

CATTLE FEEDLOT DUST: SOLUBILITY IN LUNG SIMULANT FLUID AND
STIMULATION OF CYTOKINE RELEASE FROM LUNG EPITHELIAL CELLS

by

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Abstract

Beef cattle feed lots produce significant, local point source pollution of the atmosphere. The dusts generated in the CAFOs are complex mixture of fine and ultrafine particles, organic compounds, transition metals, and adsorbed toxic gases. Since each component is toxic in itself, we do not fully understand the relative importance of each component in the dust and their interactions to inducing inflammatory changes in the lung. We did extensive literature searches to understand the mechanism of dust toxicity in respiratory system. This lead to focusing on solubility of dust in lung simulant fluid, and *in-vitro* study of release of two common biomarkers of inflammatory processes IL-6 and IL-8 from lung epithelial cells.

Various concentrations (1 to 50%) of the dust extract induced release of IL-6, and IL-8 from lung epithelial cell as indicators of pro-inflammatory changes (IL-6), and amplification and maintenance of inflammation (IL-8). IL-6 release had dose dependence; peak production was seen with 25% dust extract. IL-8 production went down as the concentration of the dust extract increased from 1% to 25%. However, 50% dust extract was cytotoxic to the cell leading to 10-15% cell viability. At non-cytotoxic concentrations for lung epithelial cells, production of IL-8 was reduced. These findings suggested that higher exposure concentration were required to initiate inflammation as indicated by IL-6 release. Lower exposure concentrations (1 and 5% extracts) were related to optimal release of IL-8 needed to amplify and maintain the inflammatory response.

Inhibition of endotoxin didn't significantly change the pattern of IL-6 or IL-8 release from epithelial cells. This finding suggested that at least a portion of the mechanism by which particle induced cytokine release from the lung epithelial cells was not endotoxin dependent. Heating samples at 120⁰C for 5 minutes modified some of the toxic properties of the dust extracts but didn't completely detoxify it. We observed that longer incubation period was required to peak release for both IL-6 and IL-8. However, the higher concentration of sample (50% extract) found to be cytotoxic in non-heat treated sample was no longer cytotoxic and induced both IL-6 and IL-8 release from the lung epithelial cells. This result suggested that heat

treatment could reduce some of the dust extract's cytotoxic properties. However, the extract's potential to induce peak cytokine release increased.

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CHAPTER 1 - CATTLE FEEDLOT DUST: SOLUBILITY IN LUNG SIMULANT FLUID AND STIMULATION OF CYTOKINE RELEASE FROM LUNG EPITHELIAL CELLS

1. Introduction

Increasing numbers of cattle and hogs are produced raised, fattened, and consumed in North America and other parts of the world. The animal confinement facilities (commonly known as Confined Animal Feeding Operations, CAFO) generate significant amount of dust particles. Dusts generated in CAFO facilities are rich source of inorganic and organic dust, allergens, endotoxin, and toxic gases such as nitrogen oxides, ammonia and hydrogen sulfide^(54, 69). Dust generated in CAFO facilities are potential health hazards to animals, CAFO workers, and people living in the vicinity of these facilities⁽⁷⁵⁾. However, neither the exposure levels, nor extent of adverse health effects in these populations after breathing CAFO air is understood. According to *Breath-taking* report by National Resources Defense council, approximately 64,000 premature deaths in the US are related to particulate matter (PM) exposure. Beef cattle feed yard dust may represent a significant, and an important local point source of pollution to the atmosphere. Understanding effects of these dust particles on environment, human, and animal health will help to prevent adverse effects on animal and human health, and the environment.

We investigated the solubility of cattle feedlot dust particles in lung simulant fluids, and stimulation of lung epithelial cells to produce cytokines when exposed to various dust extract concentration. *Our first hypothesis* was that manure mat sieved dust would be only partially soluble with 72 hrs of incubation in distilled water or in tissue culture fluids simulating epithelial lung lining fluids (ELF). We expected that partial solubility to be significant. This experiment provided important information about the extent of CAFO dust deposition in the deep lung, and clearance by alveolar macrophages. *Secondly, we hypothesized* that when incubated *in-vitro*; dust from the manure mat from cattle feedlot will stimulate lung epithelial cells (LEC) to release cytokines TNF-alpha, Interleukin-6 and Interleukin-8. Since, cattle CAFOs dust particles are complex mixture of various potential toxicants, we expected that LEC will be stimulated to

release these cytokines. *Our third hypothesis* was that heat treatment of dust extract at 120⁰C for five minute would reduce the toxic effects to LEC. This experiment was important as temperature sensitivity would indicate that the primary source of the dust need not have been produced by heat processes. It could be coarse aerosol fraction originating from the earth's crust. Alternatively, if the dust extracts were not sensitive to the heat treatment at 120⁰C, the aerosol might not be predominantly from the coarse fraction. *Our fourth hypothesis* was that if endotoxin is inhibited by treatment with antibiotic Polymixin B, there would be less or no cytokine release by LEC if the effects were driven by endotoxin. We expected that since, endotoxin is a component of livestock manure and is associated with acute inflammatory response mediated by TNF-alpha, IL-6 and IL-8, the effect should be reduced. *Our last hypothesis* was that if response is driven by endotoxin, the LEC treated with amount of pure endotoxin equivalent to the amount in dust extract, should release similar amount of cytokines.

We carried out literature reviews to find out various factors, and components involved in inflammatory pulmonary response when exposed to the dust particles. *In-vitro* and animal experiments carried out by various investigators have determined various toxic effects of agricultural dust on workers, and animals in the facilities. However, we didn't find exposure effects of cattle CAFOs dust on LEC as well as solubility of thee dust in simulant ELF. Our research findings could provide valuable information in understanding fate of cattle CAFO dust in lungs as well as inflammatory response induced by these particles in animal care takers, and animals as well as people living in the vicinity of these facilities. Within the subheading, you may want to have further subsections.

2. Literature Reviews

2.1 Respiratory system

2.1.1 Overview:

The mammalian lung contains the largest epithelial surface in the human body. It has intricate vaulted structure which can expand to 70 to 120 m² total surface area during terminal gas diffusion in young adulthood. During rest, approximately 30 to 40 m² of total surface areas is employed for gas exchange, with as few as one third of lung capillaries perfused . This surface area is approximately 40 times more than the next largest epithelial surface in the body, the skin.

Lung is capable of supporting systemic oxygen consumption ranging between 250 ml/min at rest to 5500 ml/min in adult during exercise-more than a 20-fold increase in efficiency. A matching capillary network also develops in close apposition to the alveolar surface which can accommodate a blood flow rising from 4 to 40 L/min during transition from rest to maximal exercise, roughly paralleling the increased oxygen consumption. Because of this high surface area, high volume of air exchange, and unique anatomical position, the lung is capable of absorbing large quantities of inhaled oxygen, and other substances-toxins ^(1, 2, 3).

2.1.2 Cellular Actions and Interactions

The entire airways epithelium consists of a continuous layer of cells that function both as an environmental barrier and as the first line of host defense ⁽⁴⁾. However, different parts have different types of epithelium. The bronchioles are lined by simple ciliated columnar to simple cuboidal epithelial cells, while the alveolar duct and the alveoli are lined by simple squamous epithelium. The respiratory epithelium contains specialized cells such as olfactory, ciliated, goblet, serous, basal, and Clara cells as well as type I and type II alveolar cells ^(5, 6). The different characteristic of these cells gives rise to complex functional nature of the bronchial epithelium. Olfactory cells are located within the nasal cavity and detect odor. Goblet cells are found throughout the mucous lining of respiratory passageways, and produce mucin which traps inhaled particles. Ciliated cells are responsible for propelling the tracheobronchial mucous secretions towards the pharynx and are also active in transepithelial electrolyte transport ⁽⁷⁾. The ciliated epithelium in the bronchioles are mainly composed of Clara cells which are thought to produce glycosaminoglycans (a major component of the extracellular matrix), and also metabolize airborne toxins. Metabolism is carried out by cytochrome P-450 enzymes present in the endoplasmic reticulum ⁽⁶⁾. Type I and Type II alveolar cells line the alveoli and alveolar duct. Ninety five percent of the alveolar surface is lined by type I cells which don't divide but combine or fuse with capillary endothelial cells to form a thin, blood-gas barrier through which gas exchange occurs. The blood/air barrier is normally 0.45 microns thick ⁽⁸⁾. Type II cells are actively dividing cells; possess short microvilli along their apical surfaces. These cells secrete pulmonary surfactant which decreases the surface tension of the alveolar surface, allowing the alveoli to expand during inspiration, and preventing their collapse during expiration. In addition to the epithelial cells that line the respiratory tract, and the endothelial cells that line the pulmonary capillaries, alveolar macrophages are the predominant cell type within the alveolus,

and serve as the front line of cellular defense against inhaled organisms, particulate molecules and environmental agents entering into the lungs ^(6, 7, 9).

2.1.3 Alveolar macrophage:

Pulmonary macrophages are divided into alveolar macrophages, interstitial macrophages, intravascular macrophage, and dendritic macrophages depending upon their localization in the lung ⁽¹⁰⁾. Alveolar macrophages (AM) are best characterized pulmonary macrophage population. AM are placed within the alveolar surfactant film produced by type II alveolar lining cells and is composed of phospholipids and proteins ⁽¹¹⁾. AM are primary phagocytes of the innate immune system, clearing the air spaces of infectious, toxic or allergic particles that have evaded the mechanical defenses of the respiratory tract, such as the nasal passages, the glottis, and the mucocilliary transport systems ⁽¹²⁾. Activated macrophages following phagocytosis of the foreign particle in the lungs secrete a wide array of products such as oxidants, bioactive lipids, cytokines, growth factors, proteases and antiproteases. These physiologically active substances target epithelial, endothelium, and fibroblast and activate these cells to synthesize, and release secondary mediators-cytokines ⁽¹³⁾.

2.1.4 Fibroblast

Fibroblast plays important role in pulmonary pathophysiology is fibroblast. In healing hen cells can not adequately respond, by secondary intent (pulmonary fibrosis), interstitial fibroblasts are considered as key effector cells ⁽¹⁴⁾. In fibrosis, fibroblasts proliferate to replace cells that are unable to adequately divide, and repair the lung with collagen. These fibroblasts lie beneath the basement membrane on which the epithelial cells rest ⁽⁴⁾. These fibroblasts synthesize and maintain collagen and extracellular matrix to provide support to many cells. Activated macrophages as well as other cells including neutrophils and epithelial cells releases several factors that have been shown to stimulate fibroblast chemotaxis, growth, proliferation and/ or their production of collagen ⁽¹³⁾. Fibroblasts release chemotactic factors to attract bronchial cells ⁽⁴⁾.

2.1.5 Epithelial cells

The other groups of cells that play active roles in particle toxicity are bronchial and alveolar epithelial cells. These cells have been found to release several classes of pro-

inflammatory mediators that are typically released by polymorphonuclear leukocytes cells, and macrophages-nitric Oxide, endothelins, metabolites of arachidonic acid and cytokines⁽¹⁵⁾. Until recent past, pulmonary epithelium was extensively studied to elucidate the production of pulmonary surfactant and their roles in metabolism of inhaled and systemic lipophilic substances^(16, 17). Currently we investigated their role in initiation, amplification, and down regulation of inflammatory process and tissue repair following inhalation of air pollutant^(17, 18). Interaction of epithelial cells with resident cells such as fibroblast, and underlying extracellular matrix can initiate healing of damaged cells, and tissues⁽¹⁵³⁾.

Cytokines are soluble peptides that are involved in many signal transduction pathways regulating cell growth, differentiation, and death, as well as recruitment of neutrophils, macrophages and other mobile cells to specific site of injury⁽¹⁹⁾. Pulmonary epithelial cells are capable of producing many chemotactic cytokines, multifunctional cytokines as well as growth factors and colony stimulating factors. Several studies have demonstrated that the α -chemokine interleukin-8 (IL-8), one of the most potent activators, and attractor of neutrophils, is synthesized and released in large quantity from pulmonary epithelial cells⁽²⁰⁾. Beside IL-8, human bronchial epithelial cells also produce Interleukin-6 (IL-6), Tumor Necrosis Factor-alpha (TNF- α), B-chemokine, RANTES (Regulated on Activated, Normal T cell Expressed and Secreted)⁽²¹⁾, monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein -1 α (MIP-1 α)⁽²²⁾, interleukin-1 (IL-1), granulocyte macrophage colony-stimulating factor (GM-CSF)⁽¹⁵⁾, tumor growth factor-beta (TGF- β)⁽²³⁾, interleukin-1 β (IL-1 β)⁽²⁴⁾ and interleukin-11 (IL-11), Intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1)⁽²⁵⁾. These cytokines play important role in inflammatory process by which tissues, such as lung, heal, involving eosinophil, neutrophils and other immune system cells.

2.2 Particulate matter

2.2.1 Overview

PM (particle pollution) is a mixture of solid particles and liquid droplets found in the air⁽²⁶⁾. Airborne particles come in all sorts of sizes, shapes and composition⁽²⁷⁾. However, on the basis of size distribution, it is broadly divided into coarse particles (PM_{10-2.5} μ m; particles between 2.5, and 10 microns in (airborne) diameter), intermodal particles (PM_{2.5-1} μ m; particles between 1 and 2.5 microns in diameter), accumulation particles (PM_{1-0.2} μ m; particles between 0.2

and 1 micron in diameter), and ultrafine or nanoparticles ($PM_{<0.2}$ μm ; particles less than or equal to 0.2 microns in diameter) ^(28, 29, 30). Another classification, which is related to particle formation mechanism, is the division of the size distribution into modes. According to this classification, particles are classified into nucleation or Aitken particles (0.001-0.1 microns in diameter), accumulation mode (0.1-1 microns in diameter), and coarse particles (>1 micron in diameter; usually mechanically generated) ^(31, 32). Particles in nucleation and Aitken modes are smallest of all three, and are formed by gas-to-particle conversion (GPC) which occurs in a supersaturated atmosphere, perhaps in this case within the animal. In addition, Aitken particles result from condensation of short lived nucleation particles. Accumulation mode particles originate from primary emission as well as through gas to particle conversion, chemical reactions, condensation, and particle to particle coagulations. Particles in coarse modes are the largest, and formed by mechanical breakdown of larger solid particles such as grinding weathering of soils, sea spray, volcanic activity and release from plants (e.g., pollen, and spores). The accumulation mode, which comprises most of the surface area and about half the mass of urban air particulate matter is a major constituent of air pollution ^(33, 34, 35 36). Compared with coarse particles, accumulation-mode particles contained more organic compounds, soluble inorganic species (e.g., sulfate, nitrate and ammonium) and many trace metals. Particles in these modes are too small to undergo rapid gravitational settling and are removed very slowly by rain and dry deposition. As a result they remain in the atmosphere for a long time and chance of their transportation over a long distance is greater. Particles in nucleation mode are large in number ($>10,000$ per cc) but mass concentration usually reaches only a few $\mu g/m^3$ ⁽³³⁾. These particles are inherently unstable, subject to higher rates of particle collision and coagulation resulting in increased size of individual particles, and converting them from the nucleation mode to accumulation mode ^(34, 35). Depending upon their origin, particles are also called either primary particle or secondary particles. Primary particles are emitted directly from the source such as construction site; cattle feedlot area, unpaved roads and fires. Particles formed by the complex reaction in the atmosphere are called secondary particles, the source of fine particle pollution in the USA ^(26, 36, 37).

2.2.2 Sources of Particulate Matter

PM is a heterogeneous mixture generated by different sources such as motor vehicle, power plants, small-and-large scale biomass combustion, waste incineration, industrial process,

photochemical processes, cigarette smoking, car brake-lining debris, agricultural, and livestock industries, and fugitive dust from the soil erosion^(36 38 39). PM_{0.1} and PM_{2.5} are mainly produced by power generation plants, road traffic, and ships from fuel consumption. These particles are largely dominated by carbonaceous combustion particles, mostly from transportation origin. Residual oil fly ash (ROFA) and diesel exhaust particles (DEP) are two types of particles generated by these sources⁽³⁸⁾. Coarse particles (PM-2.5-10 fraction) are primarily formed by crustal materials and predominantly consist of inorganic minerals, such as windblown dust from solid and sand⁽⁴⁰⁾. Endotoxin is found to be associated with ambient particles, predominantly in the coarse fractions⁽⁴¹⁾. Metals, polycyclic aromatic hydrocarbon (PAH) and various volatile and semi-volatile compounds also form a part of the particulate matter. Biological materials such as beta-glucans, fungal spores, allergens, and pollens are also adsorbed on their surface^(36, 42).

2.2.3 Exposure Health Effect

Particles size distributions, and correlated parameters, number concentrations, mass concentrations, mass size distributions, surface areas, shapes, and electrical charges on PM are some of the determining factors in their role to produce adverse health effects in animal and human. The capacity of particulate matter to initiate adverse health effect is dependent upon the quantity, rate and site of deposition of these particles. The most important characteristics influencing the deposition of particles in the respiratory systems are size and aerodynamic diameter⁽³⁹⁾.

The aerodynamic diameter of a particle is the diameter of a unit density sphere having the same settling velocity as the particle in question, whatever its size, shape or density⁽²⁶⁾. The capacity of the particles to increase allergic sensitization is strongly related to particle number and aggregate surface area, but not the particle mass. So, fine particles with highest surface area/unit mass elicit stronger responses than larger particles at equal mass dose because more surface area is exposed⁽⁴³⁾. Notably, ultrafine carbon black has been reported to induce stronger pulmonary inflammation than ultrafine TiO₂ with greater density⁽⁴⁴⁾. This is because smaller particles have larger surface areas which can adsorb large amount of chemical substances, allergens, endotoxin and other biological components⁽⁴⁴⁾ and can interact with large biological surfaces.

During inhalation, particle size plays important role in deposition of particles in different parts of respiratory tract. The coarse PM is mainly confined to the larger airways with very

small mass fraction reaching to smallest airways and alveoli. PM₄ is the respirable fraction so PM_{10-2.5} contains significant number of respirable fraction. In swine CAFO, respirable particles form 2-5% of total suspended particulates⁽¹⁵¹⁾. A larger part of the fine and ultrafine particles travels deep into the lungs where they are deposited⁽³⁶⁾. Particles with aerodynamic diameter less than 2.5 μm are also known as respirable particles as they can pass the proximal airway (throat and larynx), and deposit in both bronchial and bronchiolar airways or in the respiratory bronchioles, alveolar ducts and alveoli. Particles with aerodynamic diameter more than 2.5 μm are called inhalable particle as they can be deposited in the upper respiratory tract^(26, 38). Increasing numbers of studies have indicated that the level of PM_{2.5} is more closely related to adverse health effects and mortality than PM₁₀^(29, 46). Recently, research interest has been focused on the translocation of inhaled particles from lungs to other organs. Particles go to the next large vascular system usually the heart or liver. The olfactory bulb provides entry to brain and central nervous system neurons⁽¹⁷⁹⁾. Higher risks to the cardio-vascular system are caused by strong and persistent pulmonary reactions producing number of cytokines and other cellular mediators which may influence the heart, coagulation or other cardiovascular endpoints. Alternatively, these small particles can translocate from the lungs into the systemic circulation leading to cardiovascular effects^(36, 38, 47). Prolonged exposure to the increased levels of air pollution dominated by fine particles and sulfur oxide had been found to be associated with all causes, lung cancer and cardiopulmonary mortality. Fine-particulate matter and sulfate at level commonly found in the US cities have been found to be associated with increased mortality due to cardiopulmonary and lung cancer⁽⁷⁶⁾. Each 10 μg/m³ elevation in fine particulate air pollution was associated with approximately a 4%, 6% and 8% increased risk of all cause, cardiopulmonary, and lung cancer mortality, respectively⁽⁷⁷⁾. Whereas measure of coarse particle fraction and total suspended particles were not consistently associated with mortality^(77, 78, 79).

However, all the inhaled particles can't get easy access to the different parts of the respiratory systems. The mammalian lung is equipped with a variety of defense systems that include mechanical and chemical barriers (e.g.; cough reflex, mucocilliary escalator, mucus, surfactant, lysozyme, defensin) as well as mechanisms of the innate and adaptive immunity (e.g.; macrophages, dendritic cells, secretory IgA, Bronchus Associated Lymphoid Tissue (BALT))^(48, 49). The mucocilliary escalator dominates the clearance from the upper airways while macrophage phagocytosis dominates clearance from the deep lung or alveoli⁽⁴⁷⁾. The deep lung

clearance mechanism is highly efficient and capable of completely eliminating all particles smaller than 5µm as long as the airborne concentration doesn't exceed 10 particles per cubic centimeter. If the particulate concentration goes as high as 1000 particles/cubic meter, the deep lung clearance mechanisms declines to approximately 90 % elimination⁽⁵⁰⁾.

2.2.4 Mechanism of particle induced toxicity

Deposition of particulate matter initiates injury in the lung through any of the several toxic mechanisms such as lipid peroxidation, causing depletion of natural antioxidants, and mitochondrial damage. Following injury, tissue macrophage, and circulating monocytes migrate to the damaged sites where it gets activated and produces products such as reactive nitrogen species (RNS), reactive oxygen species (ROS), arachidonic acid, and proinflammatory cytokines. These substances further initiate the production of additional inflammatory mediators resulting in tissue damage^(51, 52). In this paper, we are concerned with how cattle feedlot dust dissolves in body fluids, and how it causes reactions from cultured LEC.

2.3 Cattle Feedlot Dust

2.3.1 Overview

Cattle feedlots are outdoor pens without vegetation or corrals in which dairy or beef cattle are confined, fed and watered⁽⁵³⁾. If such facilities house more than 1000 animal units (au), those are generally referred as Concentrated Animal Feeding Operation (CAFO). EPA defined 1au as roughly 1,000 pounds of animal live weight⁽⁵⁴⁾.

During 150 days of fattening period, each animal produces approximately 900 kg of dry manure solids or about 1800 dry kg per head of feed lot capacity each year⁽⁵⁵⁾.

Table 1 Manure Production per 1,000-pound animal⁽⁵⁶⁾.

	As Excreted	At time of Spreading (Nearly Dry)	Completely dried
Beef cattle	11.5 tons/year (88% water)	1.6 tons/year	1.38 tons/year
Dairy cattle	15.0 tons/year (88% water)	3.4 tons/year	1.80 tons/year

Sheep	7.3 tons/yr (75% water)	2.6 tons/year	1.83 tons/year
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As an example, every 1,000 beef cattle on feed would excrete 11,500 tons of manure, 1,600 tons at the time of spreading and 1,380 tons if completely dried.

Generally 2.5-5.0 cm of compacted manure above the mineral soil is considered a good manure management practice in cattle feedlots because it reduces nitrate leaching⁽⁵⁷⁾. Over a period of time, manure in pens becomes compact due to animal activities forming a firm surface layer. However, manures which are loose become a source of “parent material” for fugitive dust, which is generated and suspended in air due to pulverization by animal hoof and from vehicle traffic on unpaved road on feedlot⁽⁵⁸⁾. The moist content in such compacted manure in pen ranges from 35-50 % but may be as low as 10-25 % in dry weather due to evaporation and increased animal activities⁽⁵⁹⁾. Dry manure mat is more likely to generate dust by this mechanism.

Dust emission in the feed lot are function of loose manure depth on the corral surface, moisture content of the loose manure and hoof action arising from cattle activity. Loose manure depth is primarily due to stocking density (animals per unit area), animal body weight, and number of days on feed, corral surface condition and manure harvesting frequency. Animal behavior in the feed lot is dependent upon of number of animal per unit area, feeding management, breed and sex and local weather conditions⁽⁶⁰⁾. Dry, warm condition and active cattle behavior have been found to be the principal contributors to dust production in cattle feedlots. This production peaks during the evening hours when atmospheric condition at ground level are neutral or stable but animal activity increases which is characterized by increased numbers of agonistic interactions, walking and running behaviors^(58, 59, 61,62).

Agricultural dusts have much larger particle sizes than urban/industrial emissions. Typical agricultural airborne emissions such as from grain elevators, feedlots, confinement buildings, and cotton gins have been found to be composed of particles with mass-median diameter (MMD) of around 10-20 microns or greater. That means over 50% of the particle mass is below 10 micron Aerodynamic Equivalent Diameter (AED), in such situation, typically less than 5% of particle mass is smaller than 2.5 microns⁽⁶³⁾.

In a study conducted by Sweeten *et al.*⁽⁶⁴⁾ on three Texas cattle feedlots, the mean concentration of total suspended particulate (TSP) and PM₁₀ particulate matter was 700 µg/m³

(ranging from 97 to 1685 $\mu\text{g}/\text{m}^3$) and 285 $\mu\text{g}/\text{m}^3$ (ranging from 11 to 866 $\mu\text{g}/\text{m}^3$), respectively. The inhalable dust (PM_{10}) concentration was 19-40 % of mean TSP where as respirable dust (<2 micron) was 2-4.4 % of total dust on a particle volume basis. The mean diameter of particle was 8.5-12.2 micron on particle volume basis and 2.5-3.4 microns on a population basis.

The U.S. Environmental Protection Agency (USEPA) office of Air Quality Planning and Standard (OAQPS) has set National Ambient Air Quality Standards for PM. NAAQS has set primary standard for annual and 24-hour exposure period not to be exceed 15.0 $\mu\text{g}/\text{m}^3$, and 35 $\mu\text{g}/\text{m}^3$ respectively. Due to lack of evidence linking health problems to long-term exposure to coarse particle pollution, the agency revoked the annual PM_{10} standard in 2006 but has fixed the upper 24 hr exposure limit to 150 $\mu\text{g}/\text{m}^3$ ⁽⁶⁵⁾.

Cattle retain less than 30 % of their dietary nitrogen intake and most of them are excreted in the form of urea which can be rapidly hydrolyzed to ammonia. Ammonia is highly volatile and vaporizes to the atmosphere where it reacts with NO_x and SO_x to form fine particulates of diameter <2.5 μm ($\text{PM}_{2.5}$) ⁽⁶⁰⁾. Beside dust particles, cattle feed lot production systems also generate considerable amount odor, ammonia, green house gas (GHG) and pathogens ^(54, 57, 60). Microbial activity determines the amount of GHG and odor production. Microbial enzyme urease converts urea in urine to ammonia ⁽⁶¹⁾. Environmental condition such as temperature, moisture and manure content influences the microbial activity and also affect amount of the gas production and dust formation. Dust particles generated in the feedlot may contain numerous forms of microflora particularly non-pathogenic Gram-positive bacteria but not culturable gram negative bacteria ⁽⁶⁶⁾. Cell walls of Gram-negative bacteria are rich source of endotoxin. Fungi are also a constituent of the airborne microbial flora in a cattle feedlot but are non-pathogenic. But population of the micro flora varies from feedlot to feedlots ^(67, 68).

The primary respiratory hazard associated with CAFO dust are bacterial endotoxin, fungal molds, organic dusts, and the manure generated gases hydrogen sulfide and ammonia. Organic dust is an organic soup of allergens and irritants, including microbes, insect feces and components, animal and bird feces, animal dander, urinary protein antigens, animal and bird feces, storage mites, pollens, antibiotics, feed components and pesticides ^(54, 69). PM associated ammonia can impede the lungs from clearing dust particles where hydrogen sulfide cause loss of consciousness, coma, or death at high exposure level ^(142 143).

2.3.2 Effects on Health

Large numbers of literature sources indicate that workers in CAFOs have higher rates of respiratory diseases⁽³⁾. Health effects associated with breathing CAFO can be tentatively divided into respiratory disease (asthma, hypersensitivity pneumonitis, and bronchitis), cardiovascular disease (sudden death associated with particulate matter), and neuropsychiatric problems (due to odor as well as delayed effects of toxic inhalations)⁽³⁾. Particulate emission in open cattle feedlot and dairies is generating interest among environmentalists, toxicologists and public health workers. Due to emission of dust in open environment, feedlot animals as well as workers and community members in the areas are at higher risk of health effects from dust and odorous gases emanating from the feedlot. However, very little information exists about the lung function changes among populations living in the vicinity of CAFOs⁽⁷³⁾. Numerous pieces of documented evidence show that poultry and hog barn workers suffer from acute and chronic respiratory diseases and dysfunction following exposure to complex mixtures of particulates, gases and vapor within CAFO units. Common complaints among workers include sinusitis, chronic bronchitis, inflamed mucous membranes of the nose, irritation of the nose and throat, headaches, muscles aches and pains. It is estimated that 20 % to 50% of CAFO workers suffer from rhinitis, and up to 50% of workers experience bronchitis, organic dust toxic syndrome, hyper-reactive airways disease, chronic mucous membrane irritation, and other respiratory effects^(70, 71, 72, 74). Beside adverse health effects among workers, there is growing concern about health effects of people living in the vicinity of the CAFO. A study carried out by Schiffman et al.⁽¹⁴⁵⁾ on hog CAFO neighbors found that people experience more tension, depression, anger, fatigue, less vigor and more confusion than control subjects as measured by Profile of Mood States (POMS) psychological testing tool. Radon et al.⁽⁷⁵⁾ reported that people residing in the proximity to many CAFOs (>12 within a 500m radius) experienced significantly increase prevalence of self-reported wheezing and decreases forced expiratory volume in 1 second (FEV1) which is indicative of inflammatory effects of CAFO emission in the lungs. Beside human subjects, animals may be equally susceptible to dust inhalation in the feedlots MacVean et al⁽¹⁴⁴⁾ found that dust particles of size range 2.0 to 3.0 μ m in cattle feedlot are associated with the increased incidence of cattle pneumonia. The incidence was attributed to the dust overloading causing alveolar cell damage and predisposing cattle to the microbial infection.

2.4 Cytokines

2.4.1 Overview

The development of an effective immune response involves lymphoid cells, inflammatory cells, and hematopoietic cells. The complex interaction as among these cells are mediated by a group of protein collectively designated cytokines to denote their roles in facilitating cell to cell communication. Cytokines are low-molecular-weight regulatory proteins or glycoprotein secreted by white blood cells and various other cells in the body in response to a number of stimuli ⁽⁸⁰⁾. Cytokines are soluble peptides that are involved in many signal transduction pathways regulating cell growth, differentiation and death as well as recruitment of neutrophils, macrophage and other mobile cells to specific sites ^(81, 82).

2.4.2 Cytokines in the Pulmonary Inflammatory Process

The inflammatory process in the lungs is characterized by the production of leukotrienes, histamines, bradykinin and a variety of cytokine and chemokine by tissues and migrating cells. ⁽⁸³⁾. Expression of these cellular mediators is stimulated by particulate matter, bacteria and bacterial products, virus and viral products, fungi and parasite. Functionally cytokines in respect to airways disease is best categorized as proinflammatory cytokines; T cells derived cytokines, cytokine chemoattractant for eosinophils, neutrophils, monocytes/macrophages and T cell, anti-inflammatory cytokines, and growth factors ⁽⁸⁴⁾. *In-vitro* studies have considered IL-6, IL-8, and TNF- α benchmarks in the inflammatory process involving ambient and mineral particles, and air way epithelium in lungs ⁽⁸⁵⁾. So, our project will look into *in-vitro* expression of these cytokines by lung epithelial cells.

2.4.3 Interleukin-8 (IL-8)

IL-8 belongs to CXC subgroup of chemokine in which the conserved cysteines are separated by some other amino acid (X). Both leukocytic cells (monocytes, T cells, neutrophils, and natural killer cells) and non-leukocytic somatic cells (endothelial cells, fibroblasts and epithelial cells) produce IL-8 ^(86, 87). Our works examine the production of IL-8 by lung epithelial cells. According to George T. De Sanctis et al. ⁽⁸⁸⁾ IL-8 modulates IgE production, airway responsiveness, and the composition of the cells (B cells and neutrophils) recruited to the airway lumen in response to antigen. Cells don't express IL-8 constitutively but are induced by pro-

inflammatory cytokines such as TNF-alpha and IL-1⁽⁸⁹⁾. Factors such as bacteria, bacterial products, virus and viral products are principle inducer of cellular expression of IL-8 in various cells. It was originally discovered as a chemotactic factor for leukocyte and since then its various roles in other patho-physiological process have been reported^(15, 90). It has been a major chemoattractant cytokines to leukocytes such as neutrophils, monocytes, eosinophils and basophils. IL-8 causes transmigration of these cells from blood circulation into the tissue site^(91, 92). As reported by Caludai et al.⁽⁹³⁾ neutrophils are the dominant inflammatory leukocytes characterizing airway inflammation, and that IL-8 is an important mediator of this neutrophilia. IL-8 activates neutrophil degranulation, superoxide production, synthesis of platelet activating factor⁽¹⁵⁾, and mobilization of intracellular calcium production. It exhibits direct and indirect chemotactic activity against T- cells. The indirect chemotaxis activity is mediated by the production of alpha-defensins-1 and alpha defensin-2 from neutrophils which are chemoattractant to T-lymphocytes⁽⁹⁵⁾. On a molar basis, IL-8 is 10-100 fold more potent than the bacterially derived chemotactic factor fMLP (formyl-methionine-leucine-phenylalanine) in directing neutrophil chemotaxis⁽⁹⁶⁾. In addition, IL-8 is 10-100 fold more potent mediating lymphocyte compared to mediating neutrophil chemotaxis⁽⁹⁷⁾. Neutrophil-chemotaxis is mediated through a process that is both Ca⁺ and Protein Kinase C (PKC) dependent.⁽⁹⁸⁾ As reported by Simonini A. et.al.,⁽⁹⁹⁾ IL-8 may be chemotactic and mitogenic towards endothelial and smooth muscle cells as well as a signal for angiogenesis. Because of its mitogenic, angiogenic, and motogenic (chemotactic) activity, it also contributes to human cancer progression⁽⁹⁰⁾. Thomas A. Neff et al.⁽¹⁴¹⁾ have shown that Fas/FasL enhance the expression of CXC chemokine in acute lung inflammatory response by enhancing neutrophil influx and tissue damage. The release of IL-8 from cultured LEC is used in our study to predict the amplification and maintenance of lung inflammation.

Interleukin -6 (IL-6)

Interleukin -6 is a proinflammatory cytokine produced by various kinds of cells. In- vitro experiments have shown that almost all kind of cells are capable of producing and secreting proinflammatory cytokine IL-6. Monocytes, macrophages, fibroblasts, endothelial cells, epithelial cells, and lymphocytic T-cells are principal producer of this cytokine⁽⁹⁴⁾. Thus they are the principal cells capable of inducing pulmonary defense to clear foreign dust or antigen. It is also expressed and produced by PMN, tumor cell lines and mast cells lines, astrocytes and

microglial cell in CNS to mediate the same response. Rao *et al.* ⁽¹⁰⁰⁾ have demonstrated alveolar macrophage and lung fibroblast are significant sources of IL-6 in the lung. Interleukin-6 is a pleiotropic cytokine affecting various types of cells and physiological condition in paracrine as well as autocrine manner. Autocrine mechanism is considered to be important in the autonomy of proliferation of cancer cells ⁽⁸³⁾. In the work by Muraguchi *et al.* ⁽¹⁰¹⁾ human IL-6 causes activated B cells to release 3-10 fold increase in IgM, IgA and IgG antibody production. Different stages of T cell activation and differentiation are influenced by IL-6. Both thymocytes and peripheral T cells express receptors for IL-6 ⁽¹⁰²⁾. Stimulation of the receptor causes activation, proliferation and augmentation of natural killer cells ⁽¹⁰³⁾. IL-6 co-regulates the growth of committed erythroid, megakaryocytic, and myeloid progenitors as well as multipotential progenitors ⁽¹⁰⁴⁾. It has shown been shown to involve in initiation of fibroblast activation and proliferation. This fibro-proliferative response can play an important role in acute ⁽¹⁰⁵⁾, and other interstitial lung injuries ⁽¹⁰⁶⁾. Interleukin-6 is the major regulator of the acute phase response in human hepatocytes ⁽⁸³⁾. It stimulates the full spectrum of acute phase proteins seen in inflammatory state while TNF- α , and IL-1 β had only a moderate effect on the positive acute phase proteins ⁽¹⁰⁷⁾. Action of interleukin-6 on the growth and differentiation of various cells types have been well documented. According to Hajime Takizawa *et al.* ⁽¹⁰⁸⁾ IL-6 has an inhibitory effect on the proliferation of human non-small cell lung cancer cell lines which has receptor for IL-6 suggesting an autocrine mechanism of regulation. It plays important modulator role in angiogenesis of lungs however such action is not dependent upon neutrophil trapping ⁽¹⁰⁹⁾. Patient requiring high concentration of inspired oxygen can suffer from hyperoxic lung injury. Ward *et al.* ⁽¹¹⁰⁾ demonstrated that IL-6 markedly diminishes hyperoxic lung injury and such action is associated with a diminution in hyperoxia-induced cell death and DNA fragmentation. Thus stimulation of healing by secondary intent (fibrosis) reduces pulmonary edema, and per-acute toxicity, by providing an additional option for a response to toxicity. Its role has also been characterized in ischemia-reperfusion models in lungs, liver and intestine. Farivar *et al.* ⁽¹¹¹⁾ reported that IL-6 reduces the endothelial disruption and neutrophil sequestration at ischemic site via decreased expression of transcription factor NF-kB and signal transduction and activators of transcription-3 (STAT-3) in early reperfusion and diminished proinflammatory mediator secretion late in reperfusion. IL-6 also plays role in development of disease resistance. In a

murine model, expression of IL-6 in resistant strain's lungs following *Coccidiomycosis* infection was higher than in the susceptible strain ⁽¹¹²⁾.

IL-6 is used in these investigations as a pro-inflammatory indicator. The importance of production of increased amount of IL-6 by epithelial cells is to initiate inflammation to clear dust, and to initiate machinery to heal by secondary intent (fibrosis).

2.4.5 Tumor Necrosis Factor-Alpha (TNF- α)

TNF- α is a pleiotropic inflammatory cytokine and is produced by many cells including activated monocytes, fibroblasts, endothelial cells, macrophages, T-cells, mast cells, epithelial cells, smooth muscle cells, eosinophils, chondrocytes, osteoblasts, mast cells, glial cell and keratinocytes ⁽¹¹³⁾. TNF- α is also found in human breast milk ⁽¹¹⁴⁾. Macrophage inflammatory protein-2 (MIP-2), and cytokine induced neutrophils chemoattractant (CINC), members of the chemokine family of inflammatory and immunoregulatory cytokine, expression in murine lung following particulate exposure is mediated at least in part by production of TNF- α ⁽⁸¹⁾. It regulates transmigration of leukocyte from blood vessels to site of injury by increasing the expression of cell surface molecules in endothelial cells, and stimulating the adhesion and chemotaxis of neutrophils ⁽¹³⁷⁾. TNF- α plays protective role by stimulating chemotaxis of phagocytes. In addition, TNF- α stimulates antimicrobial activity of the neutrophils, macrophages, and eosinophils at lower levels. However during chronic illness, increased secretion can give rise to cachexia, hemorrhage, necrosis and, ultimately, death. ⁽¹³⁸⁾. TNF- α induces both proliferation and apoptosis of neutrophils ⁽¹⁴⁰⁾. TNF- α plays important role in the pathogenesis of endotoxin mediated septic shock ⁽¹¹⁵⁾. TNF- α regulates the inflammatory process by inducing the expression of transcription factor genes such as, nuclear factor- (NF- κ B) and activating protein-1 (AP-1)⁽¹¹⁶⁾ that induces the transcription of secondary mediators IL-1, IL-6, and IL-8, macrophage inflammatory protein-2(MIP-2), granulocyte colony stimulating factor (GCSF), intercellular adhesion molecule-1 (ICAM-1) and endothelial leukocyte adhesion molecule-1 gene ⁽⁵²⁾. Induction of IL-6 and IL-8 production by bacteria and bacterial products such as lipopolysaccharide (LPS) is thought to be mediated through initial production of TNF- α ⁽¹¹⁷⁾. TNF- α activates macrophages to produce matrix metalloproteinase, and stimulate bronchial epithelial cells to produce tenascin (extracellular matrix glycoprotein) ⁽¹¹⁸⁾. It is also involved in the activation of epidermal growth factor (EGF) which causes activation and proliferation fibroblast leading to development of peribronchiolar fibrosis ⁽⁸⁴⁾. Various roles of TNF- α have

been reported in both acute and chronic pulmonary diseases. Chronic inflammatory lung diseases, such as idiopathic pulmonary fibrosis, chronic bronchitis, adult respiratory disease syndrome, cystic fibrosis, and some forms of asthma, are associated with elevated TNF- α responses and neutrophil accumulation in lung ^(84,139). Increased level of TNF- α following inhalation of environmental agents has been observed in pulmonary inflammation from ozone, and diesel particles, as well as fibrosis from silica, and asbestos or granulomatous response from beryllium. TNF- α expression in smoker's lung is lower than it is in the non-smoker's, making them more susceptible to the infectious agents ⁽⁵²⁾.

The importance of TNF-alpha response in the lung is to support cell inflammation and lung defense. TNF-alpha is not increased in lung epithelial cells in this work. Either pulmonary epithelial cells don't support defense by this mechanism or it is produced by different cell- perhaps the pulmonary alveolar macrophage.

2.5 Endotoxin as a Biological Toxin

2.5.1 Overview

Endotoxin is part of the outer membrane of the cell wall of Gram-negative bacteria. It is invariably associated with Gram-negative bacteria whether the organisms are pathogenic or not. Although the term endotoxin is occasionally used to refer to any cell-associated bacterial toxin, it is generally reserved to refer to the LPS complex associated with the outer membrane of gram negative bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Hemophilus etc* ⁽¹¹⁹⁾.

2.5.2 Endotoxin in CAFO Environment

Gram-negative bacteria are ubiquitous in the soil, on vegetation and in water ⁽¹²⁰⁾. Exposure to endotoxin is high in certain occupational settings such as cotton mills, swine confinement barns, and in the early stage of wood processing such as debarking ^(121, 122, 123, 124). Very high endotoxin exposure occurs in livestock farming ⁽¹²⁵⁾. Elevated endotoxin levels are also present in homes where children have regular contact with farm animal and in homes where pets are present ^(126, 127). Gram-negative bacteria have been recovered from inside cattle pens ⁽¹²⁸⁾. Pathogenic gram-negative organisms such as *Salmonella sp.* and *E. coli* have been found in feedlot cattle fecal samples ⁽¹²⁹⁾. Endotoxin is stable and its toxic effects are known to

persist long time in dust⁽¹³¹⁾. Cattle feedlot dust samples are good source of endotoxin. Therefore it is possible that effects of endotoxin in conjunction with the allergic and irritant properties of the dust could predispose cattle to an inflammatory response to clear the inhaled dusts⁽⁶⁶⁾.

2.5.3 Endotoxin and Cellular Interaction

After deposition in the airways, the lipid A part of the endotoxin is opsonized by a Lipopolysaccharide binding protein (LBP) present in the fluid on the airway surface, probably produced by type II epithelial cells and exuded from the vascular compartment⁽¹³²⁾. LBP together with other protein carry endotoxin to other part of the body for metabolism and destruction⁽¹²²⁾. In lungs, alveolar macrophages carry a surface protein CD14 which binds with LBP. CD14 is also present in free form in normal alveolar fluid, where it helps the attachment of endotoxin to cells without CD14 on their cells surfaces such as epithelial cells and pulmonary dendritic cells.⁽¹³³⁾ The CD14 and LBP interaction and binding activates Toll like receptor (TLR) on the surface of macrophage⁽¹³⁴⁾ facilitating the phagocytosis of endotoxin. In the activated macrophage and other cells transcription factor nuclear factor- κ B initiates the production of variety of inflammatory cytokines, particularly IL-1 β , TNF- α and IL-6^(116, 135).

Following inhalation of endotoxin, the number of PMN cells increases in response to chemotactic substances produced by macrophages⁽¹³²⁾. The amount of E-selectin is also increased, indicating an activation of endothelia cells. C-reactive protein is increased at 24 hr after inhalation, reflecting the activation of liver cells by IL-6, IL-1 and TNF- α ⁽¹³⁶⁾. Inflammatory cytokines are generally elevated 2-6 hrs after exposure, together with neutrophils, whereas other increased at 24 hrs, a time when cytokines have returned to normal levels^(132,136).

Determining endotoxin allows us to relate it to the ability lung epithelial cells to release IL-6 and IL-8.

2.6 Deposition, Dissolution and Clearance of Inhaled Particles

Toxic effects of inhaled particles greatly depend on their patterns of deposition and the rates and pathways for their clearance from the deposition sites. The distribution of particle diameter, airway geometry, breathing pattern local flow profiles are major determinants of deposition in the lung. Particle deposition largely takes place by impaction, settling, diffusion, and interception. Large particle (>10 μ M) deposition in the lung takes place either by impaction or settling mechanisms. Deposition of small particles with aerodynamic size less than 0.5 μ m

takes place by diffusion or Brownian motion. Motion that is random (Brownian) or directed (diffusion) allows the small particles or ultrafine particles to contact walls. Deposition by interception is seen when one inhales fiber particles that don't navigate. Following deposition, particles are cleared from the airways by macrophages. Alternatively, particles may be ignored by macrophages if particle is too small to be phagocytosed. The small particles will be translocated to the lung interstitium and then to other parts of the body via blood circulation. Physicochemical properties, deposition location and clearance mechanism determines the fraction of particles being retained in the airways. The respiratory airways are lined with continuous layer of mucus. Foreign particles trapped in the mucus and slowly propelled by the mucocilliary action towards pharynx and then swallowed into the gastrointestinal tract. The mucocilliary apparatus clears deposited articles out of the respiratory systems in a matter of hours. The process is accelerated if the inhaled air contains irritating gases or aerosols. The alveoli lack ciliated cells and mucocilliary clearance is reduced. The particles depositing in alveoli either dissolved or phagocytized or pinocytized by macrophages. During phagocytosis or pinocytosis macrophages move toward the ciliated region, and are transported to lymph nodes. However, if the particles are insoluble or retained in this region for a long time, they move to the interstitium with potential of damaging the cells and possibly resulting in fibrosis. For example, silica, asbestos induce fibrosis due to their low solubility, lack of clearance and possibly high biopersistence. On the other hand, soluble or the ultrafine particles can pass through the thin alveolar membrane and can be transported to other parts of the body. But if the particle is neither soluble nor degradable in the lung it is likely to have high durability (biopersistence) with accumulation after sustained exposure^(146,150,151).

Particle dissolution in the respiratory tract has been recognized as important clearance mechanism. The respiratory epithelium is covered with lining fluids and materials that dissolve in these fluids are readily transported to the blood⁽¹⁴⁷⁾. That means particles that are dissolved in lining fluids will be cleared in solution as ions or chemical molecules. It has been now demonstrated that materials that dissolve in lung simulant fluids *in-vitro* have a high probability of undergoing similar dissolution in *in-vivo* lung lining fluids⁽¹⁴⁸⁾.

Rate of dissolution of a particle is closely related to their surface area. However, smaller particles tend to aggregate and form bigger particles reducing their total surface area. Aggregation either reduces or changes the temporal availability of particle surface area. Long *et*

al.⁽¹⁵²⁾ have shown an increase in the size of the titanium dioxide particles in solution until steady state is reached. When 5ppm of TiO₂ with initial hydrodynamic diameter 500 nm was put into tissue culture fluid, the particles aggregated till steady state was reached increasing hydrodynamic diameter to 826 nm (\approx 65%). When concentration of particle was increased from 5 ppm to 120 ppm, the steady-state aggregate size increased from 826 nm to 2368 nm (2-3 fold). Particles of larger hydrodynamic size tended to settle more quickly than did smaller hydrodynamic size particle. Thus the increased size from aggregation caused by increasing the concentration of titanium dioxide from 5 to 120 ppm was greater than that caused by steady state aggregation for 18 hrs.

3. Experimental Methods

3.1 Cell preparation

All cell exposure used in human normal bronchial epithelial cell line, BEAS-2B (American Type Culture Collection (ATCC), Manassas, VA). These epithelial cells were isolated from normal human bronchial epithelium obtained from autopsy of a non-cancerous individual. The cells were infected with an adenovirus 12-SV40 virus hybrid. These cells retain many of the characteristic of the normal type II epithelial cells, such as surfactant production and squamous cell differentiation in response to serum. Cells were cultured in Eagle's Minimum Essential Medium, EMEM ((Invitrogen Corp. Carlsbad, CA) containing 1% BSA (Fisher Inc. Palatine, IL), streptomycin, penicillin (Invitrogen Corp. Carlsbad, CA), and L-glutamine. Cells were cultured in 75 cm² cell culture flaks (Corning Inc. Corning, NY) and incubated 37⁰C in a humidified atmosphere containing 5% CO₂ till 80-90 % confluence was attained. Cells were removed from the flask by treating trypsin /EDTA (Invitrogen Corp. Carlsbad, CA) for 3 minute at 37⁰C, harvested by centrifugation at 1, 000 g for 5 minutes, washed with new medium, centrifuged and finally resuspended in fresh medium. The cell suspension was finally diluted to a concentration of 3x10⁵ cell/ml. 300 μ l of this cell suspension was then transferred to each well in 96-well microtiter plate (Nunc Inc. Naperville, IL) and incubated overnight at 37⁰C. Following overnight incubation, media was changed and each well was treated with appropriate concentration of dust extract.

3.2 Preparation of Dust Extract

Manure matt from floor of cattle feedlots in Texas was collected and finely ground in research facility at Texas A & M to be used for inhalation exposure in calves. Dust extract was prepared as described by Romberger *et al.*⁽¹⁵³⁾ 1gm of dust was placed in Hank's Balanced Salt Solution HBSS (Invitrogen Corp. Carlsbad, CA) without calcium and magnesium. The mixture underwent high speed agitation and was allowed to stand at room temperature for one hour. The mixture was then centrifuged for 10 minutes, and the supernatant fluid was collected and centrifuged again. The final supernatant fluid was sterilized by filtration using 0.22µm Millipore filter (Millipore, Billerica, MA). Samples were prepared on the same day of the cell treatment experiment. Samples were run through Affinity Pack™ Detoxi-Gel™ Endotoxin Removing Gel (Pierce Biotechnology Inc., Rockford, IL) as per manufacturer's instruction. It uses immobilized Polymixin-B to bind and remove pyrogens from solution. Polymixin B neutralizes the biological activity of endotoxin by binding to the lipid A portion of bacterial lipopolysachharide.

3.3 Cytokine Release Assay

Cytokines TNF-alpha, IL-8 and IL-6 in culture supernatant was quantified by using the sandwich ELISA assay.

3.3.1 Tumor Necrosis Factor-alpha (TNF-α)

1. Ninety- six- well flat bottomed microtiter plate were coated with 4 µmg/ml purified mouse monoclonal IgG1 (R & D systems, Minneapolis, MN) diluted in carbonate-bicarbonate buffer solution (pH 9.5) and incubated at 4°C overnight.

2. The plates were washed three times with 0.05% Tween®20 in phosphate buffered saline (PBS) (pH7.4) followed by addition of 300 µL of ELISA blocker (Pierce Biotechnology Inc., Rockford, IL) in each well.

3. The plates were incubated for 2 hours at room temperature. The plates were washed with 05% Tween®20 (Fisher Inc. Palatine, IL) in PBS (pH7.4) three times.

4. Two-fold serial dilution of recombinant human TNF-alpha (*E. Coli* derived) (R & D System, Minneapolis, MN) starting at 1 ng/ml was prepared in the diluent containing 0.1% BSA (Fisher Inc. Palatine, IL), 0.05% Tween®20 in Tris-buffered Saline (pH7.3) for the use as a standard. The range of concentration in the standard was from one thousand picogram to zero picogram per milliliter (1000-0 pg/ml).

5. One hundred (100 μ L) microliter of undiluted culture supernatant or standard was added to each well and incubated 2 hours at room temperature. Plates were washed three times with 0.05% Tween[®]20 in (PBS) (pH7.4)
6. 100 μ L of biotinylated TNF-alpha affinity purified goat IgG (R & D System, Minneapolis, MN) diluted in 0.1% BSA,.05%Tween 20 in Tris-buffered Saline pH 7.3 was added to each well and incubated at room temperature for 2 hours. The plates were washed three times.
7. 100 μ L of substrate solution streptavidin HRP (R&D System, Minneapolis, MN) diluted in 1% PBS was added to each well and incubated at room temperature for 20 minutes. The plates were washed three times.
8. 100 μ L of substrate solution (R & D System, Minneapolis, MN) was added to each well and incubated for 20-30 minutes at room temperature.
9. The reaction was stopped by stopping solution (Pierce Biotechnology Inc., Rockford, IL) at 100 μ L per well, and plates were read at 450 nm with a reference wavelength of 570 nm in an automated ELISA reader.

3.3.2 *Interleukin-6 (IL-6)*

1. Ninety- six- well flat bottomed microtiter plate were coated with 2 μ mg/ml purified mouse monoclonal IgG1 (R & D systems, Minneapolis, MN) diluted in carbonate-bicarbonate buffer solution (pH 9.5) and incubated at 4^oC overnight. The plates were washed three times with 0.05% Tween[®]20 in PBS (pH7.4).
2. 300 μ L of ELISA blocker (Pierce Biotechnology Inc., Rockford, IL) was added in each well. The plates were incubated for 2 hours at room temperature. The plates were washed with 0.05% Tween[®]20 in PBS (pH7.4) three times after incubation.
3. Two-fold serial dilution of recombinant human IL-6 (*E. coli* derived) (R & D System, Minneapolis, MN) starting at 600 pg/ml was prepared in the diluent containing 1% BSA in PBS for the use as a standard.
4. 100 μ L of undiluted culture supernatant or standard was added to appropriate well and incubated 2 hours at room temperature. The range of standard curve concentration was from six hundred to zero picogram per millimeter (600-0 pg/ml). Plates were washed three times with 0.05% Tween[®]20 in PBS (pH7.4).

5. 100 μ L of biotinylated IL-6 affinity purified goat IgG (R & D System, Minneapolis, MN) diluted in 1% BSA in PBS was added to each well and incubated at room temperature for 2 hours. The plates were washed three times.
6. 100 μ L of substrate solution streptavidin HRP (R&D System, Minneapolis, MN) diluted in 1% PBS was added to each well and incubated at room temperature for 20 minutes. The plates were washed three times.
7. 100 μ L of substrate solution (R & D System, Minneapolis, MN) was added to each well and incubated for 20-30 minutes at room temperature.
8. The reaction was stopped by stopping solution (Pierce Biotechnology Inc., Rockford, IL) at 100 μ L per well, and plates were read at 450 nm with a reference wavelength of 570 nm in an automated ELISA reader.

3.3.3 Interleukin-8 (IL-8):

1. Ninety- six- well flat bottomed microtiter plate were coated with 0.5 μ mg/ml purified mouse monoclonal IgG1 (R & D systems, Minneapolis, MN) diluted in carbonate-bicarbonate buffer solution (pH 9.5) and incubated at 4°C overnight. The plates were washed three times with .05% Tween®20 in PBS (pH7.4) followed by addition of 300 μ L of ELISA blocker in each well.
2. The plates were incubated for 2 hours at room temperature. The plates were washed with 0.05% Tween®20 in PBS (pH7.4) three times.
3. Two-fold serial dilution of recombinant human IL-8 (*E.Coli* derived) (R & D System, Minneapolis, MN) starting at 2 ng/ml was prepared in the diluent containing 0.1%BSA, 0.05%Tween®20 in Tris-buffered Saline (pH7.3) for the use as a standard. The range of the standard use was from two thousand to zero picogram per milliliter (2000-0 pg/ml).
4. One hundred microliter of (100 μ L) of undiluted culture supernatant or dust extract or standard was added to appropriate well and incubated 2 hours at room temperature. Plates were washed three times with 0.05% Tween®20 in PBS (pH7.4).
5. 100 μ L of biotinylated IL-8 affinity purified goat IgG (R & D System, Minneapolis, MN) diluted in 0.1% BSA 0.05%Tween® 20 in Tris-buffered Saline pH 7.3 was added to each well and incubated at room temperature for 2 hours. The plates were washed three times and 100 μ L of substrate solution streptavidin HRP (R&D System, Minneapolis, MN) diluted in 1% PBS was added to each well and incubated at room temperature for 20 minutes. The plates were washed three times.

6. 100 μ L of substrate solution (R & D System, Minneapolis, MN) was added to each well and incubated for 20-30 minutes at room temperature.

7. The reaction was stopped by stopping solution (Pierce Biotechnology Inc., Rockford, IL) at 100 μ L per well, and plates were read at 450 nm with a reference wavelength of 570 nm in an automated ELISA reader.

3.4 Solubility Assay

Cattle feed lot dust particles were added to colorless HBSS (Invitrogen Corp. Carlsbad, CA) at 50 mg/100 ml and 150 mg/100 ml. Then it was incubated at 37 ⁰C with gentle agitation in a shaking water bath. Samples were collected at 1, 3, 24, 48 and 72 hr. Each sample was centrifuged at 10xRPM for 20 minutes and photographed with Olympus Camedia C-750 Ultra Zoom (Olympus Incorporation, Tokyo, Japan) digital camera. The luminosity of each sample was analyzed in Adobe [®]Photoshop[®] Elements 4.0 (Adobe System Incorporated, San Jose, CA). The luminosity of test, and suspension against blank were compared and recorded.

3.5 Endotoxin Assay

Endotoxin determination in the dust extract was carried out by LAL Pyrotell[®] gel clot assay as per the manufacture's protocols. For each assay, a two-fold dilution series of control standard endotoxin was carried out to bracket the sensitivity of the Pyrotell[®]. 100 μ L of reconstituted Pyrotell[®] was added to each endotoxin free reaction tube containing 100 μ L of sample or control. The tubes were vigorously shaken for 20 seconds and incubated at 37⁰C for 60 minutes. Positive test was indicated by the formation of a gel which didn't collapse when the tube was inverted. Amount of endotoxin in the sample was calculated by multiplying the Pyrotell[®] sensitivity by the reciprocal of the dilution at the endpoint and expressed as EU/ml of the sample. All chemicals and research supplies used in endotoxin assay were purchased from Associates of Cape Cod Inc. East Falmouth, MA.

4. Results

4.1 Solubility

When cattle feed lot dust was added and mixed with water and Hank's balanced salt solution (HBSS) most of the dust floated on the surface of both solutions (Figures 1-4). During the 72 hour extraction with gentle agitation manure dust mixed with the media gradually agglomerated and settled to the bottom or remained suspended. In distilled water, and at 37°C incubation temperature, enough dust remained was suspended to reduce the luminosity by 10-15% at 1, 3, and 24 hours. However, centrifugation at 10,000g for 20 minutes didn't change the luminosity of the solutions at any of these time points suggesting that the particles were small enough to resist the sedimentation at 10,000g centrifugation. When dust was added and mixed to HBSS at 37°C and with gentle agitation, same reduction in luminosity was seen at 1hr and 3hr. Luminosity increased slightly between 3hr and 24 hours. Again, centrifugation at 10,000 g was not strong enough to cause settling of the dust particles.

After 24 hrs of incubation at 37°C and continuous gentle agitation, the luminosity measurement approximated that 40% of the total dust particles remained at the top of the media, 10% was soluble, and 50% aggregated and settled at the bottom (Figures 5-6).

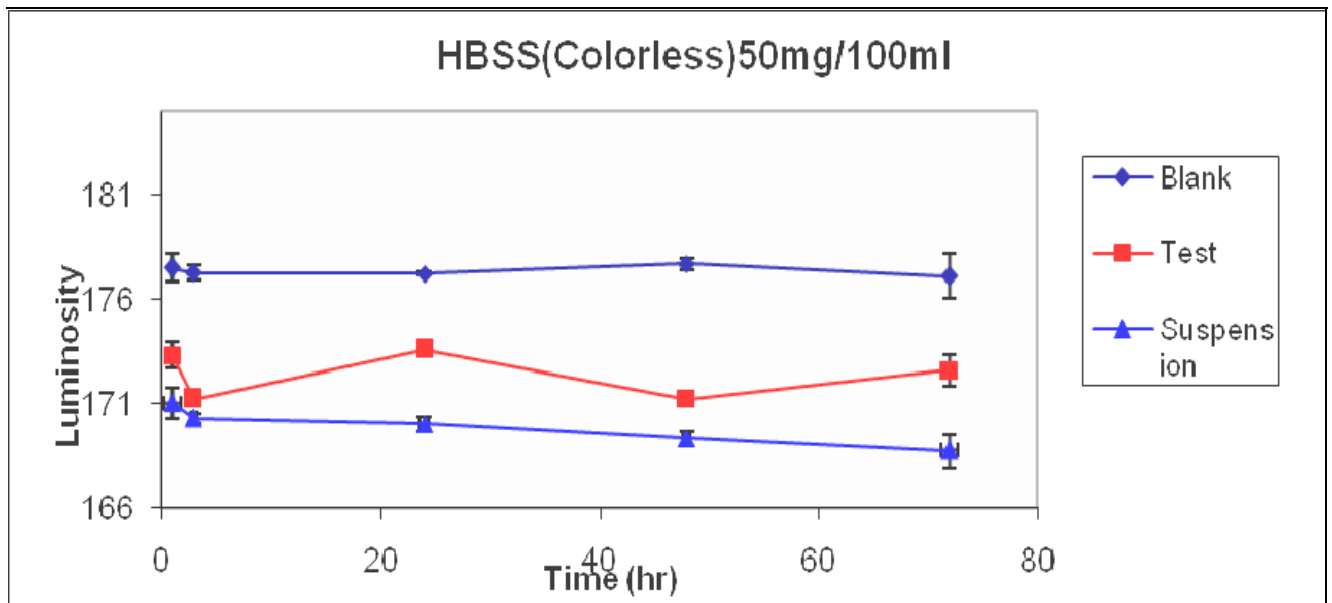


Figure 1 Luminosity of the supernatant fluid for dust particles at 50mg/100 ml in Hank's balanced salt solution (HBSS) as determined by light scattering pattern. Samples were taken at 1hr, 3hr, 24hr, 48hr and 72 hours. Values are means \pm SE (n=3).

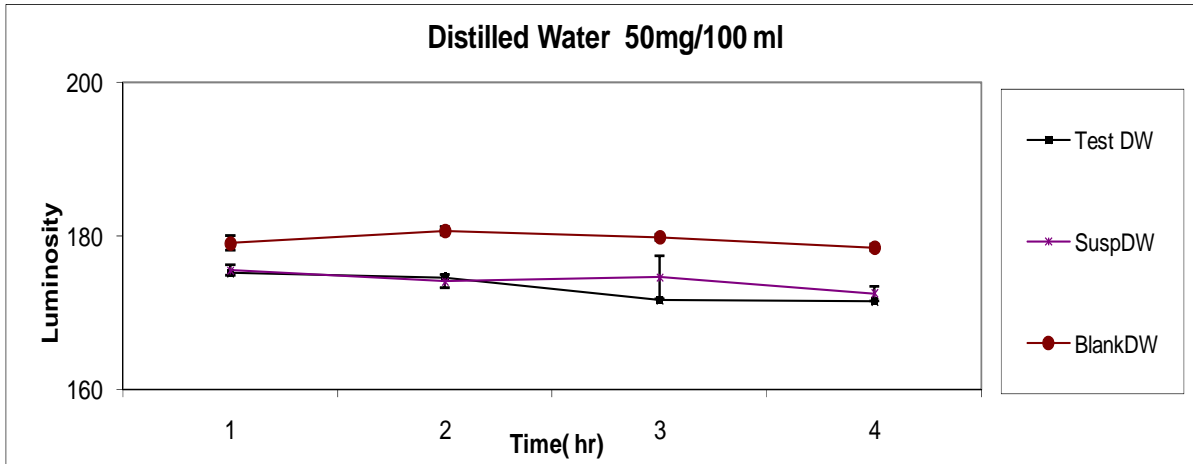


Figure 2 Luminosity of the supernatant fluid for dust particles at 50mg/100 ml in distilled water (DW) as determined by light scattering pattern. Samples were taken at 1hr, 3hr, 24hr, 48hr and 72 hours Values are means \pm SE (n=3).

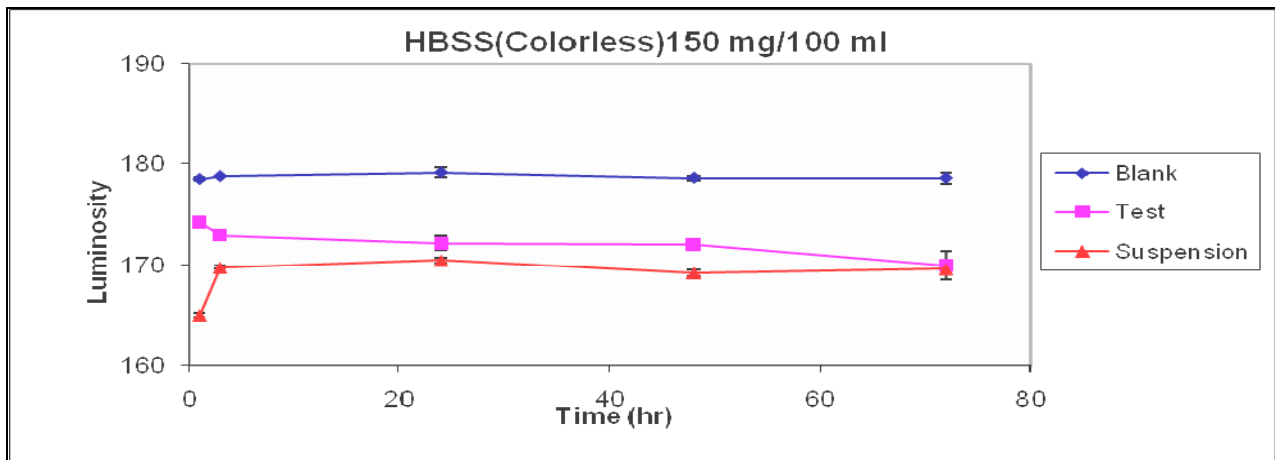


Figure 3 Luminosity of the supernatant fluid for dust particles at 150mg/100 ml in Hank’s balanced salt solution (HBSS) as determined by light scattering pattern. Samples were taken at 1hr, 3hr, 24hr, 48hr and 72 hours Values are means \pm SE (n=3).

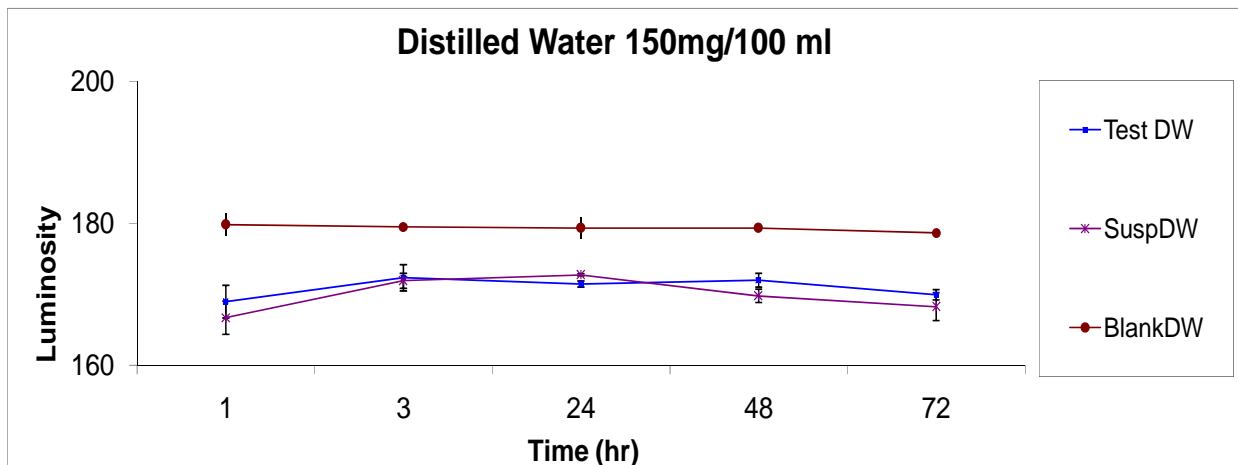


Figure 4 Luminosity of the supernatant fluid for dust particles at 150mg/100 ml in distilled water as determined by light scattering pattern. Samples were taken at 1hr, 3hr, 24hr, 48hr and 72 hours Values are means \pm SE (n=3).

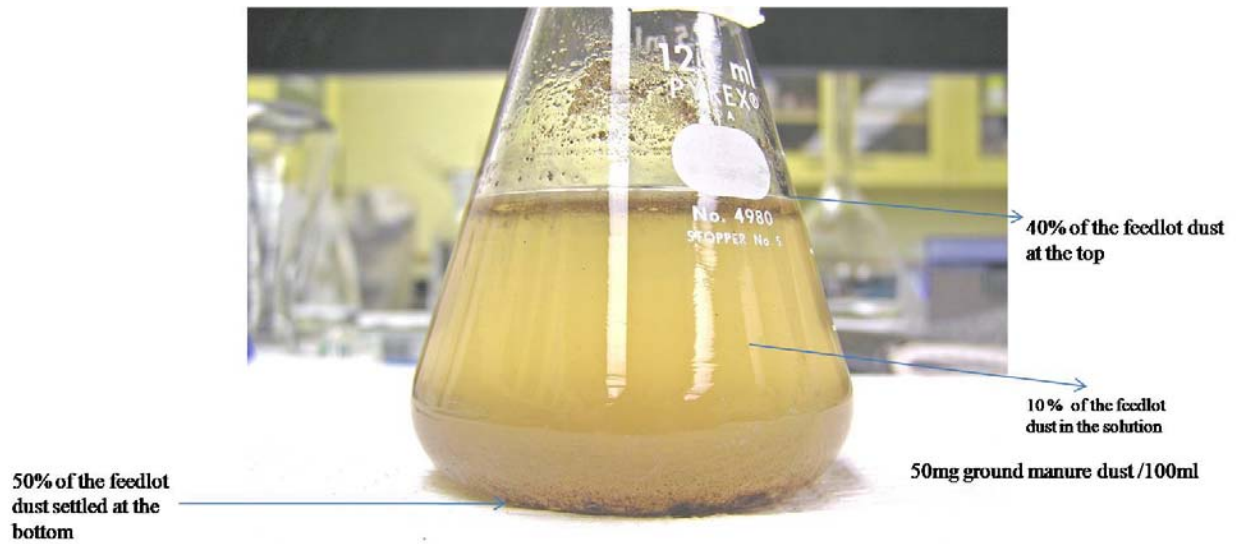


Figure 5 Erlenmeyer flask (125 ml) containing 50 mg of ground manure mat dust in HBSS. Suspension undergoing gentle agitation for 72 hours.

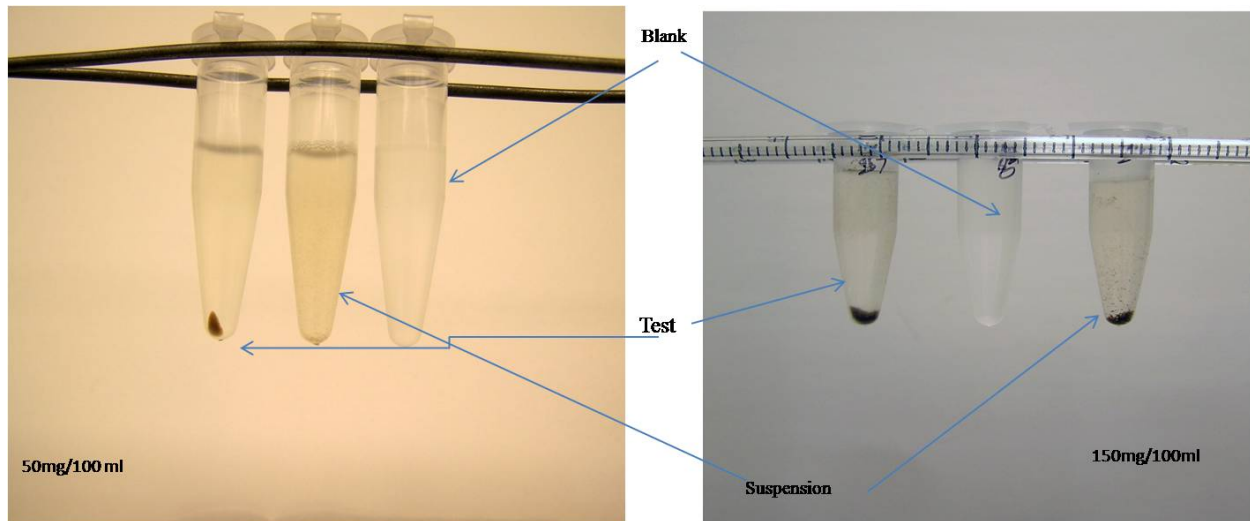


Figure 6 Solubility 150mg of cattle feedlot manure mat dust in 100 ml of HBSS (24 hr incubation) Test sample: suspended dust centrifuged and settled, Suspensions sample: uncentrifuged dust particles, and Blank was HBSS only. The test sample was compared to uncentrifuged suspension and to the blank. Change in luminosity among test, suspension and blank after 24 hr of incubation is shown in figures 1-4.

4.2 Endotoxin

The amount of the endotoxin in the sample was 2000EU/ml (20 EU/mg of dust) of the dust extract obtained by dissolving 1 gm of dust into 10 ml of endotoxin free water. Since, all samples used for endotoxin analysis were from the same lot of ground manure matt, there were less variations among sample endotoxin level.

4.3 Bioactivity (Cytokine Release)

1%, 5%, 10%, and 25 % dust extract were potent stimulators of IL-6 and IL-8 release from BEAS-2B at 6, 12 and 24 hours of culture (Figures 7 & 8). No IL-6 or IL-8 was released after 1 hours incubation of BEAS-2B with various concentration of dust extract. Release of IL-6 was dose dependent and increasing release was observed with increasing concentration. After 6 hour incubation, IL-6 release reached to peak concentration with 25 % dust sample (470±50 pg/ml) and it remained at the peak level through 24 hrs. For IL-6 release, stimulatory effects were mainly seen with 5%, 10% and 25% dust extract. For IL-8 peak release were at 1 and 5% dust extract. At 10 % and 25% dust extracts release of IL-8 were progressively less. Twelve

hours of incubation was the time of peak IL-8 release at 1450 ± 80 pg/ml. It appeared to be similar to 1470 ± 90 pg/ml in next 12 hours (24 hr of incubation). Although, effectiveness of 1% and 5% samples in inducing IL-8 release was indistinguishable ($p < 0.05$, SNK) at all time points, cell death may have limited the amount of IL-6 and IL-8 production with 50% dust extract concentration (relative cell viability 10-15%). The release of TNF- α was not detected with all sample concentrations at any time point.

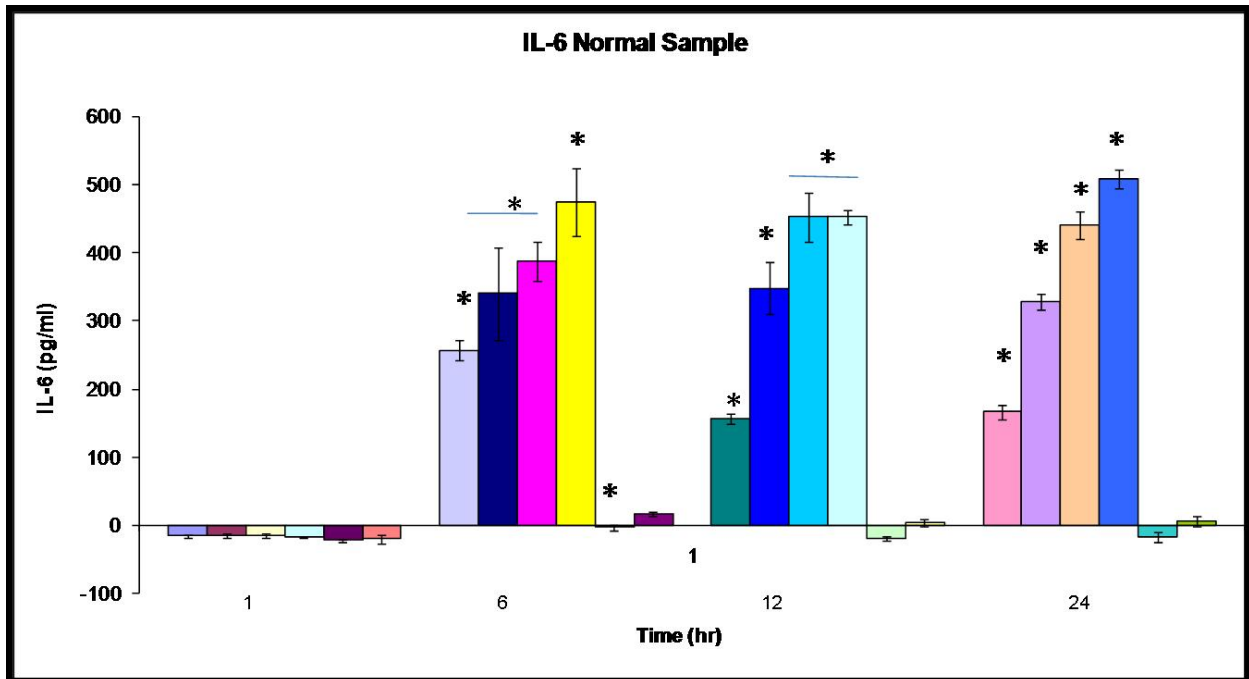


Figure 7 The release of IL-6 from BEAS-2B cell monolayers in response to 1%, 5%, 10%, 25%, 50% normal samples and control at different time points (n=9). From left to right in each group are 1%, 5%, 10%, 25%, 50%, and control at 1hr, 6hr, 12hr, and 24hr incubation period respectively. The concentration is on the ordinate, and the experimental groups are on the abscissa. * $p < 0.05$ compared with the supernatant fluids without dust extract exposure. Values are means \pm SE.

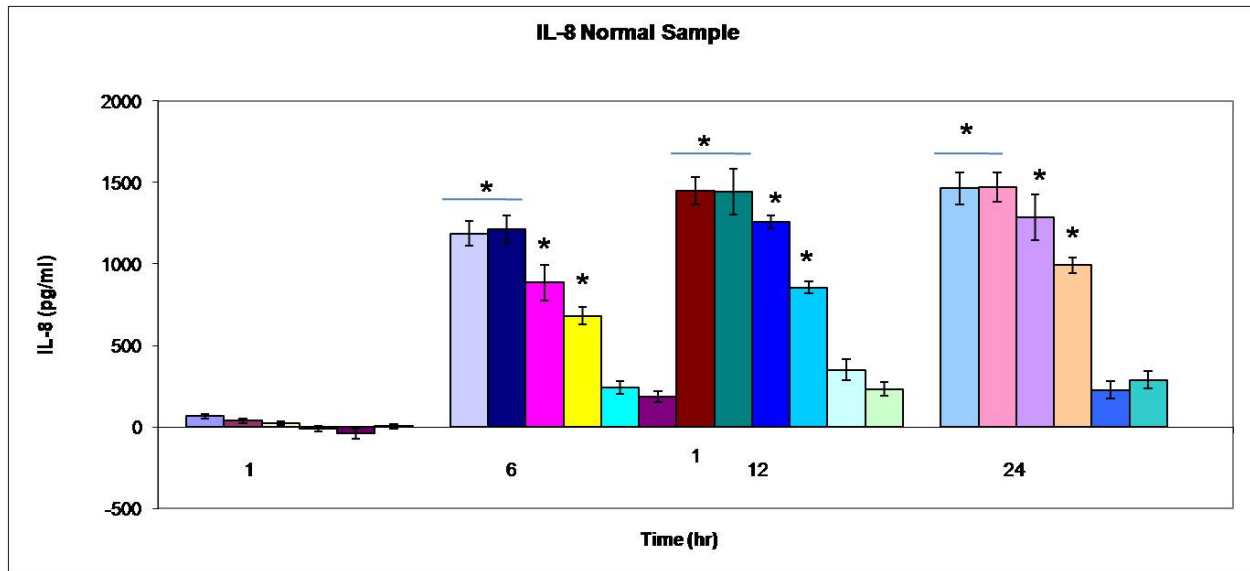


Figure 8 The release of IL-8 from BEAS-2B cell monolayers in response to 1%, 5%, 10%, 25%, 50% normal samples and control at different time points (n=9). From left to right in each group are 1%, 5%, 10%, 25%, 50% and control at 1hr, 6 hr, 12 hr, and 24 hr incubation period respectively. The concentration is on the ordinate, and the experimental groups are on the abscissa. * $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE.

Inhibition of endotoxin in dust extracts by using Polymixin B didn't seem to significantly change the pattern of IL-6 and IL-8 release from BEAS-2B cell cultures (Figures 9 and 10). However, unlike normal sample, peak IL-6 releases were not reached until 12 hrs of incubation. Similar to normal samples, peak IL-6 activities were present in 5%, 10%, and 25% concentration with 10%, and 25% inducing highest amount of IL-6 release (550 ± 27 pg/ml and 560 ± 20 pg/ml respectively) at 24 hr. The peak IL-8 release with endotoxin free sample was observed at 12 hrs with 10% dust extract (1220 ± 100 pg/ml). However, 1%, 5%, and 10% dust extracts were not significantly different in inducing IL-8 release at any time point. When cells were incubated with pure endotoxin with dose equivalent to amount of endotoxin in the dust extract, pattern of release of both cytokines were similar (Figures 11 and 12). Cytokine releases were dose dependent, and they reached to peak concentration at 12 hrs (IL-6: 444 ± 14 pg/ml and

IL-8: 1510±60 pg/ml). 5% (100 EU/ml) endotoxin sample in cell culture media was the most potent stimulator of both IL-6 and IL-8 release. 50% endotoxin sample (1000 EU/ml), though apparently toxic, didn't seem to kill cells as much as 50% dust extract and induced IL-6 and IL-8 release in dose dependent manner (range 40±20 pg/ml to 810.33±50 pg/ml for IL-8 and 50±6 pg/ml to 270±7 pg/ml for IL-6).

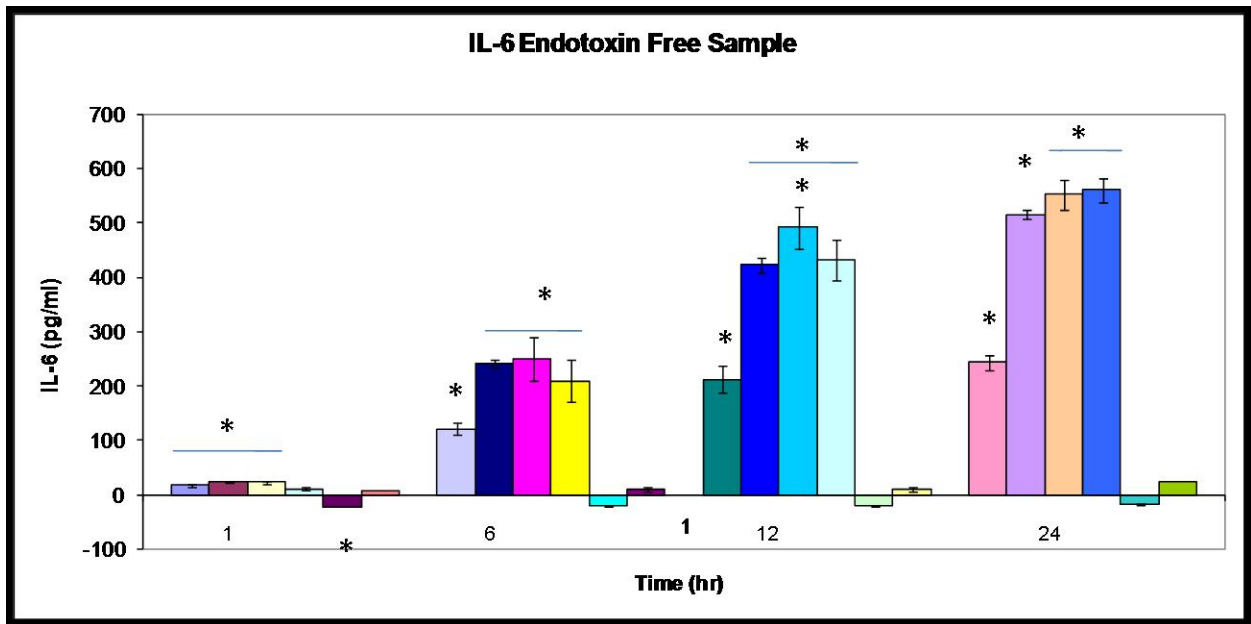


Figure 9 The release of IL-6 from BEAS-2B cell monolayers in response to 1%, 5%, 10%, 25%, 50% endotoxin free samples, and control at different time points (n=9). From left to right in each group are 1%, 5%, 10%, 25%, 50% and control at 1 hr, 6 hr, 12 hr and 24 hr incubation period respectively. The concentration is on the ordinate, and the experimental groups are on the abscissa. * $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE.

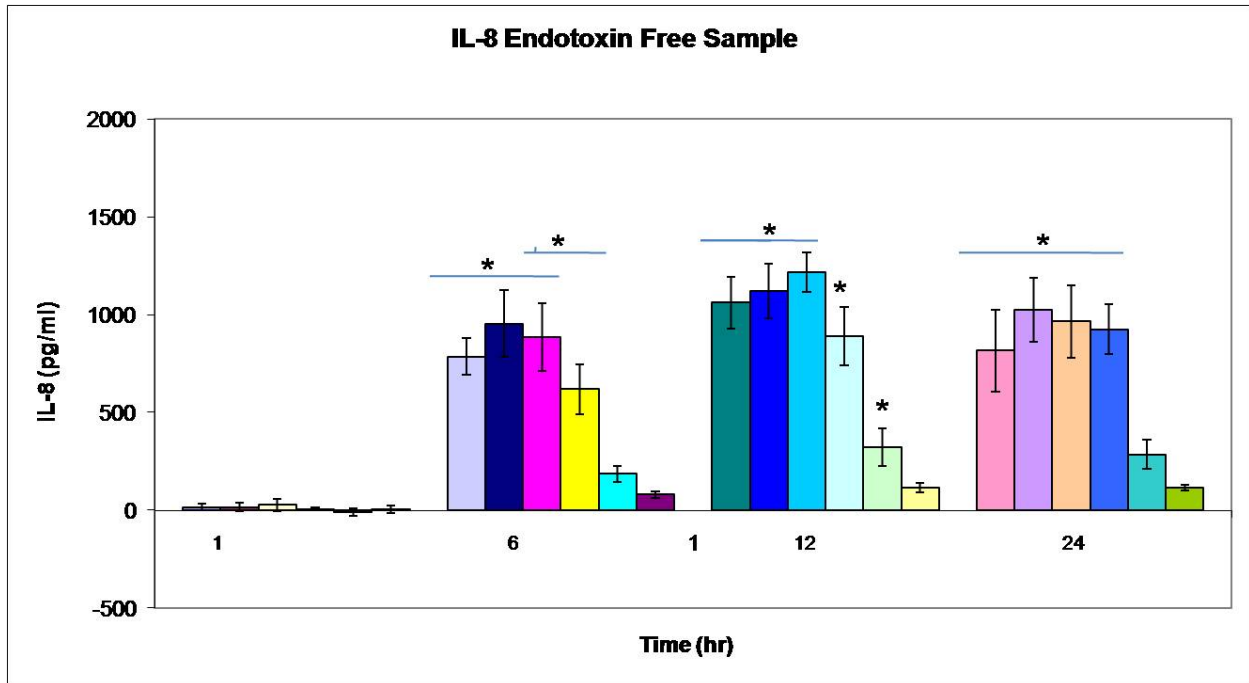


Figure 10 The release of IL-8 from BEAS-2B cell monolayers in response to 1%, 5%, 10%, 25%, 50% endotoxin free samples, and control at different time points (n=9). From left to right in each group are 1%, 5%, 10%, 25%, 50% and control at 1 hr, 6 hr, 12 hr and 24 hr incubation period respectively. The concentration is on the ordinate, and the experimental groups are on the abscissa. * $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE.

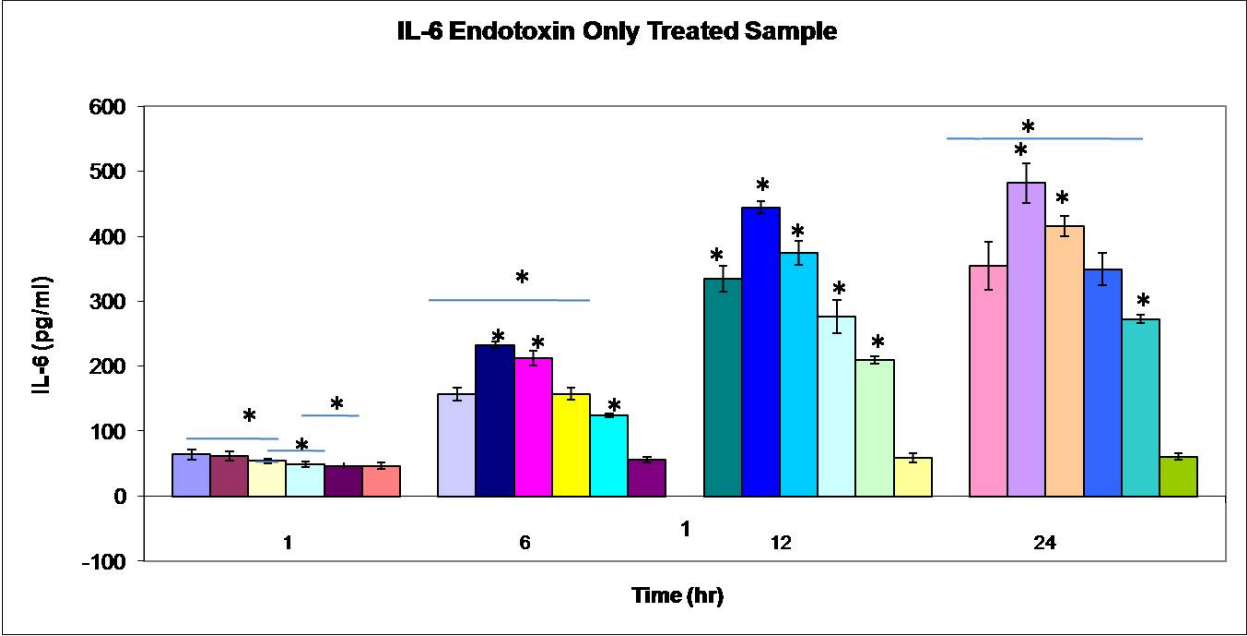


Figure 11 The release of IL-6 from BEAS-2B cell monolayers in response to 1%, 5%, 10%, 25%, 50% endotoxin only treated samples, and control at different time points (n=9). From left to right in each group are 1%, 5%, 10%, 25%, 50% and control at 1hr, 6 hr, 12 hr and 24 hr incubation period respectively. The concentration is on the ordinate, and the experimental groups are on the abscissa. * $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE

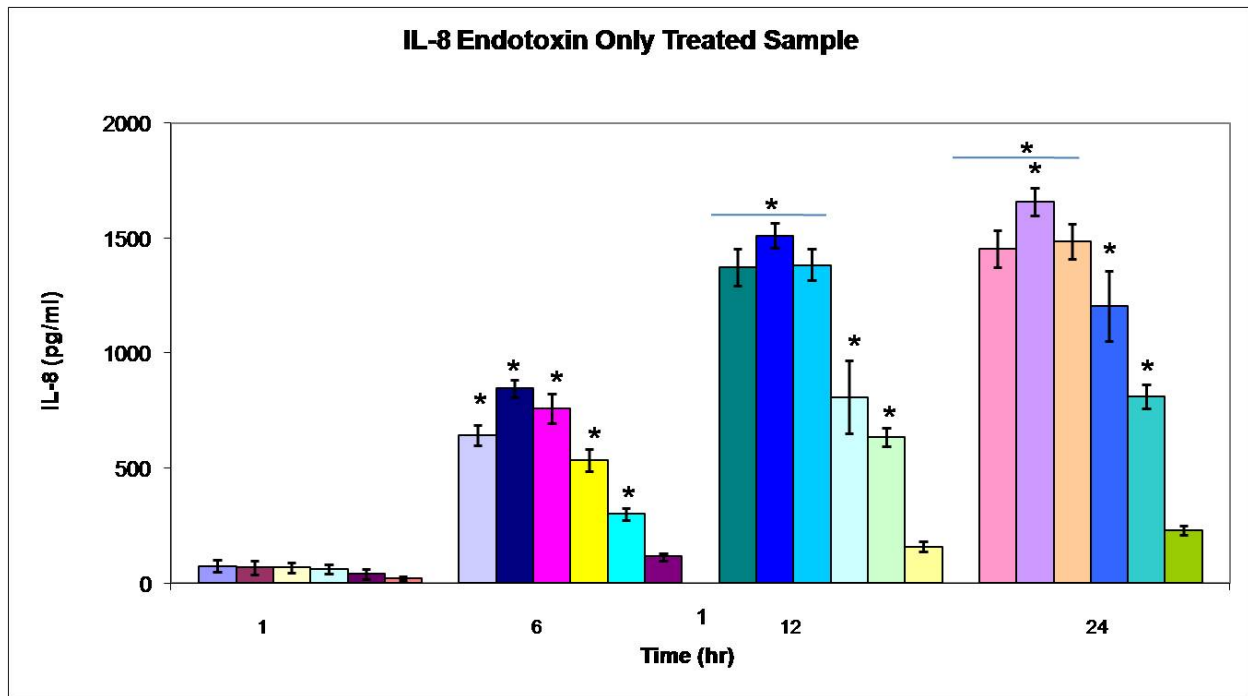


Figure 12 The release of IL-8 from BEAS-2B cell monolayers in response to 1%, 5%, 10%, 25%, 50% endotoxin only treated samples, and control at different time points (n=9). From left to right in each group are 1%, 5%, 10%, 25%, 50%, and control at 1hr, 6 hr, 12 hr, and 24 hr incubation period respectively. The concentration is on the ordinate, and the experimental groups are on the abscissa. * $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE.

Heating the sample at 120⁰C for 5 minutes didn't significantly change the pattern of cytokine release from BEAS-2B cell culture (Figures 13 and 14). All sample concentration induced IL-6 and IL-8 release in dose dependent manner. Notably, 50% dust extract was no more cytotoxic and induced both IL-6 (120 \pm 9 pg/ml at 6 hr to 270 \pm 19 pg/ml at 24hr), and IL-8 (170 \pm 40 pg/ml at 6 hr to 790 \pm 110 pg/ml at 24 hr) release from lung epithelial cells. The 50% extract's potential to induce IL-6 release was not different from 1% and 5% dust sample at 24 hr time point. IL-8 release didn't differ significantly among 5%, 10%, 25% and 50% dust samples after 12 hours of incubation. 25% dust extract was most potent stimulator of IL-6 release from bronchial epithelial cells (490 \pm 25 pg/ml) after 6 hours of incubation ($p < 0.05$).

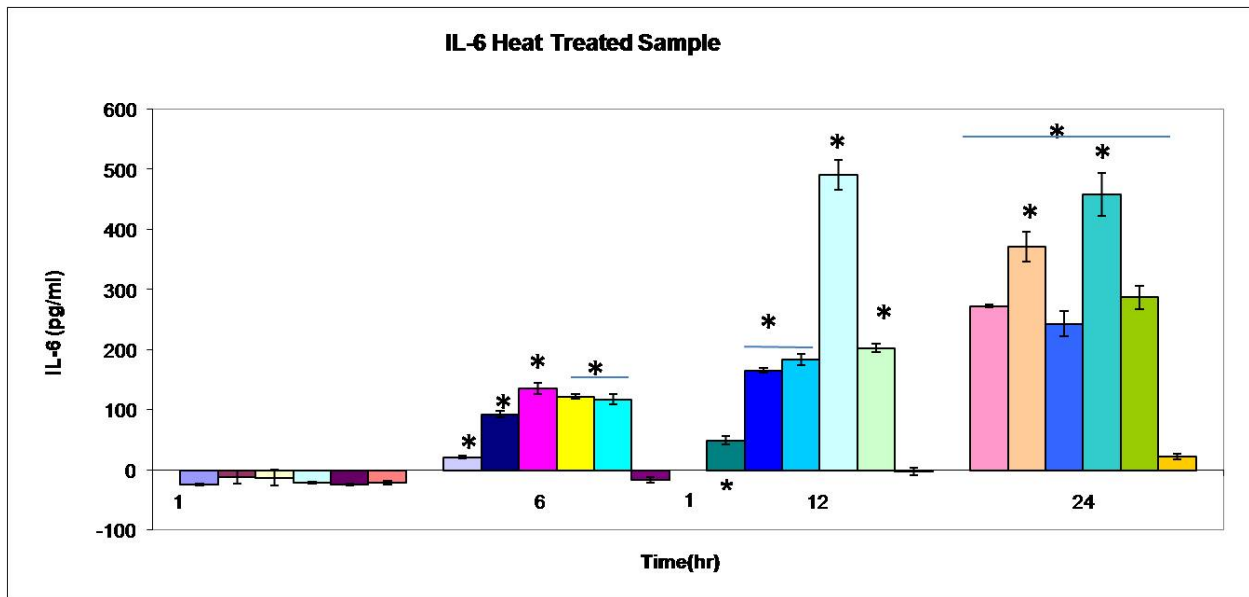


Figure 13 The release of IL-6 from BEAS-2B cell monolayers in response to 1%, 5%, 10%, 25%, 50% heat inactivated samples and control at different time points (n=9). From left to right in each group are 1%, 5%, 10%, 25%, 50% and control at 1hr, 6hr, 12hr and 24hr incubation period respectively. The concentration is on the ordinate, and the experimental groups are on the abscissa. * $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE.

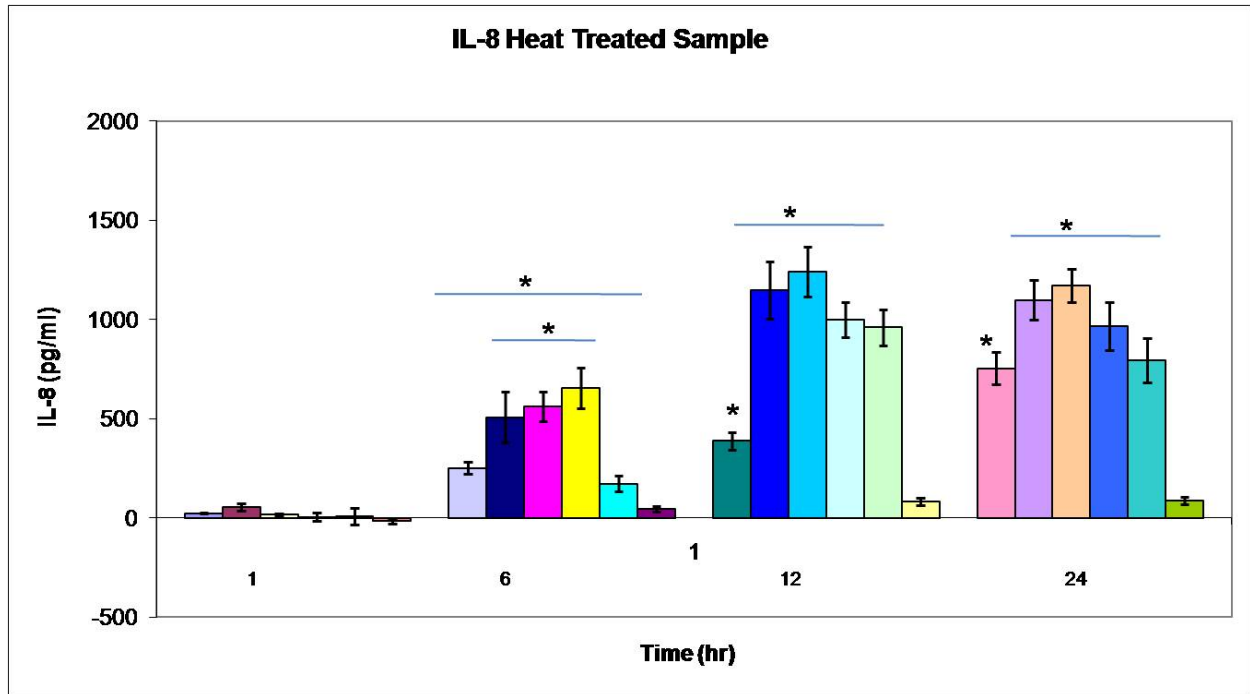


Figure 14 The release of IL-8 from BEAS-2B cell monolayers in response to 1%, 5%, 10%, 25%, 50% heat inactivated samples and control at different time points (n=9). From left to right in each group are 1%, 5%, 10%, 25%, 50% and control at 1hr, 6hr, 12hr and 24hr incubation period respectively. The concentration is on the ordinate, and the experimental groups are on the abscissa. * $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE.

Multiple comparison among different treatment groups revealed that at lower doses (1% and 5%) and short incubation period (6hrs), stimulatory effects of normal, endotoxin free, pure endotoxin, and heat treated samples were significantly different from each other (Figures 15-22). The expression of IL-8 was highest for untreated samples followed by endotoxin free, pure endotoxin, and heat treated samples respectively. However, increased concentration and incubation time leveled the strength of each sample and there were no significant difference among various groups in latter time period ($p < 0.05$). IL-6 release was highest with the untreated extracts normal sample up to 6 hr incubation period but by 12 hours, pure endotoxin and endotoxin free samples were inducing as much cytokine as by untreated samples. However, after

24 hour incubation release response were mixed and more or less indistinguishable from each other ($p < 0.05$).

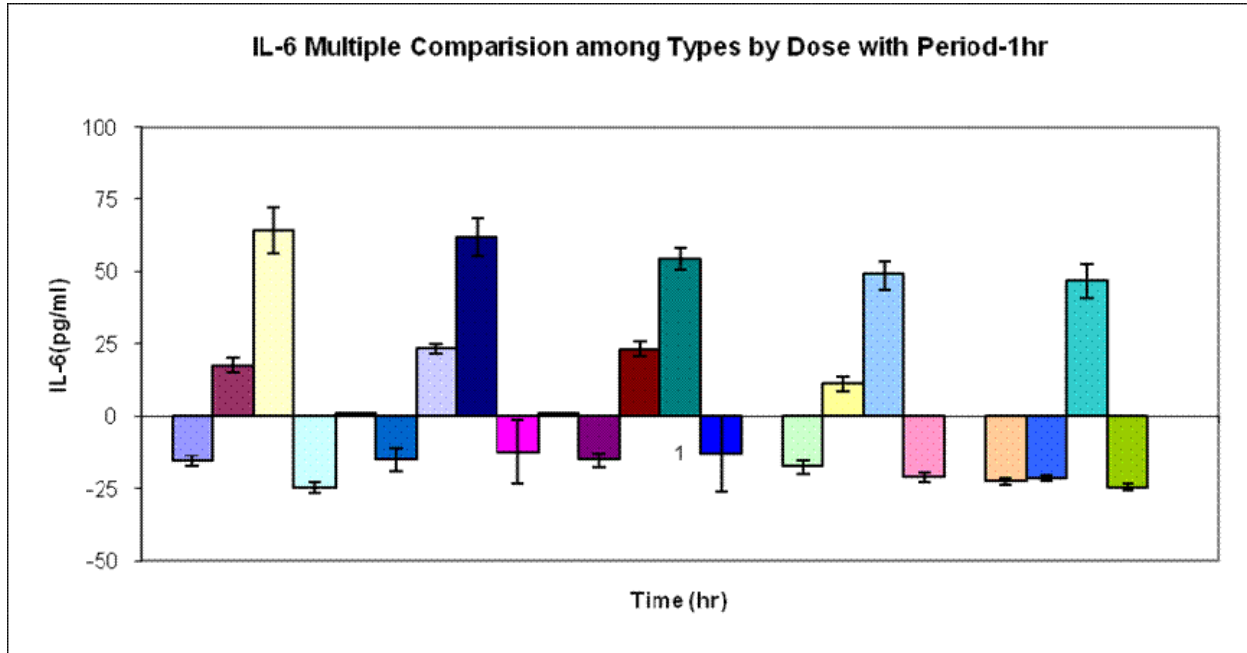


Figure 15 Multiple Comparison of IL-6 release from BEAS-2B cell monolayers among normal sample, endotoxin free sample, endotoxin only sample, and heat inactivated sample at 1hr. From left to right in each group are normal, endotoxin free, endotoxins only, and heat inactivated samples. The concentration is on the ordinate and the experimental groups are on the abscissa. * $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE (n=9).

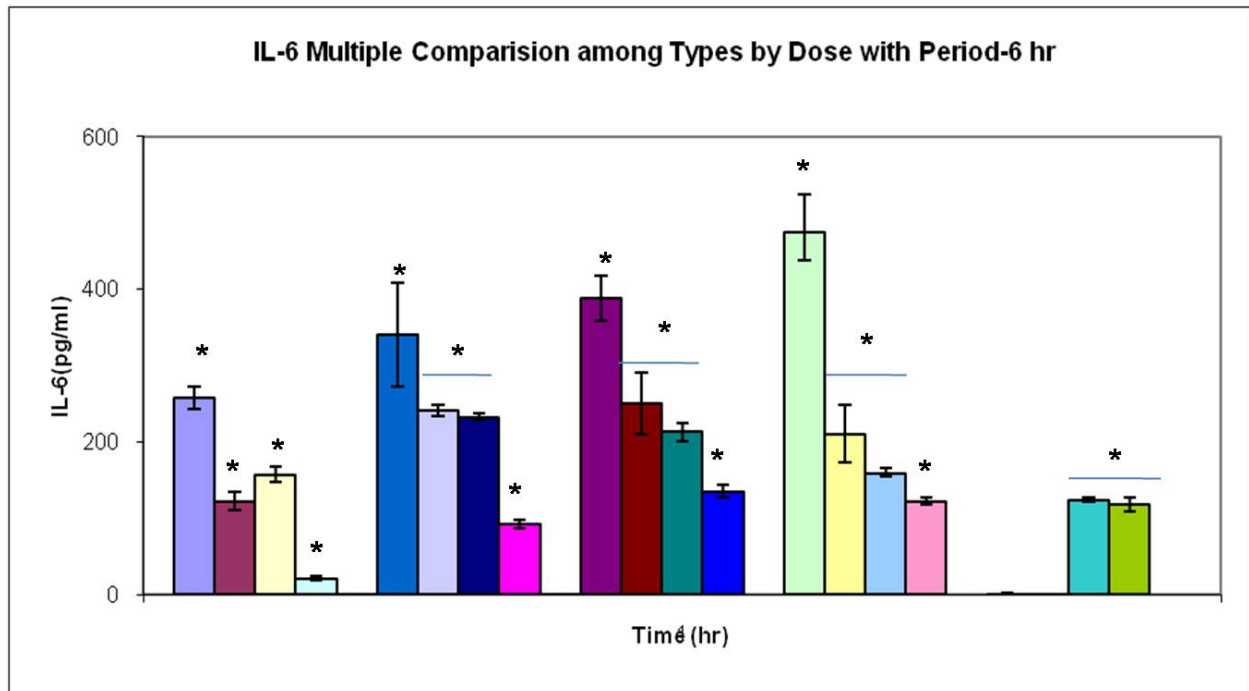


Figure 16 Multiple Comparison of IL-6 release from BEAS-2B cell monolayers among normal sample, endotoxin free sample, endotoxin only sample, and heat inactivated sample at 6hr. From left to right in each group are normal, endotoxin free, endotoxins only, and heat inactivated samples. The concentration is on the ordinate and the experimental groups are on the abscissa. * $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE (n=9).

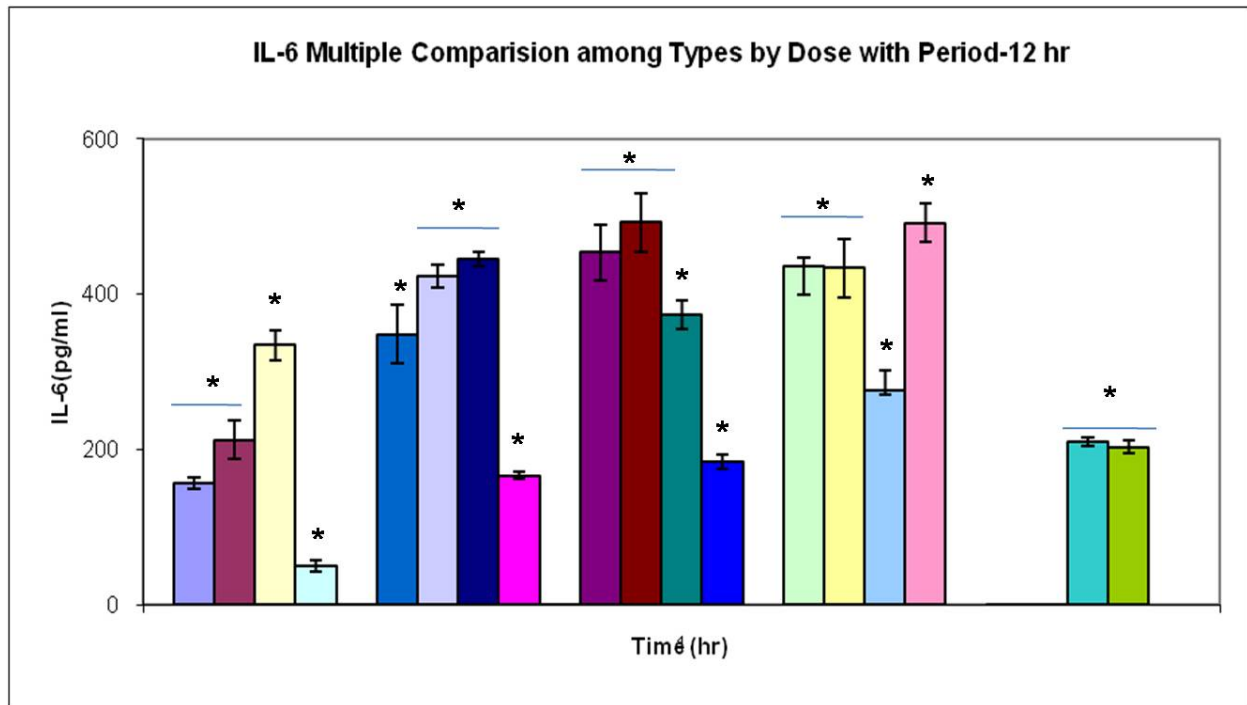


Figure 17 Multiple Comparison of IL-6 release from BEAS-2B cell monolayers among normal sample, endotoxin free sample, endotoxin only sample, and heat inactivated sample at 12hr. From left to right in each group are normal, endotoxin free, endotoxins only, and heat inactivated samples. The concentration is on the ordinate and the experimental groups are on the abscissa.* $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE (n=9).

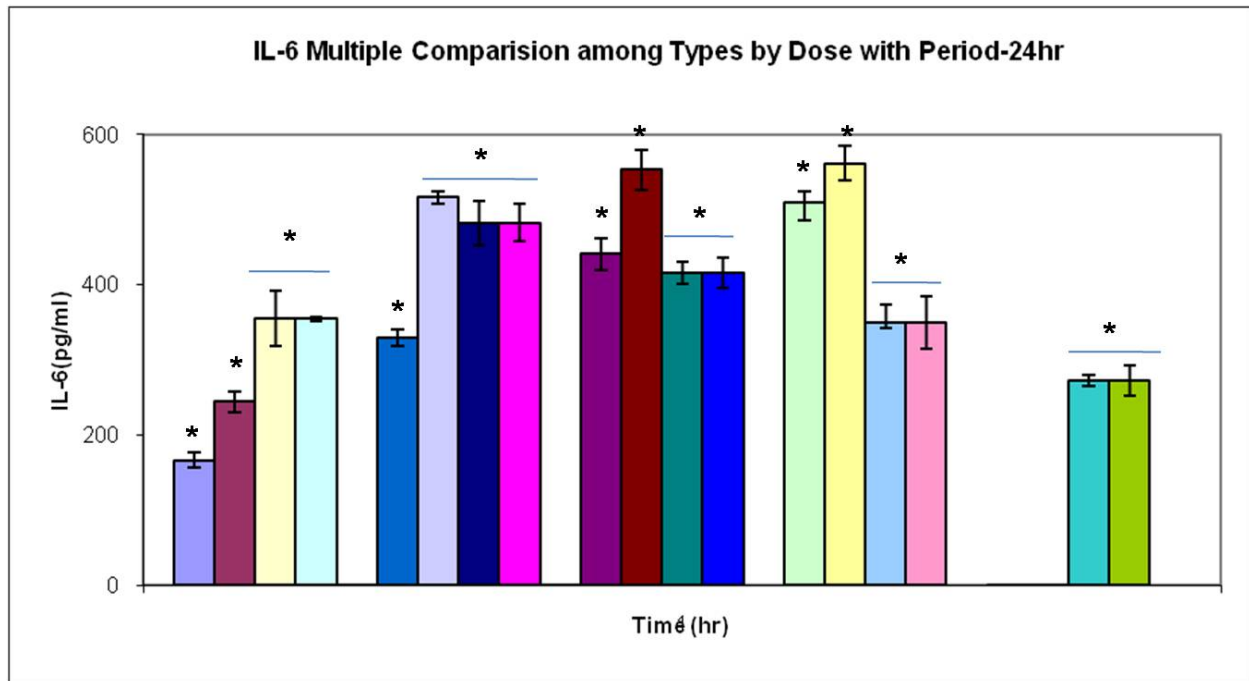


Figure 18 Multiple Comparison of IL-6 release from BEAS-2B cell monolayers among normal sample, endotoxin free sample, endotoxin only sample, and heat inactivated sample at 24hr. From left to right in each group are normal, endotoxin free, endotoxins only, and heat inactivated samples. The concentration is on the ordinate and the experimental groups are on the abscissa. * $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE (n=9).

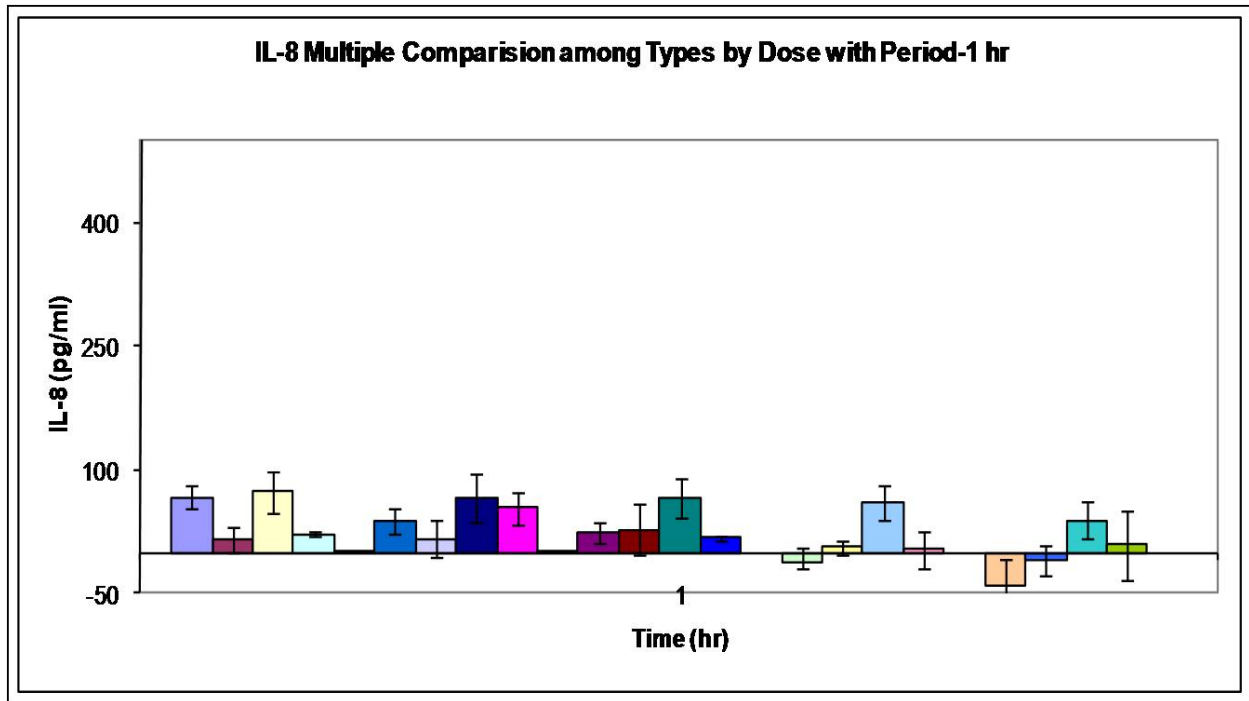


Figure 19 Multiple Comparison of IL-8 release from BEAS-2B cell monolayers among normal sample, endotoxin free sample, endotoxin only sample, and heat inactivated sample at 1 hr. From left to right in each group are normal, endotoxin free, endotoxins only, and heat inactivated samples. The concentration is on the ordinate and the experimental groups are on the abscissa.* $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE (n=9).

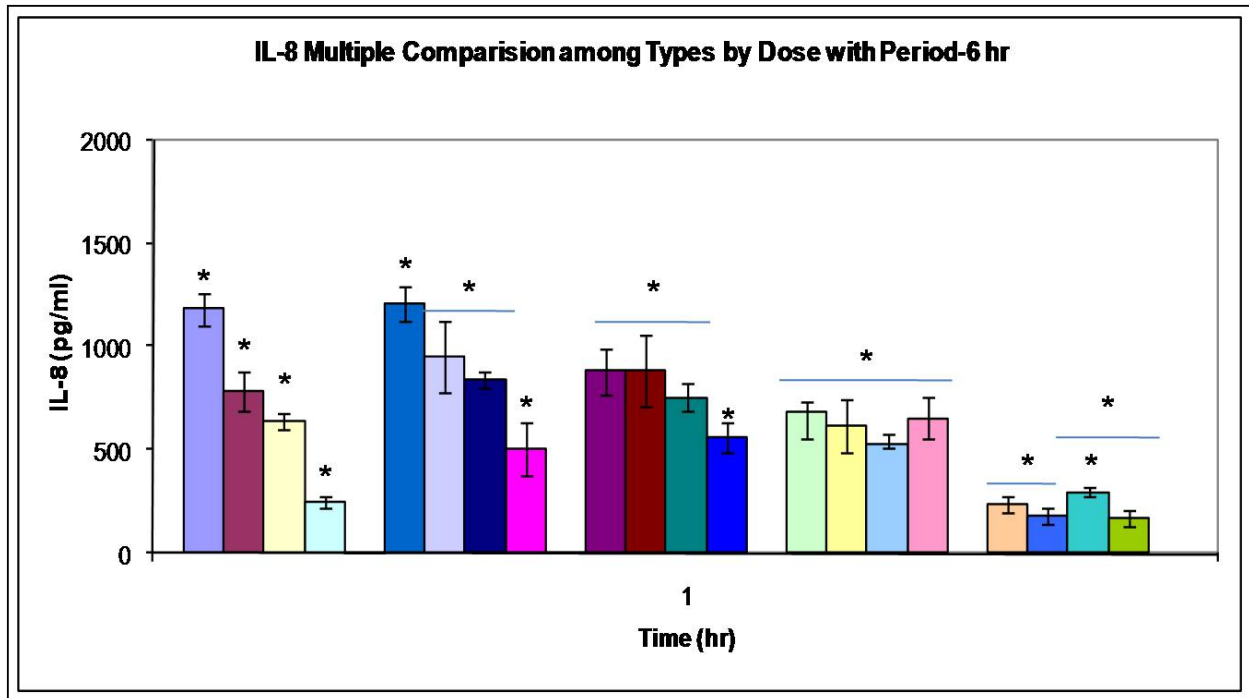


Figure 20 Multiple Comparison of IL-8 release from BEAS-2B cell monolayers among normal sample, endotoxin free sample, endotoxin only sample, and heat inactivated sample at 6hr. From left to right in each group are normal, endotoxin free, endotoxins only, and heat inactivated samples. The concentration is on the ordinate and the experimental groups are on the abscissa. * $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE (n=9).

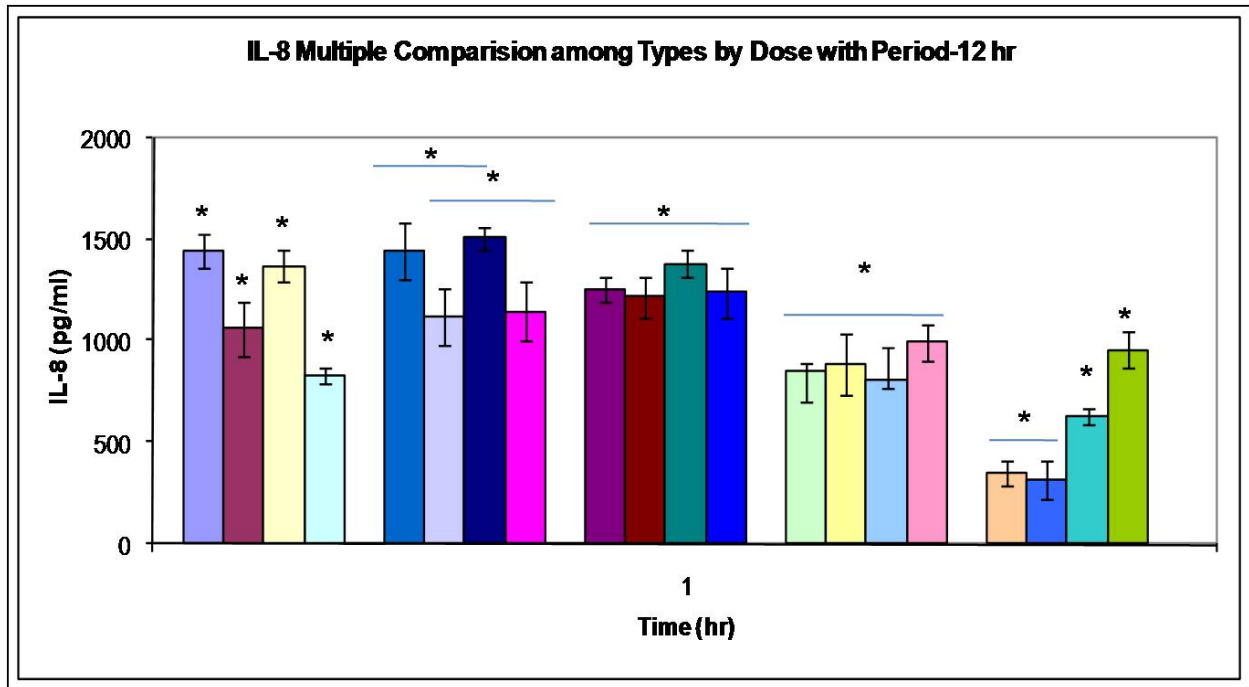


Figure 21 Multiple Comparison of IL-8 release from BEAS-2B cell monolayers among normal sample, endotoxin free sample, endotoxin only sample, and heat inactivated sample at 12hr. From left to right in each group are normal, endotoxin free, endotoxins only, and heat inactivated samples. The concentration is on the ordinate and the experimental groups are on the abscissa. * $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE (n=9).

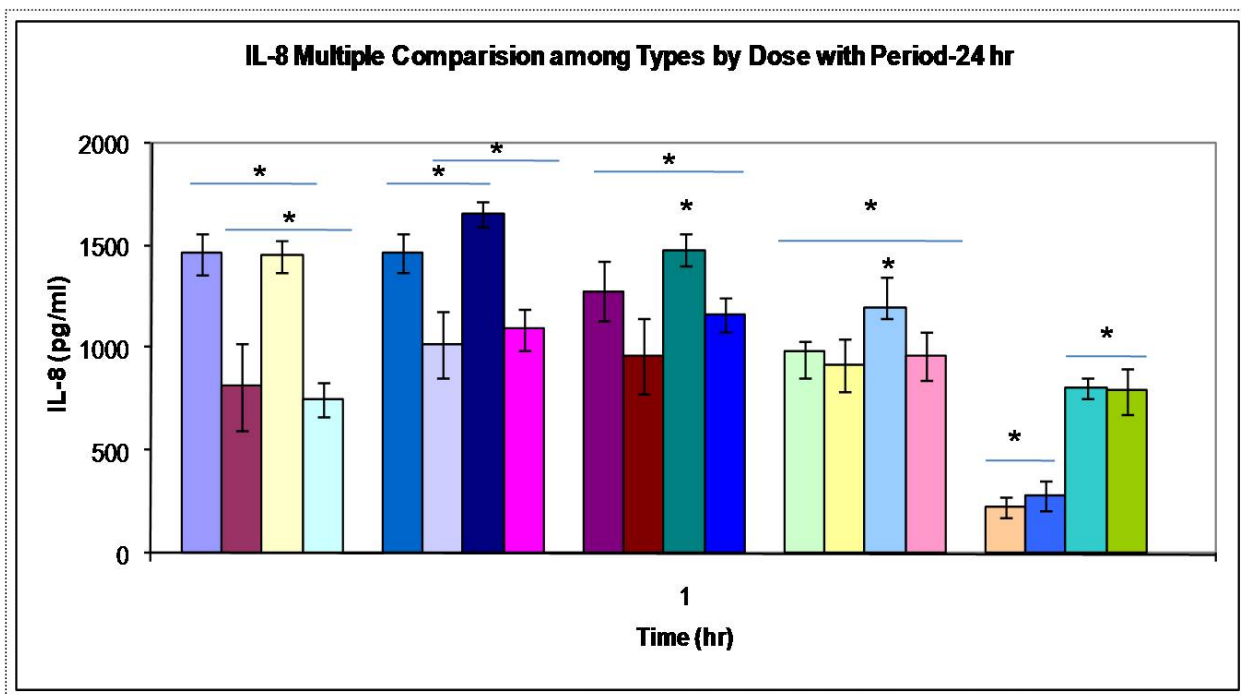


Figure 22 Multiple Comparison of IL-8 release from BEAS-2B cell monolayers among normal sample, endotoxin free sample, endotoxin only sample, and heat inactivated sample at 24hr. From left to right in each group are normal, endotoxin free, endotoxins only, and heat inactivated samples. The concentration is on the ordinate and the experimental groups are on the abscissa.* $p < 0.05$ compared with the supernatant fluids without beef cattle feedlot dust extract exposure. Values are means \pm SE (n=9).

5. Discussion

We have shown that cattle feedlot dust extracts stimulate the BEAS-2B lung epithelial cell line to produce IL-6 and IL-8 in a dose dependent manner. Interleukin-6 release was proportional to the exposure dose (1 to 25% extract). At the highest dose concentration (50% extract) production was decreased and more cell death was observed. Interleukin-8 release was highest in the 1 and 5% extract. At higher concentration less IL-8 was released. There was no significant cytotoxicity 1%, 5%, 10%, and 25% dust extracts. 50 % extract was cytotoxic having only 10-15% viable BEAS-2B lung epithelial cells. There was no detectable amount of TNF- α expression in BEAS-2B even though it initiates inflammatory process by inducing the expression of transcription factor genes such as NF- κ B and AP-1 which induces the transcription of secondary mediators IL-6 and IL-8⁽¹¹⁶⁾. Our observations were similar to those elicited by soil dust particles with BEAS-2B by Veranth *et al*⁽⁸⁵⁾. Various exposure effect experiments of

environmental toxicants to the lung epithelial cells revealed that cellular mediators such as IL-6, IL-8, and TNF- α are most commonly used parameters to assay airway inflammatory process. Saraf *et al.*⁽¹⁵⁴⁾ reported that house dust samples induced release of IL-6 and IL-8 in lung epithelial cells but effects were more potent than the swine dust. Redente *et al.*⁽¹⁵⁵⁾ showed that agriculture dust such as corn and wheat dust could induce IL-8 expression in lung epithelial cells in dose dependent manner. *In-vitro* studies by Palmberg *et al.*⁽¹⁵⁶⁾ and Wang *et al.*⁽¹⁵⁷⁾ demonstrated that hog barn dust is particularly potent stimulus of airway epithelium cell to induce IL-6 and IL-8 release. *In-vivo* studies have revealed that inhalation of swine dust can cause increased serum interleukin-6, bronchial responsiveness, decrease in vital capacity, increase in the blood granulocyte concentration, and body temperature⁽¹⁵⁸⁾.

Investigators speculate about which components in the CAFO dust extract are responsible for pathophysiological changes in the respiratory system. Some of the physical and chemical characteristic that determine the degree of the toxicity of the inhaled dust particles are particle form, surface area, surface hydrophobicity and hydrophilicity, transition metals and free radical release⁽⁴⁵⁾. Since, CAFO dust particles are complex mixture of particulate matter, microbes, volatile organic compounds, and adsorbed gases such as ammonia and hydrogen sulfide and each of these components have been proved to be toxic to the cell when taken individually; they may interact. Thus, it is extremely difficult to demonstrate a particular component that is responsible for airway inflammation. However, endotoxin, metal-dependent ROS generation, acids, bio-aerosols, ultrafine particle and organic compounds have been considered candidates for research in attempting to understand the mechanism of airway inflammation following exposure to the air pollution⁽⁶⁵⁾.

Dust is not the only potential toxicant associated with the CAFO. Odors from swine, cattle, and poultry CAFO can be overwhelming and annoying to the individuals working in the facility as well as animals and residents near to the CAFO. Sweeten *et al.*⁽¹⁵⁹⁾ reported that odor generated in the animal confinement facilities to contain ammonia, hydrogen sulfide and other as yet undefined components. Hog manure odor is composed of at least 121 different compounds⁽¹⁶⁰⁾. It has been reported that people living in the vicinity of CAFO suffered from various psychological and physiological stress such as depression, fatigue, anger and wheezing ,decreased FEV1 respectively^(75, 145). The cause of this depression is being investigated.

Due to presence of large number of coli-form bacteria in animal waste, high endotoxin levels have been found in CAFO dust in several studies. Endotoxins are components of cell wall of both pathogenic and non-pathogenic gram-negative bacteria. Their effects are known to persist for a long time⁽¹³¹⁾. We demonstrated that endotoxin in the ground manure matt from beef cattle feed contained 2000EU/ml (7×10^7 ng/ml) of the extract obtained by dissolving 1gm of dust into 10 ml endotoxin free water. Thus 1mg/ml could be expected to contain $7 \times 10^5/m^3$ ($70 \mu\text{g}/m^3$) a relatively higher level⁽¹⁷⁸⁾. The concentration of endotoxin in our sample is quite high. It reflects the nature of endotoxin content of the sample. The minimum exposure limit for endotoxin in the US is not fixed but exposure experiment by International Commission on Occupational Health (ICOH) has shown that endotoxin at 1,000 to 2,000 ng/m³ concentrations is sufficiently high to causes organic dust toxicity syndrome (ODTS). Comparatively acute bronchoconstriction occurs at levels of 100 to 200 ng/m³ and mucous membrane irritation at levels of 20 to 50 ng /m³⁽¹⁶¹⁾.

Inhibition of endotoxin by Polymixin B didn't significantly affect the expression of IL-6 and IL-8 on BEAS-2B cell line. However, it allowed peak levels of IL-6 to be released by the 5% extract suggesting proinflammatory changes could be initiated by a more dilute extract. In addition, it allowed peak IL-8 release at more concentrations (1, 5, 10 and 25 % vs. 1 and 5% extract). This suggested that maximum IL-8 release could occur over broader ranges of extracts making the maintenance of inflammation by stimulating polymorphonuclear cells and eosinophils. In both cases, endotoxins in the sample make producing lung injury by inflammation more difficult. Thus, in that sense the endotoxin in the manure mat extract is protective. This led us to postulate that PM is capable of inducing proinflammatory reactions through endotoxin-independent mechanism. A study conducted in Mexico City by Osorino-Vargas et al.⁽²⁷⁾ concluded that PM_{2.5} induced *in-vitro* cytotoxicity was through an endotoxin-independent mechanism where as cytotoxicity by PM₁₀ was endotoxin-dependent mechanism. Similar observations were reported by Von Essen et al.⁽¹⁶⁾, and Veranth et al.⁽⁸⁵⁾. So, this and other reports supports hypothesis that endotoxin is not only component in the dust extract that induces inflammatory cytokines in bronchial epithelial cells. However, release of cytokine following exposure of cells to pure endotoxin suggest that inflammatory action of endotoxin in dust extract was either masked by other components in the dust extract or the endotoxin present in our sample was not as inflammogenic as the pure endotoxin. It is evident from numerous

studies that endotoxin are well-known inflammatory agent and are consistently associated with impairment of lung functions in CAFO workers⁽⁸⁵⁾. A dose response to endotoxin and pulmonary function deterioration had been established in numerous studies. Kennedy et al.⁽¹⁶³⁾ found that exposure to endotoxin was associated with decline pulmonary functions, primarily forced expiratory volume in one second (FEV1), and development of symptoms such as chest tightness, cough, dyspnea, fever, rigors, myalgia, arthralgia and flue like symptoms. There are also some reports on beneficial effects of endotoxin. Enterline et al.⁽¹⁶⁴⁾ reported that lung cancer related mortality in cotton workers was lower than the expected mortality. This was attributed to the fact that endotoxin activates macrophages to produce TNF-alpha, a tumor killing cytokine⁽¹⁶⁵⁾. It is also found that certain level of exposure to endotoxin and subsequent secretion of inflammatory cytokines modulates the development of immune systems in the Th1 direction and suppresses the development of atopic sensitization⁽¹⁶⁴⁾.

Bronchial epithelial cells represent the first line of defense against the invading airborne pathogens and toxicants. These cells are important components of mucosal innate immunity in the respiratory systems. Mayer et al.⁽¹⁶⁶⁾ have demonstrated that bronchial epithelial cells express functional Toll Like Receptor 1-6 (TLR1, TLR2, TLR3, TLR4, TLR5, TLR6), and TLR-9 which plays important role in pathogen recognition and innate immunity. TLR are membrane spanning protein which recognize conserved microbial patterns and mediate inducible activation of innate immunity. A work by Quan Sha et al.⁽¹⁶⁷⁾ showed that TLR 1 through 10 are expressed on BEAS-2B cells. Among various TLRs, TLR-4 is expressed on the cell membrane and is key receptor for most bacterial lipopolysaccharide (LPS). When endotoxin gets deposited in the respiratory tract, lipopolysachharide binding protein (LBP) opsonises endotoxin and cluster of differentiation-14 (CD 14) or LPS receptor (LPS-R) expressed on monocytes, macrophage, granulocytes, B cells, and pulmonary dendritic cell bind with the LBP-endotoxin complex and activate TLR-4. LBP also mediates attachment of endotoxin to the cells that don't express CD14 such as epithelial cells and pulmonary dendritic cells leading to the activation of TLR-4 on cell surface. TLR-4 acts as a transducing subunit of the LPS signaling complex and induces the transcription factor NF-k β gene expression which initiates the expression of inflammatory mediators such as IL-6, IL-8, TNF- α , and IL-1 β ^(116,135,133,134).

It is speculated that in airway inflammatory conditions, soluble CD14 and LBP may become available through extravasations of serum proteins⁽¹⁵⁷⁾. Pugin et al.⁽¹⁶⁸⁾ found that

soluble CD14 is required for activation of endothelial and epithelial cells by LPS. It is thus unlikely that CD14 and LBP are involved in the LPS induced responses by epithelial cell cultured in serum free media. Zhengang et al. ⁽¹⁶⁹⁾ reported that bovine serum albumin (BSA) contains soluble CD14 and LBP which could facilitate presentation of LPS to receptive cells. Our *in-vitro* model provided this ideal environment for LPS to elucidate its inflammatory action on lung epithelial cells. However, it is of interest to note that dust, but not endotoxin induced marked IL-6 and IL-8 expression under this condition. This was evident from the fact that removal of endotoxin from the sample had little effect on expressions of both cytokines. But, contrary to the observation reported by Frampton et al. ⁽¹⁷⁰⁾ we observed expression of these cytokine in BEAS-2B which could be attributed to the addition of BSA in the cell culture media.

We observed a number of statistically significant correlations between cytokine production by bronchial epithelial cells when exposed to heat treated and non-heat treated dust extract. Heat treatment at 120⁰C had little effects on cytokine induction but it could modify some of the toxic properties of the dust extract. We observed that heat treated sample at lower concentration was not as strong as untreated sample in initiating cytokine production. However, the higher dose of sample (50% dust extract), which was otherwise toxic to the cells, could induce significantly more cytokine production suggesting that heat treatment could reduce some of its toxic properties but don't render it completely non-toxic. Vernath et al. ⁽⁸⁵⁾ suggested that heating the particles to 300⁰C or 500⁰C was required to completely remove chemical compounds or particle characteristics that induced the proinflammatory cytokines such as IL-6 and IL-8. Mathesen et al. ⁽¹⁷¹⁾ reported that heating indoor dust at 50-250⁰C resulted in reduced IL-8 and TNF-alpha response in comparison to non-heated dust. Heat treatment also may change some of the physical and chemical properties of the dust extract especially its natural crystalline structure Hemenway et al. ⁽¹⁷²⁾ reported that heat treatment of crystalline silica (cristobalite and quartz) reduced the hydrophilicity resulting in increased dust accumulation and increased in inflammatory cells enhancing the long-term clearance of the particles. It is probable that dust particles may contain some organic or inorganic substances that is resistant to the lower temperature treatment but can be inactivated at high temperature treatment.

Particle dissolution in the epithelial lining fluids (ELF) in the respiratory tract has been recognized as important clearance mechanism ⁽¹⁴⁷⁾. Solubility of the particle is dependent upon chemical nature and surface character. Fubini ⁽⁴⁵⁾ had reported that selective removal of the ion

exposed at the particle surface by chelating agent facilitates the penetration of solvent molecules in the solid and increase the solubility. Ions and molecules from the soluble particles in the lungs ELF can pass through the mucous membrane and enter into the circulation. We observed that ground beef cattle manure matt dust particles when mixed into lung simulant fluids with gentle agitation nearly mimicking the alveolar movement *in-vivo* approximately 10% dissolved into the solution, nearly 40 % formed a layer at the top of the fluid, and 50 % settled to the bottom of the flask. The dissolved particles either leave the lung completely or are redistributed via the circulation as ions or molecules to extra-pulmonary organs. The redistribution mechanism help to reduce particle load at initial deposition site and total burden to the lung. Alveolar macrophage removes the particle by phagocytosis or pinocytosis and attempts to keep the alveolar surface clean. However, if the particles are too small to be trapped by macrophage, they escape phagocytosis and enter into the interstitium or the blood circulation. Eventually particles in the interstitium are cleared either by dissolution or by lymphatic circulation ⁽⁴⁵⁾ or remain there with a chronic potential to inflame the lung.

Agricultural industries include all forms of activities connected with growing, harvesting, and primary processing of all types of crops; with breeding, raising and caring for animals. The cotton industry, forestry and fisheries as well as grain storage are also considered a part of agricultural system. So, this industry encompasses wide areas of occupational settings. It has been estimated that approximately 5 million people in the United States are involved in agriculture. In developing countries the majority of the populations depend upon agriculture for their livelihood ⁽¹⁷³⁾. For example, 90 % of the population in Nepal depends upon agriculture related enterprises for the sustainability. Most agricultural industries are potential source of dust production. Thus agriculture workers are at higher risk of developing respiratory diseases. Among the agricultural workers, health hazards in swine CAFO workers are perhaps one of the earliest identified occupational hazards ⁽¹⁷⁴⁾. Such hazards have been studied extensively. However, in the recent past beef cattle feedlots have been getting a lot of attention for its role in dust and odor generation. It is estimated that 1.38 tons of dry manure is produced from each 1000 pound beef cattle every year ⁽⁵⁶⁾. The dried manure thus produced if not compacted into a firm layer by animal activities, becomes a source of fugitive dust. Increased animal activities combined with wind blow spread dust particles and odor polluting the air in the farm as well as in surrounding environment. So, feedlot workers, feedlot owners and their families, animals, and

people who live near these farms face health risks from air pollution generated by animals, and humans in the feedlot. Besides air pollution, CAFO activities also lead to surface and ground water pollution, and development of antibiotic resistance from the antibiotics excreted in the feces ⁽¹⁷⁵⁾ .

Inflammatory responses to the inhaled particle pollutants involve production of range of cellular mediators and signaling molecules such as cytokines, chemokines, leukotrienes/prostaglandin and adhesion molecules. These molecules form a complex network to stimulate and coordinate the function of epithelial cells, alveolar macrophage, neutrophils, eosinophils, thymic lymphocytes (T cells), and other cells of immune systems ⁽¹⁷⁶⁾ . While these responses were initially developed for lung defense, excess protracted stimulation and allergic responses may lead to pulmonary damage. Depending upon the nature of these mediators, different classes of the immune cells are recruited resulting in different diseases and pathological conditions. For example in allergic asthma, eosinophils are principle cells initiating inflammatory process ⁽¹⁷⁷⁾ . The recruited immune system cells produces cytokines, ROS, lipid mediators such as leukotrienes and toxic mediators which may lead to epithelial cell damage. Injury to the epithelium will further contribute to the increased release of cytokines and chemokines from this tissue, subsequently increasing and/or prolonging the inflammatory reaction, overwhelming the defense mechanisms and possibly progressing to chronic inflammation leading to pulmonary degenerative disease such as fibrosis and scarring ⁽³⁶⁾ . These degenerative changes could lead to the pulmonary remodeling affecting the total lung capacity. Two cytokine we studied are known to initiate, and maintain the inflammatory process. IL-6 is a proinflammatory cytokine that induces acute phase proteins secretion in liver, increases vascular permeability and influences adaptive immunity by enhancing proliferation and antibody secretion from B lymphocytes ^(83,101) . The inflammatory process initiated by IL-6 stimulate macrophages phagocytosis that helps to clear dust from the respiratory tract, and activates machinery to stimulate fibroblast proliferation (healing by secondary intent-fibrosis) into areas where epithelial cells are denuded. IL-8 is a major chemoattractant cytokine to neutrophils, monocytes, eosinophils, and basophils. It amplifies inflammation and causes transmigration of inflammatory cells from blood circulation to the site of injury ^(91, 92) . IL-8 is an important mediator of neutrophilia in airway inflammation ⁽⁹³⁾ and causes degranulation of neutrophils and induces superoxide production ⁽¹⁵⁾ . IL-8 also recruits T cells at the site of injury ⁽⁹⁵⁾ .

The result of this study suggests that beef cattle feedlot dust extract contains several potent agents that trigger strong cytokine responses in the BEAS-2B to release IL-6 and IL-8. We do not understand which components in the dust extract are responsible for strong cytokine response. Moreover, in real life situations, it is the components of the air-borne dust particles which get into the respiratory systems and target different compartments and structures of the respiratory system. So, it is difficult to exactly correlate the cattle feedlot dust particles doses administered *in-vitro* to the amount of such particles coming in contact when animal or human being breathes air in the CAFO environment and depositing the dust in the deep lung. However, our study showed that beef cattle feedlot dust particles can induce proinflammatory cytokine (IL-6) that can initiate, and chemokines IL-8, a cytokine that recruits neutrophils to maintain the inflammatory process in the airway epithelium.

6. Summary

Beef cattle feedlots produce important atmospheric local point source pollution. The dust generated in these facilities are complex mixtures of ultrafine and fine particles, organic compounds, transition metals, acids, bio-aerosols, and adsorbed gases such as ammonia and hydrogen sulfide. Various epidemiological reports have indicated that dusts generated in the CAFO are potential sources of health hazards to CAFO workers, and animals. In addition, they pollute the atmosphere for the people living in the vicinity of such facilities. It is poorly understood which components in the dust extracts are responsible for driving inflammatory response in the lungs.

IL-6 and IL-8 are two inflammatory biomarkers studied in the inflammatory responses. Both cytokines are secreted by various cells of immune systems as well as pulmonary epithelial cells, and fibroblasts. IL-6 is a proinflammatory cytokine that initiates inflammation. IL-8 is a chemokine that recruits neutrophils to maintain inflammatory process in the airway epithelium.

Lung epithelial cells represent an important part of respiratory mucosal innate immunity. These cells are capable of producing varieties of cellular mediators. They express Toll like receptors (TLR) that mediate cellular response to bacteria and bacterial products such as LPS. Epithelial lining fluids (ELF) play important roles in dissolution of the inhaled particles and their subsequent removal from the lungs. Particles with higher solubility are effectively removed from

the lungs without any adverse health effects. However, if the particles are biopersistent, they are likely to induce inflammatory changes in the lung.

We studied the solubility of beef cattle dust particles in lung stimulant fluids (HBSS). We estimated that $\approx 10\%$ of the dusts was soluble, and $\approx 50\%$ settled at the bottom due to particle aggregation and $\approx 40\%$ remained at the top of the media. Dissolved particles in the media resisted centrifugation at 10,000g for 20 minutes suggesting that particles were too small to be sedimented by centrifugation. Those particles in solutions could be translocated to extra-pulmonary organs and cleared from the lungs. The settled particles could be removed by macrophage by phagocytosis and pinocytosis. However, the particles which were neither soluble nor settled are less likely to undergo early clearance.

The dust extract at various concentration induced release of IL-6 and IL-8 from BEAS-2B lung epithelial cell lines. However, TNF-alpha was not detected at all time points and from all dust extracts concentrations. IL-6 release was dose dependent and peak production was seen with 25% dust extract. However, 50% dust extract was cytotoxic to the cell (relative cell viability 10-15%). IL-8 production went down as the concentration of the dust extract increased. One, five and ten percent extract concentration caused the most IL-8 release from the lung epithelial cells. Thus, at concentration below cytotoxicity for lung epithelial cells, production of IL-8 was reduced or inhibited. These findings suggested that higher exposure concentration was required for the initiation of the inflammation as indicated by IL-6. Lower exposure concentrations were related to optimal release of IL-8 needed to maintain the inflammatory response..

Endotoxin is an important constituent of the CAFO dust particles. But our study suggested that at least a portion of particle induced cytokine release from the lung epithelial cells was not endotoxin dependent. We observed that addition of bovine serum albumin in culture media could provide necessary *in-vitro* cellular environments for the endotoxin induced cytokine release. Stimulation of cells with pure endotoxin was dose dependent for both IL-6 and IL-8 release but it was not as effective as the untreated extracts during the 12 hrs of incubation period. This corroborated similar findings of other investigators and demonstrated that beef cattle dust particles toxicity is not solely mediated by endotoxin ⁽¹⁶⁾.

Heat treatment at 120⁰C for 5 minutes didn't significantly remove the toxic potential of dust extracts. However, heat treatment modified some of the toxic properties of the dust extracts. We observed that longer incubation period was required to peak release for both IL-6 and IL-8

(12 hrs instead of 6 hr normal sample). However, the higher concentration of sample (50% extract) reported to be cytotoxic in non-heat treated sample was no longer cytotoxic and induced both IL-6 and IL-8 release from the lung epithelial cells. This result suggested that heat treatment could reduce some of the dust extract's cytotoxic properties. However the extract's potential to induce peak cytokine release didn't change.

To see the importance of this work, we extrapolated the response of lung epithelial cells to manure mat extract. From this response of LEC to these extracts, we predicted the potential health effects of animals and their human caretakers who were exposed to the dust.

First, higher concentration of CAFO feedlot dust appeared to be needed to initiate the inflammatory reactions as indicated by IL-6 release from LEC than to amplify and maintain the inflammation as indicated by IL-8 release from LEC. This trend at least quantitatively correlated to response seen the cattle and people in the feedlot and roughly to our experiences with swine CAFO⁽³⁴⁾. Thus exposure which would augment initiation of dust inflammation might have important public health implications for workers and animals.

Secondly, in both cases, IL-6 to initiate and IL-8 to maintain the inflammatory response, endotoxin in the extract appeared to protectively modulate the inflammatory response. Higher dust concentrations were needed to initiate and lower concentration to maintain peak cytokine release when the endotoxin in the extract was not inhibited by Polymixin-B. Protective roles of endotoxin have been described by other in these situations^(28, 31).

Thirdly, the extracts and thus, the dust contained thermolabile components(s). The 50% extract treated with 120⁰C for 5 minutes remove some of the thermolabile components. The removal increased the ability of LEC to release cytokines, restoring partial LEC function. In this sense, thermolabile compounds were protective. Protection was indicated by not releasing IL-6 and IL-8 and initiating and maintaining the inflammation.

Future work will determine the correspondence of this restored function to LEC activity. This research will determine the extent to which the thermolabile components reducing the release of inflammatory cytokines were balanced by contributions to the cytotoxicity to LEC, and perhaps to the animals, people, and neighbors.

CHAPTER 2 - References

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