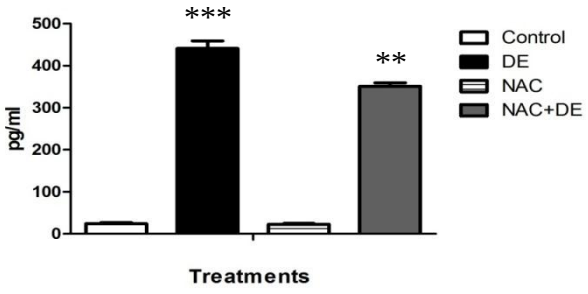
Figure 19. NO levels in airway cells post swine dust exposure. A. PTBE cells were pre-exposed to NAC (10 mM) or Sorrel (0.1%) for a one hour pretreatment then exposed to 5% DE for 4 hours. B. NHBE cells exposed to pretreatment of NAC (20mM) or sorrel (1%) following a swine dust extract (10%) exposure for 7 hours. Data are presented as mean ±SEM, n=3. *-p-value<0.05 when compared to DE. ***-p-value<0.001 when compared to the control.

5.3.6 Levels of 8-isoprostane in NHBE cells post-dust extract exposure and pretreatment of antioxidants.

8-isoprostane is a member of the arachidonic acid pathway which is known to cause inflammation. Inflammation can be known to increase oxidative stress. 8-isoprostane is commonly found in the airway and is a biomarker for oxidative stress. In this study, we tested the levels of 8-isoprostane in NHBE cells that had been exposed to 20 mM NAC (figure 20A) or 1% sorrel (figure 20B) for one hour and DE (10%) for 7 hours. The levels of 8-isoprostane were significantly higher in the DE only treated cells as depicted in figure 20A and

20B. The antioxidant pretreatment NAC significantly lowered 8-isoprostane in DE exposed cells however sorrel was able to decrease levels even lower than NAC.

A.



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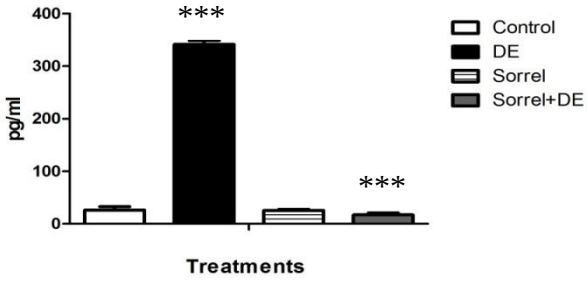

Figure 20. Levels of 8-isoprostane in NHBE cells exposed to DE for 7 hours. NHBE cells were exposed to a pretreatment of 20 mM NAC (A) or 1% sorrel (B) followed by a swine dust (10%) for 7 hours. Data presented as mean ±SEM, n=3. **-p-value<0.01 compared to DE, ***-p-value<0.001 when DE is compared to the control and when sorrel+DE is compared to DE.

5.4 Discussion

It is well established that agriculture dusts cause respiratory diseases. In this study, we evaluated the capacity of sorrel to reduce oxidative stress in airway cells caused by animal husbandry dusts from the NC A&T SU farm. We report here that airway cells exhibit signs of cytotoxicity and oxidative stress following exposure to animal husbandry dusts in vitro in a dose-dependent manner. In the NHBE and A549 cells, we observed a decrease in cells that were pretreated to 10 mM NAC. This result was unexpected. NAC is a known antioxidant scavenger and it should not alter the proliferation. Perhaps at this concentration, the NAC is not effective.

In the PTBE cells, we see a significant decrease in cells pretreated with sorrel, which is our novel antioxidant approach. This finding is interesting due to the fact, the NAC behaved in a similar manner. In the NHBE cells, we noticed decreases in proliferation 5% swine and equine dusts. On the other hand, the other dusts at 5% did not alter the proliferation. The equine and poultry 12 % dust extracts decreased the proliferation in comparison to the control. Overall, cytotoxicity and proliferation do not seem to be compromised under an agriculture dust exposure. We also did not observe any differences in prostaglandin levels between cells that were treated with agriculture dusts for 4 hours. In Sandulache at al, (2009), they noted that airway injury activates prostanglandin E2 in airway epithelium. We expected to see an increase of prostaglandin, which is a member of the arachidonic acid pathway and is prominent in inflammation. Perhaps to examine the actual levels of inflammation in these cells exposed to the various agricultural dusts, we need to consider a different means of testing as well as identify another biomarker. To look at oxidative stress more closely, we looked at the levels of free radicals being produced when the airway cells were exposed to swine dust. When we tested the levels of oxidants in NHBE cells exposed to 5% swine dust, we did not see an increase of oxidants when compared to the control. However, the pretreatment of sorrel reduced antioxidants dramatically in the sorrel and dust treated cultures. When we conducted an analysis to examine the levels of free radicals in PTBE exposed to various dust concentrations over time, we observed an increase of oxidants over time.

We also evaluated modulation of the free radical nitric oxide or NO, which has a functioning role in the airways as signaling molecule and is the precursor to nitric oxide synthase (NOS). iNOS is an inducible enzyme of NOS that is known to be prominent in airway disease and induced by a variety of airway pollutants. We did not observe any differences in PTBE cells pretreated with antioxidant and exposed to SCF DE; however, in companion studies with NHBE

cells, we observed a marked increase in NO in cells exposed to 10% DE. NAC pretreatment did not significantly decrease the levels of NO; however, sorrel attenuated these effects, suggesting that sorrel may be more efficient at preventing oxidative stress than the commercially available NAC under the conditions described herein. Nitric oxide is a known regulator of ciliary beating. In Wyatt et al. (2008), exposure to hog barn dust extract altered epithelial beating in bovine ciliated cells in vitro. In our studies, swine dust extract did not have an effect or porcine airway cells, but NHBE cells showed a response evidenced by increased NO production. However, more studies are warranted to fully understand the significance of oxidants such as NO in airway cells because NO is a potent signaling molecule. For example, it is responsible for mediating relaxation of airway smooth cells by modulating calcium (Perez-Zoghbi, Bai et al, 2010), an important signaling molecular and cofactor for numerous enzymatic reactions. Thus, it is plausible that the increased levels of NO released by agricultural dust stimulated airway epithelial cells in our study may have a beneficial effect in promoting relaxation of airway smooth muscle cells; however, this was not investigated.

Lastly, we examined the levels of 8-isoprostane, a common biomarker for lipid peroxidation, which is a form of oxidative stress. 8-isoprostane is known to regulate human airway smooth muscle function and can be upregulated in airway diseases such as asthma as well as environmental exposure (Voynow et al, 2011). In the present study, the levels of 8-isoprostane were significantly higher in DE exposed NHBE cell cultures after a 7 hour exposure compared to control cultures. Antioxidant pretreatment with NAC decreased the levels of 8-isoprostane; however, sorrel decreased the levels with better efficiency as evidenced by a complete prevention of NO induction observed by baseline (control) levels in cultures pretreated with sorrel and stimulated with SCF DE. We also note that the NAC and sorrel pretreatments

did not affect the cells adversely which corresponds with the aforementioned thought that the concentrations used are most likely optimal. In Montuschi et al, (2000), smokers who suffered from COPD had higher levels of 8-isoprostane compared to healthy non-smokers. This leads us to believe oxidative events occurring in NHBE cells *in vitro.*

Differences observed in the experiments concerning the PTBE cells, higher concentrations of dust and longer exposures should be taken into consideration. This could be phenomenon due to adaptive measures that might occur in pigs which can provide these types of cells of protection.

5.5 Conclusion

In conclusion, exposure to agriculture dusts can induce oxidative stress in respiratory cells which may help to explain the mechanism of respiratory diseases in farmers, agricultural workers and farm visitors. This study provides insight for understanding cellular and molecular events governing agriculture related respiratory diseases and a possible antioxidant preventative mechanism. While numerous studies report inflammation following exposure of bronchial cells to swine dust, our finding suggest oxidative stress as a possible mechanism for changes in airway cell viability and response. However, impact of these finding is not altogether clear at this time since it is well known that free radicals are important signaling molecules. Phytonutrients from sorrel have antioxidant properties and more studies are warranted to fully understand the antioxidant mechanism.

CHAPTER 6

Conclusions

The respiratory tract encounters many types of pollutants continuously. In this study we analyzed two environmental pollutants, agricultural dusts from animnal husbandry buildings and diepoxybutane, a known carcinogen found in cigarette smoke, on induction of oxidative stress in respiratory cells and lymphoblasts. The dusts are extremely complex and can contain various particle sizes, bacteria and other factors that could cause airway dysfunction. Animal husbandry facilities which are confinement in nature have proven to contribute to asthma, chronic bronchitis and other COPD like diseases. In this application we took a look at the variations between the confinement and semi-confinement dusts.

In this study, we determined that confinement facility dusts from North Carolina A and T State University farm contain inhalable, respiraable and thoracic particles; sizes that are well known for their ability to cause respiratory disease. Confinement dusts harbored more bacteria and smaller particles than the semi-confinement dusts. All dusts altered pH and contained levels of silicon, calcium, potassium, and phosphorus which are of respiratory importance.

We also evaluated DEB associated toxicity in lymphoblasts as well as airway cells. DEB is a known carcinogen and toxicant and is known to cause aberrant effects *in vivo* and *in vitro*. We validated that oxidative stress and apoptosis occurs in acute DEB exposures in lymphoblasts similar to the long-term exposures. On the other hand, we did not see any cellular differences (growth rate, cell integrity, free radical production) in NHBE cells that were exposed to DEB and those studies are inconclusive at this time.

Finally, we analyzed the effects of various animal hysbandry dusts on airway epithelial cells. Cellular health was compromised (proliferation, membrane integrity) and oxidative stress

occurred (hydrogen peroxide, nitric oxide, and 8-isoprostane production/release) in NHBE cells. PTBE cells and A549 cells, however, were more resistant to changes occurring after dust exposures. We also proved sorrel could be a potential natural antioxidant treatment for oxidative stress for respiratory diseases.

In conclusion, our hypothesis that confinement dusts cause more oxidative stress and inflammation and sorrel was able to prevent aspects of oxidative stress was validated. While these findings are important for understanding the molecular events governing respiratory symptoms associated with inhalation exposure to agricultural dusts, the full significance of these findings in not known. It is expected that the work summarized here would provide insight for understanding the exposures experienced by farmers, workers and farm visitors. It is hope that holistic preventatives such as sorrel may be considered as supplements to reduce the burden of agriculture-related respiratory diseases.

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Appendix

Primers used for identification of bacteria via PCR.

Organism	Primer Name	Sequence	Tm	Product Size, bp	Reference
E. coli	Ecoli670-F Ecoli670-R	5'-ACCTGCGTTGCGTAAATA-3' 3'-GGGCGGGAGAAGTTGATG-5'	58°C	670	McDaniels et al (1996)
Listeria	LM404-F LM404-R	5'-ATCATCGACGGCAACCTCGGAGAC-3' 3'-CACCATTCCCAAGCTAAACCAGTGC-5'	68°C	404	Wu et al others (2004)
Salmonella spp.	Sal284-F Sal284-R	5'-GTGAAATTATCGCCACGTTCGGGCAA-3' 3'-TCATCGCACCGTCAAAGGAACC-5'	64°C	284	Rahn et al (1992)
Bacillus	8F 1429R	5'-AGTTGATCCTGGCTCAG-3' 3'-ACCTTGTTACGACTT-5'	52°C	1554	Sacchi et al 2002
Clostridium	16SUNI-L UNI16S-R	5'-AGAGTTTGATCATGGCTCAG-3' 3'-AAGGAGGTGATCCAGCCGCA-5'	54°C	1500	Sasaki et al 2001
Actinomycetes	F243 R1378	5'-GGA TGA GCC CGC GGC CTA-3' 3'-CGG TGT ACA AGG CCC GGG AAC G-5'	72°C	1176	Heur et al 1997
Staphylococcus	Seb-1(fwd) Seb-4(rev)	5'-TCG CAT CAA ACT GAC AAA CG-3' 3'GCA GGT ACT CTA TAA GTG CCT GC-5'	55°C	477	Becker et al 1998
Pseudomonas	Ps-for Ps-rev	5'-GGTCTGAGAGGATGATCAGT-3' 3'-TTAGCTCCACCTCGCGGC-5'	55°C	1007	Widmer et al 1998

Energy Dispersive X-ray Spectroscopy on agriculture dusts collected from the animal housing units at the NC A&T SU farm.

Element (C%)	O	Na	Al	Si	P	S	Cl	K	Ca	Mg	Fe
Poultry	79.52	0.83	1.33	1.96	3.06	1.18	0.50	3.08	5.91	2.63	ND
Swine	77.96	2.52	0.62	0.91	4.35	1.63	1.69	5.11	2.57	2.55	0.09
Equine	69.01	1.23	5.62	16.32	0.25	0.36	0.31	1.13	2.01	1.35	2.42
Sm. Rum.	74.13	1.09	2.87	11.47	1.01	0.60	0.80	1.96	3.33	1.56	1.19
Dairy	87.58	1.57	0.88	2.59	1.41	0.52	0.45	1.86	1.36	1.51	0.27

ND, not detected.
C%, percent carbon atom.

ICP-OES analysis of agriculture dusts collected from the NC A&T SU animal houses.

ELEMENT conc./ppm	AL	Ca	Cr	Fe	K	Mg	Mn	Na	P	S	Si	Zn
Agriculture Unit												
Poultry	3.37	184.66	BDL	4.50	90.20	41.87	0.60	12.39	28.98	19.77	215.04	1.49
Swine	9.67	280.76	BDL	89.52	227.94	140.92	3.59	50.10	216.10	101.00	104.33	17.51
Equine	108.77	169.83	BDL	103.95	140.78	96.53	2.73	36.36	20.37	33.83	89.24	16.95
Sm. Rum.	90.11	244.38	BDL	95.69	153.32	110.22	3.47	24.08	43.07	37.03	259.91	16.30
Dairy	180.78	210.76	0.08	223.90	111.37	170.31	4.15	25.65	24.61	28.41	221.63	5.28

BDL, below detection limit. Elements that were tested, however below detection limit in all dusts were cadmium, copper, nickel, and lead. Sm. Rum., small ruminant.