## Abstract

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Effects of Ozone and Photochemically Reacted Urban Mixture on Zinc Deficient, Human Lung Cells

Several controlled exposure studies have demonstrated that humans are differentially susceptible to adverse health effects induced by air pollution exposure. Enhanced susceptibility may be caused by intrinsic factors, such as genetic background, age, and gender, or extrinsic factors, such as nutrition. Zinc (Zn) is an essential trace element and is an essential component in several hundred Zn metalloenzymes, many of which play an important role in maintaining cellular redox homeostasis and participate in Zn-finger motifs, which are important for DNA transcription. The first clinical effects of zinc deficiency were observed when studying dwarfism in 1961. Few studies have addressed the effects of zinc deficiency on the adverse effects induced by exposure to oxidant air pollutants including ozone or other photochemically reacted hydrocarbon mixtures. Epithelial cells lining the respiratory tract are one of the major cellular targets of inhaled pollutants such as ozone. The objective of this project is to examine whether Zn deficiency enhances the susceptibility of respiratory epithelial cells to pollutant-induced injury. A cell culture model of Zn-deficient (Zn-DF) and Zn-adequate (Zn-AD) respiratory epithelial cells will be exposed to ozone and photochemically reacted urban mixture in outdoor smog chambers. Inflammatory cytokine production including IL-8, IL-6, MCP-1 and cellular cytotoxicity will be analyzed for in exposed cells. Increased cytokine and cytotoxicity production will be used to determine if zinc deficiency induces greater cellular stress.

*To my parents, son and especially my wife for her endless love, support and dedication.*
*Thank you*

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# 1 Background

## *1.1 Zinc*

Malnutrition and micronutrient deficiencies are highly prevalent in developing countries and it is estimated that these deficiencies are the underlying causes for up to 50% of all deaths among children in these populations (Muller et al., 2003). Zinc plays important roles in growth, development, sexual maturity and immune system regulation. Major sources of zinc are meat, eggs, nuts, and whole grains. The United States recommended daily allowance (RDA) for zinc intake is 12 mg for women and 15 mg for men (Ames, 2001).

### 1.1.1 Characteristics

Over a quarter of all known enzymes require metallic cations to achieve full catalytic activity. Metalloenzymes contain firmly bound metal ions (cofactors) at their active sites. The ions most commonly found in metalloenzymes are transition metals such as iron, zinc, copper, and cobalt. Cofactors are required by inactive apoenzymes to convert them to active holoenzymes (Horton et al., 1996). Zinc is cofactor that is known to participate in the activation of over 300 enzymes. Some of these enzymes participate in DNA synthesis, cell division, and protein synthesis (Bao et al., 2003). Zinc absorption takes place in the small intestine where between 15% and 40% of total zinc intake is absorbed (Salgueiro et al, 2000). Most of the absorbed zinc reaches the liver where it is stored bound to hepatic metallothionein. Metallothioneins are the main proteins which bind labile zinc and they act as free radical scavengers which protects cell membranes (Salgueiro et al, 2000). Metallothioneines can also bind copper for which they have a higher affinity. The remaining absorbed zinc is more labile and rapidly exchangeable which allows it to be easily depleted by lack of continued zinc intake (Truong-Tran, 2000). Zinc absorption is dependent on serum proteins. Almost two-thirds of the zinc circulating in plasma is known to bind to albumin (Cakman et al., 1996). Zinc has been noted for its ability to participate in strong but readily exchangeable ligand binding giving strength and stability to cellular membranes (Hambidge, 2002).

The most important function of zinc is related to its antioxidant role which can prevent free radical propagation reactions. Zinc has other important roles including the activation of the thymic hormone thymulin. Thymulin is needed for T-cell maturation and differentiation (Mocchegiani et al., 2000). Zinc has a significant role in maintaining the human genome. Zinc is found in the zinc finger motif for DNA transcription. A zinc finger is part of protein that can bind to DNA. Zinc finger domains typically consist of two $\beta$ sheets; each carrying a cysteine residue and an alpha helix carrying two histidine residues. Many transcription factors and regulatory proteins that interact with DNA all contain zinc fingers. These proteins posses amino acid sequences that combine with a zinc ion. They typically interact with the major and minor groves along the double helix of DNA that is vital for DNA transcription binding and sequence reading (Moore, 2003). Zinc plays an essential role in reproduction in males and females. It is necessary for the synthesis and secretion of luteinizing and follicle stimulating hormones, gonadal

differentiation, testicular growth, formation and maturation of spermatozoa, testicular steroidogenesis, and fertilization. The biological effects of androgens and estrogens are mediated by zinc fingers which are located in highly conserved regions of their nuclear receptors (Salgueiro et al., 2000).

### 1.1.2 Zinc Deficiency

The first major recognition of the health effects of zinc deficiency was documented in 1961 with the discovery of the correlation between zinc deficiency and dwarfism in mid-Eastern countries (Hambidge 2000). Zinc deficiency is historically seen in both the elderly and young children due to a lack of intake and the ability to absorb dietary zinc (Ames, 2004, Savarion et al, 2001). Zinc deficiency causes are classified into two groups: the first group is related to genetic malfunctions resulting in acrodermatitis enteropathica, Crohn's disease, alcoholism, liver cirrhosis and chronic renal disease; the second is related to lack of nutritional intake. Clinical zinc deficiency is documented as a plasma zinc concentration of less then 10.7 $\mu$mol/L (Pepersack et al., 2001). Zinc deficiency in human cells causes oxidative DNA damage, inactivation of copper/zinc-superoxide dismutase, inactivation of tumor suppressor protein (p53) and inactivation of oxidative DNA repair (Ames, 2004, Reid (2002).

Zinc is extremely important in the development of children. During first months of birth, milk is the only source of zinc for the newborn. Lack of zinc intake can lead to low birth weights and slow development (Salgueiro et al, 2000, Osendarp et al., 2001). Dietary zinc intake has been recognized for its ability to prevent sudden infant death syndrome (Reid, 2002). Zinc deficiency in children can result in diarrhea (a leading killer of children in developing countries) and respiratory tract infections (Coovadia and Bobat, 2002). Zinc is important to bone mineralization and the lack of adequate intake has been discovered to increase the risk of dental caries (Brown et al, (1979). Zinc deficiency delays wound healing as a result of reduced expression of proinflammatory cytokines including interleukin IL-1B and TNF-alpha yielding a decrease in neutrophil infiltration during the early stage of cutaneous wound healing (Lim et al, 2004). The elderly members of the population are at risk of developing more frequent infections due to declining immune responses with advancing age. Previous studies have documented the reduction in the number of T cells and the decreased proliferative response to antigens in the elderly (Chandra, 2003).

The over supplementation of zinc can lead to problems in the absorption of iron and copper due to steric competition. The converse of this can lead to zinc deficiency (Chandra 2003). Although the zinc deficiency is recognized as a significant issue, it is difficult to conclusively measure levels of zinc in the body. The analysis of zinc in plasma, leukocytes, and other tissues requires the use of atomic absorption spectroscopy. Plasma zinc, which is most commonly measured index of zinc status, is insensitive and affected by circadian rhythms, meals, stress and other factors (Pepersack et al., (2001).

### 1.1.2.1 Zinc Deficiency in Airway Epithelial Cells

Zn-deficiency predisposes cells to apoptotic cell death via upregulation of caspase-3 activity (Chai et al., 1999, Chimienti et al., 2001). Apoptosis is an important component of naturally occurring cell turnover in the epithelium and turnover and removal of cells damaged by pollutants or inflammatory cells. Although apoptotic cell death of damaged or "old" epithelial cells is likely to be beneficial, excessive or inappropriate apoptosis may contribute to tissue injury or remodeling that is often associated with chronic inflammation (Intengan and Schiffrin, 2001). Exposure to pollutants, such as ozone causes predominantly necrotic cell death in the affected epithelium (Matsuo et al., 2003, Plopper et al., 1998). Labile zinc has been found to be localized in epithelial cells which are found in the apical and luminal sides of the entire length of the conducting airways. A549 zinc cells have been found to have low labile Zn content as compared to bronchial epithelial NCI-H292 cells. The higher Zn content in the upper conducting airway epithelium reflects the greater need to protect these cells from foreign inhaled particles and noxious agents (Truong-Tran et al., 2000).

## *1.2 Zinc Deficiency Cellular Inflammation*

While severe and moderate Zn-deficiency has significant effects on immune responses and host defense, less is known about the effects of Zn-deficiency on lung physiology and the ability to defend itself against inhaled pollutants. Chronic Zn-deficiency has been determined to cause lung inflammation in rats, as shown by large numbers of infiltrated polymorphonuclear inflammatory cells (PMNs) into the alveolar region (Gomez et al., 2003). Similarly, acute exposures to ozone caused inflammation of the lower airways is marked by infiltration of PMNs as well as upregulated expression of pro-inflammatory cytokines (Aris et al., 1993, Balmes et al., 1997, Diaz et al., 2000, Holgate et al., 2003). Although infiltration of inflammatory cells aids in clearing cells directly damaged by the pollutant exposure (Hyde et al, 1999), reactive oxygen species and other mediators released by infiltrated PMNs can contribute to further tissue injury. Hence, Zn-deficiency could further enhance pollutant-induced inflammatory responses and inflammation-associated tissue injury.

Although, the number of studies of zinc deficiency on the production of inflammatory cytokines is limited, preliminary studies have documented the production of cytokines due to zinc deficiency (Bao et al., (2003). There are numerous markers that pathways in cells use to signal inflammation. Cytokines have been identified for their ability to signal increased amounts of cellular inflammation. All chemicals other than antibodies that are secreted by leukocytes are collectively called cytokines. Cytokines have the ability to identify and attract other leukocytes to the areas of cellular inflammation. These leukocytes proceed to rid the cell of invading substances using phagocytosis and other immune response pathways (Sherwood, 1997). Interleukin 8 (IL-8) has been documented in numerous studies for signaling inflammation when exposed to

ozone and other toxic gases. IL-8, monocyte chemotactic protein one (MCP-1) and interleukin six (IL-6) are cytokines which are produced by many cells including epithelial cells (Hack et al., 1992). On a cellular level, IL-8 functions to draw neutrophils, the primary phagocyte destroyer in the body, to the site of inflammation and to activate them. MCP-1 is a cytokine which is produced by many of the same cellular types as IL-8. The cellular function of MCP-1 is to attract and to activate monocytes to sites of inflammation. MCP-1 has been documented as an important regulator of monocyte and T lymphocyte levels in the body's tissues which are vital to combating numerous diseases which include atherosclerosis, rheumatoid arthritis, and pulmonary fibrosis (Horuk, 1994). IL-6 differs from IL-8 and MCP-1 in that it elicits various effects on different types of leukocytes. IL-6 affects B cells through the stimulation of differentiation and antibody secretion (Hirano et al., 1986). Il-6 has the ability to affect T cells by acting as a co-stimulation to stimulate other cytokines including interleukin two (IL-2). IL-6 elicits hepatoctyes to produce proteins (Bauman et al., 1984) and to proliferate the growth of hematopoietic stem cells (Wong et al., 1988). The many different abilities of IL-6 to influence the entire bodies lymphatic system suggests that IL-6 will be an important regulator to fight off diseases and the invasion of foreign substances.

## *1.3 Air Toxics Exposure*

### 1.3.1 Ozone

Ozone is a deep blue explosive gas that is naturally found in the atmosphere at ambient concentrations as high as 0.20 part per million (ppm) (Koren et al., 1994). The stratospheric region of the atmosphere extends from approximately ten miles above the surface of the earth to a distance of thirty miles above the earth. Solar radiation is emitted in a range of wavelengths and energies that travel towards the earth. Non-ionizing radiation has the ability to penetrate several layers of the earth's atmosphere. The ozone in the stratosphere has a strong affinity for absorption of ultra-violet (UV) radiation that falls in the band of wavelengths that range from 100 to 400 nm. Ozone naturally occurs through photolytic reactions with oxygen gas and is produced and destroyed at a constant rate. High energy UV radiation splits the bimolecular oxygen producing a free radical that further reacts with another oxygen gas molecule to produce ozone ($O_3$). The ozone that is present in the stratosphere acts as a shield for the earth and absorbs harmful UV radiation.

The tropospheric region of the atmosphere extends from the earth's surface to approximately 10 miles above the earth. In this region, the presence of ozone acts as a pollutant and has detrimental consequences to the environment. Vehicle exhaust, industrial emissions, burning of fossil fuels, and gas vapors act as sources of nitrogen oxides ($NO_x$) and volatile organic compounds (VOC), which are ozone precursors. Hydrocarbons are photo-oxidized in the presence of $NO_x$ and VOC to form $O_3$. Ozone in

the troposphere is detrimental to the environment because it is a powerful oxidant and acts as a potent greenhouse gas by trapping reflected solar radiation from the earth's surface. Ozone has the ability to oxidize humans, animals, and plants tissues and damage their ability to survive. A correlation between human exposure to ozone and increased rates of respiratory function performance including narrowing of large airways, increased nonspecific airway reactivity, alveolar inflammation, damage to pulmonary epithelial cells, and increased leakage of vascular components into the lung has been identified (Koren et al., 1994).

### 1.3.2 Urban Mixture

The urban mixture is the composition of over 50 chemical species representative of the ambient air of an average city in the United States. The urban mixture is based from a study beginning in 1984 in which Bill Lonnemann collected hydrocarbon data from 0600 to 0900 AM in 29 cities using canisters. The data from the original study in 1984 was combined with additional data collected by Lonnemann in 1987 to represent the ambient hydrocarbon profile of 41 cities in the United States. The urban mixture is produced by combining four different phases of components including (See Table 5-1 for urban mixture speciation): a gas mixture of 14 components injected from a commercially prepared cylinder, a liquid mixture of 38 components injected from a blended mixture prepared onsite, a liquid injection of acetaldehyde, and a solid injection (by sublimation) of formaldehyde (Jeffries and Sexton, 1995). The components of the urban mixture will photochemically react and ozone will be produced. The presence of photochemically produced ozone coupled with the reacted components of the urban mixture have the potential to illicit more lethal effects on exposed subjects as compared to only ozone. Previous research has indicated that photochemical reactions of hydrocarbons and NOx generate many products, some of which are still unknown. The production of secondary products formed during photochemical reaction of hydrocarbons and NOx has been shown to illicit more inflammation in human epithelial lung cells (Sexton et al., 2004). To better understand how to protect the public from toxic air exposure, the pathway of secondary pollutant exposure and possible defense mechanisms needs to be further explored.

## *1.4 Objective*

The purpose of this study was to examine the inflammatory response of zinc deficient, human lung cells when exposed to ozone and the urban mixture. Indoor irradiation chambers and outdoor smog chambers will be used to produce photochemically reacted urban mixture and ozone for cell exposure. Cellular inflammation and cytotoxicity will be measured to determine if cellular damage has occurred.

# 2 Materials and Methods

## *2.1 Lung cell lines*

A549 cells were used as the exposure medium in these experiments. A549 cells are derived from the epithelial cells that line the respiratory airways which extend from the bronchi into the alveoli. Inhaled pollutants target the epithelial lining of the airway as the inhaled gases travel towards the alveoli (Koren et al., 1994). The origin of the A549 cell line was acquired from an adenocarcinoma of a human subject's lungs. The A549 cells exhibit large surface areas and pulmonary surfactant producing capabilities which are characteristic of alveolar type II cells. The large surface area and improved elasticity, as compared to alveolar type I cells, enables type II cells to more effectively exchange inhaled toxic gases during respiration.

### 2.1.1 Zinc Deficient Cells (Zn-DF)

The A549 cells were passaged and grown on support membranes (Transwell™ inserts) (as described in Jaspers, et al. 1997). The cells were allowed to grow on the membranes until cells covered the entire surface of the membrane, thus achieving confluency. A549 cells are normally grown in F12K with the addition of 10% fetal bovine serum (FBS) and antibiotics (Invitrogen, Carlsbad, CA). The FBS in the media formulation supplies the majority of Zn to the cells during growth. Zn-DF media was produced by incubating FBS with 10% Chelex (Bio-Rad, Hercules, CA) coupled with vigorous shaking for an approximately 18 hour period. This incubation removes Zn by chelation from the FBS. The chelex beads were removed using a 0.22 μm pore size filter. The chelation process also removes calcium from the FBS which must be replaced in order for cell growth. The final formulation for the Zn-DF media is composed of 10% of the chelex-FBS plus 200μM CaCl2 which is added to F12K basal media. Throughout the growth phase, the respective media resides on both the apical and basolateral surfaces of the A549 cells. A549 cells were allowed to grow in their respective mediums for a minimum of three weeks to achieve the shifted intracellular Zn levels. Zinc content in the cells and the respective media were analyzed (Figure 3-1) in both the deficient and adequate cells using inductively coupled plasma-absorption emission spectrometry (ICP-AES) Prior to pollutant exposure, the media residing on the apical layer was aspirated to allow the cells to have direct exchange with pollutant gases. Post-exposure, media was replaced on the apical side of the cells.

### 2.1.2 Zinc Adequate Cells (Zn-AD)

The Zn-AD cells were generated in a similar manner to the Zn-DF cells but they were supplied with chelated media which contained $ZnCl_2$. The formulation of the Zn-AD media included 10% of the chelex-FBS plus 200μM $CaCl_2$ plus 4μM $ZnCl_2$, which is added to F12K basal media. Zn concentrations in the media and were measured using inductively coupled plasma-absorption emission spectrometry (ICP-AES).

## 2.2 *Experimental Design*

### 2.2.1 Indoor Exposure

Three separate exposures occurring on January 26, 2004, February 4, 2004, and May 14, 2004 were conducted at the United States, Environmental Protection Agencies, Human Studies Research Building located on the University of North Carolina campus. The results of the indoor exposure were used to determine if measurable differences between the Zn-AD and Zn-DF cells could be observed in a more controlled laboratory setting before exposing cells in a field setting. Each exposure chamber is pressurized and has a total capacity of approximately 12 $ft^3$.

For the exposures to ozone, A549 cells will be grown on membranous support with media in the apical and basolateral compartment as described before. The transwells containing the cells are contained within a subdivided plate containing twelve wells. The basolateral side of the transwell is suspended in the media which allows the apical side of the membrane to have direct exchange. Separate sets of Zn-AD and Zn-DF cells were exposed to 200 parts per billion (ppb) of ozone and clean air for a five hour period for all exposures. Upon confluency and approximately 1 hour before exposure, the media in the apical compartment will be aspirated. This facilitates direct exposure of the cells to the gas without interference by a layer of media covering the cells, while they are continued to be supplied with nutrients from the basolateral side. Cells were exposed to air or 200 parts per billion (ppb) of ozone for 5 hours using the *in vitro* exposure chambers designed and maintained by the Human Studies Facility of the U.S. EPA. Immediately following exposure, media was added to the apical compartment and approximately eight hours post-exposure all media was collected for analysis.

### 2.2.2 Outdoor Smog Chamber Exposure

The University of North Carolina-Chapel Hill smog chamber was used for the outdoor exposure component of this project. The UNC dual chamber is located in Pittsboro, North Carolina, approximately 25 miles south of the UNC campus. The smog chamber was originally built in 1970 but has been updated several times with the last update occurring in 1994. The chamber has a rigid open A-frame structure with a total capacity of 300,000 liters.

The chamber is divided into two self contained sides which allows for a side-by-side experiment to occur. The frame is covered with 5-mil FEP Teflon film which allows the transmission of sunlight (both ultraviolet and visible regions) to penetrate the inside of the chamber and to react with injected gases. The chamber is maintained at a constant dew point temperature which is determined to be less then the early morning outside temperature. The dew point is maintained via a dehumidification system which prevents the injected gases inside the chamber from condensing on the walls and escaping the

chamber. The total loss of sample from the chamber is typically about 10% dilution of a ten hour experiment (Jeffries and Sexton 1995). The amount of dilution is measured with an injected amount of tracer chemical ($CCl_4$).

Each side of the chamber is designated as a color: red and blue. The schematic shown in Figure 2-1 illustrates the overall setup of the smog chamber and lung cell exposure system. Each chamber is connected to its incubator which contains the sample cells via glass manifolds. Each incubator contains an 8-liter, modular, cell-exposure chamber (Billups-Rothenberg, MIC-101™) that directly encloses the plate with the cell transwells. To maintain ambient and optimal viability conditions: the incubators are maintained at 37°C, a small dish of water is placed in each cell-exposure chamber to maintain humidity, and 1.0 liter per minute of medical-grade air blended with 0.05 liter per minute of $CO_2$ is used to ventilate the chamber. The transwells containing the cells are contained within a subdivided plate containing twelve wells. The basolateral side of the transwell is suspended in the media which allows the apical side of the membrane to have direct exchange. Each subdivided plate is placed within the cell-exposure chamber which resides in the incubator. The cell-exposure chamber has two openings which allows for the sample gases from the manifolds to be ventilated throughout the cell-exposure chamber during exposure. A third cell-exposure chamber was used as the control chamber. This chamber was supplied with medical grade-air and $CO_2$ throughout the entire experiment. The control cell-exposure chamber was used to hold the cells during pre- and post-exposure periods.

Two separate experiments occurring on July 9, 2004 and July 20, 2004 were conducted following the protocol outlined in Figure 2-2. At approximately 12:00 AM on the day of each experiment, the chambers were dehumidified and prepared for the experiment. In order to prevent gas loss from the chamber due to condensation, the dew point in the chamber was lowered below the expected overall nighttime low temperature. All instrumentation is checked and calibrated during chamber preparation to ensure data accuracy. At approximately 12:30 AM 2.0 ppmC of the urban mixture and 0.33 ppm of NOx was injected into blue side of the chamber. The urban mixture was allowed to photochemically react until sundown at approximately 7:00 PM. At approximately 4:00 PM, the cells, prepared using the same method used for the indoor exposure, were transported from the University of North Carolina campus to the chamber site and placed in the clean air control chamber. The amount of ozone produced in the blue side of the chamber was continuously measured throughout the day using an EPA standard reference method based on photometry with a Thermo Environmental Instruments Inc., Model 49 monitor. At approximately 7:30 PM the final amount of ozone in the blue side of the chamber was determined to be approximately 200 ppb. Approximately 200 ppb of ozone was injected into the red side of the chamber. At approximately 8:00 pm the cell plates

were transferred to the exposure chambers. Each cell plate contained both Zn-AD and Zn-DF cells. A total of six sets of cells were exposed to each side of the chamber and the air control over the course of both experiments. A set of cells was composed of one Zn-AD cell transwell and one Zn-DF cell transwell. The transwells were kept in the exposure chambers for approximately 5 hours and then returned to the clean air control chamber. The cells were transported back to the UNC campus approximately 8 hours after exposure and frozen until analysis occurred.

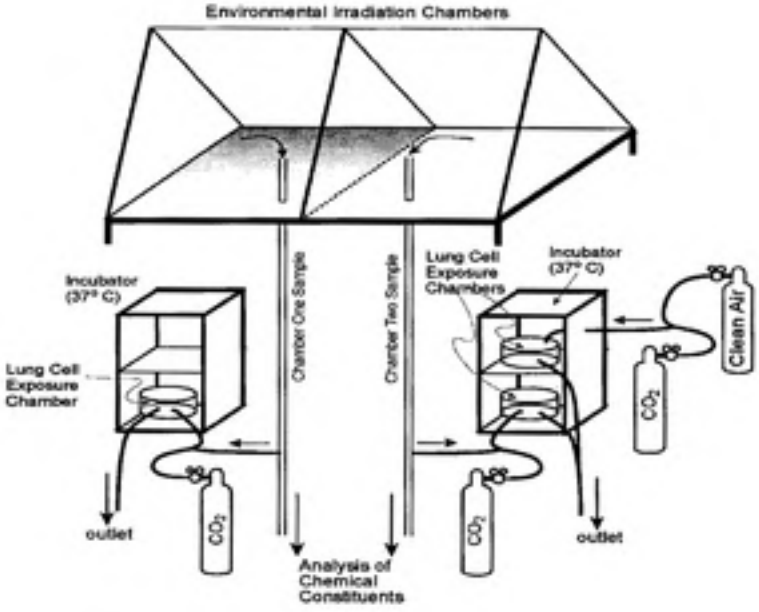


Figure 2-1 Schematic for outdoor smog chamber lung cell exposure setup

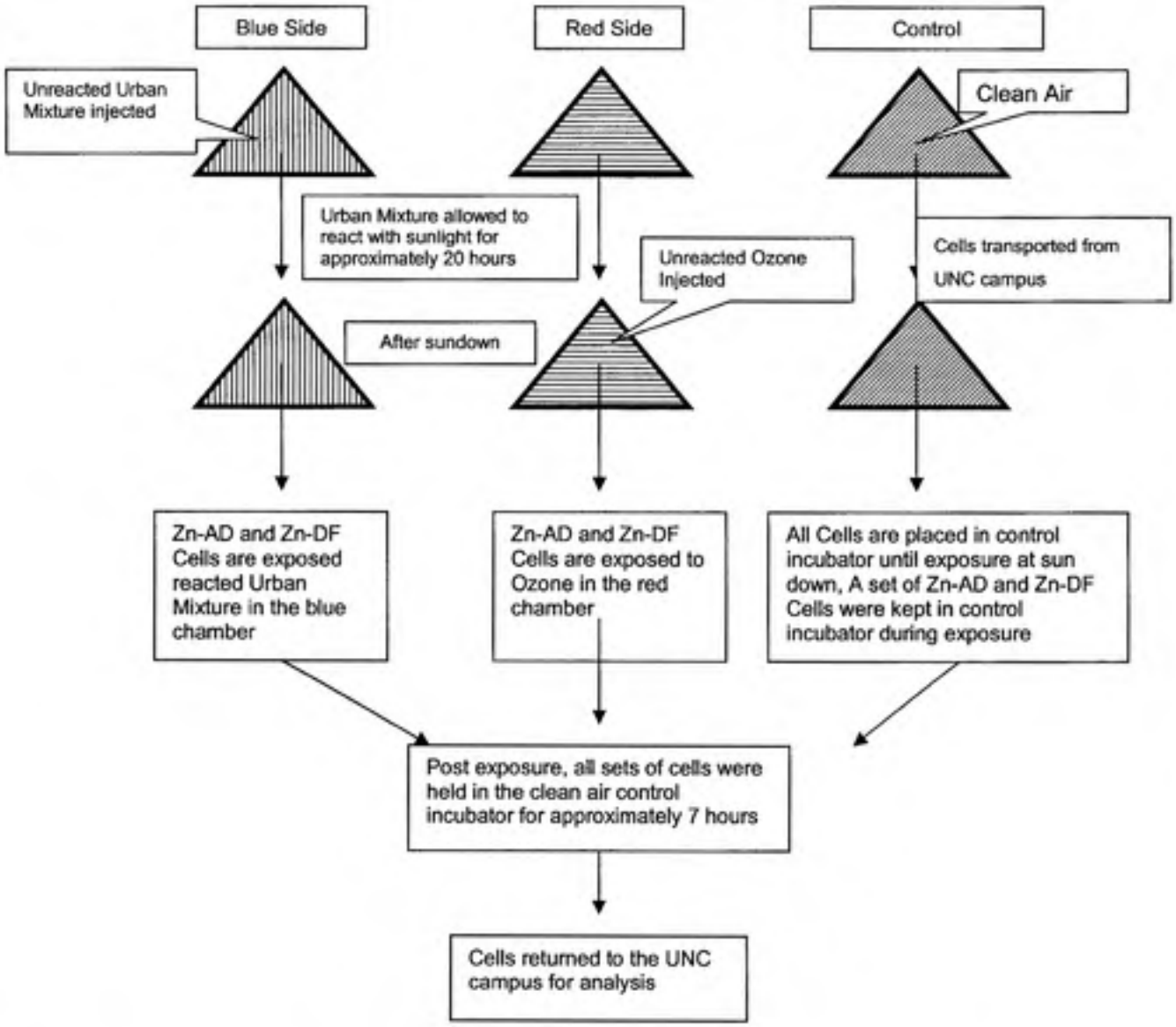


Figure 2- 2 Experimental design for Zn-AD/Zn-DF cell exposure

Shown here is the experimental design used to expose the Zn-AD and Zn-DF cells to the Urban Mixture and Ozone. Here the unreacted urban mixture is added to the blue side of the chamber and allowed to react with sunlight. Post sundown, a matched amount of ozone is injected into the red side of the chamber. Separate sets of Zn-AD and Zn-DF are then exposed to each side of the chamber. The schematic above is a description of the procedure used for the Zn-Ad/Zn-DF experiments.

## 2.3 *Analysis*

All cell functions, which include cell inflammation and cell death, are controlled by many different genes and signaling pathways. Cell functions are executed by various proteins in the cell. Mapping the intracellular protein profile in the cell is an accurate way to determine the stages of cellular inflammation and necrosis.

### 2.3.1 Inflammatory Antibody Array

Previous studies have demonstrated that A549 cells when exposed to similar levels of ozone will produce increased amounts of IL-8 (Jaspers et al. 1997). To determine if the Zn-AD/Zn-DF cells are expressing other inflammatory markers when exposed to ozone, a Human Inflammation Antibody Array (RayBiotech, Atlanta, GA) was performed. The apical supernatants of Zn-AD and Zn-DF cells that were exposed to ozone and air during the third exposure (May 20) were analyzed using the inflammatory antibody array. The antibody array is able to detect the presence of multiple cytokines in the cells as apposed to ELISA tests which allow for the analysis of only one cytokine.

### 2.3.2 ELISA

The inflammatory events that are induced in A549 cells are signaled by the increase of various inflammatory proteins and can be quantitatively measured using an ELISA test. ELISA tests are one of the most common ways to detect levels of protein cellular secretion. ELISA tests (R&D systems, Minneapolis, MN; Endogen, Rockford, IL) were the primary method of analysis to determine the relative levels of inflammation in the Zn-AD/Zn-DF cells. Several cytokines were analyzed for using ELISA which included IL-8, IL-6, and MCP-1. Both the apical and basolateral supernatants of the Zn-AD and Zn-DF cells that were exposed in both the indoor and outdoor chambers were analyzed using ELISA tests.

### 2.3.3 Cell Cytotoxicity

The basolateral layers of Zn-AD and Zn-DF cells that were exposed in both the indoor and outdoor chambers were analyzed for the release of lactate dehydrogenase (LDH) using a coupled enzymatic assay (Promega, Madison, WI) (Doyle, 2003). The amount of LDH production is representative of the degree of cell necrosis.

# 3 Results

## 3.1 Zn-DF Cells

The chelated media and cells grown in deficient media were analyzed after a period of three weeks for cellular zinc content. Figure 3-1 shows the relative amounts of zinc detected in both the deficient and adequate media and cells. The activity of Cu/Zn superoxide dismutase was measured (Figure 3-1) to determine if the activity of zinc dependent enzymes had been disrupted.

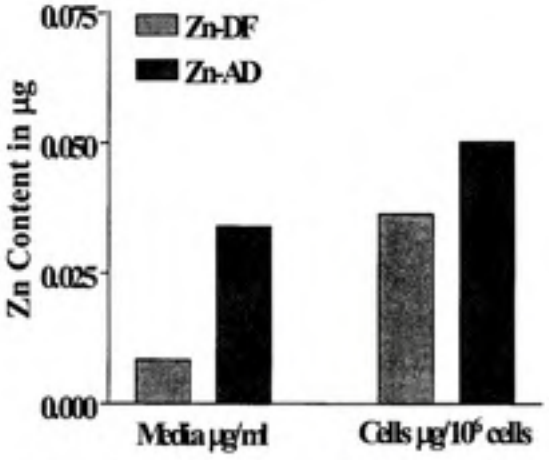


**Figure 1:** A549 cells were grown in Zn-DF and Zn-AD media for about three weeks and the Zn content of cells as well as the media was analyzed by ICP-AES.

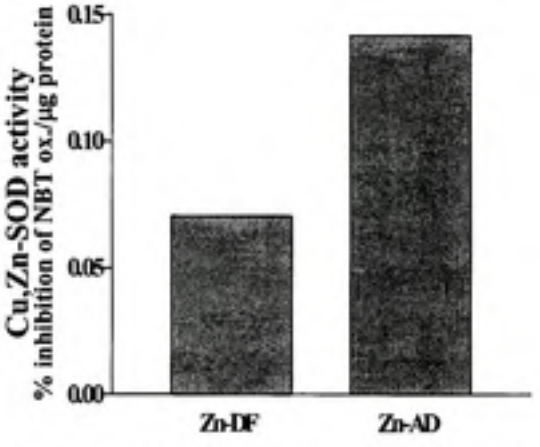


**Figure 2:** A549 cells grown in Zn-DF or Zn-AD media were analyzed for Cu,Zn-SOD activity using NBT oxidation assay.

Figure 3- 1 Relative amounts of Zn content in Zn-DF and Zn-AD media and cells

### 3.2 *Inflammatory Antibody Array*

The antibody array screened the apical serums for the production of 40 different cytokines. The detection level of the antibody array has great sensitivity and has the capability to detect some cytokines at levels as low as 25 pg/mL. The array detected the production of four different cytokines including IL-8, IL-6, MCP-1, and macrophage inflammatory protein (MIP-1$\beta$) (Figure 3-2).

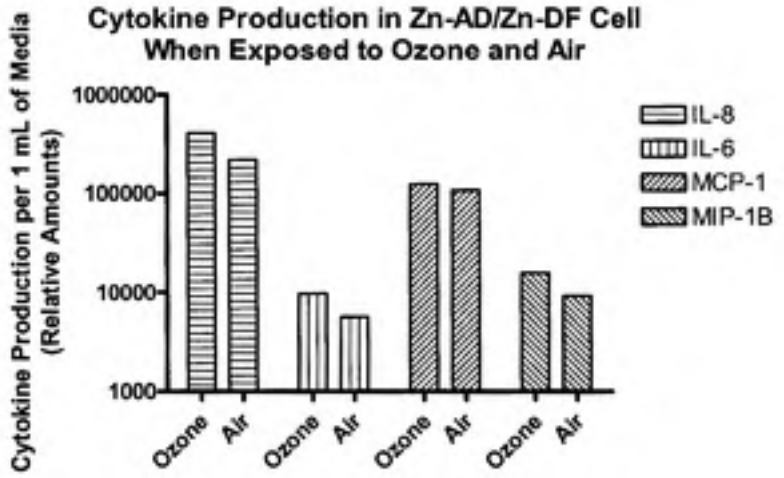


Figure 3- 2 Cytokine Production in Zn-AD/Zn-DF Cells

### 3.3 *Inflammatory Response Quantification*

ELISA tests were used to detect cytokine production levels post-exposure in the supernatants collected in both the cells exposed at the EPA Building and at the outdoor smog chamber. The supernatants were thawed a minimal number of times to minimize contamination and any further cellular stress.

### 3.3.1 Indoor Exposure

Both the apical and basolateral supernatant layers collected post the first three indoor exposures were analyzed for three different cytokines including IL-8, MCP-1, and IL-6 using ELISA tests. The results of these tests are displayed in Figure 3-3. The amount of cytokine detected is expressed per total volume of supernatant collected.

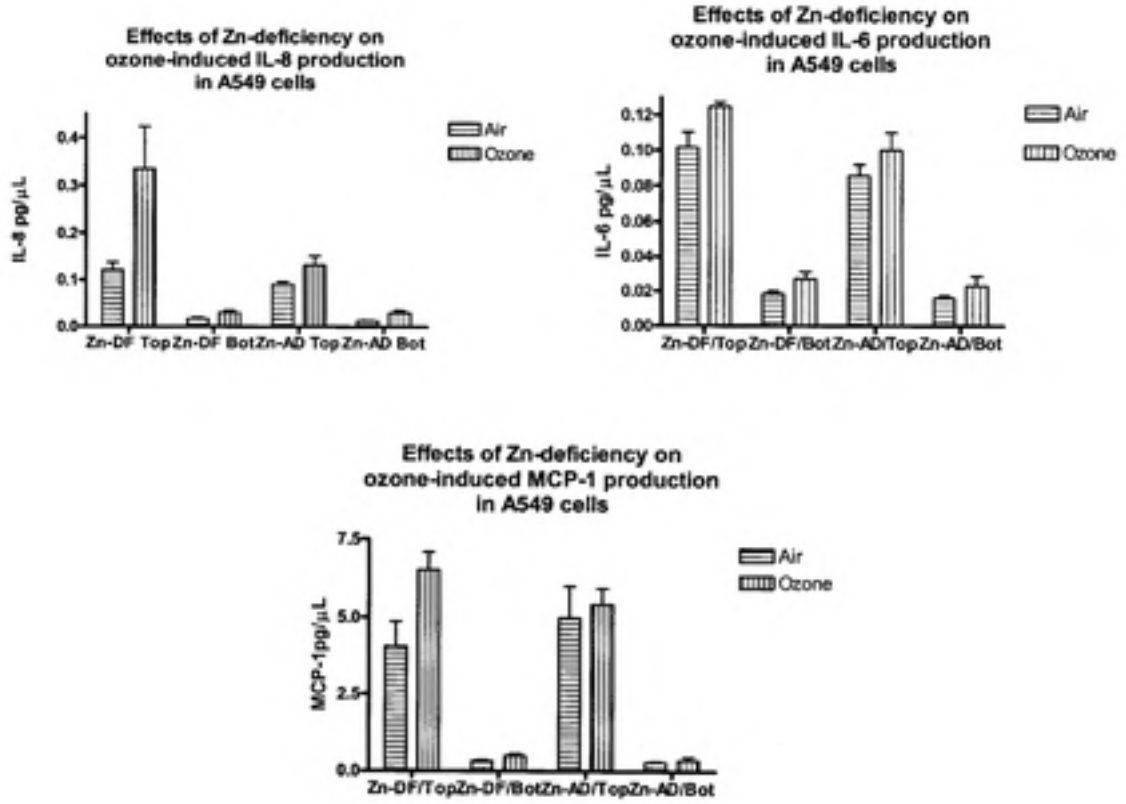


Figure 3- 3 Cytokine Production/Indoor Exposure

### 3.3.2 Outdoor Smog Chamber Exposure

Both the apical and basolateral supernatant layers collected after each of the outdoor experiments were analyzed for the same three cytokines as the indoor exposure analysis using ELISA tests. The results of these tests are displayed in Figure 3-4. The amount of cytokine detected is expressed per total volume of supernatant collected.

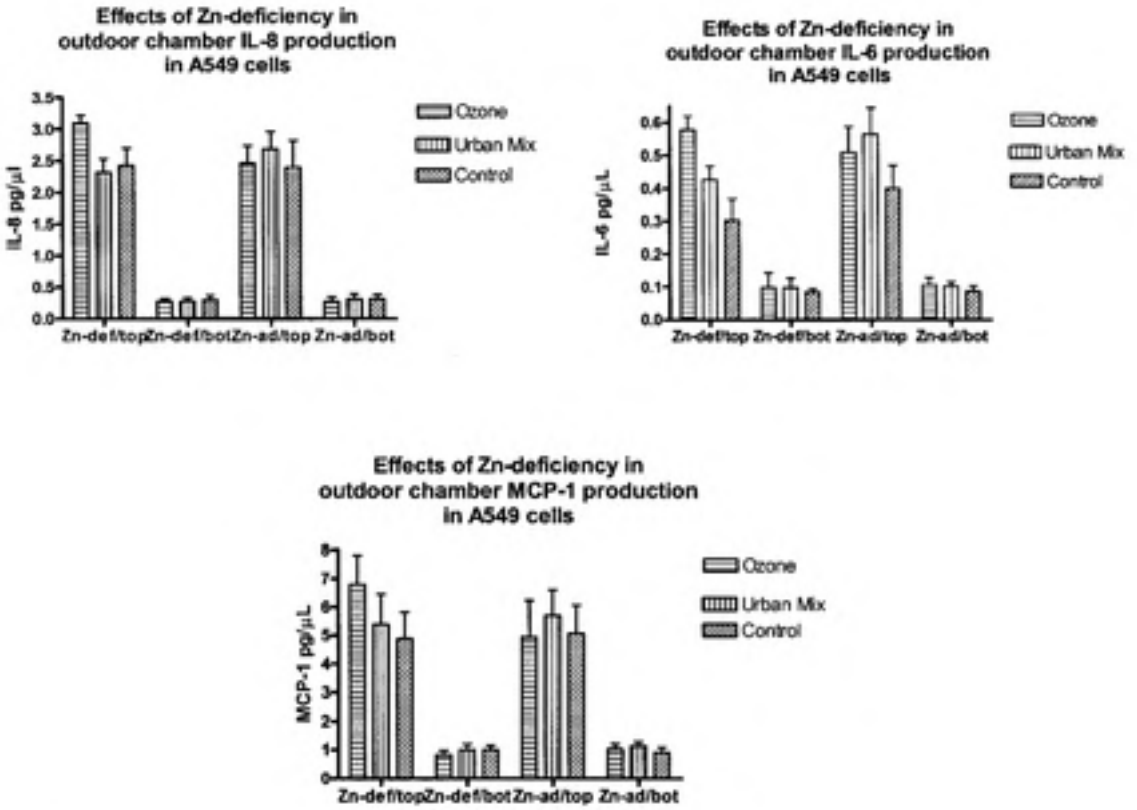


Figure 3- 4 Cytokine Production/Outdoor Chamber

### *3.4 Cell Cytotoxicity Quantification*

## 3.4.1 Indoor Exposure

An enzymatic assay was used to detect the amount of LDH produced in the basolateral layer of the collected supernatants from the cells exposed in the first and second indoor exposure. The amount of LDH produced is representative of cell necrosis. As cells die due to air toxic exposure, they will migrate based on gravity into the basolateral layer and release LDH. The amount of LDH absorption detected is represented in Figure 3-5.

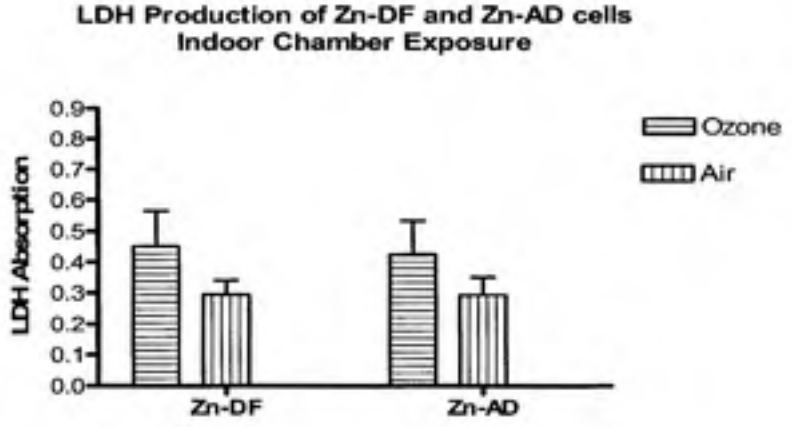


Figure 3- 5 LDH Production/Indoor Chamber Exposure

## 3.4.2 Outdoor Smog Chamber Exposure

An enzymatic assay was used to detect the amount of LDH produced in the basolateral layer of the collected supernatants from the cells exposed in each of the two outdoor smog chamber exposures. The amount of LDH absorption detected is represented in Figure 3-6.

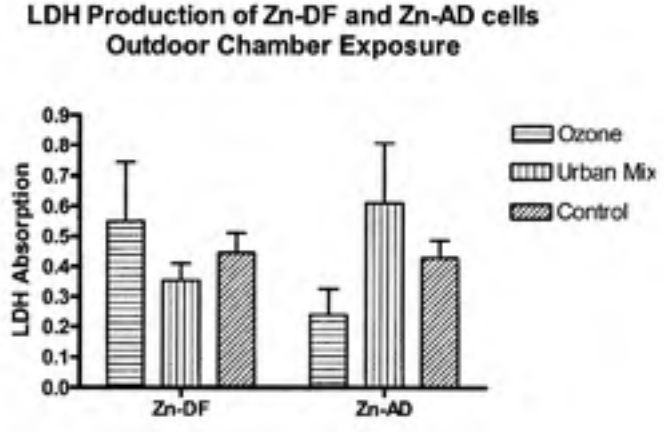


Figure 3- 6 LDH Production/Outdoor Chamber Exposure

# 4 Discussion and Conclusions

Individuals with inadequate zinc intake and/or absorption are more susceptible to infectious agents and have altered immune responses (Dardenne, 2002). In the lung, chronic zinc deficiency enhances markers of inflammation and alters markers of oxidative stress (Gomez et al., 2003).

Analysis of the Zn-DF media used for cell culture determined that the media contains about 75% less zinc than the Zn-AD medium and that cells grown in Zn-DF medium for three weeks have an intracellular zinc content that is about 30% less than their Zn-AD controls (Figure 3-1). The activity of Zn-dependent antioxidant enzymes (Figure 3-1) was analyzed to determine if the depletion of zinc in the cells affected the dependence of these enzymes. It was determined that growing A549 cells in Zn-DF media changes the activity of Zn-dependent antioxidant enzymes, such as Cu,Zn-SOD. Figure 3-1 shows that the Cu,Zn-SOD activity in Zn-DF cells was about 50% less than in the Zn-AD controls. Thus, growing respiratory epithelial cells in Zn-DF media decreases their intracellular Zn-content and suppresses the activity of Zn-dependent antioxidant enzymes.

Zn-DF and Zn-AD cells were exposed to 200 ppb of ozone and pure air for approximately five hours in the indoor exposure chambers at the EPA Building. The cytokine production profile was determined using an inflammatory antibody array (Figure 3-2). Figure 3-2 shows that IL-8, IL-6, MCP-1 and MIP-1$\beta$ were detected in the supernatants of both Zn-DF and Zn-AD cells when exposed either ozone or pure air. Statistical analysis was not conducted on the results of the antibody array procedure. This test has several limitation which makes results difficult to quantify. These results were used to determine that a trend was observed that suggests that increased cytokine production for all four detected cytokines was increased when the cells were exposed to ozone as compared to pure air. ELISA tests were employed to quantify the amount of cytokine production of IL-8, IL-6, and MCP-1. MIP-1B was eliminated from analysis to reduce project costs. Figure 3-3 shows that exposing A549 cells to 200 ppb enhances the production of IL-8, IL-6, and MCP-1 in both the apical and basolateral compartment. More interestingly, Zn-DF cells released more IL-8 towards the apical side than their Zn-AD controls possibly due to the direct exchange of gases with the apical side of the cells during exposure. The increased cytokine production in Zn-DF cells was determined to be statistically significant from the level of cytokine production in Zn-AD cells for IL-8 and Il-6. A trend appeared in all three cytokine production profiles that suggests that increased inflammation occurs in Zn-DF cells as opposed to Zn-AD cells when exposed to ozone. All cells that were exposed to air appear to have similar cytokine production without significant variances between Zn-DF and Zn-AD cells. A one way ANOVA

statistical test was used to determine statistical significance and compared to a P-value of 0.05. LDH amounts were detected in basolateral supernatants of cells which were exposed to ozone and air. The relative cytoxocity production (Figure 3-5) followed the same trend observed in the cytokine production profile. The Zn-DF cells when exposed to ozone produced increased amounts of LDH relative to Zn-AD cells. These data suggest that Zn-deficiency enhances the susceptibility of respiratory epithelial cells to ozone-induced inflammation.

## *4.1 Comparison of Results*

### 4.1.1 Ozone vs. Urban Mixture

Zn-DF and Zn-AD cells were exposed to the photochemically reacted urban mixture and 200 ppb of ozone for approximately five hours in the outdoor smog chamber. The urban mixture was allowed to photochemically react throughout the daylight hours. Post sundown, the amount of ozone produced throughout the day in the chamber containing the urban mixture was determined to be approximately 200 ppb. This same amount of pure ozone was injected into the other side of the chamber. Post exposure, the cells were examined for cytokine and LDH production. The cells which were exposed to only pure ozone exhibited the same trends observed in indoor exposures. Increased relative amounts of IL-8, IL-6, and MCP-1 (Figure 3-4) were detected in the supernatants of Zn-DF cells. Increased relative amounts of LDH (Figure 3-6) were detected in the supernatants of Zn-DF cells.

Although the cells (both Zn-AD and Zn-DF) were exposed to the same amount of ozone in each side of the chamber and the same amount of ozone used in the indoor exposures, a variance in the cytokine production profile and cytotoxicity production was observed. Increased relative amounts of Il-8, IL-6, and MCP-1 (Figure 3-4) were detected in the supernatants of the Zn-AD cells. Increased relative amounts of LDH (Figure 3-6) were detected in the supernatants of the Zn-AD cells. The increased cellular stress detected in the Zn-AD cells when exposed to the photochemically reacted urban mix suggests that the photochemically produced ozone when combined with other species found in the urban mix elicits a different cytokine production profile. It is possible that the other species in the photochemically reacted urban mixture are masking the inflammatory effects in Zn-DF cells.

## *4.2 Future Studies*

The recent explosion of alternative medicines, specifically the area of nutritional supplements demands for continued research. Currently the United States Food and Drug Administration does not place regulations on the manufacturing and outcomes of many of

these supplements. It is imperative that the area of nutritional supplements have continued research and monitoring to determine the effectiveness of these techniques. Zinc repletion studies will be conducted to determine if the addition of zinc to depleted cells will have positive outcomes. Zinc chloride ($ZnCl_2$) will be added in incremental amounts to the Zn-DF medium and added 24-72 hours prior to exposure to the pollutants. Analysis of Zn content in Zn-DF and Zn-AD media has shown that the levels of Zn are 8.3ng/ml (approximately 127nM) and 33ng/ml (approximately 500nM), respectively. Acute Zn-repletion will be performed by adding 1, 5, or 10μM $ZnCl_2$ to the Zn-DF medium. To assure Zn-repletion, Zn-repleted (Zn-RE) media and Zn-RE cells will be analyzed for Zn content. Zn-DF, Zn-AD, and Zn-RE cells will be exposed and analyzed for inflammatory cytokine production. Specifically, we will limit our analysis of Zn-RE cells to endpoints that are significantly changed in Zn-DF cells as compared to their Zn-AD control. The changes in responsiveness in Zn-RE cells as compared to Zn-DF cells will be correlated to their intracellular Zn content.

### *4.3 Conclusions*

There are significant health effects associated with human zinc deficiency. Very little is known about the damaging effects of air pollution on compromised lung cells. Zn-DF and Zn-AD epithelial lung cells were exposed to ozone and a photochemically reacted urban mixture. The inflammatory cytokine profile and relative amount of cytotoxicity was determined and compared for Zn-DF and Zn-AD cells. The increased inflammatory and cytotoxic response between the apical and basolateral supernatants suggest that the direct exposure of pollutants to the apical layer of the cells induces more cellular stress. The results of this project demonstrated that increased amounts of inflammatory cytokines and cytotoxicity result when Zn-DF cells are exposed to ozone as compared to Zn-AD cells. There appears to be unexpected correlation found that the cells, when exposed to the photochemically reacted urban mixture, induce a variance in the cytokine profile and cytotoxicity production as compared to cells exposed to pure ozone. Increased production of cytokines and LDH were detected in Zn-AD cells instead of the expected Zn-DF cells. This variance suggests that the photochemically reacted urban mixture has the ability to induce different cellular responses as compared to pure ozone. Continued research needs to focus on the health effects of photochemically reacted compounds which are more representative of the pollution exposed to the general population. Zinc deficiency has the potential to cause adverse health reactions and should be carefully monitored in the young and elderly members of the population to insure protection from the harmful pollutants in the environment.

# 5 Appendix

## 5.1 Urban Mixture Speciation

**TABLE 5-1**
Composition of synthetic urban mixture, ppbC in 1 ppmC total

| Component | ppmC | Component | ppmC | Component | ppmC |
|---|---|---|---|---|---|
| Isopentane | 86.4 | Ethane | 26.7 | Toluene | 69.0 |
| *n*-Butane | 73.5 | 2,3,3-Trimethyl-1-butene | 16.0 | 1,2,4-Trimethyberzene | 56.0 |
| Propane | 46.1 | *c*-2-Pentene | 13.5 | *m*-Xylene | 37.0 |
| Ethane | 38.5 | *t*-2-Butene | 11.6 | Benzene | 22.0 |
| 3-Methylhexane | 35.0 | Propene | 8.4 | 1,3-Diethylbenzene | 18.0 |
| Isobutene | 32.9 | 1-Octene | 8.0 | *o*-Xylene | 16.0 |
| *n*-Pentane | 31.1 | 2-Methyl-1-pentene | 8.0 | *p*-Ethyltoluene | 15.0 |
| 4-Methylnonane | 23.0 | 1-Pentene | 8.0 | Ethylbenzene | 11.0 |
| 2-Methylpentane | 22.0 | 1-Nonene | 7.0 | *n*-Propylbenzene | 11.0 |
| *n*-Decane | 20.0 | 2-Methylpropene | 4.5 | *m*-Ethyltoluene | 10.0 |
| 2,3-Dimethylpertane | 17.0 | 2-Methyl-1-butene | 4.2 | *sec*-Butylbenzene | 7.0 |
| *n*-Nonane | 16.0 | 2-Methyl-2-pentene | 3.0 | 1,2,3,5-Tetramethylbenzene | 6.0 |
| *n*-Heptane | 16.0 | Cyclohexene | 1.0 | | |
| 3-Methylpentane | 16.0 | | | | |
| *n*-Hexane | 14.0 | | | a-Methylstyrene | 3.0 |
| 2,2,4-Trimethylpentane | 13.0 | 1,3-Butadiene | 2.1 | | |
| *n*-Octane | 12.0 | Isoprene | 3.0 | Formaldehyde | 10.4 |
| 2,5-Dimethylhexane | 11.0 | a-Pinene | 6.0 | Acetaldehyde | 11.2 |
| 2,3,4-Trimethylpentane | 10.0 | | | | |
| 2,3-Dimethylbutane | 6.0 | | | | |
| Methylcyclopentane | 11.0 | | | | |
| Cyclohexane | 10.0 | | | | |
| Methylcyclohexane | 6.0 | | | | |
| | 566.4 | | 131.0 | | 302.6 |

*Note.* ppbC is parts per billion carbon; ppm C is parts per million carbon.

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