In Vitro Toxicological Evaluation of Airborne Copper-based Nanoparticles

by

Eileen Burns

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
December 2016

DEDICATION PAGE

I would like to dedicate this thesis first and foremost to my parents, Peter and Elizabeth Burns. Without their continued support (emotionally, physically, and financially) and encouragement I would not have been able to finish this master's thesis, nor would I be the person that I am today. Words cannot express the gratitude that I feel towards my parents for all that they have given me, and all that they have done and sacrificed in order for me to have been given the opportunities that I have been given. They have always taught me to think critically, speak up and be heard, dream big, and to work hard for what I am passionate about.

I can still remember being a young elementary school student working on mathematics word problems known as "Problem of the Week," and my dad telling me that I had to think critically about the problem – the answer to this problem wasn't going to be found using an equation in a textbook. Now, as a young elementary student you are not (or at least were not) often told to "think critically". In fact, I'm quite certain that if you asked elementary school children about the term, they wouldn't have the foggiest idea what you were talking about. I am beyond grateful that my dad introduced me to this invaluable life skill early on. It is without a doubt the most important skill that one needs in the research world, and I would go so far as to say in life in general. It is also a skill that needs to be used and worked on continually, and the earlier you start the better. Thank you for equipping me to be my own person, dad.

My parents have also helped me through the toughest of emotional times, and I cannot thank them enough for simply listening to me, and for striking that much needed balance between consoling me and telling me to pick myself up off the ground and getting back at it. There are times that I wanted to quit – but what graduate student doesn't experience that feeling once..or twice…or maybe a few more times than that. Thank you for always holding me up when I was broken and/or crying (both literally and figuratively), mom. Thank you for always being there mom and dad. My success is because of everything you've both done, and continue to do for me.

I would also like to mention that my whole family - Edgar, Francis, Michael, Bucky, Grandma, Grandpa, Ken, Frank, and Myrene have been sources of encouragement and support over the years. I cannot thank you all enough for what you've each done to support and encourage me. Thank you all for the kind words and I look forward to more celebrations of our family's success to come.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

A knowledge gap exists concerning the role of metal and metal oxide composition in explaining adverse pulmonary and cardiovascular health effect associations found with inhaled nanoparticles (NPs). This research investigated cardiopulmonary cellular responses resulting from exposure to airborne copper (Cu) NPs. A spark discharge generator and an *in vitro* model that mimics human lung conditions were employed to generate and deliver airborne NPs to human alveolar type-II cells (A549 cells). A significant decrease in cell viability was observed in alveolar epithelial cells exposed to Cu NPs, however no significant difference was observed in cardiovascular cells cultured in pulmonary exposure medium obtained after NP exposure to lung cells. Levels of intracellular reactive oxygen species were increased in alveolar epithelial cells exposed to NPs (as compared to NP-free air) for 4 hours exposure. This research aids in the understanding of potential toxic effects of metal-based NPs on human lung cells, and their potential to lead to adverse cardiovascular responses.

LIST OF ABBREVIATIONS USED

4-2-4 exposure	Sequential exposure (4 h exposure – 2 h rest – 4 h exposure)
4 h exposure	Single exposure
A549	Human alveolar epithelial lung cell line
AB	Alamar Blue dye
Ag	Silver
Al	Aluminum
ALI	Air-Liquid Interface
Al_2O_3	Aluminum oxide
ARDS	Adult Respiratory Distress Syndrome
Au	Gold
CASRN	Chemical Abstract Services Registry number
CD54	Cluster of Differentiation 54
CeO_2	Cerium (IV) Oxide
CO_2	Carbon Dioxide
COPD	Chronic Obstructive Pulmonary Disease
CPC	Condensation Particle Counter
Cu	Copper
DCF	Dichlorofluorescein
DMEM-HG	Dulbecco's Modified Eagle Medium – High Glucose
DMNB	Dalhousie Medicine New Brunswick
DMSO	Dimethylsulfoxide
DSL	Domestic Substances List
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
Fe	Iron
Fe_2O_3, Fe_3O_4	Iron (III) Oxide, Iron (II, III) Oxide
GSD	Geometric Standard Deviation
H9C2	Rat cardiomyoblast cell line
HBSS	Hank's Balanced Salt Solution
HEPA	High-efficiency particulate air
HCM	Human Cardiomyocytes
HUVEC	Human Umbilical Vein Endothelial Cells
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
ICAM-1	Intercellular Adhesion Molecule-1
IL	Interleukin

LDH	Lactate Dehydrogenase
MCP	Monocyte Chemotactic Protein
Mg	Magnesium
Mn	Manganese
MnO	Manganese (II) Oxide
MSDS	Material Safety Data Sheet
NAC	N-acetylcysteine
NP	Nanoparticle
NIVES	Nanoparticle *In Vitro* Exposure System
NMD	Number Median Diameter
Ni	Nickel
NiO	Nickel (II) Oxide
NO_x	Nitric Oxide and Nitrogen Dioxide
NO_y	Total Reactive Nitrogen
NO	Nitric Oxide
N_2	Nitrogen
O_2	Oxygen
O_3	Ozone
PBS	Phosphate-Buffered Saline
PET	Polyethylene Terephthalate
PI	Propidium Iodide
PM	Particulate Matter
ppb	Parts Per Billion
PS	Phosphatidylserine
ROS	Reactive Oxygen Species
SiO_2	Silicon Dioxide
SDS	Spark Discharge System
SMPS	Scanning Mobility Particle Sizer
SD	Standard Deviation
TiO_2	Titanium Dioxide
TNC	Total Number Concentration
Zn	Zinc
ZnO	Zinc Oxide
ZrO_2	Zirconium Oxide

ACKNOWLEDGMENTS

I would like to acknowledge Dr. Jason LeBlanc for providing the A549 cells used for this project, and Wanda for subculturing the cells (thank you for your continued support and willingness to answer all my A549 questions!). Thank you to the Infectious Diseases Lab students who helped me immensely as I learned the ropes of cell culture; they endured hundreds of questions and I would have been lost without their help. Specifically, Peter Robertson, Aaron Liu, and Carla Valenzuela. To all my HERC Lab-mates, thank you for all the support and laughs throughout this journey. I would like to extend a huge thank you to Tina Bowdridge for her support. You are such a valuable resource to all of us in Community Health and Epidemiology (CH&E), and have answered countless questions and searched for many answers (sorry about all the emails!). Thank you to Dr. Kathleen MacPherson (CH&E Graduate Program Director) for her guidance and support, and to funding sources (Canadian Institutes of Health Research, Nova Scotia Health Research Foundation, Lung Association of Nova Scotia and the CH&E Department) for making this project possible. I would like to thank my committee (Dr. Judy R. Guernsey and Dr. Anil Adisesh) for their support, guidance, and expert input regarding my thesis proposal and final thesis.

I must put a very special acknowledgement and thank you here to someone who has been my biggest cheerleader and support system in the lab. Crystal Sweeney, you are nothing short of a guardian angel and A LITERAL ROCKSTAR. Honestly, I was going to write this paragraph in the dedication section right after thanking my parents and family, but I (unsurprisingly) didn't want that section to exceed one page in length. I will never have the words to express to you how grateful I am for all of the skills, laughs, and boosts that you have given me throughout this journey. I say journey because it truly was – I am not the same person at the end of this degree that I was when I started. You helped me to realize my potential and to have confidence in what I do and say. You were my ally, my friend, my supporter, my reality check, and my favorite part of most days. I also must tell you that you are stuck with me for life – I may be technically leaving the lab, but I am not leaving this friendship. I'm serious about those lunches together…. I'm only a building away ☺. The most fitting quote I can think of right now is, "I'm feeling the fear, and doing it anyway". Crystal, from the bottom of my very soul, THANK YOU for absolutely everything. You truly turned my path around in this graduate marathon, and you were brought into my life at just the right time. You reminded me that no matter what people may say to me or about me, or do to me, that I am my own person, a good person, and that I will be ultimately successful. Having you see those things allowed me to also see them. Thank you for picking me up off the ground more times than I can count and reminding me that I am not alone.

Last, but certainly not least, I would like to thank my supervisor, Dr. Jong Sung Kim. I am pleased to say that I am your first graduate student here at Dalhousie, and also not your last! I am very grateful for all of the time and energy that you have invested in my project, and all of the support, guidance, and motivation that you have given me. I know at times both of us might not have thought this day would come, but here it is! I want to truly thank you for never giving up on me and believing in me throughout.

CHAPTER 1 INTRODUCTION

1.1 Characteristics and Applications of Metal-Based Nanoparticles

There are several different definitions of Nanoparticles (NPs) in use, all with two common components: a minimum of one dimension of 100 nm or less must exist, and nano-sized materials have physicochemical properties that are unique and not observed in their bulk forms (1-3). There are two main sources of NPs; natural and anthropogenic. Examples of natural NPs include those released due to forest fires, volcanoes and gas to particle conversions (3). Anthropogenic NP release or formation can either be unintentional or intentional. Examples of unintentional anthropogenic NPs include those produced as a result of internal combustion engines, airplane jets, metal fumes (i.e. welding), or power plants (3). In terms of intentionally produced or formed anthropogenic NPs, engineered NPs are designed to serve a particular function and their properties (i.e. size and shape) are controlled (3). These engineered NPs have many applications but may also have unintended health effects.

There are a growing number of applications in which NPs can be utilized due in part to their unique physicochemical properties (e.g., size, surface area, reactivity) (4). Some of these applications include biological imaging, drug delivery systems, and industrial applications such as catalysts (4, 5). Cell labeling (6), targeted drug delivery (7), medical imaging (8), cancer therapy (9-12), and biological sensors (13) are some of the applications that have used gold (Au) NPs. Another medical application, drug delivery

systems, has had aluminum (Al) NPs proposed: a hybrid Al-magnesium (Mg) NP construct encapsulates non-ionic or non-water soluble drugs in order to increase solubility, avoid clearance mechanisms, and allow site-specific drug to cell targeting (14).

In addition to the increasing number of biological and medical applications being discovered and investigated, there are a surprising number of household products used daily that actually contain NPs. For example, silver (Ag) NPs are added to toothpaste, shampoo, fabrics, deodorants, filters, paints, water purification systems, bedding, washers, kitchen utensils, toys, and humidifiers in order to take advantage of the antimicrobial properties that they impart (4). Ag NPs have even been incorporated into food packaging as a filler due to its unique antimicrobial properties (15). Ag NPs are not the only NPs used for their antimicrobial properties. Cu has been used for its antibacterial properties for centuries (16, 17), with an increase in antimicrobial activity when moving from Cu salts to Cu NPs being attributed, at least in part, due to the large surface to volume ratio of Cu NPs (16). An abundance of research has been conducted regarding the use of metal-based NPs for antimicrobial applications, where antimicrobial encompasses antiviral, antibacterial, antifouling, and anti-fungal activity (4). These applications include biocides and antibiotic treatment alternatives, as well as their addition to the household products mentioned earlier (4).

Some examples of the compositions of metal-based NPs are: Al, Al oxide (Al_2O_3), Au, Ag, copper (Cu), iron (Fe), Fe oxide (Fe_2O_3, Fe_3O_4), manganese (Mn), Mn oxide (MnO), silicon dioxide (SiO_2), titanium dioxide (TiO_2), zinc (Zn), Zn oxide (ZnO), cerium (IV)

oxide (CeO_2), nickel oxide (NiO), and zirconium oxide (ZrO_2) (4). Applications for these NPs vary greatly across the materials, as well as for each individual material. This is exemplified above by many different applications that are noted for metal-based NPs utilized for their antimicrobial properties (4).

Some further examples of applications of these metal-based NPs range from wear-resistant coating additive, magnetic imaging, gene delivery, printing inks, sterilization, and environmental remediation (4). Other common products that use the novel physicochemical properties of metal-based NPs include sunscreens, cosmetics, clothing, building products, and electronics (18). Titanium dioxide (TiO_2) and zinc oxide (ZnO) NPs have both been used to protect our skin from damage by the sun (sunscreens), as well as to kill bacteria on self-cleaning surfaces (4).

Copper and Zn NPs have demonstrated greater toxicity during *in vitro* testing than other NPs and are thought to be more toxic than other metal compositions (4, 19, 20). Zinc NPs appear to have their main applications as skin protectants in sunscreen, but they have also been used as additives in consumer or industrial products, self cleaning surfaces, dyes, paints, medical diagnostics, catalysts in tire rubber vulcanization, and cosmetics (4, 20, 21). Copper NPs are used for many applications, such as: antimicrobial, antibiotic treatment alternatives, lubricants, inks, and as filler materials that enhance conductivity and wear resistance of materials (4). Copper oxide NPs have even been used in copper intrauterine contraceptive devices (5).

1.2 Exposure Pathways of Metal-Based Nanoparticles (Specific Focus on Inhalation Exposure)

There are various pathways through which metal-based NPs may enter the environment due to their wide range of applications. While the main exposure route is *via* inhalation, there are some deliberate additions to the environment *via* soil and water remediation technologies or fertilizers that use metal-based NPs, and there are some accidental additions such as accidental spills that may occur during industrial production or transportation (22). NPs such as those based on TiO_2 and Ag may be released into both domestic and industrial wastewaters (22) and partition into sewage sludge (22) which may then be introduced to soils via agricultural land application (23-26), with up to 99% of TiO_2 NPs entering treatment plants being retained within the sewage sludge (25).

Inhalation exposure is the main route of human exposure to NPs due to their extremely small size and ability to become easily suspended in air (27, 28). Specific focus on the inhalation exposure of metal-based NPs reveals that there are several ways in which aerosolized NPs can reach the population. Three phases of the lifecycle in which NPs, such as Ag, can be aerosolized are: production, use, and disposal including waste management practices such as recycling (28). With the increase in popularity of products that contain Ag NPs (29), it is important that more research is done to fully understand the emissions of aerosolized Ag NPs across all three phases of the Ag NP lifecycle (28). Ag NPs should not be the only NPs researched, as NPs of different size, shape, surface, and composition may behave differently when airborne or when present in wastewaters.

During the production of carbonaceous NPs, aerosolized NPs have been detected and this finding may be able to be extended to metal-based NPs depending on the production techniques used (28). There are several different techniques of producing NPs, some of which occur in aqueous media (30). It is still possible for NPs that are formed in this way to be aerosolized if they are dried into a powder following their synthesis in aqueous media (28). Accidental emissions of NPs to the atmosphere may occur, through waste from the production process, direct emission of streams of the aerosolized NPs, or incineration of solid waste resulting from their production (28).

In terms of the use of NP products, it is believed that the most important products to consider are those that are intentionally releasing the NPs, such as liquid cleaning products or personal care products that are sprayed (28). There are some difficulties that arise when trying to determine precisely how the NPs are released or emitted from consumer products due to commercial sensitivities regarding how the NPs are incorporated into the products (28).

Incineration and treatment of liquid waste are said to be the two major ways in which Ag NPs are aerosolized during the disposal phase (28). When incinerated, airborne NPs can be formed when metals become vaporized and condense upon cooling (28). In one study that recreated the incineration process, a high concentration of NPs less than 10 nm in size were found (31).

In addition, NPs can be generated as a result of burning of natural materials such as wood, or mechanical wear such as erosion of tires and vehicle braking (32). Industrial burning of fossil fuels and traffic emissions are the sources from which NPs less than 100 nm in size are most often derived (32). The population as a whole may not need to be as concerned with high levels of exposures due to manufacturing if the exposure is kept limited to the site of manufacture; however, NPs in vehicle exhaust resulting from combustion are a potential hazard (32), while manufacturing emissions often pose a high occupational risk.

In addition to these sources, it is important to consider airborne metal NPs from sources such as diesel engine exhaust and traffic-related air pollution. It has been demonstrated that the particulate matter (PM) present in the exhaust of diesel engines is predominately composed of particles in the nano-size range, based on a number concentration basis (33-35). Kittelson et. al report that within diesel exhaust, typically 90% of the total particle number consists of particles within the 5-50 nm size range (33). While diesel PM consists primarily of soot, a fraction does contain metal NPs (36). These metal NPs found in diesel PM are not engineered but they are still metal NPs that humans can be exposed to through inhalation. Diesel PM has been classified by the World Health Organization as carcinogenic to humans (37), raising valid concerns as to the effects and impacts of this pollution source on human health. Unfortunately, as of yet, NPs released as a result of engine emissions have not been taken into account in emission regulations, and standards have not been established to control their release from vehicle engines (38).

Once NPs are inhaled, deposition can occur in the airways. This includes the nasal and oral cavities through to the alveoli of the lungs (28). The size of a particle affects where it will most likely deposit within the respiratory system, for example those less than 1 nm in size deposit within the nasopharyngeal-laryngeal region with a higher efficiency than within the tracheobronchial region in mathematical models (28). Particles that deposit within the alveolar region with the greatest efficiency are those sized 10 to 20 nm (28). As made clear in the literature, deposition of inhalable particles depends on their size, and due to a high level of NP deposition in the alveolar region (2, 39), it is imperative that the interaction of NPs with alveolar cells is well understood.

1.3 Exposure Scenarios for Inhalable Metal-Based Nanoparticles

Humans are exposed to NPs on a daily basis, with an estimated 5×10^{12} atmospheric NPs deposited in our lungs every day (40). Inhalation is not the only route of regular exposure; an estimated 10^{12} particles (predominantly TiO_2 or silicates), or a mass of 40 mg, enter a person's body orally through ingestion of food additives, toothpaste, pharmaceuticals, or supplements (40).

Occupational exposures, or exposures during product testing, are at the higher end of the scale when it comes to exposure ranges (40). It is important that we learn from the effects that have been observed in the past from occupational dust exposures and asbestos exposures and their resulting negative consequences, such as mesothelioma (40). Several professions deal with NPs despite a lack of exposure assessment, such as medical and dental professionals and research scientists, and despite manufacturing facilities tending

to be more stringent there are still higher concentrations associated with the handling of materials involving NPs (40).

There have been several case studies regarding exposure to metal NPs. In particular, nickel (Ni) NPs have been confirmed to be a human toxicological hazard that can cause disease resulting in death, after a 38-year old male died from adult respiratory distress syndrome (ARDS), ascribed to pulmonary Ni NP overload (41). The subject had no history of respiratory disease, was a non-smoker, and was previously healthy (41). He was exposed to Ni NPs that averaged about 50 nm in size, and were produced while operating a metal arc process used to spray Ni on bushings for turbine bearings, without the face mask provided to him (41). The exposure was estimated to have lasted 90 minutes, and immediately after the exposure, he went home due to having felt unwell (41). He was admitted to hospital 4 days after exposure with respiratory symptoms, and died of respiratory failure 13 days after the exposure (41). Samples of lung tissue obtained at the time of autopsy were re-examined in 2010, and Ni particles less than 25 nm in size were located in lung macrophages through the use of transmission electron microscopy (41).

In another case study regarding occupational Ni exposure, a 26-year-old female was exposed to Ni NP powder while weighing and manipulating the powder with no respiratory protective equipment (42). Following this exposure, the worker developed a recurring sensitivity resulting in difficulties returning to the work environment (42). The MSDS for the powder listed the aerodynamic particle size to be 20 nm and it was unclear

if the particles existed as agglomerates (42). The worker developed throat irritation, flushing of the face, skin reactions to jewellery, nasal congestion, and a post-nasal drip (42). There was no sampling done to determine the level of airborne NPs in the workplace.

These case reports highlight the need for NPs to be considered separately from their bulk counterparts when it comes to exposure and methods of protection required in the workplace.

1.4 Diseases Linked to Metal-Based Nanoparticles

Metal-based particles have been linked to increases in both pulmonary and cardiovascular morbidity and mortality (18, 33, 36, 43-49). Despite the epidemiological evidence that links exposure to increased levels of PM (<2.5 μm) to an increase in cardiovascular morbidity and mortality (18), the specific mechanisms through which these outcomes occur due to pulmonary exposure to airborne NPs has not yet been elucidated.

Inhalation of metal fumes and particles have been associated with multiple pulmonary health outcomes, including bronchitis, metal fume fever, occupational asthma, cancer and possible increases in lung tumorigenicity, suppression of lung defences, and functional changes in the lung (43-48). A rise in the levels of ambient PM have also been linked to an increase in cardiovascular morbidity and mortality through epidemiological studies (18). In addition to atherosclerosis and asthma, chronic obstructive pulmonary disease (COPD) has also been linked to a rise in ambient PM levels (18, 50-52). The major

constituents of ambient PM are the exhaust particles emitted from diesel engines (33, 49), and as mentioned earlier, a fraction of this PM is composed of metal NPs (36). These NPs are not engineered, but the link between rise in morbidity and mortality and PM containing metal-based NPs raises concerns about our exposure to metal-based NPs. Although there is a knowledge gap related to the clinical toxicity of engineered nanomaterials, there is literature regarding particles and fibers (i.e. ultra-fine particles and diesel exhaust particles) and adverse health effects and it is hypothesized that engineered NPs may have similar effects (2, 18, 53).

Severe consequences of PM exposure that have been indicated through epidemiological evidence include increased risks of myocardial infarctions (2, 18, 53), stroke (2, 18, 53), and atherosclerosis (sometimes leading to coronary artery disease) (2, 18, 53) stemming from acute, and chronic exposure to PM (2, 53). Early reports based on the toxicity of some NPs indicates that biological behavior can be affected due to NP exposure not only at the organ and tissue levels, but also at the cellular, sub-cellular, and even molecular levels as within a cell, NPs may interact with proteins, enzymes and even nucleic acids (4).

While there is a lack of human exposure data, the potential for engineered NPs to enter the cardiovascular system after penetrating the pulmonary epithelial barrier and entering the systemic circulation creates a concern regarding adverse cardiovascular impacts that could result from exposure to these NPs (18). Apart from inflammation in the respiratory system and cardiovascular system, inhalation of particles could lead to other health

effects such as asthma complications, chronic bronchitis, and respiratory tract infections and irritations (54-56).

A clinical syndrome that presents in welders, known as metal fume fever, is the result of concentrated metal oxide NPs being inhaled (18). The condition has mostly been documented following exposure to fumes of zinc and to a lesser extent copper (57). Symptoms of metal fume fever include a cough, headache, fever with a sudden onset, nausea, and vomiting (18). It has been found that these symptoms do not permanently damage the respiratory system and only occur for a short period of time (18).

Vulnerable adults, children, and those suffering from asthma are well documented as having an increased risk of suffering adverse health effects coinciding with an increase in ambient air pollution (58). In those individuals that have pre-existing conditions, such as COPD and asthma, NPs appear to exert their toxicity and provoke pulmonary health effects through an exacerbation of pulmonary inflammation (58, 59). It has been documented that chronic respiratory diseases, including COPD and asthma, that are worsened by acute inflammation have been exacerbated with corresponding changes in the levels of ambient air pollution (59-62). Ambient air pollution exposure has been documented to decrease lung function; alter mucociliary clearance; cause increased respiratory infections, COPD, increased hospitalization; and increase mortality (59-62). For those individuals with asthma or COPD, NPs have a higher deposition efficiency in the lung than those individuals who are healthy (63-65), which may explain why these individuals are more affected by ambient air pollution.

1.5 Toxicological Assessment of Metal-Based Nanoparticles

NPs display novel characteristics as compared to their large-scale counterparts, opening up the possibility for a difference to be observed between the toxicity of a particular material on a large-scale versus nanoscale. There are several physicochemical factors that contribute to the potential toxicity of a metal-based NP or NPs in general. The main factors include NP size, shape, surface, composition, and biocorona formation (40).

It has been found that the size of a particle is an important factor in the deposition patterns of NPs in the lung, with an increase in deposition occurring for NPs compared to micron-sized particles (66). While it has been difficult to determine the exact ranges of particle size that deposit in specific regions of the lung, there is evidence that smaller particles deposit lower in the respiratory tract (i.e. bronchioles and alveoli) as compared to the upper airways (i.e. oropharyngeal cavity) (66). However, sub-nm sized particles are retained in the nasopharynx, when the entire respiratory tract is considered (28). The translocation of inhaled NPs has been debated, with some animal studies and one human study finding minimal translocation of NPs from the lungs to other organs (67-69) while others (one human study, mostly animal studies) have found indications of efficient translocation (67, 70-72). As the fate of NPs following deposition has appeared to differ from the fate of particles larger in size (67), it may be important to consider NP agglomeration rates with regards to translocation. The greater the agglomeration, or build up of NPs sticking together, the larger the particles become. Agglomeration depends on the size, shape, concentration, and surface properties (73) of the NPs in question (40), and is of concern with regards to engineered NPs. Manufacturers often do not wish for their

engineered NPs to agglomerate, and thus these particles are designed so that they do not do so. A problem that this design creates for human health is that the majority of the particles persist in unagglomerated form, whilst agglomeration of particles is important for the clearance of these particles from the lung, and it is therefore crucial that this process is well understood (40). In mice exposed to Cu NPs, glomerulitis, degeneration, and necrobiosis of renal tubules was observed (4). As a comparison, these observations were not made in mice exposed to Cu microparticles, providing evidence that the size of a particle is an important toxicological characteristic (4).

The surface characteristics of a NP are related to surface area, solubility, reactivity/stability, and adsorption capacity, and these related factors' relationship to the material's composition may be minimal (74). It is possible for these factors to change throughout the lifetime of a NP, and these changes can either be intentional or unintentional (40). Surface charge of a NP can also have an effect on the toxicity of a NP (4). It has been reported that biological effects are more dependent on surface area than particle mass, and that biological activity is greater when the specific surface area per mass is greater (4).

The corona of a NP refers to the coating of proteins that bind to its surface when in the body, in the presence of biological fluids. A physical change has undergone through this corona formation, as it creates a new surface by obscuring the original surface of the NP (40). This change can affect both the agglomeration and adhesive properties of the original NP (40, 75).

The properties of a NP can be affected by factors such as length of time in suspension in aqueous solutions, dissolution, and oxidation (4). For this reason, it is important to understand the environment that the NP is in and the factors that could be changing the properties of the NP when conducting toxicity assessments. Compared to a material of the same composition on a larger scale, NPs have different properties, and it is therefore important that they are studied and their toxicity is investigated separately from their larger-scale counterparts.

1.6 Cellular Responses of Inhaled Metal-Based Nanoparticles

Reactive oxygen species (ROS) generation is one of the most commonly reported cellular responses related to NP exposure (76-79). ROS are oxygen-containing compounds that are chemically active and produced naturally in the body as a by-product of oxygen metabolism. To protect itself from ROS, the body has natural antioxidant defense mechanisms in place, using enzymes such as superoxide dismutase to convert these species into benign molecules (80). When the production of ROS overwhelms the body's antioxidant defences, a process known as oxidative stress arises (81). Oxidative stress is viewed as a key determinant of injury resulting from NP exposure, as oxidative stress responses are indications of further negative effects in the body, such as genotoxicity, inflammation, and fibrosis (76-79).

The reactive particle surface plays a significant role in the generation of ROS (82, 83), and the amount of reactive sites present on the surface of a NP is determined by its size

(84-86). Due to the increased surface area to particle mass ratio observed for NPs compared to micron-sized particles, the pulmonary responses resulting from inhalation of NPs are considered to be of greater concern than for micron-sized particles (87).

Many processes that are associated with tissue injury are involved with the oxidative stress induced by NP exposure (76). Some of these cellular processes include mitochondrial apoptosis, alteration of calcium homeostasis, and depletion of antioxidant enzymes (76). Toxic oxidative stress results in mitochondrial apoptosis, whereas mild oxidative stress is indicated through the observation of an increase in the expression of antioxidant enzymes and/or their progressive depletion (76). It is thought that pathological consequences which can be observed following exposure to NP may be due to the excessive generation of ROS, as ROS generated in response to NP exposure assists in the activation of cellular mechanisms that are associated with negative consequences such as inflammation and cancer (76). Using these negative biological responses as a screening tool, it is possible to evaluate the magnitude of NP toxicity and therefore the negative health effects resulting from NP exposure (76).

While one of the principal mechanisms of cytotoxicity for metal oxide particles is the induction of ROS (88), the ROS can precipitate the expression of inflammatory cytokines and chemokines, the activation of specific transcription factors, as well as cell signaling pathway activation (76). Due to the transcription of the genes that are involved with conditions such as inflammation, fibrosis, and cancer being closely related to the cellular

mechanisms mentioned above, ROS generation resulting from NP exposure may be what ultimately causes the adverse health effects observed after exposure (76).

It is crucial that we understand the cellular responses and mechanisms by which NPs induce disease and adverse health effects in order to be able to assess the magnitude of risk and identify and put in place practical and effective prevention strategies. A mechanism that has recently been proposed to explain the link between inhaled ambient and workplace particulate air pollution and an increase in risk of cardiovascular disease is a hepatic acute phase response induced by pulmonary inflammation (89). Excessive systemic circulation of cytokines as a result of pulmonary inflammation after the inhalation of particles is proposed to induce a hepatic acute phase response (2, 90-92). This response, despite the name, occurs during both acute and chronic inflammation and refers to systemic changes that are set in motion as a result of cytokines being released from inflamed areas (89). This work was carried out using mice, and further research needs to be undertaken to ascertain the importance of these observations in humans (89).

1.7 *In Vitro* Toxicity Testing Methods for Assessing the Toxicity of Metal-Based Nanoparticles

While it is extremely important to have *in vivo* testing when assessing the toxicity of a material, there are clear advantages to using an *in vitro* method. *In vitro* toxicity testing methods are attractive due to a lower cost, ease of control and reproducibility, and in acknowledgement of the Replacement, Reduction and Refinement principles of the Canadian Council on Animal Care (93). The use of *in vitro* testing is often a first choice

for researchers due to these reasons (94) but it cannot replace the elements that *in vivo* testing brings to the table, namely the complexity of cell-cell and cell-matrix interactions, hormonal effects, and a large diversity of cell types present in the body (94). It is therefore advisable that *in vitro* testing be used in conjunction with *in vivo* testing when assessing the toxicity of NPs.

In vitro testing can be used initially to investigate the toxicity of NPs before moving forward to more expensive *in vivo* testing. *In vitro* methods that are currently used to assess NP toxicity include assays for cell viability/proliferation, genotoxicity, hemolysis, microscopic evaluation, and mechanistic assays (e.g. ROS generation, apoptosis) (93). There are a growing number of studies that investigate *in vitro* cytotoxicity of NPs using differing cell lines and NPs (93). The use of cell-culture studies is an extremely useful tool, as it is a primary step in understanding how a particular material will react within the human body (93). When using cell cultures, it is important to be careful to avoid changes in the cell's environment (e.g. temperature) as that could affect the experimental results due to their sensitive nature.

As noted in the previous section, there are several reactions of interest between cells and NPs including cell signaling effects, chemokine and cytokine production, ROS generation, gene regulation, cell necrosis, and cell apoptosis (95) that may be investigated using *in vitro* testing. The cells selected for a particular *in vitro* assay are chosen to mimic a response that would be elicited by NPs *in vivo*, and examples of such cell types are epithelial, endothelial, red blood cells, hepatic, and neural (95). Two currently used

systems for *in vitro* testing are those involving submerged cells and those exposing cells at the air-liquid interface (96). The *in vitro* systems which involve submerged cells may not accurately reflect the reality of inhalation exposure as the solution in which the NPs are submerged may change the interaction between cell and NP due to the presence of cell culture medium and NP interactions and agglomeration of the NP within the medium (96). Furthermore, the dose of NP delivered to cells can be altered when using submerged cell exposure, as a large portion will remain in the liquid or migrate to the walls of the vessel used and be lost due to the motion of the NPs being mostly due to diffusion (random motion) (96-98). This is in contrast to larger particles, where directed sedimentation onto cells occurs (96-98).

To overcome the limitations presented by submerged cell *in vitro* exposure, systems were developed to deliver NPs directly to the cells at the air-liquid interface (96). There have been several different systems described in the literature, with some being intended for use with cigarette smoke, medical nano-powders, soot particles, and other dry substances as well as some using nebulizers to form droplets, pump-spray units to spray the NPs directly onto cells, and cloud settling systems, coupled with single particle sedimentation (96, 99-106). One system in particular, the air-liquid interface cell exposure system (ALICE) has been found to efficiently and uniformly deliver NPs in liquids to cell systems in a dose-controlled manner (96).

1.8 Exposure Risks of Nanoparticles and Susceptible Populations

There are three exposure scenarios for NP exposure that would all result in different risk-minimization strategies. These three exposure scenarios are occupational exposure, exposure to NPs present in ambient air pollution, and exposure to NPs through the use of various commercial products. When assessing those most at risk for exposure to NPs, the first line of susceptibility is the occupational setting, and the workers present in these settings. For engineered NPs, this is where the first and greatest exposures are most likely to occur (107). Generally speaking, the guidelines for the use of, and exposure to, NPs are related to occupational health, not the general population.

The risk to the general public requires clarification in view of the presumptive regulatory approach. As mentioned previously, the exhaust from diesel engines and other traffic-related sources leads to airborne exposure of metal-based NPs in the general population. The effects of these repeated low-dose exposures are not well understood and the lack of knowledge and regulation poses a risk to the public. The public may not be aware of their exposures, whether they are from an ambient airborne source, or engineered NPs from a source such as commercial products. Those with pre-existing conditions such as asthma and COPD, and other vulnerable individuals (i.e. children, elderly) may be at an increased risk of adverse health effects due to airborne exposure to metal-based NPs (58, 59).

In the absence of a routine air monitoring program to characterize NPs in ambient air, there may be public safety concerns. Without the ability to observe increases in the levels

of NPs in ambient air, it is difficult to determine why the increases are occurring, and to take steps to minimize exposure. These measures need to be put in place, to improve the protection of human health including individuals that are more sensitive to NP exposure, to the best of our ability.

1.9 Current Policies and Regulations Regarding Nanoparticles

In Canada, nanomaterials that are imported or manufactured in Canada are regulated by Environment Canada and Health Canada under the Canadian Environmental Protection Act of 1999 (108). Within the act, there is a legal regime established specifically for the management of new and existing substances, which includes nanomaterials (108). This requires specific information to be provided to government officials by importers or manufacturers of these new substances, in order for an evaluation of the substance's potential effects on the environment and human health to be carried out (108).

With the shared responsibility to assess threats posed by these substances, Environment Canada focuses on environmental risks, while Health Canada focuses on human health risks (108). Specifically, Health Canada uses the following existing regulatory and legislative frameworks to manage substances and products that may contain nanomaterials: Food and Drugs Act; Cosmetic Regulations; Food Additive Regulations; Food and Drug Regulations; Medical Devices Regulations; Natural Health Products Regulations (108). There is no explicit reference to nanomaterial in the Acts and Regulations administered by Health Canada, and it is possible that the regimes that are

currently in place are not completely effective for all nanomaterials (108). Therefore, further adaptations are necessary in order to address specific nanomaterial properties.

The Domestic Substances List (DSL) is a listing of substances used, imported or manufactured in Canada for commercial purposes at a quantity exceeding 100 kilograms per year (109). The listing was compiled before the Canadian Environmental Protection Act came into existence, and includes approximately 23,000 substances used, imported, or manufactured in Canada between January 1st, 1984, and December 31st, 1986 (109). Currently, any substance that is not listed on the DSL is subject to notification under the New Substances Notification Regulations (110). Under these regulations, it is possible to address the unique properties that nanomaterials exhibit using Significant New Activity Notices, where substances are flagged to report to the Government of Canada if there are any major changes in the way that the substance is used (109). Substances listed on the DSL are described using their chemical name and a unique identification number, regardless of whether they are in their nanoscale form or non-nanoscale form (111). This unique identification number is referred to as the Chemical Abstract Services Registry number, or CASRN. The CASRN does not make any distinctions between substances with the same chemical composition, but differing particle sizes or substances which display differences in properties between their nanoscale and non-nanoscale forms (111). Due to this limitation, an assessment of risks to human health and the environment has not been carried out for nanomaterials falling under a CASRN already listed on the DSL, as they are not considered new substances (111). Environment and Climate Change

Canada and Health Canada are working together to develop an approach to address this gap regarding the nanoscale form of substances already listed on the DSL (111).

The physicochemical properties, as well as *in vivo* kinetics, of NPs can differ drastically from those of the bulk form of the same compound; this would likely result in a different hazard assessment for the NPs (2). It is important that research be carried out to understand the toxicity associated with NP exposure to inform regulations and policies related to the use of nanotechnology and subsequent exposure to NP, as well as exposure to NP in ambient air. With projects such as this one, an efficient and cost-effective screening tool for NP toxicity can be brought into use to evaluate metal-based NPs of various compositions as they are developed and brought into use.

1.10 Future Directions for Research (Current Gaps in the Literature)

Current gaps in the literature include the lack of confirmation of the mechanisms by which NP pulmonary exposure may result in increased risk of developing cardiovascular disease. More research needs to be done in order to elucidate this mechanism, and this project will be a stepping stone towards that goal. There is a large gap regarding the regulations and guidelines for nanomaterials, particularly those materials that are already listed on the DSL in their bulk form. Further, there is a current lack of available human exposure data, as well as a lack of understanding regarding the chronic effects of exposure to NPs and a standardization of toxicity testing methods (40). This makes it

more difficult to understand the relationship that exists between NP exposure and chronic diseases, in particular, respiratory diseases.

CHAPTER 2 OBJECTIVES AND HYPOTHESIS

The long-term goal of this research is to provide more information on the mechanisms in which adverse health effects are caused by exposure to airborne metal-based NPs. The primary objective of this project is to further the knowledge of the mechanisms in which systemic toxic responses, in particular cardiopulmonary responses, are produced as a result of airborne NP exposure. To achieve this primary objective, three specific aims were used. A schematic summary of these objectives is shown in **Figure 2-1**, as well as a schematic outlining the translocation paradigm regarding cardiovascular effects produced via the inhalation of airborne metal NPs (**Figure 2-2)** addressed in objective 3.

The following specific aims and their related sub-hypotheses are central to the main hypothesis of this work. These sub-hypotheses will be used in conjunction with the main hypothesis in order to achieve the primary objective of this project, and to work towards the long-term goal of this project. The main hypothesis for this body of work is presented immediately below.

H_0: Airborne exposure to Cu NPs induces cytotoxicity in both human alveolar epithelial cells and cardiovascular cells.

2.1 Specific Aim 1: Determine the characteristics of airborne Cu NPs in an *in vitro* NP exposure system

H_0: An in vitro NP exposure system using NP spark generation that *generates and delivers fresh NPs directly onto human alveolar epithelial cells is a useful model to assess toxicity of airborne Cu-based NPs.*

The first specific aim of this work was to characterize the NPs produced using the spark NP generation system. This characterization comprised of two aspects: size, and by-products of NP generation including total reactive nitrogen (NO_y), nitric oxide (NO), and ozone(O_3) levels. This characterization used Cu electrodes to generate metal-based NPs. By characterizing the NPs produced, we were comfortable that the NPs delivered throughout the exposures were the correct size, consistent, and any chemical species delivered alongside the NPs were not at a level which would cause the toxic responses observed. Additionally, as it is possible that synergistic effects could exist between NO_x and O_3, and the NPs produced, it was important that their levels were measured to aid in reproducibility and future, continuing research. It was expected that 5 nm Cu NPs would be produced, and O_3, NO_y, and NO levels would be well below the limits for cellular damage.

2.2 Specific Aim 2: Evaluate the primary (pulmonary) toxic responses of human alveolar epithelial cells after airborne Cu NP exposure

H_0: Exposure to airborne Cu NPs induces cytotoxicity and intracellular ROS in human alveolar epithelial cells.

The second specific aim of this work was to evaluate the primary cellular toxicity response after repeated exposure to airborne Cu NPs. Human alveolar epithelial lung cells (A549 cells) were sequentially exposed to NPs in a 4-2-4 fashion, where the 4 represents 4 hours of NP delivery and the 2 represents a 2-hour period where the cells were allowed to rest in a CO_2 incubator. This sequential exposure allows a more realistic simulation of human exposure scenarios, as compared to a single exposure. Biological responses were determined following the completion of the 4-2-4 exposure. In order to assess the toxic response, data regarding the level of ROS generation, cell viability, as well as NP concentration in the exposure medium were analyzed. As Cu NPs have been observed to have a higher toxicity (4, 19, 20), it was important that Cu NPs were investigated to determine their pulmonary toxic response in a scenario that mimics realistic human exposure. It was expected that an elevation in the concentrations of ROS and lactate dehydrogenase, and a decrease in cell viability post exposure would be observed.

2.3 Specific Aim 3: Assess secondary (systemic) toxic responses in cardiovascular cells after *in vitro* inhalation exposure of NPs

H_0: Pulmonary exposure to Cu NPs induces adverse effects on cardiovascular cells cultured in the exposure medium.

The third specific aim of this work is to assess the systemic response, specifically the cardiovascular impact of Cu NPs, after repeated exposure to airborne Cu NPs.. Due to the fact that the mechanism as to how cardiovascular responses are related to pulmonary exposures to NP has not yet been elucidated, it is crucial that a foundation of information

is acquired in order to aid in investigating the mechanism further. The "exposure medium" obtained during specific aim 2 was used to culture cardiovascular cells. This work was carried out in collaboration with three researchers who have extensive experience related to cardiovascular pathophysiology; Dr. Pulinilkunnil, Dr. Kienesberger, and Dr. Brunt at Dalhousie Medicine New Brunswick (DMNB). It was expected that a decrease in the cell viability of cardiomyocytes cultured in the exposure medium resulting from specific aim 2 would be observed.

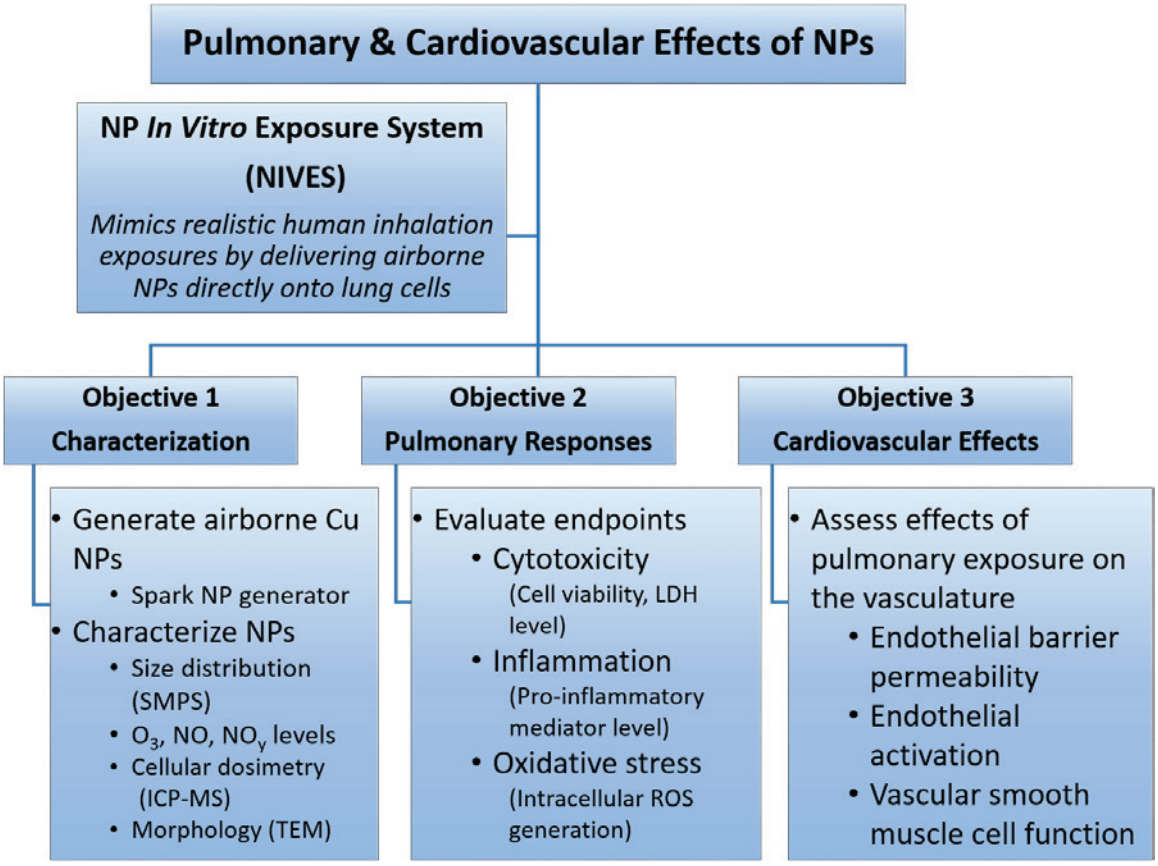

Figure 2-1: Schematic summary of objectives.

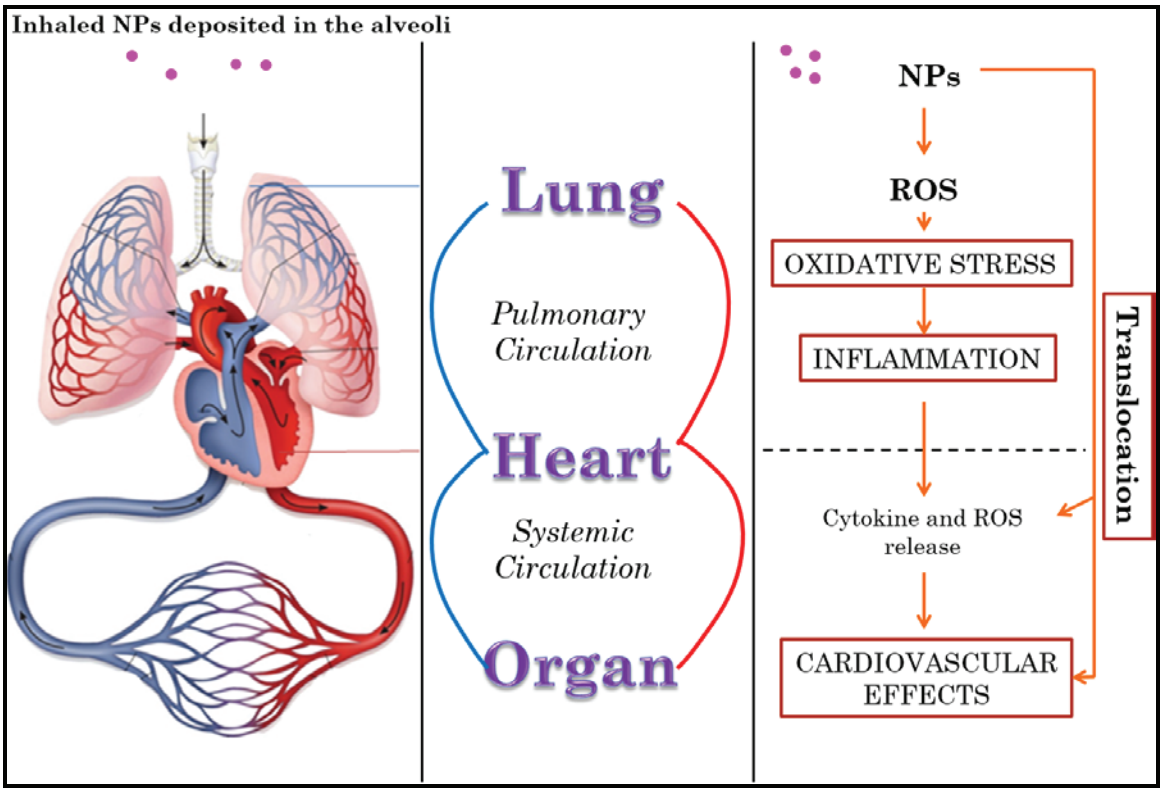

Figure 2-2: Schematic of the translocation paradigm regarding cardiovascular effects produced via the inhalation of airborne metal nanoparticles.

CHAPTER 3 METHODS

3.1 CHARACTERIZATION

3.1.1 Particle Distribution

Nanoparticles were generated using a Spark Discharge System (SDS). High voltage (HV Rack System, Ultravolt) was applied to copper (99.999%) electrodes, thus generating a spark between the electrodes. The generated NPs were carried through tubing via nitrogen, oxygen, and carbon dioxide gases in a ratio similar to the composition of air and delivered to the Scanning Mobility Particle Sizer (SMPS, TSI 3080) and Condensation Particle Counter (CPC, TSI 3775), and the exposure chamber.

The size distribution of the spark-generated Cu NPs was characterized using the SMPS and CPC. Optimal conditions for generation of monodispersed NPs with consistent number concentrations, size, and geometric standard deviation were determined by alterations in voltage and current within the spark discharge system. Once the distributions of produced NPs were Gaussian, monodispersed, and appeared consistent, the system was run for 4 hours in order to confirm the consistency of NPs produced. This characterization was carried out prior to cell exposure.

3.1.2 Total Reactive Nitrogen (NO_y), Nitric Oxide (NO), and Ozone (O_3) Levels

Levels of NO_y and NO were determined using an NO_y Analyzer (Thermo Scientific Model 42i-Y NOy NO-DIF-NOy Analyzer) and measured over a period of 4 hours. The system was run on three separate occasions to strengthen reliability of the data.

Levels of O_3 were determined using an Ozone Monitor (2B Technologies) and measured over a period of 4 hours. The system was run on three separate occasions to strengthen reliability of the data.

3.2 Primary Toxicity Response (Pulmonary)

The *in vitro* testing system used in this project allows for the prediction of *in vivo* repeated low-dose toxicity through the sequential (repeated) exposure of lung cells to NPs using the set up described in specific aim 1. This procedure simulated the protocol of an *in vivo* repeated low-dose study of NPs. The sequential NP exposure was done in a 4 h-2 h-4 h fashion, meaning that the cells will be exposed to particles for 4 h in the system, followed by an incubation period for 2 h (referred to as resting) in the cell incubator (37°C in 5% CO_2), and finally they were exposed for another 4 h in the system. Following the second exposure, the transwell inserts (Corning) were removed from the exposure chamber and analyzed as described in the following sections. The *in vitro* testing system produced exposure media (containing biological mediators, as well as NPs) that was used to simulate the manner in which NPs deposited on the respiratory

epithelial tract may reach organs other than the lungs by crossing the air-blood barrier (*via* the alveolar region) and enter systemic circulation.

The principal responses to Cu NP exposures evaluated in this work were oxidative stress and inflammatory responses of human lung epithelial cells (A549 cells), as well as the systemic responses resulting from pulmonary exposure to Cu NP. This analysis allowed the oxidative stress/inflammation paradigm induced by NPs to be revealed and provide insight as to the mechanisms of NP toxicity.

3.2.1 Cell Selection and Culture

A549 cells were selected to appropriately represent the lung epithelium in an *in vitro* model for lung cell NP toxicity at the ALI. Alveolar epithelial cells are some of the first lung cells exposed to inhaled toxicants, and A549 cells are a reasonable surrogate cell line for the ideal primary human lung epithelial cells. A549 cells retain several features of type II alveolar epithelial cells, such as the production of alveolar surfactant (112). Cells were obtained from Dr. Jason LeBlanc (Halifax, NS), and cultivated in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (herein referred to as culture medium) at 37°C under humidified atmosphere containing 5% CO_2.

To prepare for exposure, cells were harvested with 0.25% phenol red-free trypsin-EDTA (Gibco®) and counted before 1 mL of cell suspension was seeded onto the apical side of the transwell membrane inserts (Cat. 3450, polyester, 24 mm diameter, 4.7 cm^2, 0.4 µm pore size, Corning, USA) at a concentration of 450,000 cells/mL. Cells were allowed to

attach to the transwell for 24 h under submerged condition with 2 mL basal culture medium. Apical medium was removed and cells were washed twice with 500 µL of phosphate-buffered saline (PBS, Gibco®), 12 h prior to exposure. The PBS used to wash the cells was removed, and no apical medium was added to cells following the wash.

Immediately prior to exposure, cells (approximately 95% confluence) were again washed twice with 500 µL of PBS on the apical side and transferred to the exposure chamber wells (Vitrocell®), where the basal medium provided to the cells was 17 mL. All solution was removed from the apical side of the insert, thus only a thin film of surface liquid produced by the cells was present during exposure. At the time of exposure, approximately 900,000 cells were present on the surface area (4.7 cm^2) of the membrane.

3.2.2 Airborne Delivery of Cu NPs to A549 Cells

Once cells were placed in the exposure chamber, the exposure chamber was connected to the aerosol flow. Aerosol flow rates for individual transwell inserts were 10 mL/min, obtained using a vacuum pump (EW-07061-11, Cole-Parmer). Aerosol flow was comprised of 3.80 L/min N_2, 0.95 L/min O_2, 0.25 L/min CO_2, and Cu NPs generated using the SDS. N_2 gas was passed through a diffusion dryer (Drierite Laboratory Gas Drying Unit) and a HEPA filter (1602051, TSI) before passing through the SDS. Aerosol flow was passed through an X-ray neutralizer (model # 308702, TSI) prior to delivery to the cells. In air exposure experiments only, aerosol flow was passed through a HEPA filter (PN 12144, PALL) to remove Cu NPs prior to delivery to the cells. Throughout exposure (both air and NP) aerosol size distribution was continually measured using the

SMPS. Cells were maintained at 37ºC using a waterbath (Alpha A-6, Lauda) that circulates water through the exposure chamber's walls. A schematic of the setup outlines all flowrates, aerosol flow pathways and direction (**Figure 3-1**), as well as the ALI exposure scenario (**Figure 3-2**). Following exposure, well established assays were used to assess cytotoxic and immunotoxic effects as outlined in sections 3.2.3 – 3.2.6. For all experiments (except dosimetry experiments), 1 mL of PBS wash solution and 17 mL of basal media was kept and frozen for later analyses. The 1 mL of PBS wash solution originated from 2 separate 500 µL PBS washes, where the PBS was added following the completion of exposure and collected from the cells immediately following the wash (i.e. the PBS did not remain on the cells).

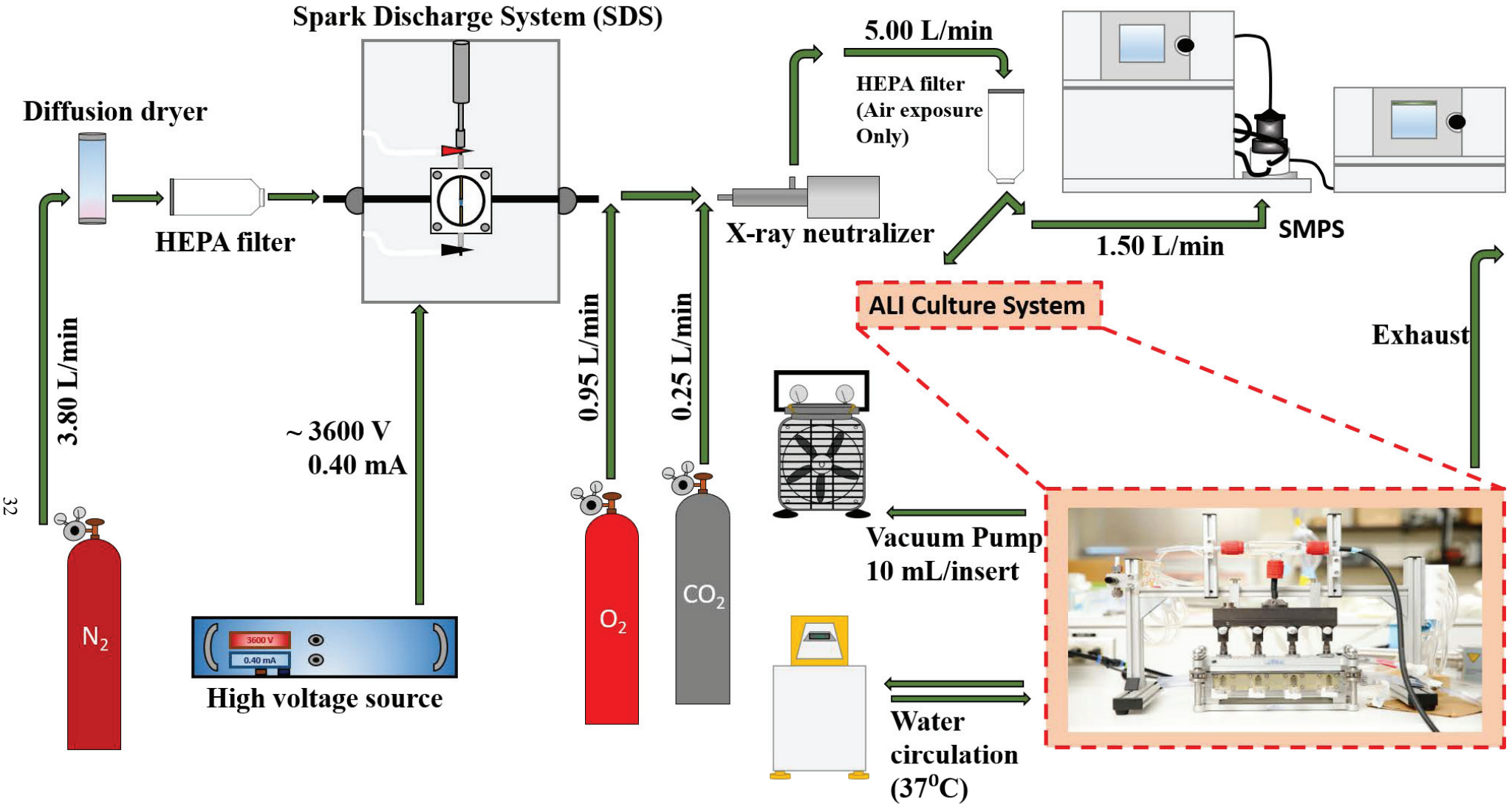

Figure 3-1: Schematic of the experimental setup.

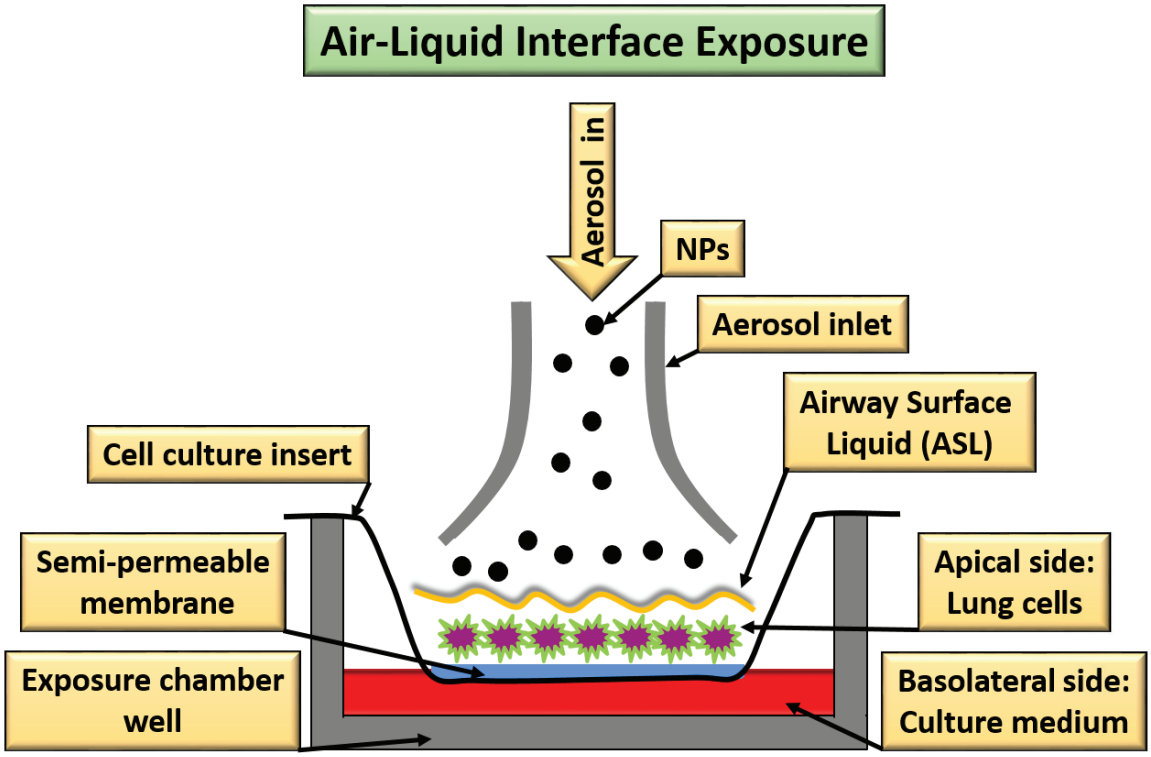

Figure 3-2: Schematic of the Air-Liquid Interface Exposure.

3.2.3 Cellular Viability

Cellular viability was measured using AlamarBlue® (AB, Invitrogen™, DAL1025) Cell Viability Reagent. AB dye is proven safe and non-toxic and is widely used to quantify cellular viability (113). AB dye is a redox indicator, and produces both a colorimetric change and a fluorescent signal proportional to the metabolic activity of the cells being analyzed. AB dye is reduced by healthy cells, visualized via a change in the dye's color from blue to pink. This allows for quantification of the change in cellular viability as a result of exposure to NPs (114).

Following exposure (either air or NP), cells on inserts were transferred to a fresh 6-well plate and washed twice with 500 µL of PBS on the apical side. A 1:10 solution of AB dye in culture media was prepared, and 1 mL of this solution was placed on the apical side of each insert containing washed cells. The 6-well plate was then incubated (37ºC, 5% CO_2) under dark conditions for 2 hours. Following incubation, 100 µL was taken from each sample and placed in a 96-well plate and absorbance values were read at 570 nm and 600 nm using a microplate reader (Benchmark Plus™ microplate spectrophotometer, BIORAD). Each sample was read using 4 replicates. Fresh culture medium was used as a blank, and AB dye in culture medium (cell-free) was used as a negative control. Values were then normalized to the control condition (cells on insert maintained in the incubator under standard cell conditions), which were set at 100% viability. Cellular viabilities were expressed as percentage of the control.

Cellular viability was also measured using Trypan Blue assay. Trypan Blue dye is not absorbed by viable cells with intact membranes, but is able to enter dead cells and cause them to appear blue under a microscope. Cellular viability can then be quantified by counting the live versus dead (or non-stained versus stained) cells on a hemocytometer. Following exposure (either air or NP), cells on inserts were transferred to a fresh 6-well plate and washed twice with 500 µL of PBS on the apical side. Cells were then harvested using 200 µL of 0.25% phenol red-free trypsin-EDTA (Gibco®) and counted using a hemocytometer viewed under a microscope. Cellular viability was again normalized to the control condition (cells on inserts maintained in the incubator under standard cell conditions), which were set at 100% viability. Cellular viabilities were expressed as percentage of the control.

3.2.4 Intracellular ROS Generation

Carboxy-H_2DCFDA (Life Technologies, C400) was used to determine levels of intracellular ROS generation. Carboxy-H_2DCFDA can be used to quantify the intracellular levels of ROS generated due to its ability to passively enter the cell and react with ROS to form dichlorofluorescein (DCF) – a highly fluorescent compound. Carboxy-H_2DCFDA, the carboxy derivative of fluorescein, carries an additional negative charge that improves cellular retention of the dye as compared to the non-carboxylated forms (115).

Following exposure (either air or NP), cells on inserts were transferred to a fresh 6-well plate and washed twice with 500 µL of PBS on the apical side to remove extra-cellular

ROS. A working solution of Carboxy-H_2DCFDA reconstituted in DMSO (concentration of 1 mM) was diluted in HBSS to a final concentration of 25 µM. 1 mL of final solution (Carboxy-H_2DCFDA in HBSS) was placed on the apical side of inserts before they were incubated under dark conditions for 30 minutes (37 ºC, 5% CO_2) to load the fluorescent dye. Following this incubation, the dye solution was discarded, and cells were washed twice with 500 µL of PBS on the apical side to remove any residual extra-cellular dye. Cells were then incubated under dark condition in 1 mL of Triton X-100 (0.1% solution in PBS) for 30 mins. Following this incubation, cells were lysed using a cell scraper under dark conditions and the solution was collected into an amber microcentrifuge tube. Lysates were centrifuged at 14,000 g and 100 µL of supernatant was transferred to a 96-well plate. Each lysate's fluorescence intensity was measured in duplicate with excitation of 485 nm and emission at 530 nm using a microplate reader (VICTOR X5 Multilabel Plate Reader, Perkin Elmer). The values of cells exposed to particle-free air or NPs in the system were normalized to the control condition.

3.2.5 Lactate Dehydrogenase (LDH) Analysis

Lactate Dehydrogenase (LDH) levels were measured using a commercial colorimetric assay kit (Cytotoxicity Detection Kit^{PLUS} (LDH), Roche Diagnostics, Sigma). LDH can be found in most living cells. LDH levels are released during tissue damage, and can therefore be used to quantify cellular membrane damage (116).

PBS washes collected from apical washes following air and NP exposures were thawed and used for this analysis. Sample volumes of 100 µL were placed in a 96-well plate

before having 100 µL of reaction mixture (reaction catalyst and dye solution) added to each well. The 96-well plate was then incubated in dark conditions for 10 minutes before absorbance values were read on a microplate reader **(**Infinite® M200 Pro, Tecan**)** at a wavelength of 490 nm, with a reference wavelength of 610 nm. All samples were run in duplicate, and were normalized to the control condition (incubator control samples).

3.2.6 Dosimetry

Samples for dosimetry were collected and subsequently digested on the same day using a Discover® SP-D Microwave Digestor (CEM). Inserts were cut using a sterile single use stainless surgical blade (no. 11, 0086, Lance) and digested in 10 mL of concentrated nitric acid (A509-P212, trace metal grade, Fisher Chemical). Samples were digested at a temperature of 210 ºC, pressure of 400 PSI, and power of 300 W. Stirring was set to medium, ramp time for samples was 5 minutes, and hold time was 4 minutes. Following digestion, samples were diluted to 2% nitric acid and stored in the fridge for later analysis. Cu levels were measured using an Inductively Coupled Plasma-Mass Spectrometer (iCAP Q ICP-MS, Thermo Fisher Scientific).

3.3 Secondary Toxicity Response (Cardiovascular and Systemic)

This project sought to identify specific effects of inhaled NPs on the cardiopulmonary system. To determine the causative components, additional work must be undertaken. Using the set-up described earlier, the exposure medium of lung cells exposed to NPs was collected and used for *in vitro* assessments to determine the effect of first-pass

exposure of pulmonary NPs on coronary vasculature and underlying cells (i.e. smooth muscle, cardiomyocytes). This allowed for an examination of the influence of inhalation of NP on the metabolism and function of vasculature and cardiac muscle. These studies were performed in collaboration with researchers with extensive cardiovascular pathophysiology experience - Drs.Pulinilkunnil, Kienesberger, and Brunt. The methodology used in elucidating vascular response to NP exposure follows below.

3.3.1 Cytotoxicity

Cell viability was investigated in HUVEC and H9C2 cells using FACS analysis, and for H9C2 rat cardiomyoblasts using Presto Blue Viability Reagent as well. HUVEC cells (human umbilical vein endothelial cells) and H9C2 cells were both chosen as a way to illustrate the effects on the entire cardiovascular system. The H9C2 rat cardiomyoblasts represent a homogeneous cardiac-derived cell line, whereas the HUVEC cells represent the vasculature of the body (a single layer of endothelial cells comprises the inner layer of blood vessels in the body – this is the interface between blood circulating through the body and the remainder of the vessel wall). As our primary goal for this objective was to investigate whether there are any cytotoxic effects to cardiovascular cells after airborne exposure of pulmonary cells to NPs, it was imperative that we had representative cells of the entire cardiovascular system. Used together, it was possible to obtain a clearer picture of how airborne exposure to NPs can affect the cardiovascular system, as well as an indication of where those effects may be more likely to occur (i.e. to heart tissue, vasculature, or both). H9C2 rat cardiomyoblasts were specifically looked at due to their significance in contractile function, whereas HUVEC cells were looked at due to their implication in atherosclerosis. Furthermore, a homogenous cell line was chosen over a

primary cell culture so that it would be possible to elucidate molecular pathways involved in potential detrimental effects. Cardiac muscle is comprised of several cell types (fibroblasts, erythrocytes, cardiomyocytes and cardiomyoblasts, etc) and therefore if an effect was observed using a primary cell culture, we would be unable to determine if the contents of the exposure media were affecting the cardiomyocytes directly or through other cells (i.e. fibroblasts signaling cardiomyocyte cell death as opposed to NPs directly causing death of cardiomyocytes).

H9C2 rat cardiomyoblasts were chosen over human cells due to financial and logistical concerns such as timeline. Human cells (HCM, or human cardiomyocytes) are very expensive, and take a very long time to grow. As a primary investigation into the effects of airborne exposure to NP and the resulting effects on the cardiovascular system, it was appropriate to use an acceptable substitute (the H9C2 cells for HCM). H9C2 cell lines were easier to work with, as they were easier to grow and exhibited faster growth. Following work with this cell line, a recommendation could be made concerning further development of the study and work in primary cells (HCM).

For FACS analysis, HUVEC cells (400, 000 cells per plate) were plated the day before treatment in 35 mm dishes with 2.0 mL of main media (HUVEC cell growth factor media). Treatment lasted for 24 hours, and was done with 150 µL of exposure media for all three media types (incubator control, air exposure, NP exposure). Cells were then harvested and FACS analysis was done using Annexin V and propidium iodide. H9C2 cells (500,000 cells per plate) were plated the day before treatment in 60 mm dishes with

2.0 mL of main media (Dulbecco's Modified Eagle Medium-High Glucose (DMEM-HG) media). Treatment lasted for 24 hours and was done with 100 µL of exposure media for all three media types (incubator control, air exposure, NP exposure). Cells were then harvested and FACS analysis was done using Annexin V and propidium iodide.

Treatment of cells was carried out using a ratio of 10% exposure media (collected from previous pulmonary exposure) to 90% main media (for HUVEC cells, this would be HUVEC cell growth factor media; for H9C2 cells, this would be DMEM-HG media). H9C2 cells were treated with 100 µL, as compared to the 150 µL for HUVEC cells due to limitations in exposure media available. The overall proportion of 10% exposure media to 90% main media was preserved (i.e. 900 µL main media added to 100 µL exposure media for H9C2 cells, and 1350 µL main media added to 150 µL exposure media added to HUVEC cells). This proportion was chosen due to the fact that the exposure media from pulmonary exposure was a different type of media that the media that HUVEC and H9C2 cells are grown in. In order to ensure that any cytotoxicity observed was not due to the change in media, the proportion of 10% was chosen. For example, HUVEC is a very sensitive cell line, and won't grow in different media than its own, as it requires specific growth factors.

Following the 24 hours of treatment, cells were harvested for FACS analysis. To do so, they were washed with normal PBS before the cells were incubated with 0.5 mL of 0.05% Trypsin EDTA for 5 minutes. Following this incubation, cells were removed from the incubator and 1 mL of exact media was added to the cells. This cell suspension was

then transferred to a 60 mm diameter FACS tube. Cells were centrifuged down before being washed twice with PBS. Following the second PBS wash, 1% BSA in PBS was added to the cells before they were centrifuged down again. 2 µL of Annexin V in 100 µL of 1% BSA in PBS was added to the cells before they were incubated on a shaker for 15 minutes. Next, 1 µL of propidium iodide was added and cells were again incubated in a shaker for another 15 minutes. Finally, cells were washed with normal PBS twice before 0.5 mL of BSA was added and cells were directly analyzed with a FACS machine (Gallios™ Flow Cytometer 10 colors, 4 lasers from Beckman Coulter).

For Presto Blue analysis, 12,000 H9C2 rat cardiomyoblasts per well (well volume of 100 µL) were seeded for 75% confluency two days (day -1) prior to treatment in 100 µL of DMEM-HG media (supplemented with 10% FBS). To initiate H9C2 differentiation, the media in all wells was replaced the following day (day 0) with DMEM-HG media that was free of FBS. This media was renewed on day 3. Treatment began on day 6, where media was replaced with different ratios of exposure media to main media (DMEM-HG (0% FBS) for an additional 24 h. The ratios were: DMEM-HG (0% FBS) only, ¼ exposure media to ¾ main media, ½ exposure media to ½ main media, or exposure media only. The total volume per well was 100 µL (therefore, a ratio of ¼ to ¾ would mean 25 µL to 75 µL). Following 24 h of treatment, media was replaced with DMEM-HG (0% FBS) with 10% Presto Blue Viability Reagent for 3 h; fluorescence intensity was measured with excitation at 560 nm and emission at 590 nm using the Synergy H4 Hybrid Reader.

3.4 Data Analysis

The statistical analysis of results was carried out using a two-tailed Student's t-test. The t-test type was two sample equal variance. Results were interpreted using the mean ± SD and the level of statistical significance ($p < 0.001$, $p < 0.01$, $p < 0.05$, or not statistically significant).

CHAPTER 4 RESULTS AND DISCUSSION

4.1 CHARACTERIZATION

4.1.1 Particle Size Distribution

Optimal conditions for generation of approximately 5 nm sized NPs occurred at approximately 3600 V and 0.40 mA. Peaks produced were distributed in a Gaussian fashion, and monodispersed with a geometric standard deviation (GSD) less than 1.6 in accordance with the literature (117-119). **Figure 4-1** shows a representative peak of NPs produced.

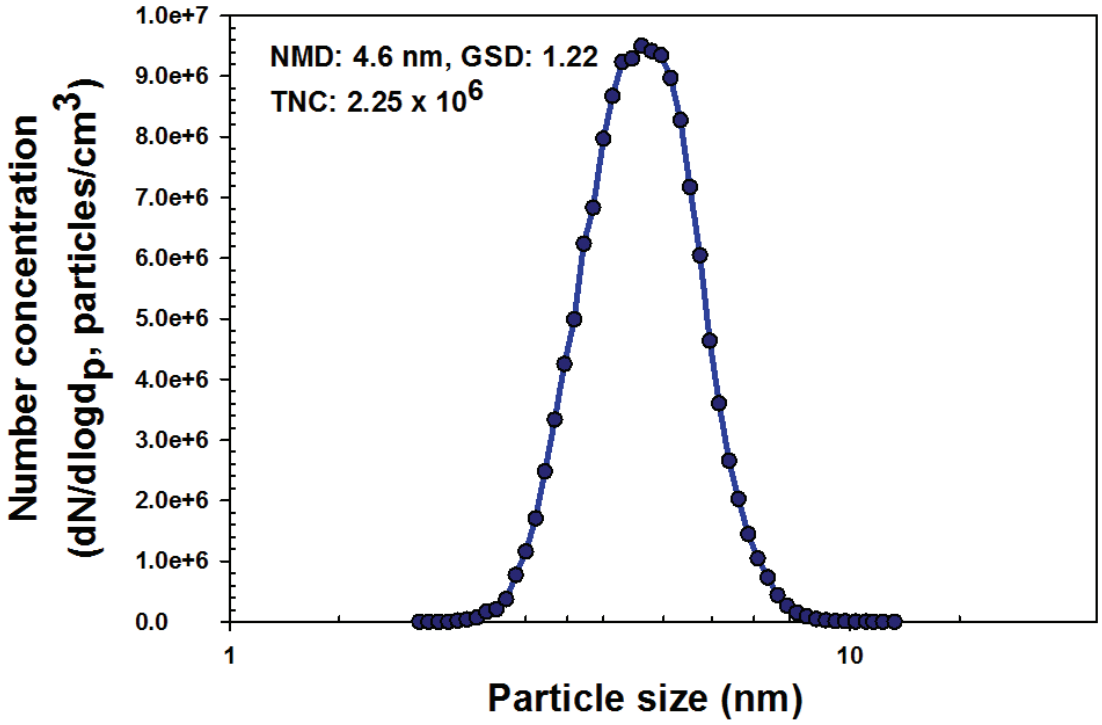

Figure 4-1: Representative size distribution of copper nanoparticles produced using the Spark Discharge System showing a number mean diameter of 4.6 nm with a geometric standard deviation of 1.22.

4.1.2 Total Reactive Nitrogen (NO_y), Nitric Oxide (NO), and Ozone (O_3) Levels

Levels of O_3, NO_y, and NO were measured over a 4 h period of NP generation, with NPs produced using the SDS. Levels of O_3, NO, and NO_y were found to be 17.2 ± 0.3 $\mu g/dm^3$, 4.6 ± 3.5 $\mu g/dm^3$, and 19.7 ± 8.8 $\mu g/dm^3$, respectively. Results are shown in **Table 4-1.**

Gas Measured	NO_y	NO	O_3
Concentration ($\mu g/dm^3$)	19.7 ± 8.8	4.6 ± 3.5	17.2 ± 0.3

Table 4-1: Levels of O_3, NO_y, and NO measured over a 4 h period of nanoparticle generation, with nanoparticles produced using the Spark Discharge System.

4.2 PRIMARY TOXICITY RESPONSE (PULMONARY)

4.2.1 Cell Viability

Sequential exposure (4-2-4) of A549 cells to Cu NPs (approximately 5 nm in size) resulted in a significant reduction in cellular viability as compared to NP-free (filtered) air of 6.9%. Single exposure (4 h) of A549 cells to Cu NPs (approximately 5 nm in size) resulted in a significant reduction in cellular viability as compared to NP-free (filtered) air of 9.6%. Cell viability for sequential exposure is shown in **Figure 4-2**. Cell viability for single exposure is shown in **Figure 4-3**. Contrary to the original hypothesis in this

study, there was no observable difference in the cell viability between A549 cells that were sequentially exposed (4-2-4) versus those that had only a single exposure (4 h). Single exposure (4 h) of A549 cells to Cu NPs resulted in a reduction in cell viability similar to the sequential exposure, when compared to NP-free (filtered) air. As compared to incubator controls, there was a decrease in cell viability for NP-free (filtered) air that was statistically significant for both the sequential (4-2-4) exposure and single (4 h) exposure. This difference could likely be attributed to the low levels of O_3, NO, and NO_y that were generated during exposure. The NP-free (filtered) air exposure and the NP exposure groups would both be exposed to the same levels of these gases, therefore the difference observed in the cell viability between these two groups gives an indication of the effect of NPs on cell viability.

The decrease in cell viability observed was lower than expected, however there are several hypotheses as to why this may have been the case. As will be noted in the dosimetry results, the dose of airborne Cu NPs delivered was very low. Comparing the NPs delivered in this study to those delivered in a study with a similar setup by Jing et al. (120), we can see that the TNC of particles delivered in their study was 2.27×10^7 particles/cm^3, whereas it was 2.25×10^6 particles/cm^3 in this study. This 10-fold decrease in NP dose delivered could have contributed to the higher than expected viability observed. To further the comparison of the study by Jing et al., the NPs generated in their study had a number mean diameter of 9.2 nm, as compared to the 4.6 nm NPs produced in this study. Taken together, the lower dose and the smaller size of NP have resulted in a smaller effect on cellular viability than was expected.

A study described by Wongrakpanich et al. (121), notes that smaller sized CuO NPs were not observed to elicit a high level of cytotoxicity as compared to larger NPs. Wongrakpanich et al. observed a significant difference in the viability of A549 cells that were exposed to 24 nm sized NPs as compared to those exposed to 4 nm sized NPs. They hypothesized that the cellular uptake of these 4 nm CuO NPs was lower due to the smaller size of the NP resulting in different, and less efficient routes of entry into the cells (121). There has been evidence that active cellular uptake of NPs is size dependent, and that it is possible that the size range for optimal uptake may differ between NPs of different compositions (122). A study investigating the optimal NP radius for endocytosis found that the optimal NP radius was approximately 25-30 nm, and noted that a previous study had observed that endocytosis of Au NPs with a radius of 7 nm only occurred when at least six of them were clustered together producing a cluster radius of approximately 20 nm (123, 124).

As was hypothesized, cells that were exposed to NPs in the presence of the reactive oxygen species scavenger N-Acetyl Cysteine (NAC) had an observable and significant increase in their viability as compared to NP-free (filtered) air of 9.6%. This evidence further suggests that cytotoxicity of NPs is ROS-mediated, in accordance with what has been observed in the literature. While the effects on the cell viability were much greater in the Jing et al. study with the larger NPs, they similarly observed that there was no reduction in cellular viability, as compared to controls, when A549 cells were concurrently exposed to NPs and NAC (120).

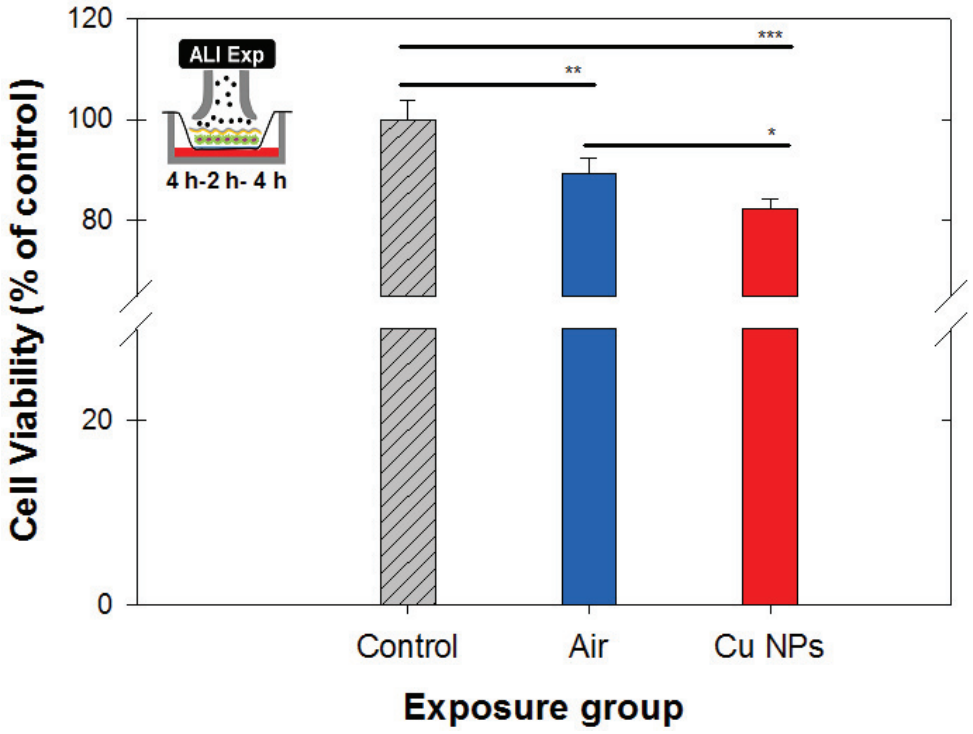

Figure 4-2: Cellular viability for control (incubator), air (particle-free), and copper nanoparticle (Cu NPs) exposure groups for A549 cells sequentially exposed at the air-liquid interface (ALI) as evaluated by the Alamar Blue assay.

Note: A549 cells in the air and Cu NP exposure groups were sequentially exposed with an aerosol flow rate of 10 mL/min.

Post-exposure (0 h), Alamar Blue assay was used to evaluate cellular viability. Cellular viability was then normalized to the control cells (maintained in the incubator at 37ºC and 5% CO_2).

Data are expressed as mean ± SD; ***statistically significant difference compared to control (***$p < 0.001$; ** $p < 0.01$; *$p < 0.05$).

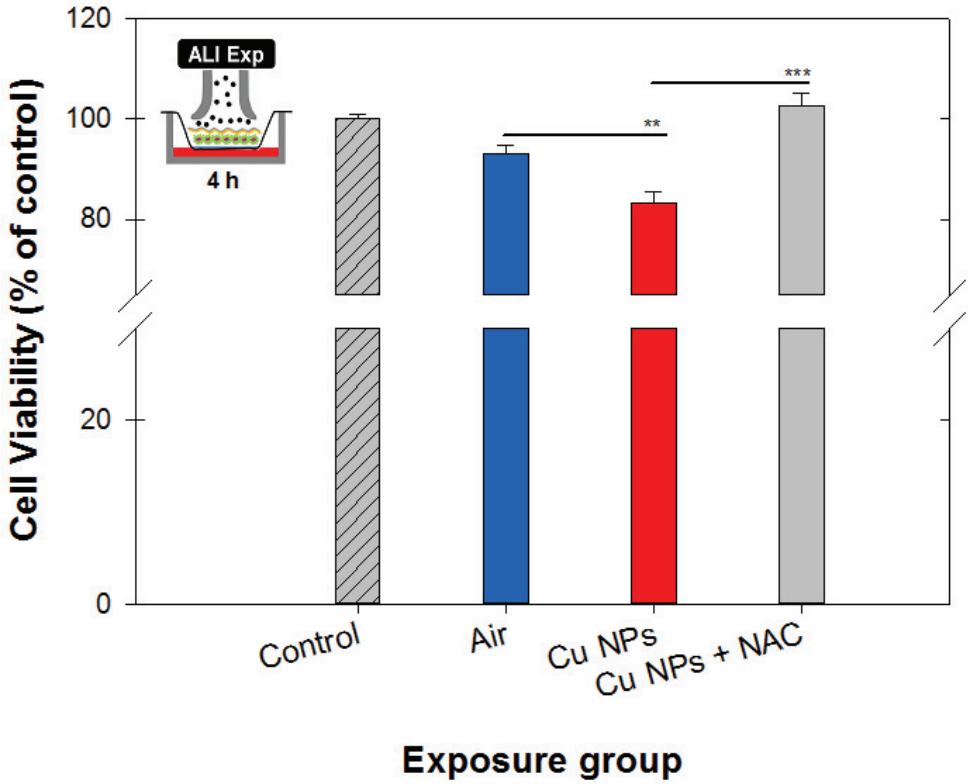

Figure 4-3: Cellular viability for control (incubator), air (particle-free), copper nanoparticle (Cu NPs), and copper nanoparticle with N-Acetyl Cysteine (Cu NPs + NAC) exposure groups for A549 cells exposed for 4 h at the air-liquid interface (ALI) as evaluated by the Alamar Blue assay.

Note: A549 cells in the air and Cu NP exposure groups were subjected to a single exposure for 4 h with an aerosol flow rate of 10 mL/min.

Post-exposure (0 h), Alamar Blue assay was used to evaluate cellular viability. Cellular viability was then normalized to the control cells (maintained in the incubator at 37ºC and 5% CO_2).

Data are expressed as mean ± SD; ***statistically significant difference compared to control (***$p < 0.001$; ** $p < 0.01$).

4.2.2 Intracellular ROS Generation

Intracellular ROS generation was measured for sequentially exposed A549 cells (4-2-4), as well as for A549 cells with a single exposure (4 h). The intracellular levels of ROS generated in A549 cells after a single exposure (4 h) were markedly different than those generated after a sequential (4-2-4) exposure. After a sequential exposure, there was a statistically significant difference in the level of intracellular ROS generated during a NP-free (filtered) air exposure compared to incubator controls, however there was no significant difference between cells that had been exposed to air vs those that had been exposed to the NPs. A possible explanation for the increase in intracellular ROS generation in the NP-free (filtered) air exposure is that the second 4 hours of exposure resulted in too much stress on the cells, and they were unable to efficiently scavenge the intracellular ROS produced. There are two possible contributors to the stress conditions potentially experienced by the cells – no humidity control was present in the exposure system, and there were low levels of O_3, NO, and NO_y present during exposure (as was well characterized prior to exposure). The lack of an optimal level of humidity (as would be present in the incubator) may have presented additional detrimental stress conditions to the cells. The low levels of O_3, NO, and NO_y present during exposure may have contributed to the difference observed in the intracellular ROS levels generated in the NP-free (filtered) air exposure and incubator control groups. Expressed as a fraction of the level of intracellular ROS generated by incubator control cells, sequential (4-2-4) exposure resulted in intracellular ROS levels of 1.4 for NP-free (filtered) air exposed cells, and 1.4 for NP exposed cells. However, after a single exposure, the NP-exposed cells showed a significant increase in intracellular ROS levels as compared to the air-

exposed cells. Expressed as a fraction of the level of intracellular ROS generated by incubator control cells, single (4 h) exposure resulted in intracellular ROS levels of 0.97 for NP-free (filtered) air exposed cells, and 1.7 for NP exposed cells. Intracellular ROS levels for sequential exposure are shown in **Figure 4-4**, and intracellular ROS levels for single exposure are shown in **Figure 4-5**. While there was no humidity control present in the exposure system and there were low levels of O_3, NO, and NO_y present during NP-free (filtered) air and NP exposures, these conditions were consistent between these two groups. The NP-free (filtered) air exposure group was an appropriate control to mitigate these concerns regarding the results observed in the NP exposure group, and therefore the effect due to NP exposure is still able to be ascertained.

The experiments undertaken in this study point further towards ROS-mediated toxicity of Cu NPs, in accordance with the literature. This can be observed in the set of experiments using NAC, which is a scavenger of ROS compounds. When A549 cells were exposed to NPs in the presence of NAC, the levels of intracellular ROS generated were not significantly different than the levels generated during an air exposure, nor were they significantly different than the levels generated in the incubator controls. Expressed as a fraction of the level of intracellular ROS generated by incubator control cells, single (4 h) exposure resulted in intracellular ROS levels of 0.97 for NP exposed cells in the presence of NAC, and 1.1 for NP exposed cells allowed to rest for 2 hours following exposure.

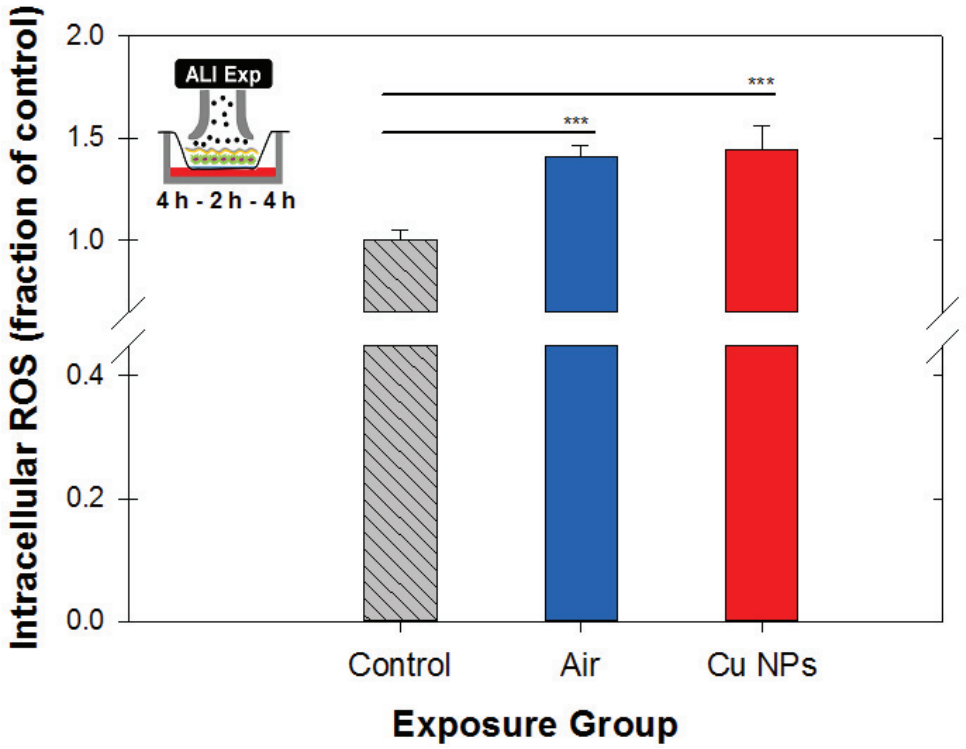

Figure 4-4: Intracellular reactive oxygen species (ROS) generation for control (incubator), air (particle-free), and copper nanoparticle (Cu NP) exposure groups for A549 cells sequentially exposed at the air-liquid interface (ALI) as evaluated by fluorescence detection with Carboxy-H_2DCFDA.

Note: A549 cells in the air and Cu NP exposure groups were sequentially exposed with an aerosol flow rate of 10 mL/min.

Post-exposure (0 h), carboxy-H_2DCFDA fluorescence detection was used to evaluate ROS generation. ROS Levels were then normalized to the control cells (maintained in the incubator at 37ºC and 5% CO_2).

Data are expressed as mean ± SD; ***statistically significant difference compared to control (***$p < 0.001$).

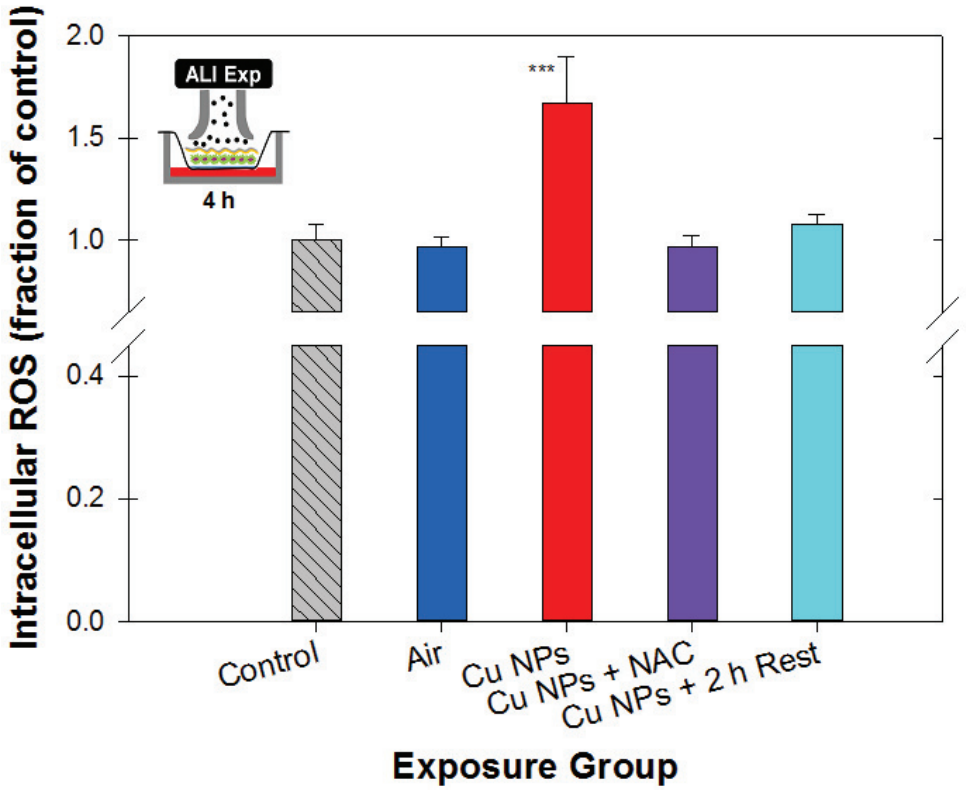

Figure 4-5: Intracellular reactive oxygen species (ROS) generation for control (incubator), air (particle-free), and copper nanoparticle (Cu NP), copper nanoparticle with N-Acetyl Cysteine (Cu NPs + NAC), and copper nanoparticle with rest (Cu NP + 2h rest) exposure groups for A549 cells exposed for 4 h at the air-liquid interface (ALI) as evaluated by fluorescence detection with Carboxy-H_2DCFDA.

Note: A549 cells in the air and Cu NP exposure groups were subjected to a single exposure for 4h with an aerosol flow rate of 10 mL/min.

Post-exposure (0 h), carboxy-H_2DCFDA fluorescence detection was used to evaluate ROS generation. ROS Levels were then normalized to the control cells (maintained in the incubator at 37ºC and 5% CO_2).

Data are expressed as mean ± SD; ***statistically significant difference compared to control (***$p < 0.001$).

4.2.3 Lactate Dehydrogenase (LDH)

LDH analysis revealed a similar trend to that which was observed in the analysis of intracellular ROS generation. Expressed as a fraction of the level of LDH generated by incubator control cells, sequential (4-2-4) exposure resulted in LDH levels of 10.63 for NP-free (filtered) air exposed cells, and 2.0 for NP exposed cells. Expressed as a fraction of the level of LDH generated by incubator control cells, single (4 h) exposure resulted in LDH levels of 2.8 for NP-free (filtered) air exposed cells, 35.7 for NP exposed cells, and 1.8 for NP exposed cells in the presence of NAC. LDH generated by NP exposed cells was significantly higher than LDH generated by NP-free (filtered) air exposed cells for single exposure only. LDH levels resulting from sequential exposure are shown in **Figure 4-6**, and **Figure 4-7** shows results from a single exposure.

While it was expected that a significant difference in LDH levels would be observed between NP and NP-free (filtered) air exposures, it was not originally expected that there would be a significant difference between these two exposure groups for single exposure (4 h) only, and not sequentially (4-2-4) exposed cells. However, after the results were obtained for the intracellular ROS levels, it was believed that LDH levels would follow a similar pattern. Single exposure (4 h) cells did follow this similar pattern, where NP exposed cells had a significantly higher level of LDH generated compared to NP-free (filtered) air exposed cells. Most important to note for this pattern is that cells that were exposed to NPs in the presence of a ROS scavenger (NAC) displayed levels of LDH comparable to that of NP-free (filtered) air exposed cells. This result lends further

evidence to the hypothesis that the mechanism of NP toxicity is ROS mediated. The higher LDH levels for NP-free (filtered) air exposed cells compared to NP exposed cells observed for sequentially exposed (4-2-4) cells may be due to the lack of centrifugation of the apical PBS wash collected following the NP-free (filtered) air exposure. This is the only set of samples for which there was an omission of the centrifugation step – all other samples were centrifuged, and their corresponding exposure groups were unaffected by this one-time omission. Without the centrifugation of this apical wash, it is possible that there were cells present in this wash, and these samples would have measured LDH found within those cells in addition to the LDH that was excreted from any damaged cells. Despite this fact, there is still a significant 12.5-fold increase in LDH levels for single (4 h) NP exposed cells, as compared to single (4 h) NP-free (filtered) air exposed cells. The difference between sequentially (4-2-4) NP exposed cells and sequentially (4-2-4) NP-free (filtered) air exposed cells was not significant.

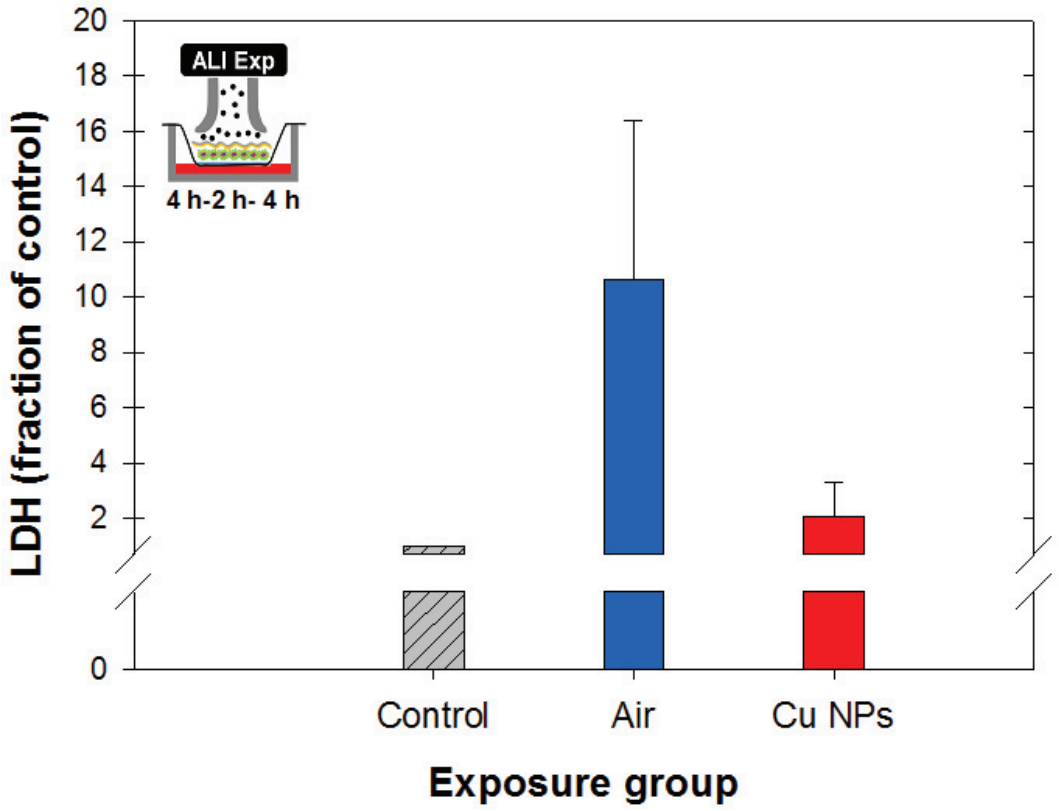

Figure 4-6: Apical lactate dehydrogenase (LDH) levels resulting from a sequential exposure.

Note: A549 cells in the air and Cu NP exposure groups were sequentially exposed with an aerosol flow rate of 10 mL/min.

Post-exposure (0 h), a commercial kit was used to evaluate LDH levels. LDH levels were then normalized to the control cells (maintained in the incubator at 37ºC and 5% CO_2).

Data are expressed as mean ± SD.

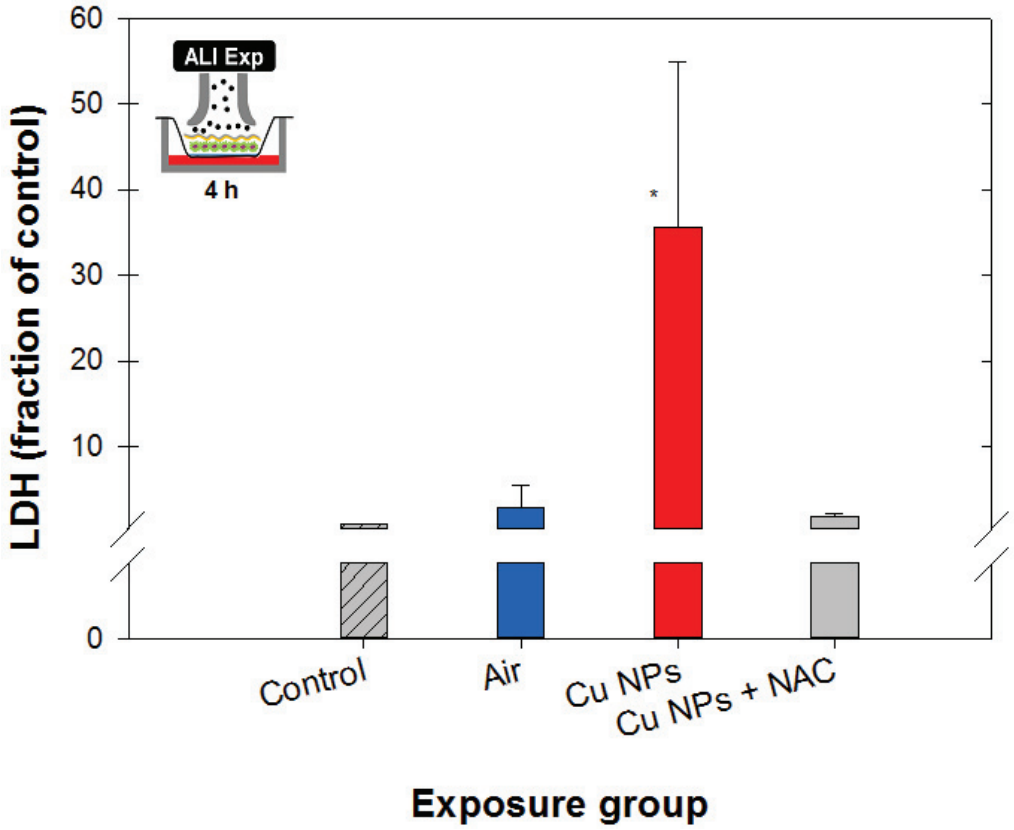

Figure 4-7: Apical lactate dehydrogenase (LDH) levels resulting from a single exposure**.**

Note: A549 cells in the air and Cu NP exposure groups were subjected to a single exposure for 4h with an aerosol flow rate of 10 mL/min.

Post-exposure (0 h), a commercial kit was used to evaluate LDH levels. LDH levels were then normalized to the control cells (maintained in the incubator at 37ºC and 5% CO_2).

Data are expressed as mean ± SD; *statistically significant difference compared to NP-free (filtered) air exposure (*$p < 0.05$).

4.2.4 Dosimetry

Cellular dosimetry was measured, and the dose of Cu NPs delivered to the monolayer of A549 cells was found to be 0.1 μg/cm^2 over a 4 hr period. This low dose is of extreme importance, as the manner in which traditional toxicology studies are carried out misses the real-world scenarios that afford a more realistic simulation of the low-dose human exposures that would occur in the real world. Traditional studies typically have an acute exposure with a high dose of NP. While these studies may indicate a large toxic response observed in the cells, this information is not as easily related to the real world human exposures that we need to have more insight into.

4.3 Secondary Toxicity Response (Cardiovascular and Systemic)

4.3.1 Cardiac Cytotoxicity

Exposure medium containing Cu ions and biological mediators released from A549 cells after NP delivery was collected to investigate its potential cytotoxicity on cardiovascular cells. Two cell lines (HUVEC and H9C2) were grown in the exposure medium. There did not appear to be any effect on the viability of HUVEC or H9C2 cells as a result of treatment with NP exposure media. A summary of cell viability determined by FACS analysis for HUVEC cells is shown in **Figure 4-8**. The FACS analysis used to produce Figure 4-8 is shown in **Figure 4-9**. A summary of cell viability determined by FACS analysis for H9C2 cells is shown in **Figure 4-10**. The FACS analysis used to produce Figure 4-10 is shown in is shown in **Figure 4-11**.

Through FACS analysis, it is possible to ascertain the extent and type of (early or late/necrotic) apoptosis in the exposed cells. Translocation of membrane phospholipid

phosphatidylserine (PS) from the inside to the outside of the plasma membrane is one of the earlier events of apoptosis, whereas later stages of cell death (due to apoptotic or necrotic processes) are accompanied by the loss of membrane integrity (122). Annexin V is a binding protein with a high affinity for PS that gives an indication of early apoptosis, as fluorochrome-labelled Annexin V allows detection of exposed PS using flow cytometry (122). Vital dyes such as propidium iodide (PI) are used to illustrate the later stages of cell death, due to the fact that PI permeates the membranes of dead or damaged cells, but is excluded by viable cells with intact membranes (122). Thus, staining with both Annexin V and PI allow for distinction between cells that are considered viable, cells in early apoptosis, and cells that are either in late apoptosis or dead. Cells that are considered viable are Annexin V and PI negative, cells in early apoptosis are Annexin V positive and PI negative, damaged/necrotic cells are Annexin V negative and PI positive, while cells that are in late apoptosis are Annexin V and PI positive (122). With this in mind, Figures 4-9 and 4-11 will show alive or viable cells in the bottom left quadrant, cells in early apoptosis in the upper left quadrant, cells in late apoptosis in the upper right quadrant, and damaged/necrotic cells in the lower right quadrant. Figures 4-9 and 4-11 also show that no significant difference in cell viability, early apoptosis, late apoptosis, and damaged/necrotic cells was observed for HUVEC or H9C2 cells.

CD54 is also known as Intercellular Adhesion Molecule 1 (ICAM-1), and is a protein that is normally present in low concentrations on the surface of endothelial cells and is important for the transendothelial migration of leukocytes to sites of inflammation (125). Concentrations are greatly increased as a result of cytokine stimulation (125), and it is

possible that cytokines (small proteins that are secreted by cells and elicit an effect on other cells (126)) could be present in higher concentrations in NP exposure media as compared to NP-free (filtered) air exposure media. Effects of NP exposure on the expression of Cluster of Differentiation (CD54) in HUVEC cells is shown in **Figure 4-12**. It is shown in Figure 4-12 that there was no difference in CD54 expression between the incubator control, NP-free (filtered) air exposure, and NP exposure groups

Figure 4-13 shows microscopic images of H9C2 cells before and after treatment with exposure media. Additionally, cellular viability for H9C2 cells using the Presto Blue viability reagent is shown in **Figure 4-14**. No significant difference was observed in cell viability for H9C2 cells using Presto Blue viability reagent, which is in agreement with the FACS analysis done for H9C2 cells (Figures 4-10, and 4-11).

The lack of effect on the viability of HUVEC and H9C2 cells cultured in sequential (4-2-4) exposure media may be evidence that cardiovascular damage resulting due to NP exposure may be more closely linked to the levels of ROS and LDH produced due to exposure. The pulmonary pattern observed was that there was a much larger increase in ROS and LDH levels produced during a single exposure (4 h) as compared to a sequential exposure (4-2-4) for NP exposed cells compared to NP-free (filtered) air exposed cells. Considering this pattern, it is possible that there was no effect on the viability of the HUVEC or H9C2 cells due to the fact that there was not a significant difference in ROS or LDH levels for sequentially (4-2-4) NP exposed cells compared to sequentially (4-2-4) NP-free (filtered) air exposed cells.

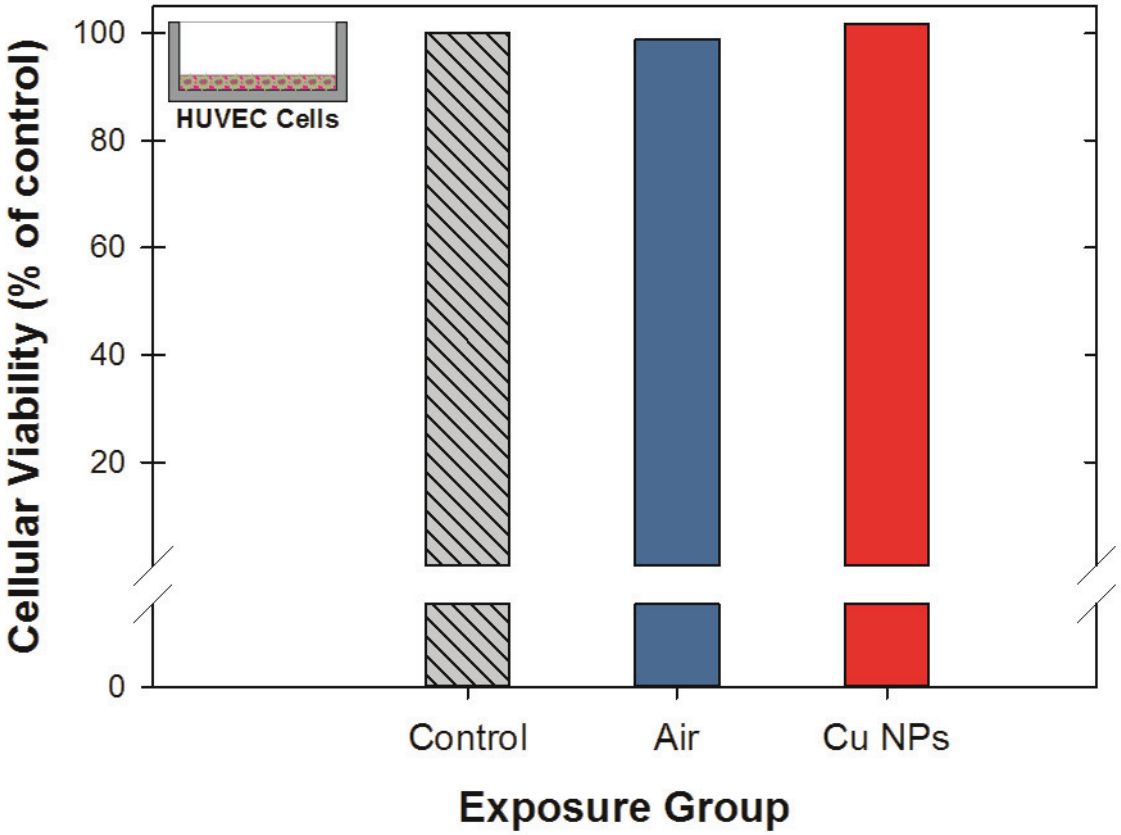

Figure 4-8: Cell viability determined by fluorescence-activated cell sorting (FACS) analysis for HUVEC cells cultured in control (incubator), air (particle-free), or copper nanoparticle (Cu NP) 4-2-4 exposure medium.

Note: HUVEC cells were plated in 2.0 mL of media in 35 mm dishes 24 h prior to treatment with 150 µL of each exposure group's media for 24 h.

Cells were harvested and FACS analysis was done using Annexin V and propidium iodide. Cellular viability was then normalized to the control cells (cultured in incubator control exposure medium).

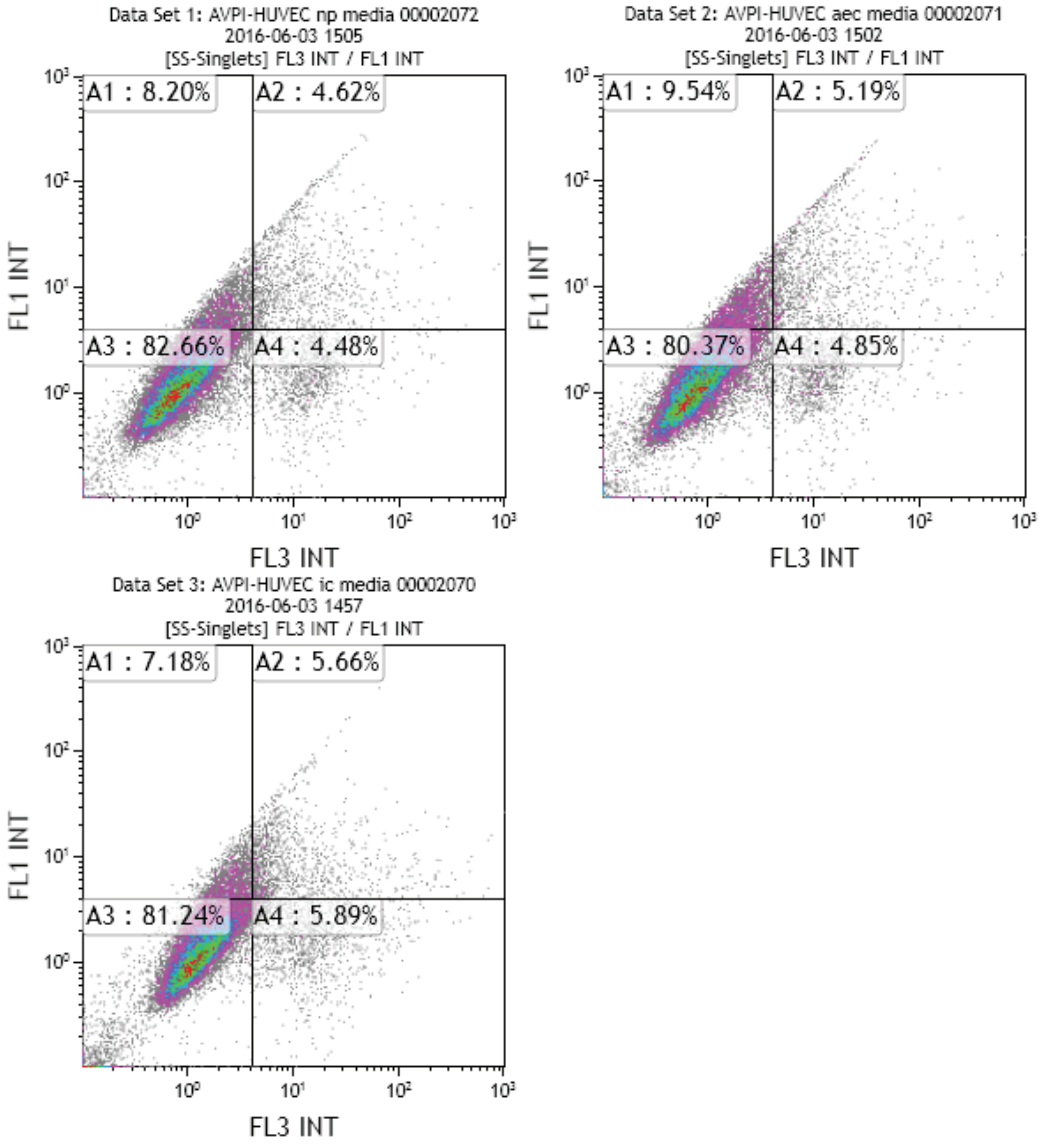

Figure 4-9: Fluorescence-activated cell sorting (FACS) analysis using Annexin V and propidium iodide for HUVEC cells cultured in control (incubator), air (particle-free), or copper nanoparticle (Cu NP) 4-2-4 exposure medium.

Note: HUVEC cells were plated in 2.0 mL of media in 35 mm dishes 24 h prior to treatment with 150 µL of each exposure group's media for 24 h.

Cells were harvested and FACS analysis was done using Annexin V and propidium iodide.

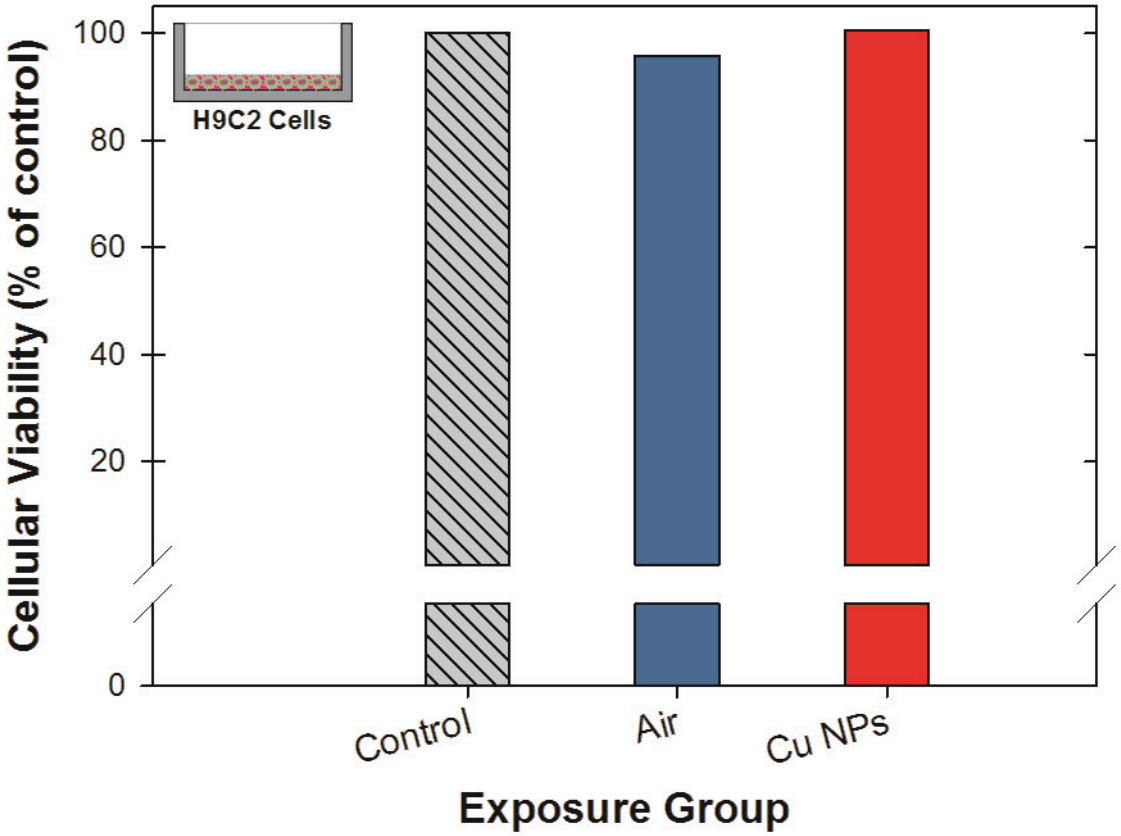

Figure 4-10: Cell viability determined by fluorescence-activated cell sorting (FACS) analysis for H9C2 cells cultured in control (incubator), air (particle-free), or copper nanoparticle (Cu NP) 4-2-4 exposure medium.

Note: H9C2 cells were plated in 60 mm dishes 24 h prior to treatment with 100 µL of each exposure group's media for 24 h.

Cells were harvested and FACS analysis was done using Annexin V and propidium iodide. Cellular viability was then normalized to the control cells (cultured in incubator control exposure medium).

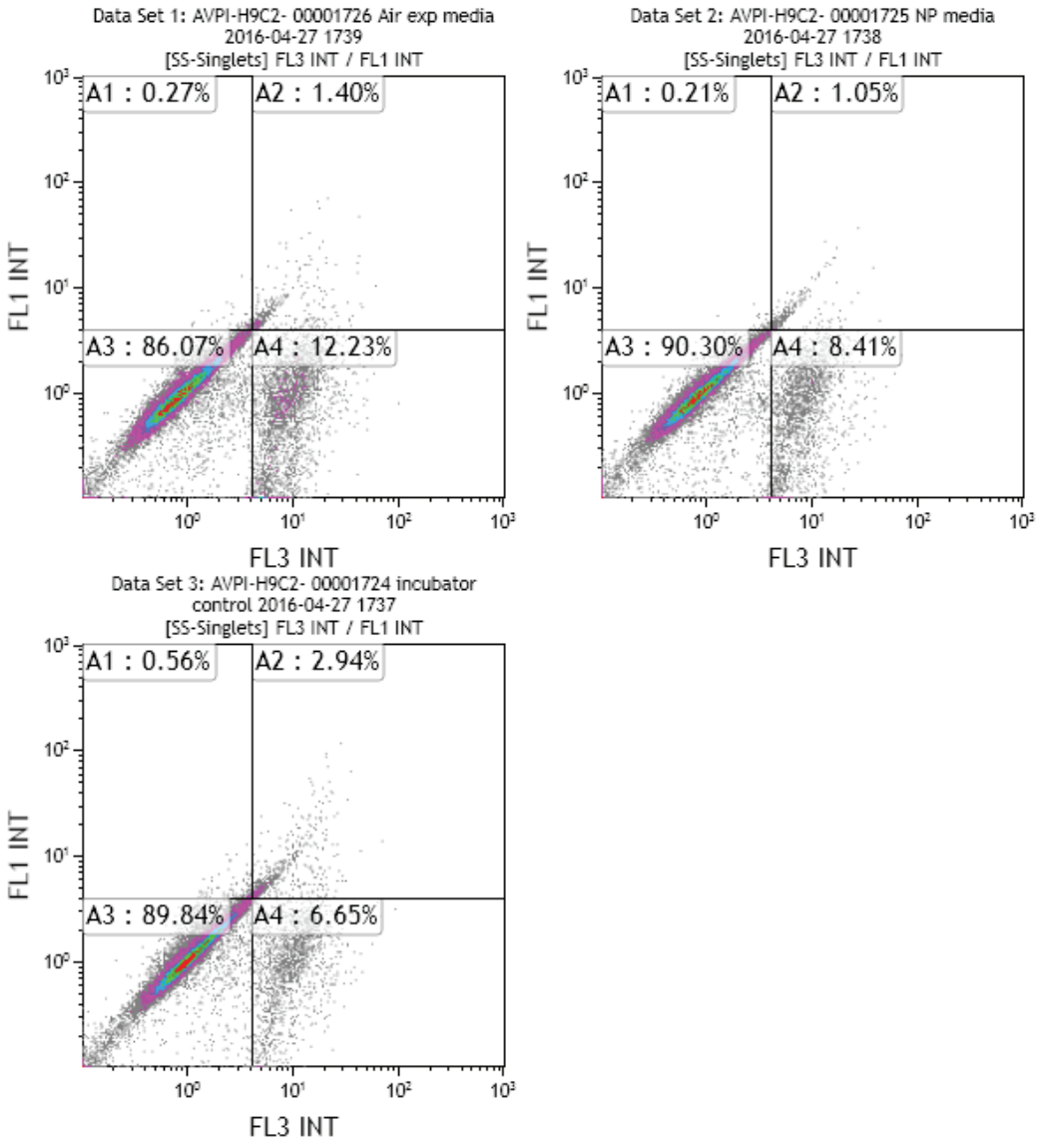

Figure 4-11: Fluorescence-activated cell sorting FACS analysis using Annexin V and propidium iodide for H9C2 cells cultured in control (incubator), air (particle-free), or copper nanoparticle (Cu NP) 4-2-4 exposure medium.

Note: HUVEC cells were plated in 2.0 mL of media in 35 mm dishes 24 h prior to treatment with 150 µL of each exposure group's media for 24 h.

Cells were harvested and FACS analysis was done using Annexin V and propidium iodide.

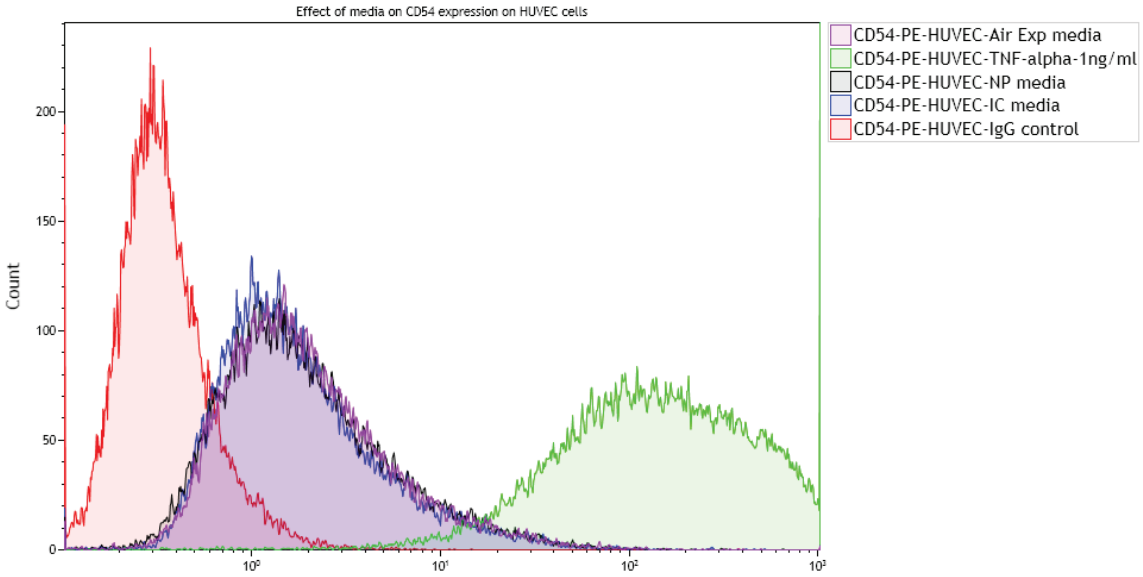

Figure 4-12: Expression of CD54 determined by fluorescence-activated cell sorting (FACS) analysis for HUVEC cells cultured in control (incubator), air (particle-free), or copper nanoparticle (Cu NP) 4-2-4 exposure medium.

Note: One day prior to treatment 500,000 HUVEC cells were plated in a 35 mm dish. The following day, cells were treated with various treatment media (10% treatment media in main media) for 8 h, and FACS analysis was done using PE-anti human CD 54 as a positive control, and PE-anti Mouse IgG as a negative control to observe CD54 expression.

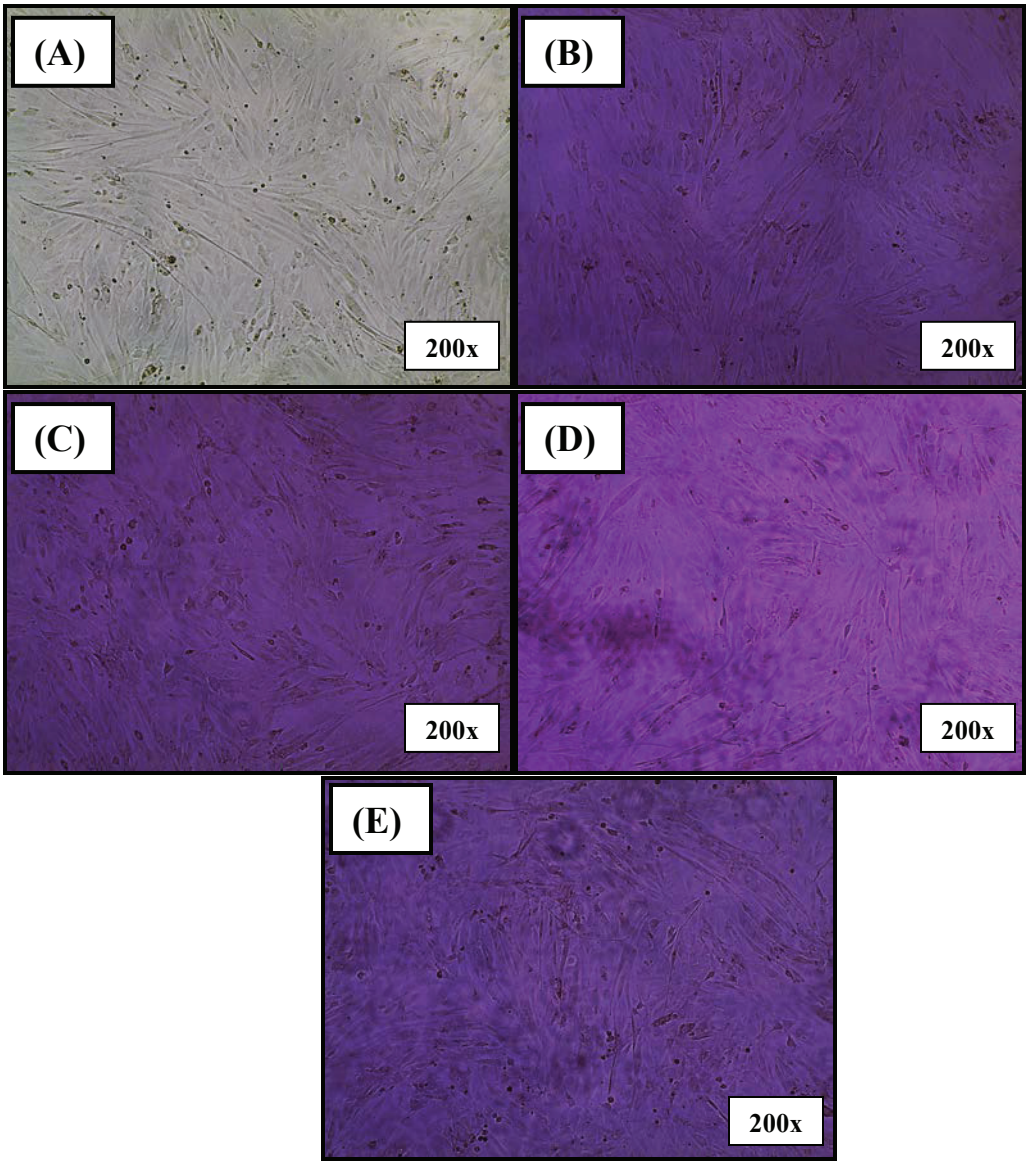

Figure 4-13: (A) H9C2 Pre-Treatment, (B) DMEM control treatment, (C) Incubator Control treatment, (D) Air treatment, and (E) Nanoparticle (NP) treatment; imaged at 200x magnification.

Note: 12,000 H9C2 rat cardiomyoblasts per well were seeded for 75% confluency two days (day -1) prior to treatment in 100 µL of DMEM-HG media (supplemented with 10% FBS). To initiate H9C2 differentiation, the media in all wells was replaced the following day (day 0) with DMEM-HG media that was free of FBS. to initiate H9C2 differentiation. This media was renewed on day 3. Treatment with exposure media began on day 6, where media was replaced with either DMEM-HG (0% FBS), 1/4 treated media in 3/4 DMEM-HG (0% FBS), 1/2 treated media, or 100% treated media for an additional 24 h. Following 24 h of treatment, media was replaced with DMEM-HG (0% FBS) with 10% Presto Blue Viability Reagent for 3 h; spectral colorimetric analysis (560,590 reading) was conducted on the Synergy H4 Hybrid Reader.

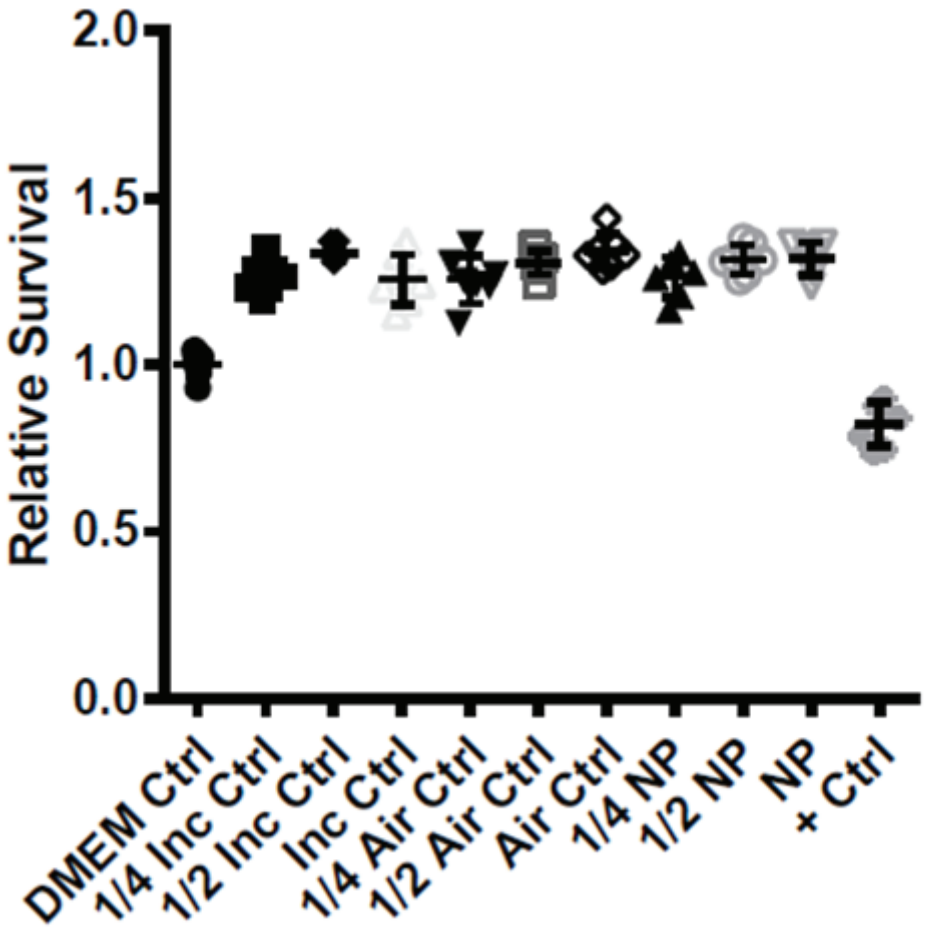

Figure 4-14: Cell viability determined by Presto Blue Viability Reagent in H9C2 rat cardiomyoblasts cultured in control (incubator), air (particle-free), or copper nanoparticle (Cu NP) 4-2-4 exposure medium.

Note: 12,000 H9C2 rat cardiomyoblasts per well were seeded for 75% confluency two days (day -1) prior to treatment in 100 µL of DMEM-HG media (supplemented with 10% FBS). To initiate H9C2 differentiation, the media in all wells was replaced the following day (day 0) with DMEM-HG media that was free of FBS. to initiate H9C2 differentiation. This media was renewed on day 3. Treatment began on day 6, where media was replaced with either DMEM-HG (0% FBS), 1/4 treated media in 3/4 DMEM-HG (0% FBS), 1/2 treated media, or 100% treated media for an additional 24 h. Following 24 h of treatment, media was replaced with DMEM-HG (0% FBS) with 10% Presto Blue Viability Reagent for 3 h; spectral colorimetric analysis (560,590 reading) was conducted on the Synergy H4 Hybrid Reader.
Treatment groups and the positive control containing main media and cells (+ Ctrl) were normalized to the main media only control (DMEM Ctrl). FBS is not present in the media of treatment or control groups, as differentiation requires serum deprivation.

CHAPTER 5 STRENGTHS AND LIMITATIONS

5.1 LIMITATIONS

Limitations of this work include using NPs of only one size, and of only one metal composition, only a single dose was used, and that the environmental dose was not verified. A complete toxicological investigation of Cu-based NPs should include multiple doses in order to understand the dose-response of these NPs. While the potential environmental dose (dose expected from airborne exposure in the environment, separate from occupational settings) is low and more closely mimicked through this study, it is important that further work be carried out to verify and compare how closely the environmental dose is mimicked. Ideally, in order to gather a full picture of how these Cu-based NPs affect human health and well-being, it is important that further investigations are carried out.

Despite *in vivo* work being the gold standard for toxicological assessments, the use of rodents (such as mice, rats) does not provide information specifically on how human cells interact with NPs. Use of these rodents does has benefits, however there are important differences in physiology between these species and humans and it is crucial that an understanding of how specifically human cells react is obtained. This limitation leads directly into the strengths of this study.

5.2 Strengths

The major strength of this study is that the experimental set up used is an innovative way to investigate the cellular responses of human lung cells and cardiovascular cells as a result of airborne NP exposure. Using the set up described earlier, the NPs can be delivered to the cells without a significant change in their properties due to the delivery method (as can be the case in submerged cell exposure). The experimental set up used enables mimicking the *in vivo* scenario of inhalation exposure more realistically, specifically the repeated low-dose exposure scenario. Instead of looking at the negative consequences of a one time, high dose of NPs, the approach enables the investigation of how problematic a continual, low exposure to NPs can be to human health. This is especially important, as NP-containing products and technology are on the rise, and presumably our exposures to NPs will increase and persist. The experiments undertaken in this project has furthered our understanding of the mechanisms of cardiovascular effects as a result of airborne NP exposure, and they are a stepping stone towards future work as well as an indication of the direction of future work that should be carried out.

This project has helped to aid in the development of an efficient way to screen metal-based NPs for pulmonary and cardiovascular toxicity, as well as provided an appropriate way to observe a more realistic exposure scenario using cells. As was mentioned earlier in the introduction, the design of the system used in the experiment provides a useful tool to overcome some of the main limitations present in current *in vitro* models for inhalation exposure. It more accurately reflects the realistic human physiological exposure scenario.

CHAPTER 6 CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

In conclusion of this thesis project, it is prudent to identify that more work must be undertaken to further the knowledge regarding cardiopulmonary toxicity resulting from the inhalation exposure of airborne NPs that are of this miniscule size. Compared to NPs of similar composition but larger size, the NPs studied in this project had produced different biological responses. While this study has indicated that perhaps the immediate effects of inhalation exposure to Cu NPs that are approximately 4 nm in size may not be as detrimental as previously postulated, it is possible that the longer term effects could be abundant and overlooked in traditional investigations.

This project has opened the doorways in terms of perspectives on toxic response to NPs. Perhaps the most important measure of NP toxicity is not, in fact, cellular viability. While the cellular viability did not appear to change between a single exposure and a sequential exposure, the levels of intracellular ROS generated are significantly different between these two exposure scenarios. This highlights the importance of evaluating toxic responses using assays that measure endpoints outside of cellular viability, in order to fully understand the true toxic response of certain NPs. This work has also given indication and reason to believe that immediately observable toxic effects are not the only effects that should be observed. For example, the effects of airborne NP exposure on cell cycle such as the ability or a cell to function normally and replicate, as well as the ability of a cell to recover from injury.

With further investigations into the long-term and potentially lasting effects of NP exposure, it will be possible to have a more complete picture as to the true toxic potential of metal NPs.

To compliment the work that has been undertaken for this master's thesis, there are several types of experiments that are proposed to move forward and gain further insight into the effects resulting from airborne exposure to metal NPs.

6.2 Future Work

6.2.1 Cell Cycle Analysis

In order to investigate the long-term effects of inhalation exposure to airborne Cu NPs, it is important that a cell cycle analysis be done on cells that have been exposed to these NPs. This information will provide insight into the cell's ability to recover from NP toxicity, and/or the permanent impairments that may be caused due to NP exposure. For example, perhaps the cell cycle will be interrupted at certain checkpoints due to DNA damage. Investigations into the dose required to cause anomalies in the cell cycle, as well as the amount of time post-exposure that the anomalies occur would aid in the understanding of the toxicity associated with these NPs. Additionally, it is vital that an understanding of the ability of lung cells to recover is obtained. For example, do only a small number of cells show an anomaly in their cell cycle analysis, with most cells recovering, or do all cells eventually become unable to replicate and divide normally.

6.2.2 Colony Formation Analysis

In addition to cell cycle analysis, colony formation analysis would be of benefit in understanding the long term effects of inhalation exposure to airborne Cu NPs. The results of this project have shown that a small percentage of cells die outright as a result of NP exposure, but it is still largely unknown as to what acute or persistent problems are arising in the cells that survive exposure. The ability of these cells to survive and proliferate are of interest; the percentage of cells that are able to survive and proliferate normally would give an indication as to how dangerous NP exposure is further down the road once exposure has ceased and the body has had time to recover. In order to truly understand the level of danger that these NPs pose to human health, an understanding of how lasting their effects are must be obtained.

6.2.3 Differing Metal Compositions

Building on this work, this system can be used as a screening method for NP toxicity of varying compositions. The metal electrodes found within the SDS can be exchanged for electrodes of differing compositions with ease, allowing for an appropriate way to deliver the NPs in the same fashion and changing only metal composition while maintaining other factors. With this in mind, it will be possible to determine which metals appear to be the most toxic and detrimental to human health, and appropriately and efficiently recommend which metals should be investigated in *in vivo* animal models first.

6.2.4 Cardiovascular Analysis of 4hr Exposure Media

While the exposure media obtained during the 4-2-4 exposure of A549 cells to Cu NPs did not appear to have any effects on the viability of HUVEC or H9C2 cells, it is important to note that there was an observed and significant difference between the intracellular levels of ROS generated by A549 cells during 4-2-4 exposure and 4 h exposure. Due to this difference, further work should be done to test the viability of cardiovascular cells treated with this 4 h exposure media. This work would help to further our knowledge of the mechanisms through which cardiovascular morbidity and mortality is increased in response to inhalation exposure of metal NPs. The 4-2-4 exposure media has twice the amount of Cu NPs present as compared to the 4 h single exposure media; therefore, if there were an observable and significant difference in the viability of cardiovascular cells treated with 4-2-4 media as compared to 4 h media it would appear that the NPs themselves were not the major cause of the cardiovascular toxicity. It would be reasonable to conclude in that case that the oxidative stress or inflammatory mediators released by lung cells were the main cause of cardiovascular toxicity. It is possible that less ROS formation and LDH release were observed in the case of sequential exposure, as compared to single exposure (despite twice the amount of copper being present) due to an adaptive response by the A549 cells. Following the first exposure, the cells are given a "rest" period in the incubator where there are no new NPs being delivered to the cells. Perhaps during this time, the NP-exposed cells are able to partially recover and therefore the ROS levels following the second exposure are not as high as would be expected.

BIBLIOGRAPHY

1. Lövestam G RH, Roebben G, Klüttgen BS, Gibson N, Putaud JP, et al. Considerations on a Definition of Nanomaterial for Regulatory Purposes. Luxembourg: European Commission Joint Research Centre. 2010.

2. Oberdorster G, Oberdorster E, Oberdorster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect. 2005 Jul;113(7):823-39.

3. Oberdorster G, Stone V, Donaldson K. Toxicology of nanoparticles: a historical perspective. Nanotoxicology. 2007;1(1):2-25.

4. Schrand AM, Rahman MF, Hussain SM, Schlager JJ, Smith DA, Syed AF. Metal-based nanoparticles and their toxicity assessment. Wiley interdisciplinary reviews Nanomedicine and nanobiotechnology. 2010;2(5):544-68.

5. Fahmy B, Cormier SA. Copper oxide nanoparticles induce oxidative stress and cytotoxicity in airway epithelial cells. Toxicology in Vitro. 2009;23(7):1365-71.

6. Yi H, Leunissen J, Shi G, Gutekunst C, Hersch S. A novel procedure for pre-embedding double immunogold-silver labeling at the ultrastructural level. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 2001;49(3):279-84.

7. Voskerician G, Shive MS, Shawgo RS, von Recum H, Anderson JM, Cima MJ, et al. Biocompatibility and biofouling of MEMS drug delivery devices. Biomaterials. 2003;24(11):1959-67.

8. Sharma P, Brown SC, Bengtsson N, Zhang Q, Walter GA, Grobmyer SR, et al. Gold-Speckled Multimodal Nanoparticles for Noninvasive Bioimaging. Chemistry of materials : a publication of the American Chemical Society. 2008;20(19):6087-94.

9. Hirsch LR, Stafford RJ, Bankson JA, Sershen SR, Rivera B, Price RE, et al. Nanoshell-Mediated Near-Infrared Thermal Therapy of Tumors under Magnetic Resonance Guidance. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(23):13549-54.

10. Loo C, Lin A, Hirsch L, Lee MH, Barton J, Halas N, et al. Nanoshell-enabled photonics-based imaging and therapy of cancer. Technology in cancer research & treatment. 2004;3(1):33-40.

11. Hainfeld JF, Slatkin DN, Smilowitz HM. The use of gold nanoparticles to enhance radiotherapy in mice. Physics in medicine and biology. 2004;49(18):N309-15.

12. Pissuwan D, Valenzuela S, Killingsworth M, Xu X, Cortie M. Targeted destruction of murine macrophage cells with bioconjugated gold nanorods. J Nanopart Res. 2007;9(6):1109-24.

13. Chen J, Wiley B, Li ZY, Campbell D, Saeki F, Cang H, et al. Gold Nanocages: Engineering Their Structure for Biomedical Applications. Advanced Materials. 2005;17(18):2255-61.

14. Tyner KM, Schiffman SR, Giannelis EP. Nanobiohybrids as delivery vehicles for camptothecin. Journal of Controlled Release. 2004;95(3):501-14.

15. Cushen M, Kerry J, Morris M, Cruz-Romero M, Cummins E. Evaluation and Simulation of Silver and Copper Nanoparticle Migration from Polyethylene Nanocomposites to Food and an Associated Exposure Assessment. Journal of Agricultural and Food Chemistry. 2014;62(6):1403-11.

16. Chatterjee AK, Sarkar RK, Chattopadhyay AP, Aich P, Chakraborty R, Basu T. A simple robust method for synthesis of metallic copper nanoparticles of high antibacterial potency against E. coli. Nanotechnology. 2012;23(8):085103.

17. Borkow G, Gabbay J. Putting copper into action: copper-impregnated products with potent biocidal activities. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2004 Nov;18(14):1728-30.

18. Xia T, Li N, Nel AE. Potential health impact of nanoparticles. Annual review of public health. 2009;30:137-50.

19. Lanone S, Rogerieux F, Geys J, Dupont A, Maillot-Marechal E, Boczkowski J, et al. Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines. Particle and Fibre Toxicology. 2009;6(1):14.

20. Wang B, Feng W-Y, Wang T-C, Jia G, Wang M, Shi J-W, et al. Acute toxicity of nano- and micro-scale zinc powder in healthy adult mice. Toxicology Letters. 2006;161(2):115-23.

21. Colvin VL. The potential environmental impact of engineered nanomaterials. Nature biotechnology. 2003 Oct;21(10):1166-70.

22. Tourinho PS, van Gestel CAM, Lofts S, Svendsen C, Soares AMVM, Loureiro S. Metal-based nanoparticles in soil: Fate, behavior, and effects on soil invertebrates. Environmental Toxicology and Chemistry. 2012;31(8):1679-92.

23. Mueller NC, Nowack B. Exposure Modeling of Engineered Nanoparticles in the Environment. Environmental science & technology. 2008;42(12):4447-53.

24. Gottschalk F, Nowack B. The release of engineered nanomaterials to the environment. Journal of Environmental Monitoring. 2011;13(5):1145-55.

25. Johnson AC, Bowes MJ, Crossley A, Jarvie HP, Jurkschat K, Jürgens MD, et al. An assessment of the fate, behaviour and environmental risk associated with sunscreen TiO2 nanoparticles in UK field scenarios. Science of The Total Environment. 2011;409(13):2503-10.

26. Gottschalk F, Sonderer T, Scholz RW, Nowack B. Modeled Environmental Concentrations of Engineered Nanomaterials (TiO2, ZnO, Ag, CNT, Fullerenes) for Different Regions. Environmental science & technology. 2009;43(24):9216-22.

27. Bakand S, Hayes A, Dechsakulthorn F. Nanoparticles: a review of particle toxicology following inhalation exposure. Inhal Toxicol. 2012;24(2):125-35.

28. Quadros ME, Marr LC. Environmental and Human Health Risks of Aerosolized Silver Nanoparticles. Journal of the Air & Waste Management Association (Air & Waste Management Association). 2010;60(7):770-81.

29. Maynard A. Nanotechnology: a Strategy for Addressing Risk. Woodrow-Wilson International Center for Scholars: Washington, DC. 2006.

30. Howe PDS. Silver and silver compounds : environmental aspects. Geneva: World Health Organization; 2002.

31. Carbone F, Barone A, Pagliara R, Beretta F, D'Anna A, D'Alessio A. Ultrafine Particles Formed by Heating Droplets of Simulated Ash Containing Metals. Environmental Engineering Science. 2008;25(10):1379-88.

32. Miller MR, Shaw CA, Langrish JP. From particles to patients: oxidative stress and the cardiovascular effects of air pollution. Future cardiology. 2012;8(4):577-602.

33. Kittelson DB. Engines and nanoparticles: a review. Journal of Aerosol Science. 1998;29(5–6):575-88.

34. Hesterberg TW, Long CM, Lapin CA, Hamade AK, Valberg PA. Diesel exhaust particulate (DEP) and nanoparticle exposures: What do DEP human clinical studies tell us about potential human health hazards of nanoparticles? Inhalation Toxicology. 2010;22(8):679-94.

35. U.S. EPA. Health Assessment Document for Diesel Engine Exhaust (Final 2002). US Environmental Protection Agency, Office of Research and Development, National Center for Environmental Assessment, Washington Office, Washington, DC,EPA/600/8-90/057F. 2002.

36. Liati A, Schreiber D, Dimopoulos Eggenschwiler P, Arroyo Rojas Dasilva Y. Metal particle emissions in the exhaust stream of diesel engines: an electron microscope study. Environmental science & technology. 2013;47(24):14495-501.

37. Organization WH. International Agency for Research on Cancer. Press release No 213. 2012.

38. Rönkkö T, Pirjola L, Ntziachristos L, Heikkilä J, Karjalainen P, Hillamo R, et al. Vehicle Engines Produce Exhaust Nanoparticles Even When Not Fueled. Environmental science & technology. 2014;48(3):2043-50.

39. Protection ICoR. Human respiratory tract model for radiological protection. A report of a Task Group of the International Commission on Radiological Protection. Annals of the ICRP. 1994;24(1-3):1-482.

40. Kendall M, Holgate S. Health impact and toxicological effects of nanomaterials in the lung. Respirology (Carlton, Vic). 2012;17(5):743-58.

41. Phillips JI, Green FY, Davies JC, Murray J. Pulmonary and systemic toxicity following exposure to nickel nanoparticles. Am J Ind Med. 2010;53(8):763-7.

42. Journeay WS, Goldman RH. Occupational handling of nickel nanoparticles: A case report. American Journal of Industrial Medicine. 2014;57(9):1073-6.

43. Antonini JM, Lewis AB, Roberts JR, Whaley DA. Pulmonary effects of welding fumes: Review of worker and experimental animal studies. American Journal of Industrial Medicine. 2003;43(4):350-60.

44. Antonini JM, Taylor MD, Millecchia L, Bebout AR, Roberts JR. Suppression in lung defense responses after bacterial infection in rats pretreated with different welding fumes. Toxicology and Applied Pharmacology. 2004;200(3):206-18.

45. Contreras GR, Chan-Yeung M. Bronchial Reactions to Exposure to Welding Fumes. Occupational and Environmental Medicine. 1997;54(11):836-9.

46. d'Errico A, Pasian S, Baratti A, Zanelli R, Alfonzo S, Gilardi L, et al. A Case-Control Study on Occupational Risk Factors for Sino-Nasal Cancer. Occupational and Environmental Medicine. 2009;66(7):448-55.

47. Hannu T, Piipari R, Kasurinen H, Keskinen H, Tuppurainen M, Tuomi T. Occupational asthma due to manual metal-arc welding of special stainless steels. European Respiratory Journal. 2005;26(4):736-9.

48. Langard S. Nickel-related cancer in welders. The Science of the total environment. 1994;148(2-3):303-9.

49. Kittelson D. On-road particles-characteristics and measurement. In: Proceedings of the International Workshop on Vehicle-related Nonoparticles and Environmental Health Seoul, Korea. 2007:47–71.

50. Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M, et al. Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. Circulation. 2004 Jun 1;109(21):2655-71.

51. Dockery DW, Pope CA, Xu X, Spengler JD, Ware JH, Fay ME, et al. An Association between Air Pollution and Mortality in Six U.S. Cities. New England Journal of Medicine. 1993;329(24):1753-9.

52. Samet JM, Dominici F, Curriero FC, Coursac I, Zeger SL. Fine Particulate Air Pollution and Mortality in 20 U.S. Cities, 1987–1994. New England Journal of Medicine. 2000;343(24):1742-9.

53. Nel A, Xia T, Mädler L, Li N. Toxic Potential of Materials at the Nanolevel. Science. 2006;311(5761):622-7.

54. Soto K, Garza KM, Murr LE. Cytotoxic effects of aggregated nanomaterials. Acta Biomaterialia. 2007;3(3):351-8.

55. Asgharian B, Price OT. Deposition of Ultrafine (NANO) Particles in the Human Lung. Inhalation Toxicology. 2007;19(13):1045-54.

56. Ayres JG, Borm P, Cassee FR, Castranova V, Donaldson K, Ghio A, et al. Evaluating the Toxicity of Airborne Particulate Matter and Nanoparticles by Measuring Oxidative Stress Potential—A Workshop Report and Consensus Statement. Inhalation Toxicology. 2008;20(1):75-99.

57. Baxter P. Hunter's Diseases of Occupations. 10th Edition ed: CRC Press; 2010.

58. Gwinn M, Vallyathan V. Nanoparticles: Health Effects - Pros and Cons. Environ Health Perspect. 2006;114(12):1818-25.

59. Silkoff PE, Zhang L, Dutton S, Langmack EL, Vedal S, Murphy J, et al. Winter air pollution and disease parameters in advanced chronic obstructive pulmonary disease panels residing in Denver, Colorado. The Journal of allergy and clinical immunology. 2005 Feb;115(2):337-44.

60. Gong H, Jr., Linn WS, Clark KW, Anderson KR, Geller MD, Sioutas C. Respiratory responses to exposures with fine particulates and nitrogen dioxide in the elderly with and without COPD. Inhal Toxicol. 2005 Mar;17(3):123-32.

61. Koenig JQ, Mar TF, Allen RW, Jansen K, Lumley T, Sullivan JH, et al. Pulmonary effects of indoor- and outdoor-generated particles in children with asthma. Environ Health Perspect. 2005;113(4):499-503.

62. Pietropaoli AP, Frampton MW, Hyde RW, Morrow PE, Oberdorster G, Cox C, et al. Pulmonary function, diffusing capacity, and inflammation in healthy and asthmatic subjects exposed to ultrafine particles. Inhal Toxicol. 2004;16 Suppl 1:59-72.

63. Byrne JD BJ. The significance of nanoparticles in particle-induced pulmonary fibrosis. Mcgill J Med 2008;11(1):43-50.

64. Oberdorster G, Oberdorster E, Oberdorster J. Nanotoxicology: An Emerging Discipline Evolving from Studies of Ultrafine Particles. Environmental Health Perspectives. 2005;113(7):823-39.

65. Anderson PJ, Wilson JD, Hiller FC. Respiratory tract deposition of ultrafine particles in subjects with obstructive or restrictive lung disease. Chest. 1990;97(5):1115-20.

66. Carvalho TC, Peters JI, Williams Iii RO. Influence of particle size on regional lung deposition – What evidence is there? International Journal of Pharmaceutics. 2011;406(1–2):1-10.

67. Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, et al. Translocation of inhaled ultrafine particles to the brain. Inhal Toxicol. 2004;16(6-7):437-45.

68. Kreyling WG, Semmler M, Erbe F, Mayer P, Takenaka S, Schulz H, et al. Translocation of Ultrafine Insoluble Iridium Particles from Lung Epithelium to Extrapulmonary Organs is Size Dependent but Very Low. Journal of Toxicology and Environmental Health, Part A. 2002;65(20):1513-30.

69. Brown JS, Zeman KL, Bennett WD. Ultrafine particle deposition and clearance in the healthy and obstructed lung. American journal of respiratory and critical care medicine. 2002 Nov 166(9):1240-7.

70. Oberdorster G, Finkelstein JN, Johnston C, Gelein R, Cox C, Baggs R, et al. Acute pulmonary effects of ultrafine particles in rats and mice. Research report (Health Effects Institute). 2000 Aug(96):5-74; disc 5-86.

71. Nemmar A, Hoet PH, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, et al. Passage of inhaled particles into the blood circulation in humans. Circulation. 2002;105(4):411-4.

72. Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Lunts A, et al. Extrapulmonary translocation of ultrafine carbon particles following whole-body inhalation exposure of rats. Journal of toxicology and environmental health Part A. 2002;65(20):1531-43.

73. Bantz C, Koshkina O, Lang T, Galla H-J, Kirkpatrick CJ, Stauber RH, et al. The surface properties of nanoparticles determine the agglomeration state and the size of the particles under physiological conditions. Beilstein Journal of Nanotechnology. 2014;5:1774-86.

74. Xia T, Kovochich M, Liong M, Meng H, Kabehie S, George S, et al. Polyethyleneimine Coating Enhances the Cellular Uptake of Mesoporous Silica Nanoparticles and Allows Safe Delivery of siRNA and DNA Constructs. ACS Nano. 2009;3(10):3273-86.

75. Kendall K, Kendall M, Rehfeldt F. Adhesion of Cells, Viruses and Proteins Dordrecht: Springer; 2011. Available from: http://public.eblib.com/EBLPublic/PublicView.do?ptiID=645897.

76. Manke A, Wang L, Rojanasakul Y. Mechanisms of Nanoparticle-Induced Oxidative Stress and Toxicity. BioMed Research International. 2013;2013:15.

77. Anreddy RN, Yellu NR, Devarakonda KR. Oxidative biomarkers to assess the nanoparticle-induced oxidative stress. Methods in molecular biology (Clifton, NJ). 2013;1028:205-19. PubMed PMID: 23740122. Epub 2013/06/07. eng.

78. Sarkar A, Ghosh M, Sil PC. Nanotoxicity: oxidative stress mediated toxicity of metal and metal oxide nanoparticles. Journal of nanoscience and nanotechnology. 2014 Jan;14(1):730-43. PubMed PMID: 24730293. Epub 2014/04/16. eng.

79. Kim S, Choi JE, Choi J, Chung K-H, Park K, Yi J, et al. Oxidative stress-dependent toxicity of silver nanoparticles in human hepatoma cells. Toxicology in Vitro. 2009 9//;23(6):1076-84.

80. Fridovich I. The biology of oxygen radicals. Science. 1978;201(4359):875-80.

81. Burton GJ, Jauniaux E. Oxidative stress. Best Practice & Research Clinical Obstetrics & Gynaecology. 2011;25(3):287-99.

82. Donaldson K, Tran CL. Inflammation caused by particles and fibers. Inhal Toxicol. 2002 Jan;14(1):5-27.

83. Schins RP. Mechanisms of genotoxicity of particles and fibers. Inhal Toxicol. 2002;14(1):57-78.

84. Wilson MR, Lightbody JH, Donaldson K, Sales J, Stone V. Interactions between ultrafine particles and transition metals in vivo and in vitro. Toxicol Appl Pharmacol. 2002;184(3):172-9.

85. Sioutas C, Delfino RJ, Singh M. Exposure Assessment for Atmospheric Ultrafine Particles (UFPs) and Implications in Epidemiologic Research. Environmental Health Perspectives. 2005;113(8):947-55.

86. Stone V, Shaw J, Brown DM, Macnee W, Faux SP, Donaldson K. The role of oxidative stress in the prolonged inhibitory effect of ultrafine carbon black on epithelial cell function. Toxicology in vitro : an international journal published in association with BIBRA. 1998;12(6):649-59.

87. Donaldson K, Stone V, Clouter A, Renwick L, MacNee W. Ultrafine particles. Occupational and Environmental Medicine. 2001;58(3):211-6.

88. Risom L, Moller P, Loft S. Oxidative stress-induced DNA damage by particulate air pollution. Mutation research. 2005;592(1-2):119-37.

89. Saber AT, Jacobsen NR, Jackson P, Poulsen SS, Kyjovska ZO, Halappanavar S, et al. Particle-induced pulmonary acute phase response may be the causal link between particle inhalation and cardiovascular disease. Wiley interdisciplinary reviews Nanomedicine and nanobiotechnology. 2014;6(6):517-31.

90. Fang SC, Cassidy A, Christiani DC. A Systematic Review of Occupational Exposure to Particulate Matter and Cardiovascular Disease. International Journal of Environmental Research and Public Health. 2010;7(4):1773-806.

91. Packard RR, Libby P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. Clinical chemistry. 2008;54(1):24-38.

92. Taubes G. Cardiovascular disease. Does inflammation cut to the heart of the matter? Science. 2002;296(5566):242-5.

93. Arora S, Rajwade JM, Paknikar KM. Nanotoxicology and in vitro studies: The need of the hour. Toxicology and Applied Pharmacology. 2012;258(2):151-65.

94. Dhawan A, Sharma V. Toxicity assessment of nanomaterials: methods and challenges. Analytical and bioanalytical chemistry. 2010 Sep;398(2):589-605.

95. Jones CF, Grainger DW. In vitro assessments of nanomaterial toxicity. Advanced Drug Delivery Reviews. 2009;61(6):438-56.

96. Lenz A, Karg E, Lentner B, Dittrich V, Brandenberger C, Rothen-Rutishauser B, et al. A dose-controlled system for air-liquid interface cell exposure and application to zinc oxide nanoparticles. Particle and Fibre Toxicology. 2009;6(1):32.

97. Limbach LK, Li Y, Grass RN, Brunner TJ, Hintermann MA, Muller M, et al. Oxide nanoparticle uptake in human lung fibroblasts: effects of particle size, agglomeration, and diffusion at low concentrations. Environmental science & technology. 2005;39(23):9370-6.

98. Teeguarden JG, Hinderliter PM, Orr G, Thrall BD, Pounds JG. Particokinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments. Toxicological sciences : an official journal of the Society of Toxicology. 2007;95(2):300-12.

99. Bitterle E, Karg E, Schroeppel A, Kreyling WG, Tippe A, Ferron GA, et al. Dose-controlled exposure of A549 epithelial cells at the air-liquid interface to airborne ultrafine carbonaceous particles. Chemosphere. 2006 Dec;65(10):1784-90.

100. Aufderheide M, Mohr U. CULTEX--an alternative technique for cultivation and exposure of cells of the respiratory tract to airborne pollutants at the air/liquid interface. Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie. 2000 June;52(3):265-70.

101. Fiegel J, Ehrhardt C, Schaefer UF, Lehr CM, Hanes J. Large porous particle impingement on lung epithelial cell monolayers--toward improved particle characterization in the lung. Pharmaceutical research. 2003 May;20(5):788-96.

102. Savi M, Kalberer M, Lang D, Ryser M, Fierz M, Gaschen A, et al. A novel exposure system for the efficient and controlled deposition of aerosol particles onto cell cultures. Environmental science & technology. 2008;42(15):5667-74.

103. Grainger CI, Greenwell LL, Martin GP, Forbes B. The permeability of large molecular weight solutes following particle delivery to air-interfaced cells that model the respiratory mucosa. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV. 2009 Feb;71(2):318-24.

104. Schreier H, Gagne L, Conary JT, Laurian G. Simulated lung transfection by nebulization of liposome cDNA complexes using a cascade impactor seeded with 2-CFSME0-cells. Journal of aerosol medicine : the official journal of the International Society for Aerosols in Medicine. 1998;11(1):1-13.

105. Knebel JW, Ritter D, Hoffmann K, Lödding H, Windt H, Koch W, et al. Development and Validation of a Semiautomatic System for Generation and Deposition of Sprays on Isolated Cells of the Respiratory Tract. Toxicology Mechanisms and Methods. 2001;11(3):161-71.

106. Blank F, Rothen-Rutishauser BM, Schurch S, Gehr P. An optimized in vitro model of the respiratory tract wall to study particle cell interactions. Journal of aerosol medicine : the official journal of the International Society for Aerosols in Medicine. 2006;19(3):392-405.

107. Schulte P, Geraci C, Zumwalde R, Hoover M, Kuempel E. Occupational Risk Management of Engineered Nanoparticles. Journal of Occupational and Environmental Hygiene. 2008;5(4):239-49.

108. Government of Canada. NanoPortal - Regulations and Standards 2014 [cited 2016]. Available from: http://nanoportal.gc.ca/default.asp?lang=En&n=23410D1F-1.

109. Government of Canada. Chemical Substances - Glossary 2014 [cited 2016]. Available from: http://www.chemicalsubstanceschimiques.gc.ca/glossary-glossaire-eng.php#d.

110. Environment and Climate Change Canada. Nanomaterials Government of Canada,; 2016 [cited 2016]. Available from: https://www.ec.gc.ca/subsnouvelles-newsubs/default.asp?lang=En&n=D179F162-1.

111. Environment and Climate Change Canada. Proposed prioritization approach for nanoscale forms of substances on the Domestic substances list: Government of Canada, ; 2016 [cited 2016]. Available from: https://www.ec.gc.ca/lcpe-cepa/default.asp?lang=En&n=FA3C8DBF-1.

112. Stringer B, Imrich A, Kobzik L. Lung epithelial cell (A549) interaction with unopsonized environmental particulates: quantitation of particle-specific binding and IL-8 production. Experimental lung research. 1996;22(5):495-508.

113. Rampersad SN. Multiple Applications of Alamar Blue as an Indicator of Metabolic Function and Cellular Health in Cell Viability Bioassays. Sensors (Basel, Switzerland). 2012;12(9):12347-60.

114. ThermoFisher Scientific. AlamarBlue® Cell Viability Reagent 2016 [cited 2016]. Available from: https://www.thermofisher.com/order/catalog/product/DAL1100?ICID=search-product.

115. ThermoFisher Scientific. Carboxy-H2DCFDA (general oxidative stress indicator) 2016 [cited 2016]. Available from: https://www.thermofisher.com/order/catalog/product/C400?ICID=search-product.

116. Sigma-Aldrich. Cytotoxicity Detection KitPLUS (LDH) 2016 [cited 2016]. Available from: http://www.sigmaaldrich.com/catalog/product/roche/cytodetro?lang=en&region=CA.

117. Bisgaard H, O'Callaghan C, Smaldone GC. Drug Delivery to the Lung. New York, NY, USA: Marcel Dekker; 2001.

118. Salem H, Katz SA. Inhalation Toxicology, Second Edition. Salem H, Katz SA, editors: CRC Press; 2005.

119. Fuchs NA SA. Aerosol Science: New York: Academic Press; 1966.

120. Jing X, Park JH, Peters TM, Thorne PS. Toxicity of copper oxide nanoparticles in lung epithelial cells exposed at the air-liquid interface compared with in vivo assessment. Toxicology in vitro : an international journal published in association with BIBRA. 2015 Apr;29(3):502-11. Pubmed Central PMCID: PMC4373347.

121. Wongrakpanich A, Mudunkotuwa IA, Geary SM, Morris AS, Mapuskar KA, Spitz DR, et al. Size-dependent cytotoxicity of copper oxide nanoparticles in lung epithelial cells. Environmental Science: Nano. 2016;3(2):365-74.

122. Hingorani R, Deng J, Elia J, McIntyre C, Mittar D. Detection of Apoptosis Using the BD Annexin V FITC Assay on the BD FACSVerse System BD Biosciences. 2011.

123. Zhang S, Li J, Lykotrafitis G, Bao G, Suresh S. Size-Dependent Endocytosis of Nanoparticles. Advanced materials (Deerfield Beach, Fla). 2009;21:419-24.

124. Chithrani BD, Chan WC. Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes. Nano letters. 2007 Jun;7(6):1542-50.

125. Roebuck KA, Finnegan A. Regulation of intercellular adhesion molecule-1 (CD54) gene expression. Journal of leukocyte biology. 1999;66(6):876-88.

126. Zhang J-M, An J. Cytokines, Inflammation and Pain. International anesthesiology clinics. 2007;45(2):27-37.