

# INTRATRACHEAL INSTILLATION OF SILVER NANOPARTICLES EXACERBATES CARDIAC ISCHEMIA/REPERFUSION INJURY IN MALE SPRAGUE-DAWLEY RATS

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The uses of engineered nanomaterials have expanded in biomedical technology and consumer manufacturing. Exposure to particulate matter has been demonstrated to negatively influence cardiovascular health and expand myocardial infarction. Furthermore, pulmonary exposure to various engineered nanomaterials has, likewise, demonstrated the ability to exacerbate cardiac ischemia reperfusion (I/R) injury. We hypothesized that pulmonary exposure to AgNP induces cardiovascular toxicity in the form of expanded I/R injury, electrical dysfunction and inducing a persistent increase in circulating proinflammatory cytokines. To test this hypothesis, we exposed male SD rats to an intratracheal (IT) instillation of 200  $\mu$ g of 20 or 110 nm polyvinylpyrrolidone (PVP) or citrate capped AgNP, in 200  $\mu$ l of the respective PVP or citrate vehicle. Serum samples were collected prior to instillation and 1, 3, 6, 24, 48, 72, and 168 hours following instillation. Serum samples were analyzed by multiplex assay for concentrations of: G-CSF, GM-CSF, MIP-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-5, IL-6, IL-10, IL-13, IL-17 $\alpha$ , IL-18, MCP-1, IFN $\gamma$ , RANTES, and TNF- $\alpha$ . Twenty four and 168 hours after IT exposure, cardiac ischemia was induced by left anterior descending coronary artery ligation for 20 minutes followed by 2 hours of reperfusion. Intraoperative ECG was monitored throughout cardiac I/R surgery for heart rate (HR), PR interval, and QT

interval. To test the impact of silver ion exposure on cardiac I/R injury we administered 200  $\mu$ l of 0.01 mg/mL, 0.1 mg/mL, or 1 mg/mL silver acetate (AgAc) and induced cardiac I/R 24 hours later. Intratracheal instillation of AgNP resulted in expansion of I/R injury for both sizes of citrate and PVP capped AgNP at both 24 hours and 168 following instillation; exposure to 0.1 and 1 mg/mL AgAc also resulted in expansion of I/R injury. Intratracheal instillation of AgNP did not result in increased serum concentrations of selected proinflammatory cytokines, however post I/R serum levels of IL-2, IL-6, and IL-18 were significantly elevated in rats exposed to 20 nm PVP capped AgNP compared to vehicle controls at 24 hours post instillation. Instillation of AgNP had no impact on HR or QT interval. However, exposure to 20 nm AgNP resulted in a differential prolongation or shortening of PR interval during reperfusion based on capping agent. In conclusion IT instillation of AgNP exacerbates cardiac I/R injury 24 and 168 hours following instillation, without inducing a strong systemic inflammatory response or electrical dysfunction. Exposure to AgNP may result in a sensitization of the immune system in response to a secondary insult (e.g., cardiac I/R) which are largely correlated with capping agents and particle size and may drive expansion of I/R injury at 24 and 168 hours following IT instillation of AgNP.



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CARDIAC ISCHEMIA/REPERFUSION INJURY IN MALE SPRAGUE-DAWLEY RATS

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by

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## **DEDICATION**

*This thesis is lovingly dedicated to my parents,*

*Jim and Donna Holland*

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## LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
AgAC	Silver acetate
AgNP	Silver nanoparticle
Ang-II	Angiotensin II
ANS	Autonomic nervous system
ATP	Adenosine triphosphate
AV	Atrioventricular
BALF	Bronchoalveolar lavage fluid
BPM	Beats per minute
Ca <sup>++</sup>	Calcium
CAD	Coronary artery disease
Citrate	Sodium citrate
CNS	Central nervous system
DI	Deionized
ECG	Electrocardiogram
FIO <sub>2</sub>	Fraction of inspired oxygen
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H <sub>2</sub> O	Water
H <sup>+</sup>	Hydrogen
HR	Heart rate
IFN $\gamma$	Interferon gamma
IL	Interleukin
I/R	Ischemia reperfusion
IT	Intratracheal

K <sup>+</sup>	Potassium
LAD	Left anterior descending coronary artery
LV	Left ventricle
MCP-1	Monocyte chemotactic protein-1
MI	Myocardial Infarction
MIP-1 $\alpha$	Macrophage inflammatory protein-1 $\alpha$
mPTP	Mitochondrial permeability transition pore
Na <sup>+</sup>	Sodium
O <sub>2</sub>	Oxygen
PE	Polyethylene tubing
PM	Particulate matter
PR	PR interval
PVP	Polyvinylpyrrolidone
MI	Myocardial infarction
MWCNT	Multiwalled carbon nanotubes
SD	Sprague-Dawley
SEM	Standard error of the mean
RANTES	Regulated on activation, normal T cell expressed and secreted
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
Th	T helper
TNF $\alpha$	Tumor necrosis factor $\alpha$
TTC	2,3,5-Triphenyltetrazolium chloride
QTc	QT interval with Bazette's correction

# **CHAPTER 1**

## **INTRODUCTION**

### **1.1. PROBLEM and APPROACH**

Cardiovascular disease remains the leading cause of mortality worldwide. Recent investigations have demonstrated that pulmonary exposure to particulate matter can negatively impact cardiovascular health and may result in an expansion of subsequent myocardial infarction (13, 14, 29, 67). Ambient particulate material impact on cardiovascular health has served as a backdrop concern raising questions regarding the potential exposure risks to the plethora of engineered particle materials (10, 11). In recent decades, the uses of engineered nanomaterial rapidly expanded, and investigation into the potential toxicity of new forms of particulate matter has grown in kind. Inhalation of particulate matter can be a primary route of exposure and is the subject of much current investigation. The information regarding any potential cardiovascular impact of exposure to engineered nanomaterial remains limited. Furthermore, the mechanisms, which underlie cardiovascular toxicity, are poorly defined or investigated. This thesis sets out a study designed to evaluate the expansion of myocardial injury following acute occlusion and reperfusion after exposure to two sizes of silver nanoparticles (AgNP) capped with either polyvinylpyrrolidone (PVP) or citrate, and to understand the potential role of systemic inflammatory markers or electrical conductivity as underlying the cardiovascular toxicity.

### **1.2. BACKGROUND**

#### **1.2.1. SILVER NANOPARTICLES**



In recent decades advances in materials sciences and engineering have yielded a new class of nano-sized materials. These nanomaterials have a size range of between 1 nm and 100 nm in at least one dimension, and are characterized by high surface to mass ratio (81, 105). Of the various forms of engineered nanomaterials, silver nanoparticles (AgNP) have garnered much attention for potential uses in both industry and medicine. There has been long standing knowledge that silver exhibits innate antimicrobial properties (94) which have made AgNP an attractive particle for biomedical and consumer applications, including but not limited to: wound dressings silver impregnated catheters, vascular prosthetics, surgical mesh (136), clothing and undergarments, air filters, laundry detergents, toiletries, and water taps (22). However, despite the seemingly ubiquitous potential of AgNP, the toxicity of these engineered nanomaterials, particularly the cardiovascular toxicity, is poorly understood. Furthermore, mechanisms by which AgNP may interact with biological interfaces through size or surface characteristics have yet to be fully elucidated.

Toxicologically, AgNP have become the target of investigation due to the high reactivity of silver ( $\text{Ag}^+$ ) ions.  $\text{Ag}^+$  ions bind to both sulfur and phosphate molecules (22), which may interrupt normal cellular membrane function as well as disrupt DNA and RNA binding (138). Silver ion dissolution may be a key to understanding the underlying toxicological mechanisms of AgNP. Nanoparticle size may be another important factor in determining toxicological impact of AgNP exposure, particularly in metal derived nanomaterials. The smaller the particle diameter, the more silver atoms are externalized, which may impact the degree of biological interactions by increasing the rate of ionic dissolution (102). Free  $\text{Ag}^+$  ions may contribute to the generation of oxidative stress as well as binding to sulfhydryl groups of proteins changing their function and activity (65). Beyond the question of the contribution of  $\text{Ag}^+$  ion toxicity, interactions of AgNP *in vivo* also are proposed to play a crucial role in particle toxicity. Surface

charge of AgNP is thought to be a key factor in the toxicity associated with exposure to AgNP which may influence cellular membrane degradation or permeability (34). Surface oxidation of AgNP may also lead to oxidative stress (97) and interactions with macromolecules that may reflect altered signaling processes contributing to cardiovascular dysfunction following pulmonary exposure to AgNP (71).

Inhalational exposure to aerosolized particles is a chief concern regarding the safety of engineered nanomaterials. Aerosolization of particles may occur at several phases of AgNP manufacture and utilization. Inhalational exposure to AgNP is most likely to occur during particle synthesis, handling of dry powders, aerosolization of liquid suspensions, as well as the manufacture and machining of composites containing AgNP (102). Exposure is also possible outside of the occupational exposure realm. Use of AgNP in consumer products such as disinfecting sprays or deodorants have the potential to directly aerosolize AgNP, which could lead to a direct pulmonary exposure to AgNP (102).

### **1.2.2. PULMONARY EXPOSURE to PARTICULATE MATTER**

There has been wide investigation into the impact of exposure to various forms of particulate matter (PM) and their impact on the health of multiple organ systems. One of the primary areas of investigation has included pulmonary toxicity of PM. Pulmonary exposure serves as a primary route of human exposure to PM and pulmonary detriments from exposure to air pollution are well known. Particulate matter with a diameter of approximately  $< 100$  nm has the ability to deeply infiltrate the lungs at the same time avoiding many mechanisms for clearance of larger foreign debris (66). The interaction with cells and tissues deep within the lungs may initiate a response leading to downstream cardiovascular detriments. Pulmonary exposure to PM has been demonstrated to not only induce a primary inflammatory response in

the lungs, but also prime the immune system to over-respond to an insult following PM exposure (81, 126). Exposure to ultrafine particles had been shown to alter inflammatory responses through calcium signaling in pulmonary monocytes (33). Furthermore, the true impact of PM was unseen until secondary stimulation with thapsigargin whereby PM exposure induced a 2.5 fold increase in cytosolic calcium influx (33). The additional calcium may act as a second messenger following PM exposure inducing heightened inflammatory responses. For example, A basophilic cell line exposed to AgNP was demonstrated to stimulate degranulation following AgNP induced calcium influx (137).

Murine exposure to PM induced inflammatory responses in the lungs of exposed animals, triggering ROS production (127). Increases in ROS may alter redox sensitive transcription of cytokines (44). Exposure to PM has been shown to induce increases in IL-6 concentrations in BALF. Localized cytokine secretion in the lungs has been theorized to lead to systemic inflammation (60). Furthermore, key pro-inflammatory mediators in the lungs, IL-1 $\beta$ , IL-6, and TNF $\alpha$  also are known to have an impact on the heart. Given the inflammatory response to PM in the lungs and the proximity and physiologic relationship between the heart and lungs, it seems likely that inflammatory responses in the lung may lead to effects in the heart and vasculature.

### **1.2.3. THE HEART- LUNG CONNECTION**

It is very difficult to separate the lungs from the heart when evaluating pulmonary PM exposure and subsequent cardiovascular dysfunction. The heart and lungs are greatly interconnected in terms of anatomy and physiological function. There is a clear relationship between pulmonary exposure to a toxicant and cardiovascular compromise (10, 125). Long term exposure to PM and air pollution drastically increase cardiovascular morbidity and

mortality in a dose-dependent manner (57). Pulmonary exposure to diesel particles has been shown to induce a variety of cardiac effects. In one such study, ApoE<sup>-/-</sup> mice were placed on a western diet and then exposed to either diesel exhaust particles or saline. The mice exposed to diesel exhaust particles had significantly greater vascular plaque formation than mice exposed to only saline (68). In an experiment with heavy metal laden PM, pulmonary exposure exacerbated myocardial mitochondria injury (43). Furthermore, exposure to diesel has been shown to impact measures of autonomic control of the cardiovascular system (19). Despite well-known associations between pulmonary exposures and negative cardiac outcomes, the precise mechanisms remain unclear.

In addition to systemic inflammation, translocation of particles out of the lungs to the cardiovascular system is another proposed mechanism of interaction between pulmonary exposures and cardiovascular toxicity. Ultrafine particles are inhaled deeply into the lungs are able to interface with pulmonary vasculature (60, 76). Studies have demonstrated that both instillation and inhalation of nano sized materials result in a fraction of the nanomaterials crossing the blood alveolar barrier and entering systemic circulation (72, 78). PM translocating from the lung would next reach the heart where the PM may act locally, stimulating direct cardiac toxicity however, this has yet to be demonstrated. There is a paucity of data regarding the degree to which particles are able to translocate from the lungs and to what organs, tissues, and cells are most susceptible to potential translocation.

A third hypothesis regarding how pulmonary exposure impacts cardiovascular end points involves dysfunctional feedback in the ANS (77). Exposure to PM has been shown to induce changes in heart rate variability, indicative of changes in autonomic balance (6, 19). In a study of healthy North Carolina State Highway patrol officers exposed to ultrafine particles

during a normal work shift, changes in heart rate variability as well as increased inflammatory blood markers were demonstrated (74). This study is of interest not only because it demonstrates the ability of pulmonary exposure to PM to impact normal cardiac physiology in healthy individuals, but demonstrates a link between: pulmonary exposure, autonomic function, inflammation and the cardiovascular system. In recent years our understanding of immunity has expanded to include reflex control of immune responses from the CNS. Pulmonary inflammation resulting from particulate exposure may increase vagal sensory input to the CNS resulting in increased cardiac vagal stimulation (74) and attenuate the inflammatory response to PM. ANS mediation of inflammation may also be impacted by adjustment to regulatory set points where prolonged decreased vagal outflow may induce a proinflammatory phenotype in the face of physiological insult (121). The exact mechanism by which PM may modulate the ANS through pulmonary exposure is not well known. Particulate matter in the lung triggers activation of pulmonary C-fibers. Autonomic feedback from C-fibers can impact cardiac function by influencing vagal tone and may be a key component of the relationship between pulmonary exposures and cardiovascular outcomes (56, 66).

#### **1.2.5 MYOCARDIAL INFARCTION and ISCHEMIA/REPERFUSION INJURY**

The incidence of myocardial infarction is high, an estimated 1,255,000 new or recurrent events of myocardial infarction occur per year in the United States (100). The processes by which a vessel may become occluded are varied but often results from a blockage of the lumen of a coronary artery by, somewhat paradoxically, the repair response to a ruptured atherosclerotic plaque (107), whereby initial recruitment of platelets to the site of injury begins to block the lumen of a coronary artery and ultimately reduces or altogether stops blood flow to distal portions of the myocardium. Although the most common pathological mechanism for initiation of myocardial infarction, rupture of atherosclerotic plaques are not the only means of

inducing myocardial ischemia or infarction. If oxygen ( $O_2$ ) demand outpaces  $O_2$  supply, such as during strenuous exercise (39), worsening cellular hypoxia can lead to ischemia. Furthermore, cardiac ischemia can be induced by coronary vasospasm (96). It is important to note that total luminal occlusion of a coronary artery is not required to induce infarction, as decreased coronary flow may result in inadequate  $O_2$  distribution to cardiac tissue, resulting in ischemia and ultimately cardiomyocyte death.

The cellular mechanisms that ultimately lead to cardiomyocyte death are strongly tied to hypoxia. During ischemia adequate  $O_2$  can no longer be delivered to tissues. The heart is an organ with high metabolic demand; as such, delivery of  $O_2$  to the myocardium via the coronary arteries in support of normal cardiac function is crucial (16). Occlusion of one or more coronary arteries leads to ischemia in areas distal to the blockage, whereby metabolism is compromised. The inability to generate adenosine triphosphate (ATP) via oxidative phosphorylation causes a shift to anaerobic glycolysis (17). Anaerobic glycolysis increases intracellular  $H^+$  ion concentration inducing acidosis that then disturbs the  $Na^+/H^+$  exchanger. ATP depletion inactivates  $Na^+/K^+$  ATPase. The combined effect results in  $Na^+$  overload,  $Na^+-Ca^{++}$  exchanger attempts to compensate for the  $Na^+$  overload by pumping  $Ca^{++}$  into the cytoplasm, however the  $Ca^{++}$  overload induces myocardial contracture and cell death (71). The cytosolic oversaturation with  $Na^+$  or  $Ca^{++}$  results in an osmotic force resulting in cellular edema (17). Activation of phospholipases by intracellular calcium degrades cardiomyocyte cell membranes (17, 84) contributing to cell death and myocardial infarction.

In order to restore normal cardiac function it is imperative to restore coronary blood flow. Clinically, blood flow can be restored through medical interventions such as thrombolytic therapy or percutaneous coronary intervention (135), but the restoration of blood flow, known

as reperfusion, can also result in further myocardial injury, known as reperfusion injury.

Reperfusion injury is a broad term that encompasses several processes including arrhythmia, myocardial stunning, and microvascular injury leading to no -reflow phenomenon (17, 91). The mechanisms of reperfusion injury are diverse, and in many respects mirror processes witnessed in ischemic injury.

Reactive oxygen species (ROS) are thought to play a key role in mediating reperfusion injury (52). The surge of  $O_2$  associated with reperfusion generates superoxide anion or peroxynitrate by cardiomyocyte mitochondria (123). The impact of ROS on reperfusion injury is varied and thought to involve triggering at least one of the following: protein kinases and various genomic pathways (91, 84, 6), peroxidation of lipid membranes diminishing cell membrane integrity (70), triggering apoptosis (15, 16, 30), and dysfunction in  $Ca^{++}$  handling (37).

Calcium handling in reperfusion injury is further influenced by influxes in  $Na^+$ . Much like in ischemia, exchange of  $Na^+$  for  $Ca^{++}$  leads to high cytosolic  $Ca^{++}$ . The overload of calcium leads to rigor type contracture, where high levels of  $Ca^{++}$  leads to excessive activation of myocardial contraction. Rigor type contraction contributes to the development of myocardial dysfunction and induces apoptosis (30). Additionally, fluctuations in  $Ca^{++}$  from the sarcoplasmic reticulum stimulate opening of the mPTP (1, 32). Once the mPTP opens, the proton gradient required for synthesis of ATP rapidly dissipates and  $H_2O$  is able to flood into mitochondria, causing mitochondrial swelling and rupture and triggering apoptosis as well as necrosis (85). Production of Ang-II by RAS induces increases in intracellular  $Ca^{++}$  of cardiomyocytes and smooth muscle, impairing diastolic function and inducing coronary vasoconstriction, respectively (30).

Leukocyte trafficking is another key component of reperfusion injury that occurs shortly after return of blood flow (30). During reperfusion neutrophils move to areas of ischemia in order to phagocytose dead tissue, releasing cytokines to further mediate the immune response (115). The factors secreted by neutrophils may be damaging to viable tissue and include ROS, cytokines and chemokines (51). Neutrophils collect in post-capillary venules causing obstruction and contribute to microvascular dysfunction (51,106). It is thought that microvascular dysfunction mediates no-reflow phenomenon associated with reperfusion injury (93). Platelets recruited to sites of injury also may contribute to reperfusion injury and microvascular dysfunction by secreting vasoactive thromboxane A<sub>2</sub> and 5-HT. Monocytes infiltrating the zone of infarction following reperfusion further augmenting the inflammatory response to ischemia and reperfusion by releasing proteases capable of infarct expansion through proteolysis (43).

### **1.2.6 CYTOKINE MEDIATORS OF MYOCARDIAL DYSFUNCTION**

There is a strong association between elevated levels of circulating cytokines and cardiovascular disease. In humans with coronary artery disease (CAD), it has been shown that elevated serum concentrations of IL-1 $\beta$ , TNF $\alpha$ , and IL-8 are present during events of unstable angina (87). IL-1 $\beta$  and TNF $\alpha$ , in addition to IL-18 are responsible for enhanced surface expression of selectins, VCAM-1 and ICAM-1 (62, 53), which indicate endothelial activation due to inflammation (132) and which may play a role in vascular changes associated with particle exposure and no-reflow phenomenon (62, 67). IL-1 family cytokines, including IL-18, have been shown to contribute to LV dysfunction post-MI (86, 130). The dose-dependent impairment of myocardial dysfunction is synergistic with cardio depression caused by TNF $\alpha$  (63, 132). Interleukin-18 activates T-cells in response to interferon (INF) $\gamma$  which then induces release of IL-6. Interleukin-6 a prototypical cytokine and key mediator of acute phase



response as well as stimulated release of TNF $\alpha$ . Elevated concentrations of circulating proinflammatory cytokines, such as TNF $\alpha$  and IL-6 by activation of innate immune cells has been associated with a worsening of heart failure (19, 73, 103). During ischemia, IL-6 and TNF $\alpha$  are released into ischemic and border zone areas of the heart as a response to myocardial stretch and stress (80). IL-6 is associated with cardiac hypertrophy, and myocardial wasting and dysfunction (132). Acute coronary syndrome also is associated with a rise in plasma concentrations of IL-6 and IL-2 (35, 42). IL-2 is a proinflammatory cytokine key to T cell differentiation promoting T helper (Th)1 and Th2 while inhibiting Th17 and T follicular helper cells (91). Infusion of exogenous IL-2 has been shown to induce heart failure and myocarditis (36, 104). Considering the overall impact on cardiovascular dysfunction directly attributed to pro-inflammatory cytokines, in addition to secondary damage from leukocyte recruitment, cytokines may play a role in mediating cardiovascular dysfunction and expansion of I/R injury.

### **1.3. HYPOTHESIS and AIMS**

There is strong evidence that exposure to PM can exacerbate cardiovascular disease. The mechanisms by which pulmonary exposure to PM can mediate cardiac injury are unclear. We hypothesize that intratracheal (IT) instillation of AgNP induces a proinflammatory response and autonomic dysfunction that augments cardiac I/R injury for up to seven days following exposure.

#### **1.3.1. AIM 1:**

Determine the extent of cardiac I/R injury on SD rats 24 hours and 168 hours after IT instillation of AgNP.

### **1.3.2. AIM 2:**

Compare the impact of IT instillation of AgNP on electrical and autonomic dysfunction in SD Rats prior to and during I/R injury with ECG.

### **1.3.3. AIM 3:**

Profile the circulating cytokines in serum: 0, 1, 3, 6, 24, 48, 72, 168 hours and following I/R injury after IT exposure to AgNP to determine the inflammatory impact of particle exposure.

## **CHAPTER 2**

### **METHODS and MATERIALS**

#### **2.1. EXPEREMENTAL DESIGN**

In order to determine the impact of pulmonary exposure to silver nanoparticles (AgNP) we performed intratracheal (IT) instillation of male Sprague-Dawley (SD) rats with 20 nm or 110 nm AgNP capped with PVP or citrate, or a respective PVP or citrate vehicle. By evaluating two sizes of AgNP as well as capping agents we are able to begin to define the physiochemical properties of the materials that impact cardiovascular toxicity (40,3). An additional cohort of animals was instilled with silver acetate AgAc in order to determine the contribution of ionic silver to cardiac injury.

Twenty four or 168 hours post IT instillation rats underwent a 20 minute occlusion of the LAD followed by 2 hours of reperfusion. Following reperfusion the heart were stained with TTC and analyzed for the extent of myocardial infarction (MI)(47). (Aim 1) ECG was monitored for a baseline period then throughout occlusion and reperfusion in order to evaluate the potential contribution of autonomic dysfunction to I/R injury by measuring heart rate (HR) (57, 132), PR interval, and QT interval (21). (Aim 2) Blood was collected prior to instillation and 1, 3, 6, 24, 48, 72 and 168 hours after IT instillation to examine concentrations of circulating cytokines and chemokines over time. A final blood sample following I/R injury was collected to examine differences in circulating cytokines released in response to cardiac I/R injury following exposure to AgNP. (Aim 3)

#### **2.2. ANIMALS**

Male (SD) rats between the age of 51 and 54 days and weighing between 201-225 grams were purchased from Charles River Laboratory (Raleigh, NC, USA). Rats were housed two per cage under 12 hour light/dark cycles. Standard rat chow and water were provided *ad libidum*. All animals were allowed a 1 week acclimatization period in the East Carolina University (ECU) Department of Comparative Medicine vivarium prior to any experimentation. Animals were randomly assigned to 10 experimental groups: 1 or 7 day naïve, small PVP vehicle, small AgNp, large PVP vehicle, and large AgNp, citrate, small citrate, and large citrate. A second cohort of SD rats was randomized into 3 groups and received 1 mg/mL, 0.01 mg/mL, or 0.001 mg/mL AgAc. ECU Institutional Animal Care and Use Committee approved all animal handling and experimental procedures.

### **2.3. NANOMATERIAL and VEHICLES**

For the purposes of investigation both PVP and Citrate coated AgNP were used for instillation. To test toxicity of exposure to ionic silver AgAc was obtained as a dry powder from RTI International (Research Triangle Park, NC). 20 nm and 110 nm AgNp were manufactured and provided to the investigators by nanoComposix (San Diego, CA) through the NCNHIR U19 Consortium funded through NIEHS. The prepared nanomaterials were characterized by the National Characterization Laboratory associated with the National Cancer Institute.

#### **2.3.1 PVP CAPPED AgNP**

20 nm and 110 nm AgNp were capped with polyvinylpyrrolidone (PVP) to prevent particulate agglomeration. Each AgNp size was evaluated for hydrodynamic diameter utilizing dynamic light scattering (DLS), as well as transmission electron microscopy (TEM) for determining core diameter. Zeta potential analysis was utilized for determination of surface charge. Silver concentration was determined by inductively coupled plasma mass spectrometry (ICP-MS).

Endotoxin levels were also characterized and found to be below acceptable limits. Table 2.1 provides characterization of AgNP. Aliquots of PVP capped AgNP showed plating on their containers, which may indicate a breakdown of the nanomaterial. More frequent characterization of nanomaterials may be necessary once stored to ensure structural stability.

### **2.3.2 PVP VEHICLES**

The vehicles for PVP control groups were created by adding sterile saline to a 10 or 40 kDa PVP dry powder to yield a 1.4% PVP/saline solution. The 10 kDa PVP/saline mixture was used as the vehicle control for the 20 nm AgNP and the 40 kDa PVP/saline mixture was used for the 110 nm AgNP.

### **2.3.3 CITRATE CAPPED AgNP**

20 nm and 110 nm AgNP were coated with citrate to prevent particulate agglomeration. Each AgNP size was evaluated for hydrodynamic diameter by DLS as well as TEM determined the core diameter. Zeta potential analysis was utilized for determination of surface charge. Silver concentration was determined ICP-MS. Endotoxin levels were also characterized and found to be below acceptable limits. Table 2.1 provides characterization of AgNP.

### **2.3.4 CITRATE VEHICLE**

The vehicle control for citrate AgNP was created with a 2 mM solution of sodium citrate dissolved in deionized H<sub>2</sub>O. Unlike PVP capped AgNP groups, one citrate vehicle was sufficient for both 20 nm and 110 nm particles.

### **2.3.4 AgAc**

Silver Acetate was provided by RTI International (Research Triangle Park, NC) as purchased from Sigma-Aldrich (St. Louis, MO, USA). 1.62 mg of AgAc was dissolved in sterile H<sub>2</sub>O to create an ionic silver concentration of 1 mg/mL. Dilutions were made from an aliquot of the 1

mg/mL AgAc to yield 0.1 mg/mL AgAc and 0.01 mg/mL AgAc. All silver solutions and dry AgAc were stored at 4°C and kept from light.

#### **2.4. AgNP PREPARATION, DOSING, and INTRATRACHEAL INSTILLATION**

AgNP aliquots were cup-horn sonicated for 30 seconds at 50% amplitude (Misonix Model 1510r-MTH, Branson Ultrasonics Corp. Danbury, CT). Aliquots were vortexed prior to instillation for 30 seconds. Rats were anesthetized by inhalation of a 50:50 isoflurane propylene-glycol mixture in an induction chamber. Once an adequate plane of anesthesia had been achieved and assessed by physical examination, the rat was moved from the induction chamber and suspended by the frontal incisors on a inclined board. The tongue of the rat was anteriorly displaced with padded forceps and a pipette tip was placed into the laryngopharynx, just superior to the glottis. 200  $\mu$ L of AgNP, AgAc, or vehicle was dispensed into the glottal opening and the rat was stimulated to breathe while securing the tongue with forceps, ensuring pulmonary aspiration. Following instillation the rats were returned to their home cage and monitored until they resumed normal grooming behavior.

#### **2.5. SERIAL BLOOD COLLECTION**

Rats were anesthetized by inhalation of 2-3% isoflurane, dispersed in medical grade oxygen, until an adequate plane of anesthesia had been achieved and assessed by physical examination. The rats were transferred to a nose only inhalation system to deliver 1% isoflurane dispersed in medical grade oxygen to ensure maintenance of anesthesia throughout blood collection. The rats were placed in a lateral recumbent position on a heating pad. The tail was prepared for venipuncture by cleaning with a sterile 70% isopropyl saturated prep pad. Location of the lateral tail vein was obtained by visual inspection. Venipuncture was achieved with a BD Vacutainer 25 g needle collection set (Becton, Dickinson, and Company, Franklin

Lakes, NJ). Blood from the tail was collected into BD microtainer serum separator tubes (Becton, Dickinson, and Company, Franklin Lakes, NJ). Approximately 200-300  $\mu$ L was collected at each time point. Time points for collection were: prior to AgNp exposure and 1, 3, 24, and 168 hours following exposure. Following the ischemia-reperfusion surgery a final blood sample was obtained from the inferior vena cava. All samples were allowed to sit at room temperature for approximately 30 minutes before being centrifuged at 2000 rcf at room temperature for 2 minutes. Serum supernatant was removed from the sample, snap frozen in liquid N<sub>2</sub> and stored at - 80 °C.

## **2.6. CARDIAC ISCHEMIA/REPERFUSION and QUANTIFICATION of INFARCT SIZE**

Twenty-four or 168 hours following IT instillation rats were anesthetized with an intramuscular injection of ketamine/xylazine (90/10 mg/kg, respectively). Anesthesia was maintained throughout the procedure with supplemental injections of ketamine/xylazine. Body temperature was maintained at 37°C with a heating pad and TC-1000 Temperature Controller (CWE, Inc., Ardmore, PA). Once an adequate plane of anesthesia was achieved the rats were intubated via tracheostomy with a 16 gauge angiocatheter. Rats were ventilated with fraction of inspired oxygen (FiO<sub>2</sub>) of 1 via an Inspira Advanced Safety Ventilator (Harvard Apparatus, Holliston, MA) with a 3 mL tidal volume at 81 breaths per minute.

Each animal was allowed at least 15 minutes of equilibration prior to any baseline collection prior to ischemic manipulation. Following the baseline period the heart was visualized by an anterolateral partial sternotomy. The LAD was visualized and occluded with 6-0 prolene suture and PE 90 with a flared end as a reversible tourniquet. The tourniquet was secured in place for a 20 minute period of ischemia by Kelly hemostatic forceps. Successful occlusion of the LAD was confirmed by: A.) blanching of the myocardium distal to the occlusion, B.) myocardial

dyskinesia, and C.) ECG changes (Fig. 2.1). Following 20 minutes of ischemia the reversible tourniquets was released allowing two hours of reperfusion.

Following reperfusion the rat was exsanguinated by transection of the inferior vena cava. The descending thoracic aorta was then cannulated with PE 90 and advanced to the coronary ostia. The heart was flushed with approximately 5 mL 0.09% normal saline followed by 5 mL of 0.25% TTC (Sigma-Aldrich, St. Louis, MO) to determine viable from infarcted tissue.

Following infusion of TTC the LAD is re-occluded and the heart is infused with 1% Evans blue dye in order to demarcate the remote myocardium. Following Evans blue infusion the heart was excised and sliced into approximate 1mm sections distal to the occlusion. (Fig. 2.2) The slices were sandwiched between two glass slides and both sides were digitally photographed. Image J was obtained from the National Institutes of Health was used to determine the area of the left ventricle, area at risk, and area of infarction.

## **2.7. ECG MONITORING and ANALYSIS**

ECG recordings during the surgical procedure were obtained by PowerLab 8/35 data acquisition interface (AD Instruments, Colorado Springs, CO, USA), Animal Bio Amp (ADInstruments), and LabChart 7 Pro (ADInstruments). Anesthetized rats were placed supine and four total needle electrodes were inserted subcutaneously into one in each limb and secured with surgical tape. Electrode placement provided lead II for analysis. Each animal was allowed at least 15 minutes of baseline rhythm collection prior to ischemic manipulation. Intervals and segments were measured at several time points throughout the baseline period and surgical procedure. At each time point, 10 seconds of ECG recording were analyzed for average: HR, QT Interval (with Bazette's correction for heart rate), and PR interval. For each 10 second period, ectopic beats or sections of artifact were excluded from analysis. In addition,



in order to reduce the overall number animals required for experimentation, rhythm conversion was attempted when the duration of VT or VF was deemed life threatening.

## **2.8. QUANTIFICATION of SERUM CYTOKINES**

Concentrations of serum: IL -1 $\beta$ , IL-2, IL-5, IL-6, IL-10, IL-13, IL-18, MCP-1, G-CSF, GM-CSF, IFN $\gamma$ , MCP-1, MIP-1 $\alpha$ , RANTES, and TNF $\alpha$  were measured at selected time points with a 7-plex Milliplex MAP Cytokine/Chemokine Panel and Immunoassay (EMD Millipore, Billerica, MA) according to the manufacturer's instructions. The naïve and PVP capped AgNP and respective vehicle groups were run on a Luminex 200 (Luminex, Austin, TX). Citrate capped AgNP and vehicle groups were run on a MagPix system (Luminex, Austin, TX) and all results were reported and analyzed by Milliplex Analyst version 5.1(EMD Millipore, Billerica, MA).

## **2.9. STATISTICAL ANALYSIS**

All data are presented as mean value  $\pm$  SEM. a P-value of  $< 0.05$  indicated statistical significance unless otherwise noted. GraphPad Prism software version 6 (LaJolla, CA) was used for the purposes of statistical analysis and graphing. Cardiac I/R data were compared by one-way ANOVA with Bonferroni's post-hoc test. Differences between time and particle capping were performed by t-test. Group size was calculated based on power analysis of cardiac I/R experiments. ECG data was analyzed by one-way ANOVA with Tukey post-hoc test. Cytokines were compared to their respective vehicle by t-test.

**Table 2.1:** NCL particle characterization data for PVP and citrate capped AgNP.

Hydrodynamic size reported was determined from Z-average. Data are as presented as a mean  $\pm$  SEM of replicates.

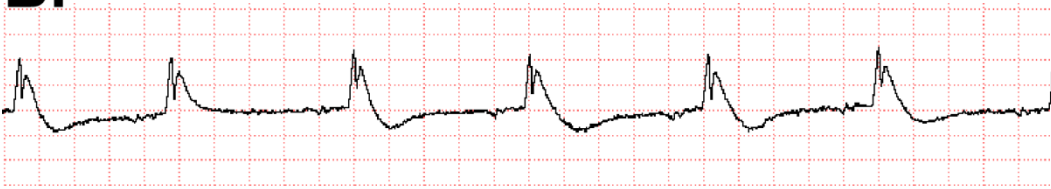
	PVP Capped AgNP		Citrate Capped AgNP	
	20 nm	110 nm	20 nm	110 nm
Hydrodynamic Size (nm)	26.00 ± 0.089	112.3 ± 0.149	23.98 ± 0.0477	104.2 ± 0.120
Core Diameter (nm)	20.95 ± 0.311	114.2 ± 1.421	20.28 ± 0.229	111.3 ± 1.970
Zeta Potential (mV)	- 37.12 ± 1.136	- 25.92 ± 1.237	- 48.50 ± 2.059	- 43.02 ± 1.472
Silver Concentration (mg/g)	1.090 ± 0.001	1.101 ± 0.003	1.105 ± 0.007	0.980 ± 0.014
Endotoxin Concentration (EU/mL)	1.133 ± .291	<0.5 ± 0	<0.5 ± 0	<0.5 ± 0

**Figure 2.1:** ECG changes indicative of positive occlusion of the LAD. Panel A contains a representative sample of ECG recording prior to occlusion of the LAD. Panel B displays classical post-occlusion ST segment elevation morphology. Panel C illustrates a high level of myocardial ectopy (arrows) resulting from myocardial ischemia induced by ligation of the LAD. Presence of ST segment elevation and myocardial ectopy indicate proper occlusion of the LAD.

**A.**



**B.**

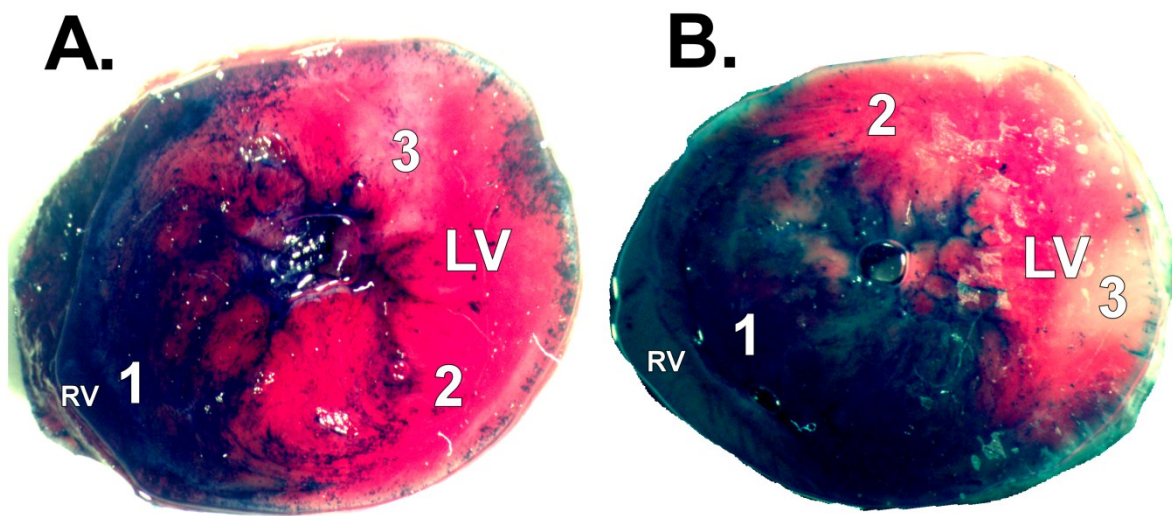


**C.**



0.5 sec

**Figure 2.2:** Representative naive (panel A) and particle exposed (panel B) transverse myocardium slices having undergone I/R protocol. Following I/R protocol panel B. shows a greater area of infarct within the area at risk (areas appear white or unstained). 1 indicates Evans blue counterstain to demarcate remote myocardium uninvolved in the ischemic event. 2 identifies TTC positive staining indicating the myocardial area at risk for ischemic injury that remained viable. 3 indicate TTC negative staining denoting areas of myocardial subject to infarction. Left ventricle (LV) and right ventricle (RV) are indicated.



## **CHAPTER 3**

### **EXPANSION OF CARDIAC I/R INJURY FOLLOWING INTRATRACHEAL EXPOSURE to AgNP.**

#### **3.1 INTRODUCTION**

Exposure to particulate matter (PM) has been linked with cardiovascular disease and dysfunction. Exposure to air pollution has been associated with an acute rise in incidence of myocardial infarction (MI) (10). Pulmonary exposure to diesel particulate has been implicated in expansion of ischemia reperfusion (I/R) injury in male Wistar rats as early as 6 hours following PM exposure (29, 99, 120). The apparent cardiovascular toxicity of pollution associated PM has raised concerns regarding the cardiovascular toxicity of engineered nanomaterials. In a murine model pulmonary instillation of MWCNT resulted in persistent expansion of I/R injury up to 28 days following exposure (124). Likewise, intratracheal (IT) instillation of C<sub>60</sub> fullerenes resulted in expansion of cardiac I/R injury in SD rats 24 hours following pulmonary exposure (119). Given the apparent ability of a range of PM to exacerbate I/R injury (64, 109), this thesis investigated the impact of pulmonary exposure to AgNP on cardiac I/R injury 24 hours and 7 days following an IT instillation of 200 µgs of AgNP. We hypothesized that pulmonary exposure to AgNP would result in expansion of cardiac I/R injury 24 hours and 7 days post instillation.

#### **3.2. RESULTS**

##### **3.2.1 AgNP EXPANDS CARDIAC I/R INJURY 24 HOURS FOLLOWING IT INSTILLATION**

Ventricular heart segments were analyzed following a protocol of 20 minutes of ischemia and 2 hours of reperfusion following IT exposure to 200 µgs of 20 nm or 110 nm citrate or PVP capped AgNP. Instillation of both 20 nm and 110 nm PVP capped AgNP



resulted in a significantly elevated percentage of infarcted myocardium within an area at risk,  $41.65 \pm 2.05$  % and  $36.82 \pm 2.75$  %, respectively when compared to naïve,  $22.30 \pm 1.57$  % or corresponding vehicles, small PVP,  $21.59 \pm 1.27$  and large PVP,  $23.65 \pm 2.43$  (Figure 3.1). Similarly, both sizes of citrate capped AgNP induced expansion of MI 20 nm,  $37.48 \pm 2.61$  % and 100 nm,  $30.51 \pm 1.58$  % as compared to citrate vehicle,  $24.05 \pm 1.28$  % and naïve 24 hours following IT instillation (Figure 3.2). At 24 hours no differences exist in the extent of myocardial infarction between particles sizes within a capping group, however there was a difference in the extent of I/R injury between 20nm PVP capped and 110nm citrate capped AgNP. At 24 hours following IT instillation, 110 nm citrate capped AgNP induced less myocardial injury than the 20 nm PVP capped AgNP.

### **3.2.2. AgNP EXPANDS CARDIAC I/R INJURY 168 HOURS FOLLOWING IT INSTALLATION**

IT instillation of PVP capped AgNP demonstrated persistent I/R injury 7 days post exposure,  $37.79 \pm 2.74$  % and  $42.06 \pm 4.71$  %, respectively compared to naïve,  $16.99 \pm 0.64$  % or small PVP  $23.27 \pm 0.89$  %, and large PVP,  $27.21 \pm 2.20$  % (Figure 3.3). There was no difference in the extent of cardiac injury between 24 and 168 hours post instillation in rats exposed to PVP capped AgNP (Figures 3.1 and 3.3). Citrate capped AgNP demonstrate the ability to expand myocardial infarction 7 days following IT instillation: 20 nm,  $36.85 \pm 1.39$  % and 110nm  $28.30 \pm 2.24$  % compared to citrate vehicle  $20.83 \pm 1.28$  (Figure 3.4). The expansion of the I/R Injury at 168 hours post instillation was greater with the 20nm citrate capped AgNP than that of the 110 nm citrate capped AgNP. 168 hours following IT exposure to 110 nm AgNP resulted in a significantly smaller I/R injury with in the 110 nm citrate capped AgNP as compared to the I/R injury associated with 110 nm PVP capped AgNP.

### **3.2.3. IT INSTILLATION of AgAc RESULTS in EXACERBATION of I/R INJURY**

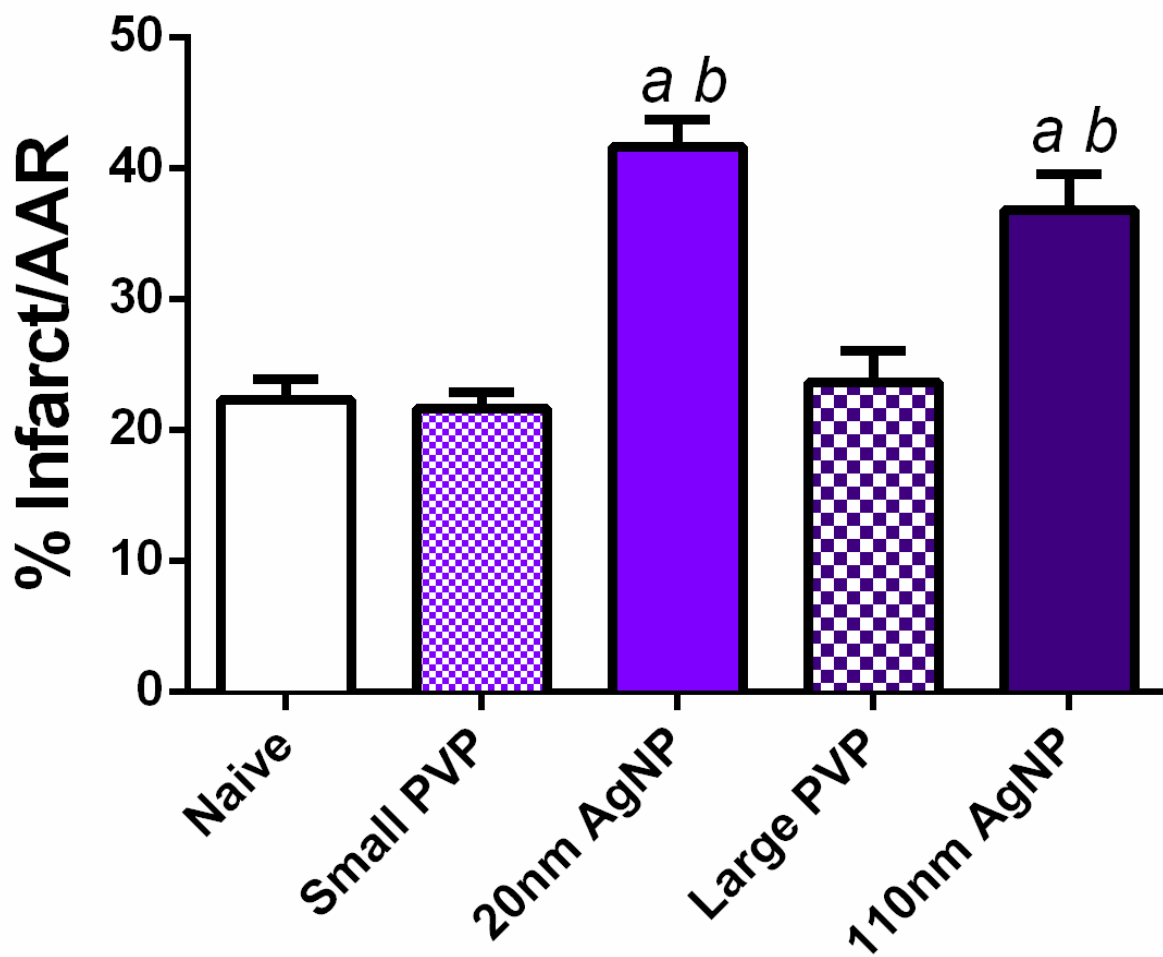
Dose response to administration of 200  $\mu$ l of 0.01 mg/mL, 0.1 mg/mL, and 1 mg/mL AgAc demonstrated an increase in I/R injury 24 hours following IT instillation (Fig 3.5). The lowest concentration AgAc administered did not result in any statistical increase in infarction percent  $21.62 \pm 1.9$  % compared to the naïve group  $16.99 \pm 0.64$  %. The highest concentration of AgAc resulted in a mean injury of  $37.55 \pm 4.15$  % and the median concentration of AgAc resulted in  $28.20 \pm 0.17$  % of the area at risk infarcting.

### **3.2.4 IT INSTILLATION of 1mg/mL AgAc DECREASES POST OCCLUSION SURVIVAL**

Sprague Dawley rats IT instilled with 1mg/mL AgAc exhibited higher post occlusion mortality than any other group (Fig 3.6). Intratracheal instillation of 1mg/mL AgAc resulted in premature mortality for 3 of the 4 rats within the cohort, dying only 5, 15, and 30 minutes into reperfusion. No other experimental group experienced such a profound mortality rate associated with the ischemia reperfusion protocol.

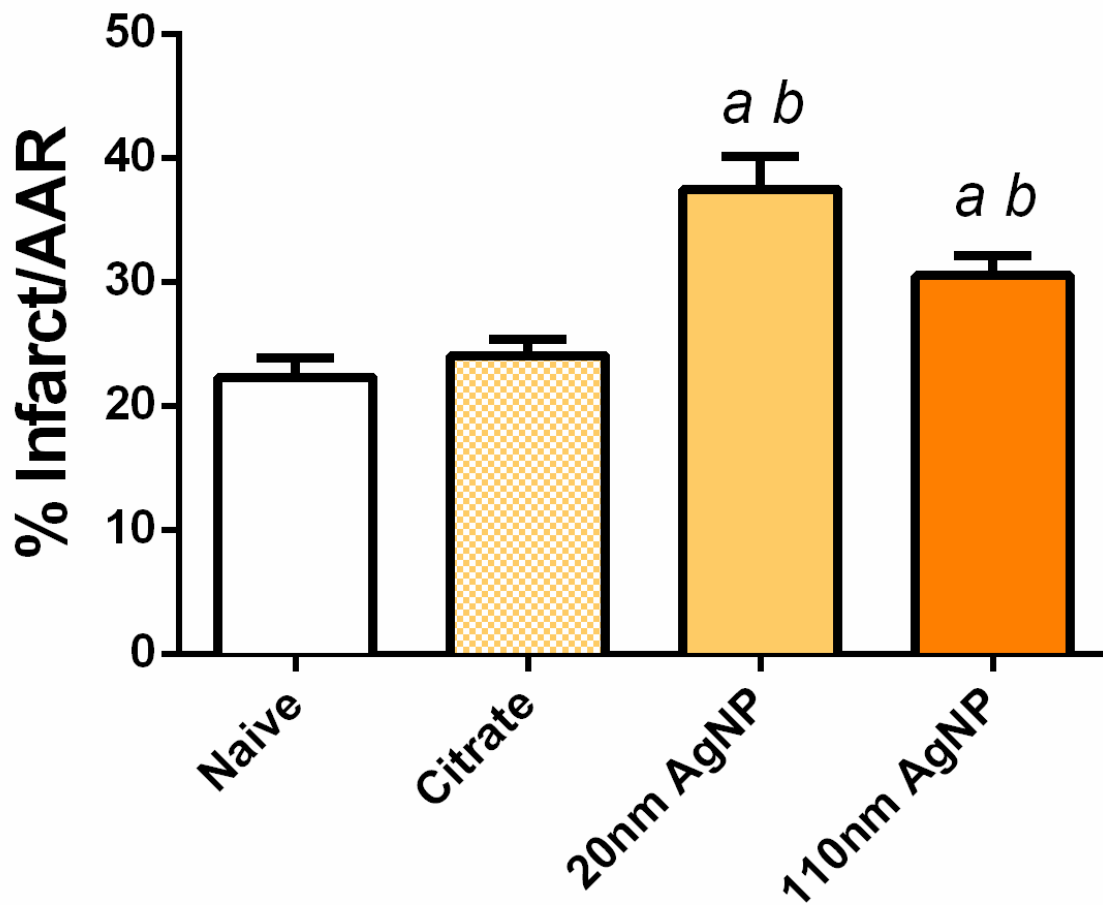
**Figure 3.1:** Male SD rats exposed to IT instillation of 20nm or 110 nm AgNP, or PVP vehicle. I/R injury is expanded 24 hours following IT exposure to AgNP. Statistical significance,  $P < 0.05$  determined by one-way ANOVA with Bonferonni post hoc test. *a* significant versus naïve, *b* significant vs vehicle.  $n = 3-4$

# PVP Capped AgNP 24 Hours Post Instillation



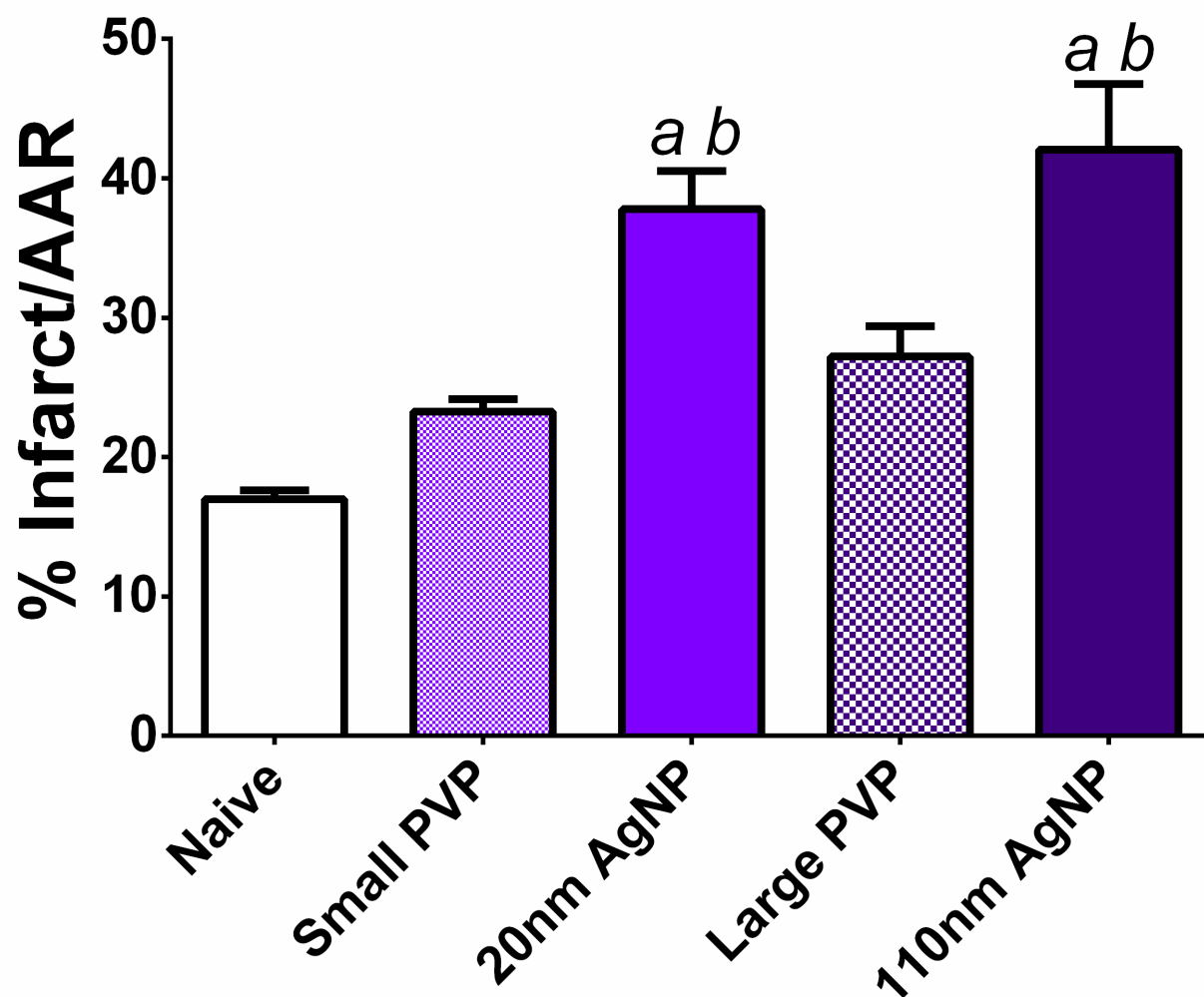
**Figure 3.2:** Male SD rats exposed to IT instillation of 20nm or 110 nm AgNP, or citrate vehicle. I/R injury is expanded 24 hours following IT exposure to AgNP. Statistical significance,  $P < 0.05$  determined by one-way ANOVA with Bonferonni post hoc test. *a* significant versus naïve, *b* significant vs vehicle  $n = 3-4$ .

## Citrate Capped AgNP 24 Hours Post Instillation



**Figure 3.3:** Male SD rats exposed to IT instillation of 20nm or 110 nm AgNP, or PVP vehicle. I/R injury is expanded 168 hours following IT exposure to AgNP. Statistical significance,  $P < 0.05$  determined by one-way ANOVA with Bonferonni post hoc test. *a* significant versus naïve, *b* significant vs vehicle.  $n = 3-4$

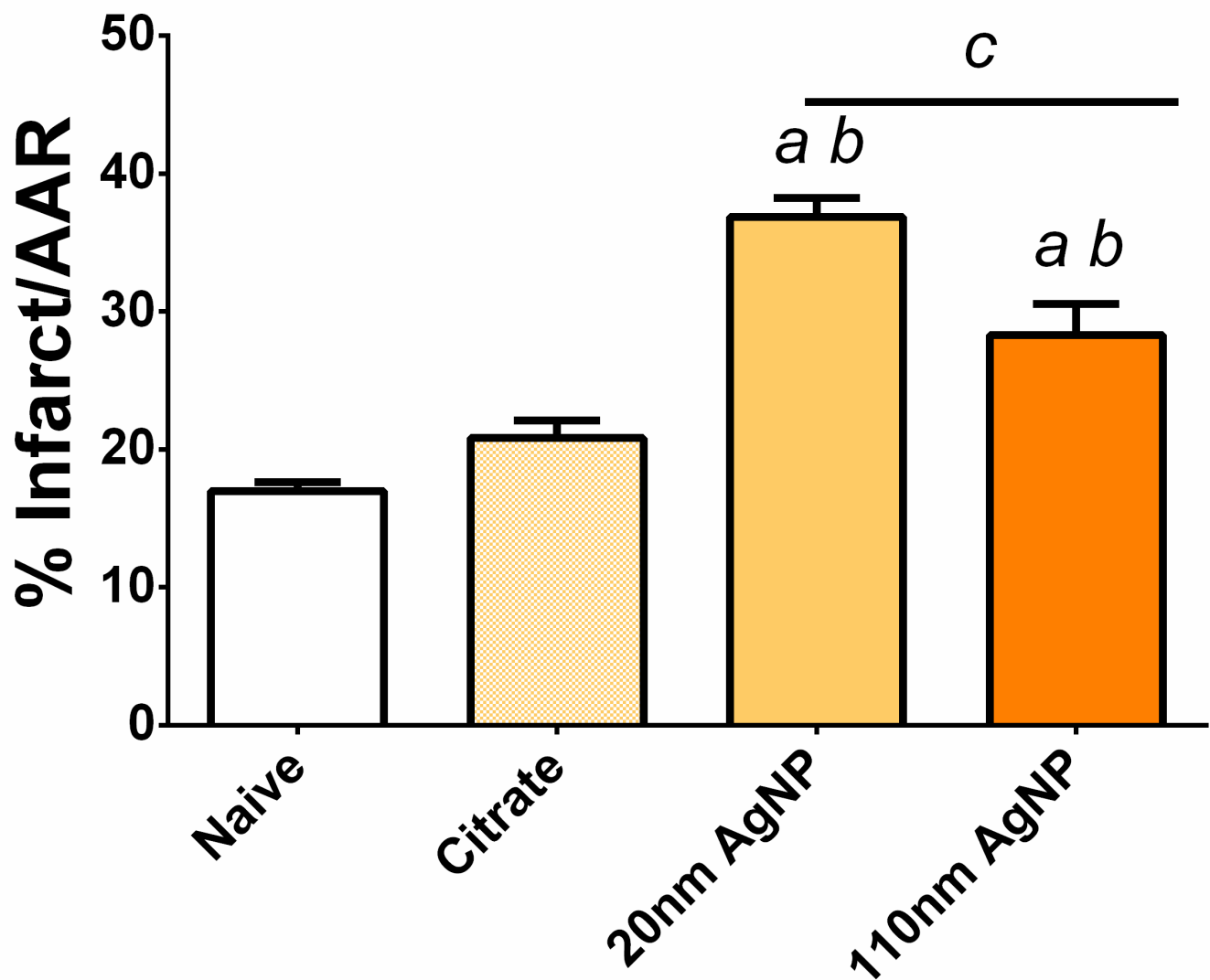
# PVP Capped AgNP 168 Hours Post Instillation





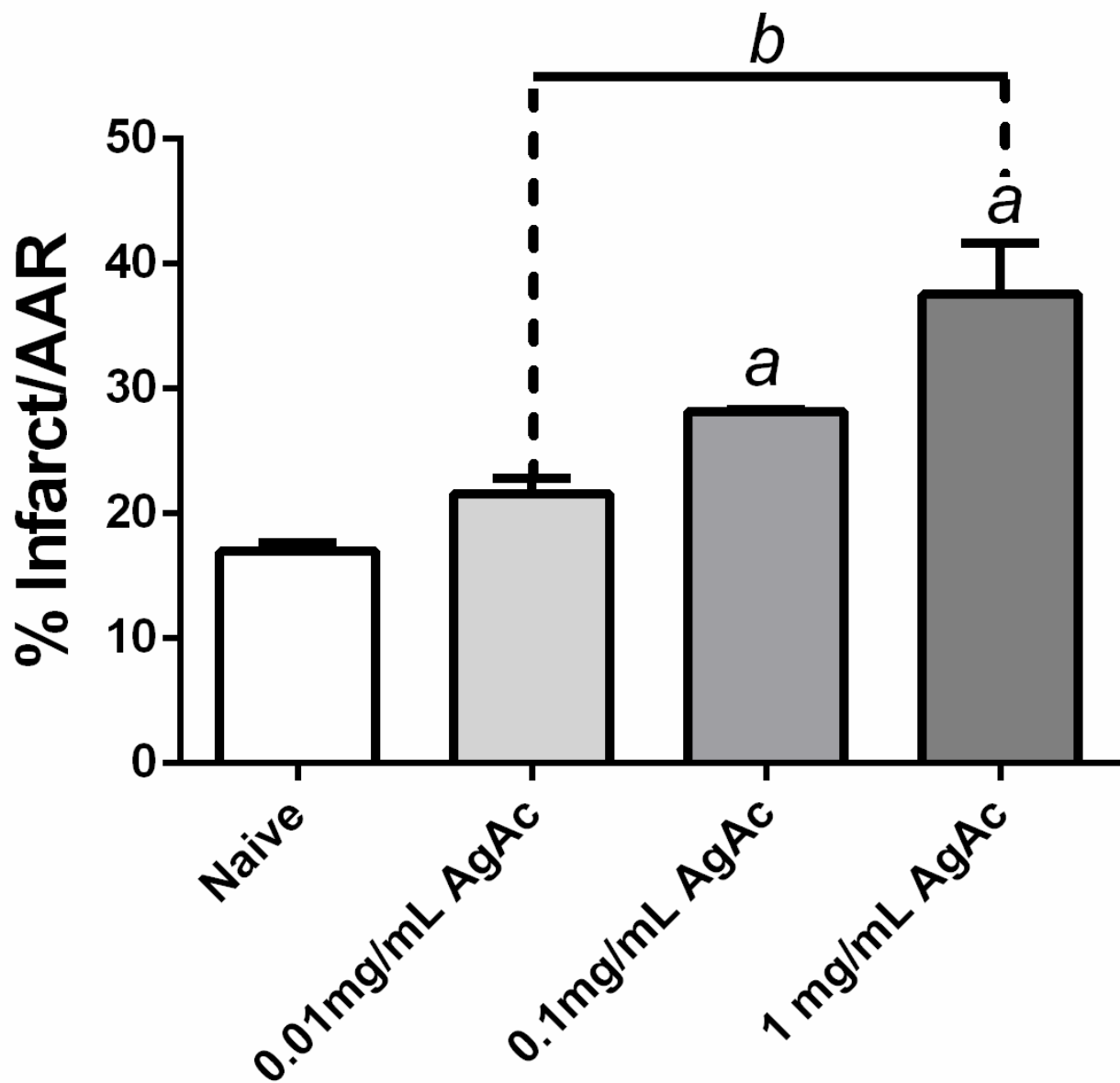
**Figure 3.4:** Male SD rats exposed to IT instillation of 20nm or 110 nm AgNP, or citrate vehicle. I/R injury is expanded 168 hours following IT exposure to AgNP. Statistical significance,  $P < 0.05$  determined by one-way ANOVA with Bonferonni post hoc test. *a* significant versus naïve, *b* significant vs vehicle, *c* significant vs other particle size. n= 3-4

# Citrate Capped AgNP 168 Hours Post Instillation



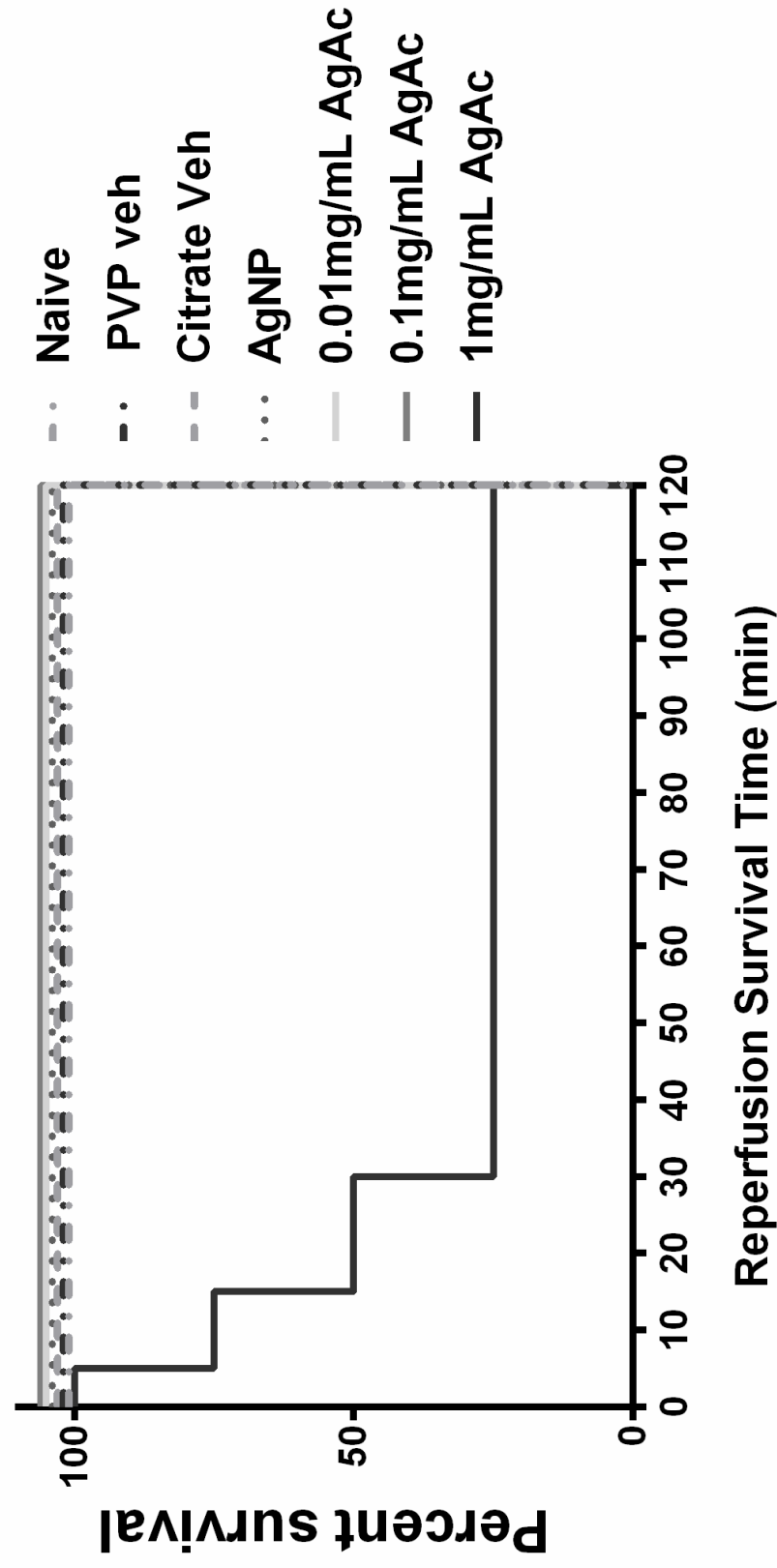
**Figure 3.5:** Male SD rats exposed to IT instillation of 0.01 mg/mL, 0.1 mg/mL, or 1 mg/mL AgAc. I/R injury is expanded 24 hours following IT exposure to the two highest concentrations of AgAc. Statistical significance,  $P < 0.05$  determined by one-way ANOVA with Bonferonni post hoc test. *a* significant versus naïve, *b* significant vs AgAc concentration.  $n = 3-4$

# AgAc 24 Hours Post Instillation



**Figure 3.6:** Survival plot of all groups demonstrates animal survival time into reperfusion. Only animals from the group instilled with the highest concentration of AgAc failed to survive to the end of the designated reperfusion protocol, with deaths at 5, 15, and 30 minutes into reperfusion. n= 3-4

# Survival Time After Reperfusion



### 3.3. DISCUSSION

These data reveal that pulmonary exposure to AgNP results in exacerbation of myocardial infarction following a 20 minute period of ischemia and two hours of reperfusion in Sprague-Dawley-Rats up to 7 days following instillation. Although there are no statistical differences between the 20 nm and 100 nm AgNP I/R Injuries at 24 hours the slight differences measured are suggestive that capping agents may influence the downstream toxicity associated with a pulmonary AgNP exposure (114). The apparent difference in I/R injury observed at 7 days post instillation may be mediated by an interaction of particle size as well as capping agents. Twenty four hours following IT exposure, both size particles in both capping agents have a similar impact on expansion of I/R injury. These findings are congruent with other studies evaluating expansion of cardiac I/R injury following pulmonary exposure to various engineered nanomaterials in rats and mice (13, 14, 118). At 7 days post exposure a trend begins to develop where exposure to 20 nm AgNP maintains an augmented I/R injury independent of capping agent. However by 7 days post exposure, the impact of 110 nm AgNP on I/R injury appears to be capping agent dependent. At 7 days post exposure PVP capped 110nm AgNP results in an expansion of I/R injury that appears to be slightly larger than that seen at 24 hours, whereas extent of I/R injury with citrate capped AgNP is lower. It appears that any initial insult to the lungs by pulmonary exposure to AgNP induces expansion of MI independent of particle size or capping. However, unique biophysical interactions between particle sizes and capping agents may trigger unique downstream pathways of toxicity capable of inducing differential responses at later time points (22).

A secondary question can be raised regarding AgNP and potential cardiovascular toxicity: What, if any, contribution do silver ions have in the exacerbated myocardial I/R injury? In order to address that question, male SD rats were instilled with 1 mg/mL, 0.1 mg/mL, or 0.01

mg/mL concentrations of AgAc, a silver salt that dissociated to form  $\text{Ag}^+$  in an aqueous solution. These concentrations represent a range of potential silver ion dissolution from the nanoparticles in the lungs ranging from 100% dissolution to 1%. Reports from the NIH Centers for Nanotechnology Health Implications Research Consortium meetings suggest that the dissolution rate from these nanomaterials in *in vitro* settings can range from 1-30% dependent on the particle size and the initial suspension concentration. Comparing I/R injury expansion from AgAc to AgNP we observe similar levels of injury expansion at the highest concentration of AgAc as induced by AgNP. However, 1 mg/mL AgAc represents a higher than expected *in vivo* dissolution rate. Instillation of 0.1 mg/mL AgAc also induced I/R injury equivalent to all but 20 nm PVP capped AgNP. These data allow us to suggest, based on comparisons of AgNP to AgAc concentration dependent I/R injury, particle dissolution rates are likely greater than 10% within the first 24 hours. The *in vitro* dissolution rate is dependent on concentration, so it may be the expansion of I/R injury at 24 hours post exposure is associated with processes effected by the rate of dissolution of  $\text{Ag}^+$ . Alternatively, particle size and capping agent may impact the concentration of silver ions able to interact with cells and tissue *in vivo*. It is apparent that  $\text{Ag}^+$  ions are at least partially responsible for the cardiovascular toxicity of AgNP due to their concentration dependent ability to expand I/R injury 24 hours post IT instillation. It may be that  $\text{Ag}^+$  ions, alone, are more detrimental than AgNP as evidenced by the high intraoperative mortality only seen in the 1mg/mL AgAc cohort.



## **CHAPTER 4**

### **ELECTRICAL and AUTONOMIC RESPONSE to IT INSTILLATION of AgNP**

#### **4.1 INTRODUCTION**

Previous studies have demonstrated the ability for pulmonary exposure to particulate matter (PM) to impact autonomic function (19, 21). In order to determine the impact of pulmonary exposure to AgNP on cardiac autonomic function we analyzed electrocardiogram (ECG) data recorded during the ischemia reperfusion (I/R) experimental protocol. In order to measure the potential influence of AgNP exposure on autonomic function we evaluated: heart rate (HR), PR interval, and corrected QT interval (QTc). Changes in HR may indicate whether AgNP exposure results in changes to parasympathetic and sympathetic balance and regulation of HR. PR interval is the ECG surface reading of atrial depolarization as well as conduction through the atrioventricular (AV) node interval (75) which are subject to control of the autonomic nervous system (ANS) whereby increased vagal tone slows AV conduction and increases PR interval. QT interval with Bazett's correction for HR is used to determine the duration of ventricular action potential via surface ECG (101, 140). Additionally, prolongation of QT interval beyond the compensatory influence of autonomic tone places the heart at significant risk for ventricular arrhythmias which may contribute to the development of ischemia as well as sudden cardiac death (38).

#### **4.2 RESULTS**

##### **4.2.1 AgNP SIZE AND CAPPING DO NOT IMPACT HEART RATE 7 DAYS POST EXPOSURE**

In order to determine the potential impact of IT instillation of AgNP seven days following exposure on cardiac autonomic function we evaluated the HR of male SD rats by sampling a

baseline period of 15 minutes for 10 seconds at 1, 5, 10, and 15 minutes prior to ischemic manipulation. Exposure to PVP capped AgNP had no impact on mean baseline HR (Fig 4.1). IT instillation of citrate capped AgNP also resulted in no differences in HR within baseline (Fig 4.2). Likewise, there were no differences in HR following occlusion of the LAD or reperfusion in either PVP (Fig 4.1) or citrate (Fig 4.2) capped groups.

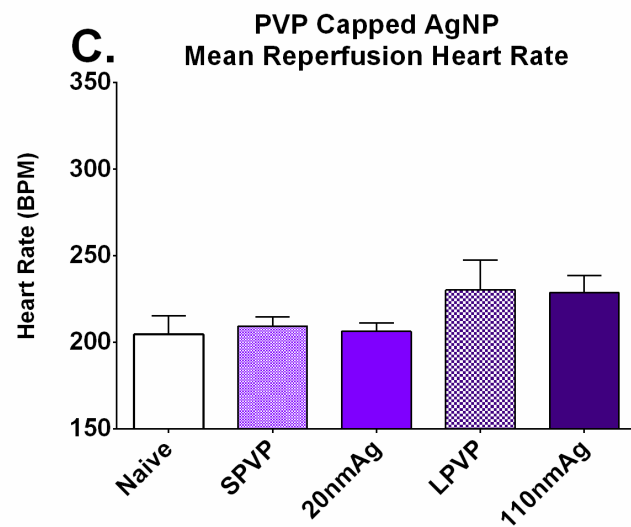
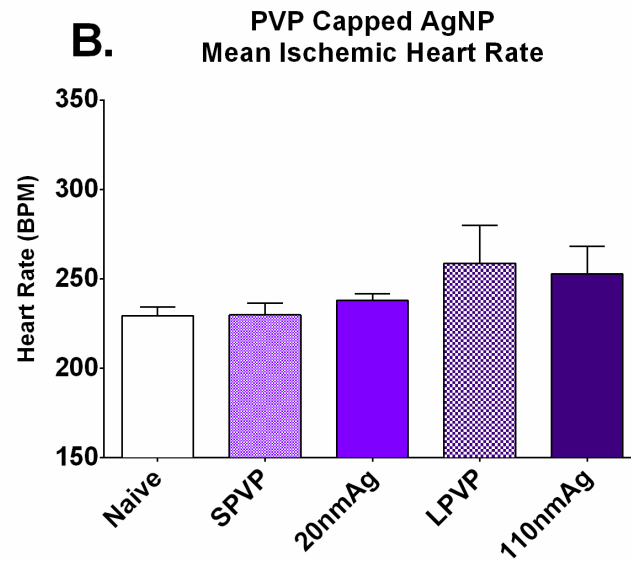
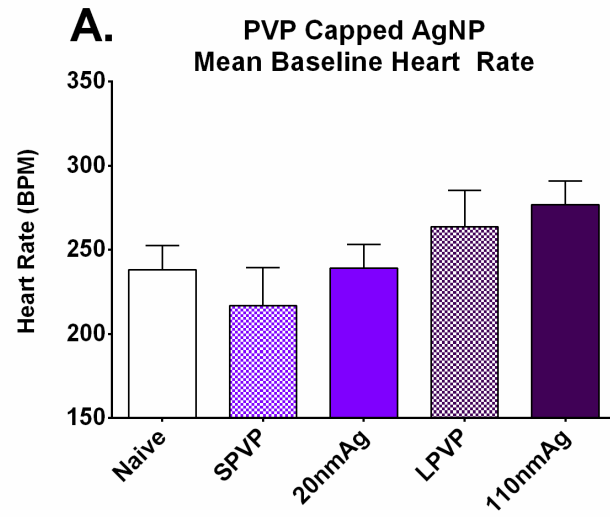
#### **4.2.3 AgNP DOES NOT IMPACT QTc 7 DAYS POST EXPOSURE**

Analysis of ECG revealed no differences in mean QTc between AgNPs, naïve, or controls, during baseline, ischemia, reperfusion for PVP capped AgNP (Fig 4.3). Citrated capped AgNP also demonstrated no differences in mean QTc during baseline, ischemia, and reperfusion (Fig 4.4).

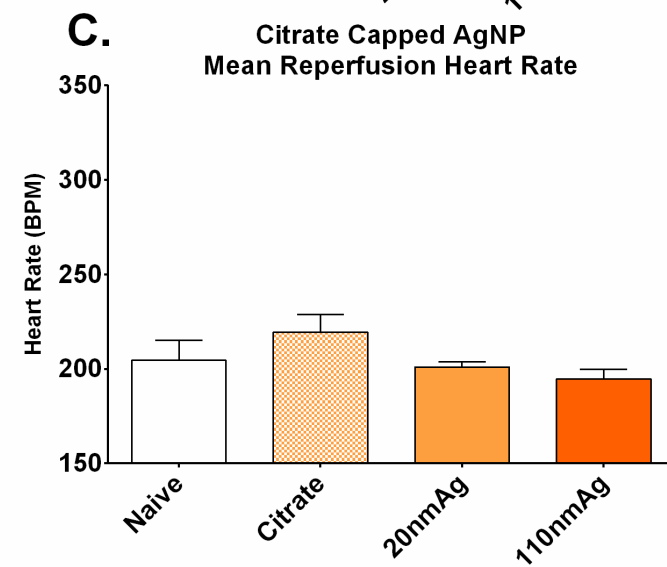
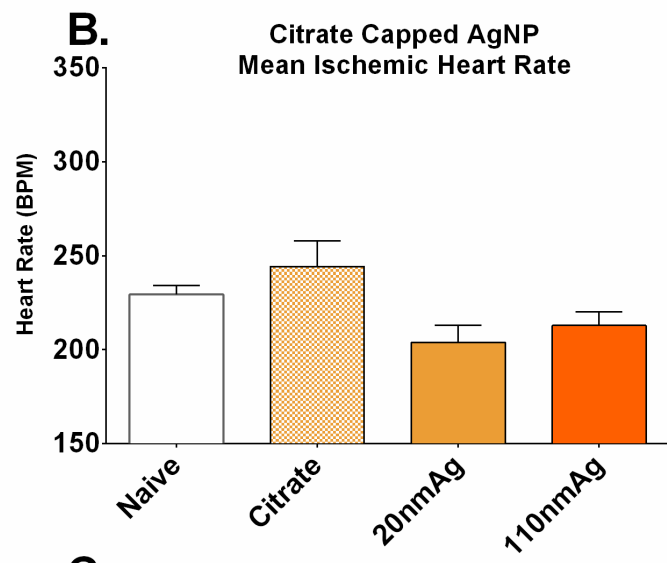
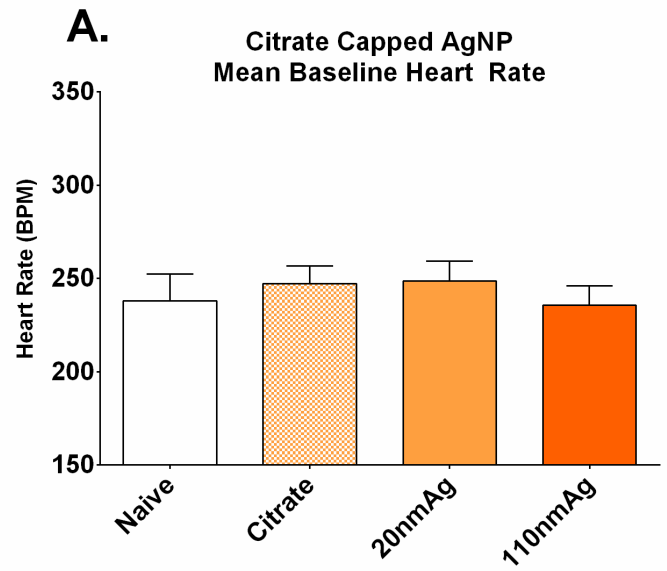
#### **4.2.2 AgNP SIZE and CAPPING DIFFERENTIALLY IMPACT PR INTERVAL.**

Intratracheal instillation of PVP capped AgNP does not significantly alter baseline or ischemic PR interval at 7 days following exposure (Fig. 4.3). Likewise, IT instillation of citrate capped AgNP resulted in no differences in baseline or ischemic PR interval compared to vehicle or naïve at 7 days post exposure (Fig 4.4). Mean reperfusion PR interval was longer in 20 nm PVP capped AgNP,  $0.052 \pm 0.002$  seconds, than SPVP vehicle,  $0.045 \pm 0.002$  seconds, but not significantly different than naïve,  $0.053 \pm 0.001$  seconds. 110 nm PVP capped AgNP was not different from LPVP vehicle or naïve. (Fig. 4.5) PR interval was decreased in 20nm citrate capped AgNP,  $0.048 \pm .001$ , compared to citrate vehicle,  $0.055 \pm 0.002$  seconds, but not naïve,  $0.053 \pm 0.001$  seconds, 110 nm AgNP was not statistically different from naïve or vehicle (Fig 4.6).

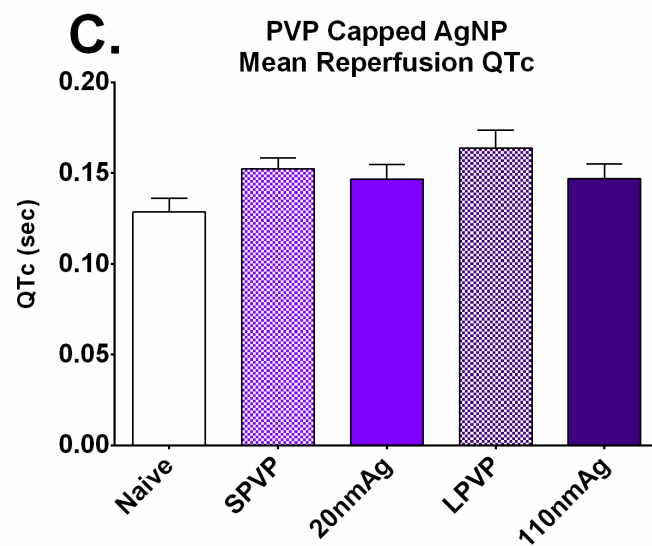
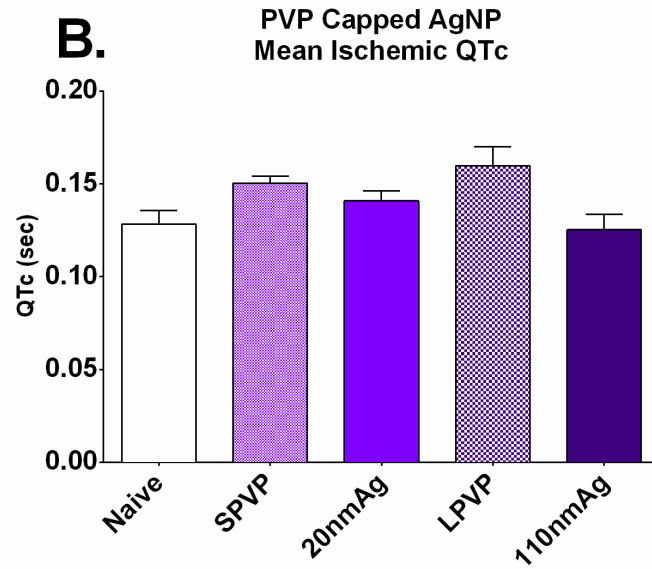
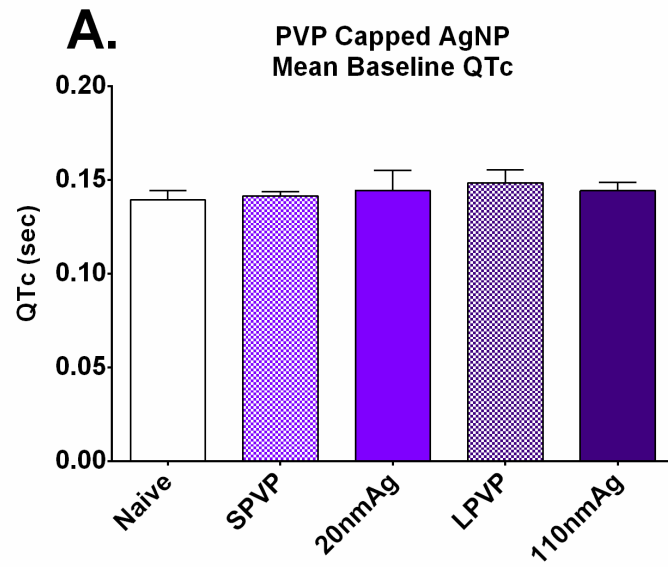
**Figure 4.1:** Heart rates for male SD rats exposed to PVP capped AgNP 7 days prior to assessment. Mean values displayed based on a 10 second sample at: 1, 5, 10, 15 minutes into baseline (A) and occlusion (B). 10 second samples were taken at: 1, 5, 10, 15, 25, 30, 45, 60, 75, 90, 105 and 120 minutes during reperfusion (C). No differences in HR exist.  $P < 0.05$  determined by two-way ANOVA with Tukey post hoc test,  $n=3-4$ .



**Figure 4.2:** Heart rates for male SD rats exposed to citrate capped AgNP 7 days prior to assessment. Mean values displayed based on a 10 second sample at: 1, 5, 10, 15 minutes into baseline (A) and occlusion (B). 10 second samples were taken at: 1, 5, 10, 15, 25, 30, 45, 60, 75, 90, 105 and 120 minutes during reperfusion (C). No differences in HR exist.  $P < 0.05$  determined by two-way ANOVA with Tukey post hoc test,  $n=3-4$ .

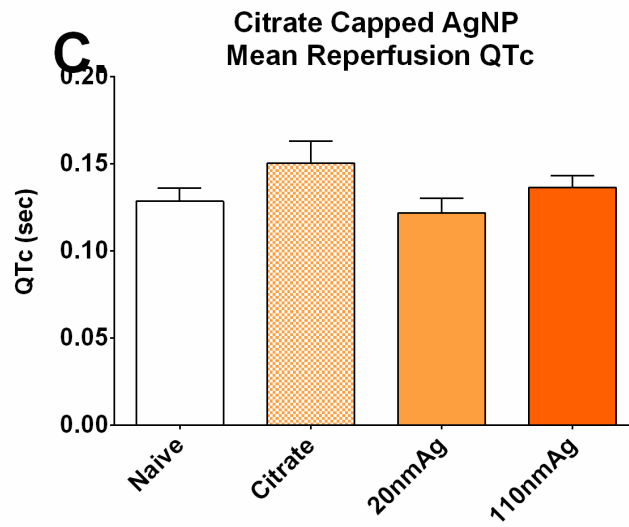
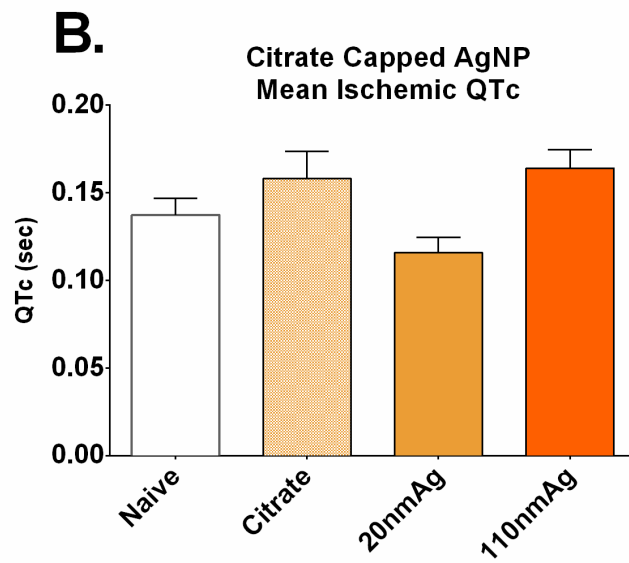
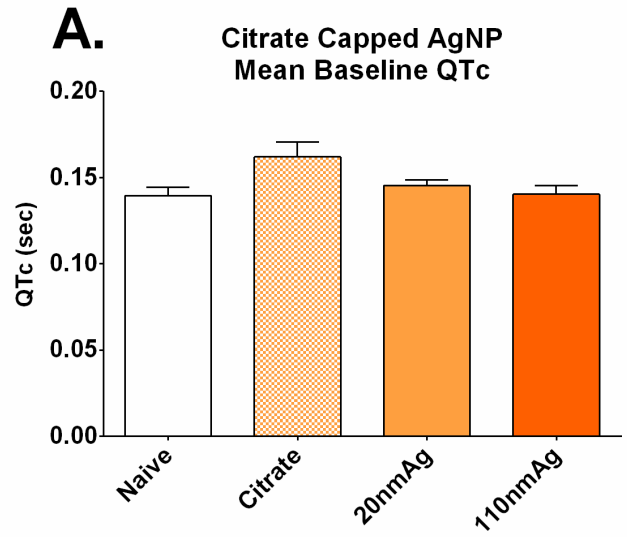


**Figure 4.3:** Corrected QT interval for male SD rats exposed to PVP capped AgNP 7 days prior to assessment. Mean values displayed based on a 10 second sample at: 1, 5, 10, 15 minutes into baseline (A) and occlusion (B). 10 second samples were taken at: 1, 5, 10, 15, 25, 30, 45, 60, 75, 90, 105 and 120 minutes during reperfusion (C). No differences in PR interval exist.  $P < 0.05$  determined by two-way ANOVA with Tukey post hoc test,  $n = 2-4$ .

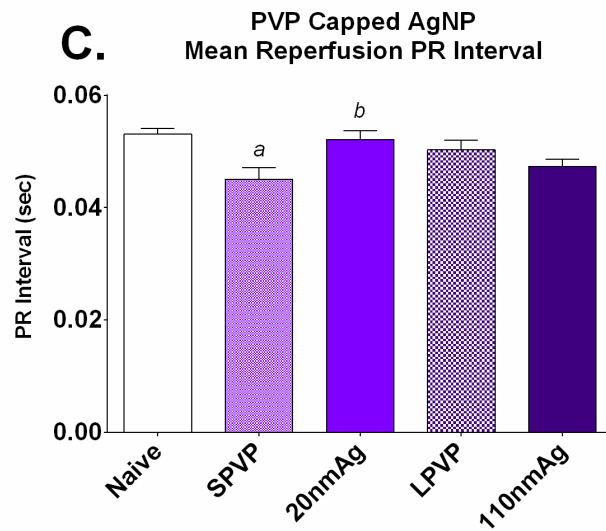
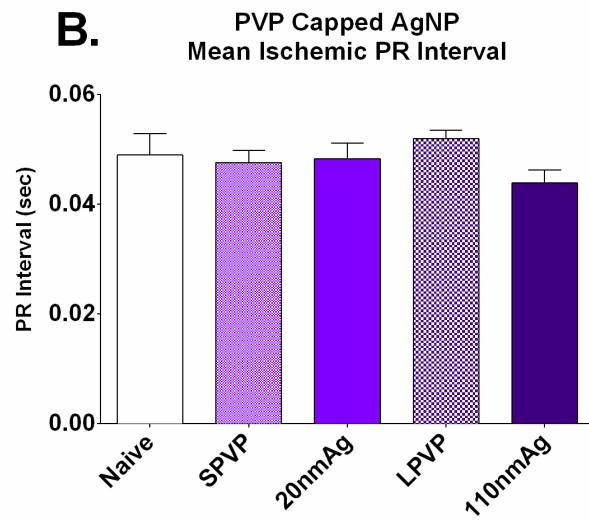
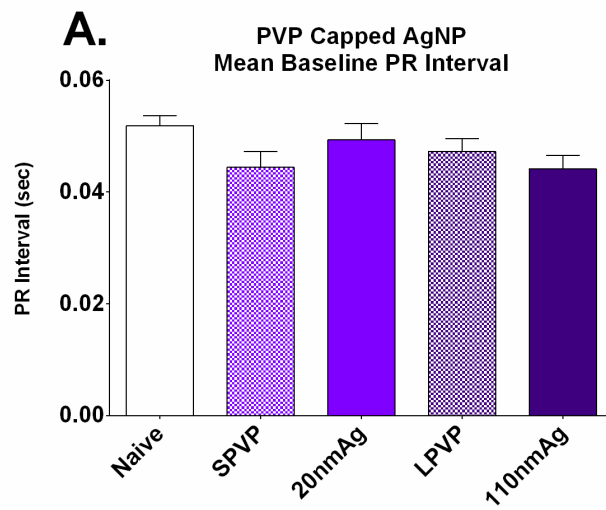




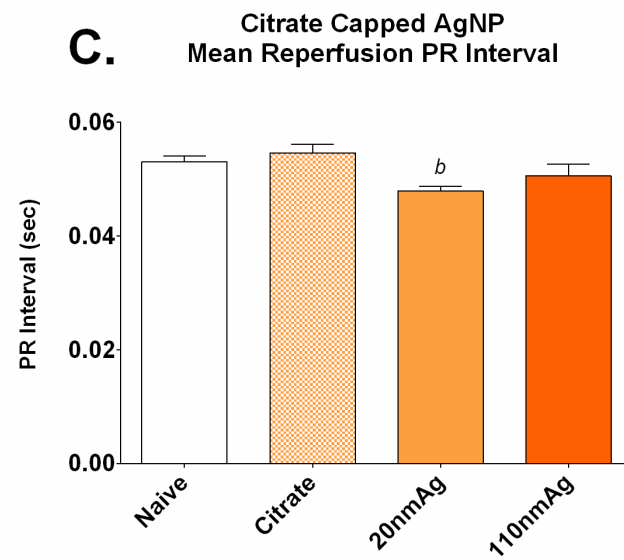
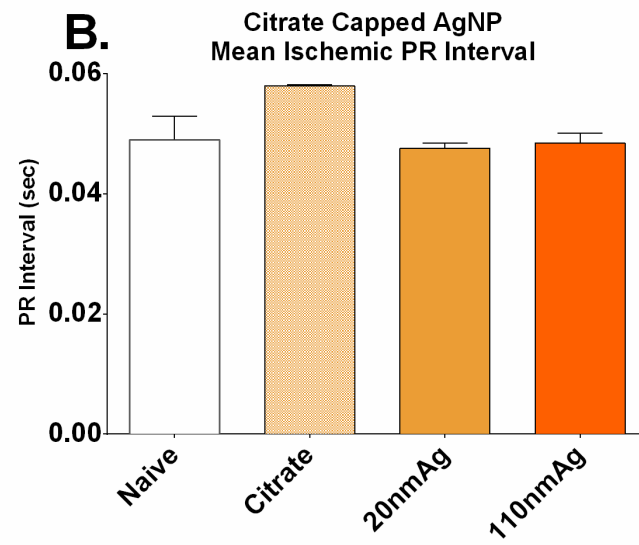
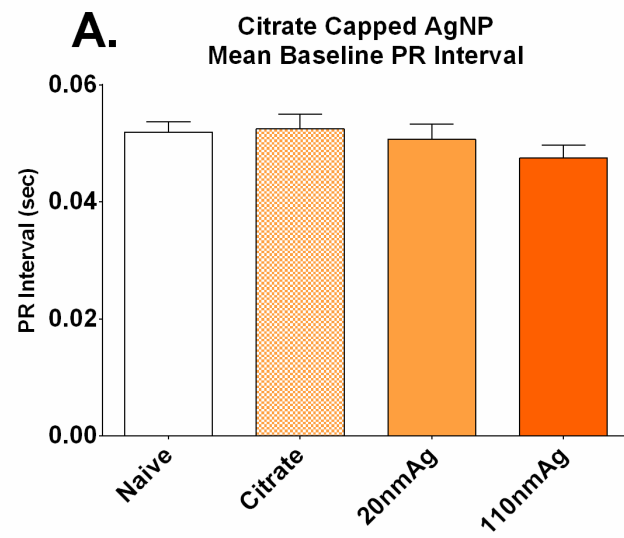
**Figure 4.4:** Corrected QT interval for male SD rats exposed to citrate capped AgNP 7 days prior to assessment. Mean values displayed based on a 10 second sample at: 1, 5, 10, 15 minutes into baseline (A) and occlusion (B). 10 second samples were taken at: 1, 5, 10, 15, 25, 30, 45, 60, 75, 90, 105 and 120 minutes during reperfusion (C). No differences in PR interval exist at any time point.  $P < 0.05$  determined by two-way ANOVA with Tukey post hoc test,  $n=2-4$ .



**Figure 4.5:** PR interval for male SD rats exposed to PVP capped AgNP 7 days prior to assessment. Mean values displayed based on a 10 second sample at: 1, 5, 10, 15 minutes into baseline (A) and occlusion (B). 10 second samples were taken at: 1, 5, 10, 15, 25, 30, 45, 60, 75, 90, 105 and 120 minutes during reperfusion (C). No differences in PR interval during baseline or ischemia exist. Mean PR was lengthened during reperfusion by 20nm AgNP compared to vehicle but not naïve.  $P < 0.05$  determined by two-way ANOVA with Tukey post hoc test,  $n = 2-4$ .



**Figure 4.6:** PR interval for male SD rats exposed to citrate capped AgNP 7 days prior to assessment. Mean values displayed based on a 10 second sample at: 1, 5, 10, 15 minutes into baseline (A) and occlusion (B). 10 second samples were taken at: 1, 5, 10, 15, 25, 30, 45, 60, 75, 90, 105 and 120 minutes during reperfusion (C). 20 nm AgNP reduces PR interval compared to vehicle.  $P < 0.05$  determined by two-way ANOVA with Tukey post hoc test,  $n = 2-4$ .



### 4.3 DISCUSSION

Despite previous studies demonstrating the ability for pulmonary exposure to PM to induce changes in autonomic balance as measured by HR and HR variability (12, 19, 21) seven days following post exposure there appears to be no influence on HR. Neither PVP nor citrate capped AgNP were demonstrated to induce a significant change in HR. Despite the body of evidence that exposure to PM induces an autonomic imbalance, evidence of AgNP influencing autonomic balance is both sparse and conflicting. Exposure to aerosolized AgNP has been shown to have no basal impact on HR 7 days following exposure (98). The same study did find that a significant increase in HR could be induced with isoproterenol following exposure to high doses of AgNP. A zebrafish embryo model of AgNP exposure demonstrated a size dependent impact on HR, whereby smaller AgNP were able to induce a greater reduction in resting HR (90). Our data is congruent with a lack of particle response in respect to HR 7 days post exposure. This thesis only evaluated HR 7 days post exposure, it is possible that any changes in autonomic balance do not persist 7 days post exposure but may be evident at earlier time points.

Much like HR, exposure to both PVP and citrate capped AgNP resulted in no changes in corrected QT interval. At present, there have been no published studies evaluating the impact of exposure to AgNP on QT interval. Much like with HR, data regarding the impact of PM exposure on QT interval are conflicting. Pulmonary exposure to ambient traffic-related particulate matter, namely black carbon, has been demonstrated to be associated with an increase in QT interval. (8) Interestingly, the same study was unable to show a statistically significant change in QT interval following exposure to ultrafine was not associated with the same prolongation of QT interval. The impact of PM exposure on ventricular repolarization and QT interval may be delayed in onset, as demonstrated by a human study which showed

the greatest changes to QT interval occurred 3 to 4 hours following exposure to ambient fine PM (59). A lack of particle impact on QT interval is also supported by a study of spontaneous heart failure prone rats whereby exposure to whole diesel exhaust was unable to induce changes in QT interval (20). Pulmonary exposure to PM collected from an urban area on the east coast was determined to result in prolonged QT intervals compared to control (134). Despite evidence suggesting that exposure to PM may impact ventricular depolarization or repolarization events as measured by QT interval, our results demonstrate that exposure to AgNP does not result in alterations to QT interval 7 days post exposure.

Finally, PR interval appears to be differentially impacted by exposure to AgNP. Previous studies have found an association between exposure to PM and increased PR interval (20, 60). Our study demonstrates that exposure to 20 nm AgNP results in capping dependent changes in PR interval. Exposure to PVP capped AgNP results in a prolongation of the PR interval when compared to vehicle. This response is similar to literature reports regarding prolongation of PR intervals following exposure pulmonary exposure to PM. Citrate capped AgNP also alter PR interval compared to vehicle control, however unlike PVP citrate results in a shortened PR interval, a somewhat surprising response contradictory to previous studies of PM as well as to the responses seen in PVP capped AgNP. It may be that surface modification of capping agents play an important role on mediating biological responses to AgNP. It may be important to note that although some statistical differences were seen in PR intervals these only were present in 20nm AgNP and only compared to their direct control. It is suggestive of the fact that even at 7 days following instillation of AgNP the 20nm particle is able to elicit a small response. It may also be important to note that differences were only detected during reperfusion suggesting that particle responses may require a stressor to manifest themselves.



It is unclear whether or not changes observed were a result of stress of reperfusion injury or a delayed response to ischemic insult.

## **CHAPTER 5**

### **INFLAMMATORY RESPONSE to IT INSTILLATION of AgNP**

#### **5.1 INTRODUCTION**

The ability for various PM to induce an inflammatory response has been well documented. Exposure to diesel particles has been shown to increase secretion of TNF- $\alpha$ , as well as IL-6 and IL-1 $\beta$  from human alveolar macrophages (46). Pulmonary responses to PM have been shown to stimulate release of polymorphonuclear leukocytes in bone marrow (117) which may contribute to a systemic inflammatory response (128). Furthermore, differential cellular response to AgNP may induce a release of cytokines and chemokines from endothelial cells which could contribute CV toxicity associated with exposure to AgNP (122). In order to evaluate the inflammatory response to exposure to AgNP we exposed male SD rats to two sizes of AgNP with two different capping agents. We measured concentration of several circulating cytokines before and after instillation of PM, as well as immediately following I/R Injury.

#### **5.2 RESULTS**

##### **5.2.1 IT INSTILLATION of AgNP DOES NOT RESULT IN INCREASED CONCENTRATION of CIRCULATING CYTOKINES**

No differences were noted in serum cytokine concentrations for: G-CSF, GM-CSF, IL-1 $\beta$ , IL-2, IL-5, IL-6, IL-10, IL-13, IL-17, IL-18, IFN $\gamma$ , MIP-1 $\alpha$ , TNF $\alpha$  or RANTES at 1, 3, 6, 24, 28, 48, 72, and 168 hours following IT instillation of AgNP or vehicle. Concentrations of serum cytokines at baseline and following IT instillation are listed for: small PVP (Table 5.2), PVP capped 20 nm AgNP (Table 5.3), large PVP (Table 5.4), PVP capped 110 nm AgNP (Table 5.5), citrate (Table 5.6), citrate capped 20 nm AgNP (Table 5.7) and citrate capped 110 nm AgNP (Table 5.8).

### **5.2.2 PVP and CITRATE CAPPED AgNP ELICT DIFFERENTIAL INFLAMMATORY RESPONSE FOLLOWING I/R INJURY**

Following I/R injury 7 days post IT instillation of AgNP the greatest changes in inflammatory cytokine levels from baseline are seen in the 20 nm PVP capped AgNP. Several proinflammatory cytokines including IL-2, and IL-18 are significantly increased from baseline compared to vehicle (Fig 5.1). Following I/R injury, 7 days post IT instillation, circulating serum concentrations of IL-2 and IL-18 were significantly elevated from baseline  $138.1 \pm 34.4 \%$  and  $153.1 \pm 54.0$  respectively in the 20 nm PVP capped AgNP. Whereas the corresponding vehicle demonstrated a  $16.6 \pm 17.9 \%$  decrease from baseline in serum IL-2 and only a  $5.1 \pm 20.0 \%$  increase from baseline serum IL-18. Likewise, at 7 days serum IL-6 is elevated  $211.0 \pm 56.4 \%$  from baseline in the 20 nm AgNP versus a depression of  $14.7 \pm 34.6$  from baseline in the small PVP vehicle (Fig 5.2). These difference are only demonstrated in the PVP capped AgNP and only in the 20 nm variety. No differences were detected between 110 nm PVP capped AgNP and the respective vehicle. Regarding the citrate capped AgNP no differences in inflammatory response to I/R exist at 7 days post exposure between 20 nm and 110 nm AgNP and citrate vehicle.

### **5.2.3 TIME POST IT EXPOSURE IMPACTS INFLAMMATORY RESPONSE TO I/R INJURY**

Serum cytokine profile response to I/R injury following IT instillation of AgNP is attenuated at 7 days as compared to the profile and levels seen from 24 hours post AGNP exposure. At 24 hours following IT instillation of AgNP induction of I/R injury results in a  $89.1 \pm 33.9 \%$  increase in serum IL-6 concentration from baseline compared to a  $-69.4 \pm 14.5.0 \%$  decrease from baseline following IT instillation of small PVP vehicle . At 24 hours cytokine release in response to I/R injury following IT instillation of PVP capped 110 nm appears to be blunted from baseline compared to large PVP vehicle,  $-87.9 \pm 12.1 \%$  versus an increase of

26.4  $\pm$  87.2 %, respectively (Fig 5.2), however no statistical significance exists. No statistical differences were found between citrate, citrate capped 20 nm AgNP and citrate capped 110 nm AgNP cytokine levels, 24 post exposure, in response to I/R injury. When the cytokine response to I/R injury was evaluated 7 days following IT instillation the relationship between 20 nm AgNP and small PVP remains consistent; 20 nm AgNP was significantly elevated from baseline 211  $\pm$  34.5 % compared to small PVP -14.7  $\pm$  56.4 %. Citrate capped AgNP groups had no statistical differences 7 days following pulmonary exposure from the vehicle control levels. Despite similarities in pattern of inflammatory response to I/R injury 24 hours and 7 days following IT instillation of AgNP, independent of capping agent, the magnitude of response had diminished.

**Table 5.1:** Mean serum cytokine concentrations at baseline. Baseline serum cytokine concentration determined by calculating the mean value of cytokine concentrations at time 0 across individuals for all treatment groups with respective capping agent or vehicle. N/A indicated cytokine was not measured for a particular capping agent. Values of cytokine concentrations are displayed as mean  $\pm$  SEM, n=12-16.

<b>Cytokine</b>	<b>PVP Baseline Mean <math>\pm</math> SEM (pg/mL)</b>	<b>Citrate Baseline Mean <math>\pm</math> SEM (pg/mL)</b>
<b>G-CSF</b>	246.8 $\pm$ 24.3	36.67 $\pm$ 8.6
<b>GM-CSF</b>	N/A	1.023 $\pm$ 1.0
<b>IL-1<math>\beta</math></b>	64.9 $\pm$ 8.0	7.6 $\pm$ 4.8
<b>IL-2</b>	459.6 $\pm$ 45.8	161.7 $\pm$ 24.5
<b>IL-5</b>	N/A	300.2 $\pm$ 33.8
<b>IL-6</b>	1559.0 $\pm$ 226.3	366.4 $\pm$ 125.5
<b>IL-10</b>	N/A	44.9 $\pm$ 10.6
<b>IL-13</b>	N/A	15.6 $\pm$ 4.3
<b>IL-17</b>	N/A	79.7 $\pm$ 10.1
<b>IL-18</b>	391.5 $\pm$ 38.3	151.8 $\pm$ 23.3
<b>IFN<math>\gamma</math></b>	N/A	38.1 $\pm$ 21.3
<b>MIP-1<math>\alpha</math></b>	N/A	15.7 $\pm$ 3.6
<b>MCP-1</b>	2296.0 $\pm$ 78.1	N/A
<b>TNF<math>\alpha</math></b>	49.9 $\pm$ 6.3	16.6 $\pm$ 2.2
<b>RANTES</b>	N/A	963.3 $\pm$ 124.1

**Table 5.2:** Serum cytokine concentrations at baseline (0 h) and 1, 3, 6, 24, 48, 72, and 168 hours following IT instillation of small PVP vehicle. N/A indicates a cytokine was not examined at a time point. ND indicates a cytokine fell below detection range. Values are represented as a mean of concentration (pg/mL) at each time point  $\pm$  SEM, n= 4.

<b>Small PVP</b>	<b>0h</b>	<b>+1h</b>	<b>+3h</b>	<b>+6h</b>	<b>+24h</b>	<b>+48h</b>	<b>+72h</b>	<b>+168h</b>
<b>G-CSF</b>	253.4 ± 23.8	163.7 ± 48.4	123.4 ± 40.7	178.9 ± 37.6	246.7 ± 22.1	140.0 ± 33.1	118.6 ± 46.5	119.9 ± 39.3
<b>IL-1<math>\beta</math></b>	58.9 ± 13.9	63.7 ± 13.00	58.9 ± 13.9	58.0 ± 4.3	57.1 ± 7.3	51.0 ± 8.2	41.1 ± 9.4	40.67 ± 11.4
<b>IL-2</b>	358.7 ± 37.1	219.3 ± 80.3	151.5 ± 29.2	251.4 ± 50.2	294.6 ± 13.3	229.1 ± 17.5	188.6 ± 58.8	194.9 ± 61.6
<b>IL-6</b>	846.6 ± 151.6	824.7 ± 211.2	558.3 ± 198.8	455.7 ± 160.1	497.8 ± 100.4	197.4 ± 123.6	277.0 ± 102.1	222.3 ± 162.7
<b>IL-18</b>	293.9 ± 30.9	183.5 ± 55.2	128.5 ± 25.5	226.6 ± 24.5	210.0 ± 15.8	195.3 ± 26.0	149.2 ± 47.2	160.4 ± 54.4
<b>MCP-1</b>	2109 ± 103.5	1820 ± 279.0	1580 ± 277.4	1909 ± 185.2	1935 ± 77.0	1668 ± 118.1	1418 ± 327.5	1358 ± 207.6
<b>TNF<math>\alpha</math></b>	82.6 ± 7.9	61.8 ± 16.1	47.5 ± 13.2	63.2 ± 12.1	67.5 ± 3.7	49.3 ± 2.9	37.1 ± 13.7	41.8 ± 11.6



**Table 5.3:** Serum cytokine concentrations at baseline (0h) and 1, 3, 6, 24, 48, 72, and 168 hours following IT instillation of PVP capped 20 nm AgNP. N/A indicates a cytokine was not examined at a time point. ND indicates a cytokine fell below detection range. Values are represented as a mean of concentration (pg/mL) at each time point  $\pm$  SEM, n= 4.

<b>20 nm AgNP/PVP</b>	<b>0h</b>	<b>+1h</b>	<b>+3h</b>	<b>+6h</b>	<b>+24h</b>	<b>+48h</b>	<b>+72h</b>	<b>+168h</b>
<b>G-CSF</b>	342.6 ± 50.8	328.8 ± 45.8	329.8 ± 40.1	316.6 ± 83.8	281.8 ± 27.9	256.9 ± 26.4	208.0 ± 34.3	312.9 ± 107.9
<b>IL-1<math>\beta</math></b>	40.7 ± 11.42	64.4 ± 23.66	53.2 ± 11.17	48.3 ± 16.41	48.0 ± 18.1	21.37 ± 15.2	39.58 ± 27.5	42.47 ± 15.6
<b>IL-2</b>	658.7 ± 110.4	691.9 ± 111.4	618.2 ± 39.58	563.2 ± 120.7	637.7 ± 29.62	465.2 ± 160.9	513.2 ± 91.29	703.4 ± 213.0
<b>IL-6</b>	2380 ± 567.9	2382 ± 699.3	2111 ± 255.7	1984 ± 667.1	1260 ± 316.1	992.6 ± 380.0	1114 ± 505.5	2228 ± 1086
<b>IL-18</b>	547.0 ± 88.6	540.9 ± 78.7	479.1 ± 28.5	469.6 ± 87.2	466.2 ± 21.5	332.1 ± 133.8	426.6 ± 102.7	589.2 ± 192.6
<b>MCP-1</b>	2592 ± 122.6	2676 ± 196.2	2629 ± 88.59	2484 ± 257.5	2494 ± 139.0	1954 ± 371.9	2146 ± 215.6	2606 ± 419.8
<b>TNF<math>\alpha</math></b>	42.6 ± 6.0	47.3 ± 5.9	46.8 ± 4.0	41.3 ± 8.2	42.6 ± 4.4	28.5 ± 9.4	32.9 ± 5.9	48.7 ± 15.9

**Table 5.4:** Serum cytokine concentrations at baseline (0h) and 1, 3, 6, 24, 48, 72, and 168 hours following IT instillation of large PVP vehicle. N/A indicates a cytokine was not examined at a time point. ND indicates a cytokine fell below detection range. Values are represented as a mean of concentration (pg/mL) at each time point  $\pm$  SEM, n=4.

<b>Large PVP</b>	<b>0h</b>	<b>+1h</b>	<b>+3h</b>	<b>+6h</b>	<b>+24h</b>	<b>+48h</b>	<b>+72h</b>	<b>+168h</b>
<b>G-CSF</b>	178.8 ± 23.4	220.1 ± 62.3	110.2 ± 44.2	91.8 ± 54.3	206.6 ± 81.0	266.5 ± 52.9	158.2 ± 24.8	237.6 ± 30.4
<b>IL-1β</b>	72.3 ± 13.0	83.8 ± 29.7	32.5 ± 19.3	33.2 ± 19.9	100.4 ± 24.1	99.7 ± 17.3	47.2 ± 18.7	105.2 ± 14.8
<b>IL-2</b>	395.7 ± 89.5	403.1 ± 106.2	203.2 ± 64.0	182.9 ± 91.2	348.9 ± 117.3	503.3 ± 93.9	306.1 ± 54.8	433.5 ± 76.0
<b>IL-6</b>	1174 ± 354.4	1101 ± 509.5	243.2 ± 172.6	284.9 ± 271.9	900.4 ± 550.2	1302 ± 290.3	705.4 ± 368.8	1549 ± 236.8
<b>IL-18</b>	343.2 ± 83.3	315.1 ± 105.1	189.3 ± 71.5	153.0 ± 68.0	254.8 ± 110.8	402.0 ± 74.8	263.3 ± 38.5	385.1 ± 60.3
<b>MCP-1</b>	2333 ± 194.6	2278 ± 336.3	1687 ± 234.1	1617 ± 279.3	1840 ± 681.1	2496 ± 227.7	2022 ± 139.1	2633 ± 353.2
<b>TNFα</b>	42.95 ± 13.3	48.64 ± 14.4	22.67 ± 9.9	13.22 ± 10.4	73.45 ± 28.8	60.20 ± 12.2	32.10 ± 6.2	50.52 ± 10.7

**Table 5.5:** Serum cytokine concentrations at baseline (0h) and 1, 3, 6, 24, 48, 72, and 168 hours following IT instillation of PVP capped 110 nm AgNP. N/A indicates a cytokine was not examined at a time point. ND indicates a cytokine fell below detection range. Values are represented as a mean of concentration (pg/mL) at each time point  $\pm$  SEM, n= 4

<b>110 nm AgNP/PVP</b>	<b>0h</b>	<b>+1h</b>	<b>+3h</b>	<b>+6h</b>	<b>+24h</b>	<b>+48h</b>	<b>+72h</b>	<b>+168h</b>
<b>G-CSF</b>	212.3 ± 55.8	174.1 ± 14.6	93.3 ± 21.8	143.0 ± 19.6	129.4 ± 27.5	177.5 ± 33.5	181.2 ± 21.4	123.9 ± 24.3
<b>IL-1β</b>	87.8 ± 19.9	97.9 ± 13.2	40.5 ± 14.1	82.5 ± 16.2	68.6 ± 6.5	101.9 ± 10.3	95.4 ± 17.0	57.3 ± 11.0
<b>IL-2</b>	425.3 ± 45.2	419.6 ± 25.5	256.7 ± 32.0	379.3 ± 41.8	356.2 ± 35.7	453.7 ± 60.8	465.2 ± 55.7	321.8 ± 33.9
<b>IL-6</b>	1715 ± 321.4	1749 ± 126.5	584.1 ± 313.0	1501 ± 289.6	875.3 ± 321.0	1713 ± 545.7	1914 ± 285.9	1167 ± 518.4
<b>IL-18</b>	382.0 ± 39.0	411.0 ± 50.0	226.5 ± 46.2	331.8 ± 51.17	286.0 ± 39.9	412.1 ± 45.9	417.1 ± 51.2	315.6 ± 49.7
<b>MCP-1</b>	2149 ± 98.8	2204 ± 93.3	1615 ± 194.8	1958 ± 49.4	1860 ± 114.1	2122 ± 190.4	2103 ± 127.6	1816 ± 171.0
<b>TNFα</b>	31.7 ± 3.2	30.8 ± 2.0	17.2 ± 4.6	23.8 ± 2.1	22.9 ± 3.5	34.3 ± 7.1	33.7 ± 5.4	22.2 ± 3.8

**Table 5.6:** Serum cytokine concentrations at baseline (0h) and 1, 3, 6, 24, 48, 72, and 168 hours following IT instillation of citrate vehicle. N/A indicates a cytokine was not examined at a time point. ND indicates a cytokine fell below detection range. Values are represented as a mean of concentration (pg/mL) at each time point  $\pm$  SEM, n= 4

<b>Citrate</b>	<b>0h</b>	<b>+3h</b>	<b>+24h</b>	<b>+168h</b>
<b>G-CSF</b>	31.9 ± 10.4	45.6 ± 16.1	59.0± 21.5	23.2 ± 10.5
<b>GM-CSF</b>	ND	ND	ND	10.9± 10.9
<b>IL-1<math>\beta</math></b>	5.1 ± 5.1	7.605 ± 3.8	1.685 ± 1.4	11.6 ± 11.6
<b>IL-2</b>	138.1 ± 14.01	150.5 ± 31.7	109.6 ± 29.3	175.1 ± 65.3
<b>IL-5</b>	299.1 ± 27.7	305.6 ± 43.5	243.1 ± 43.6	351.2 ± 79.6
<b>IL-6</b>	299.3 ± 136.7	587.8 ± 234.4	43.3 ± 43.3	590.1 ± 484.2
<b>IL-10</b>	38.1 ± 8.2	42.6 ± 11.6	23.9± 8.1	51.3 ± 31.2
<b>IL-13</b>	14.4 ± 7.5	19.5 ± 8.2	ND	20.66 ± 20.7
<b>IL-17</b>	74.5 ± 5.5	83.9± 17.9	54.62 ± 18.2	94.7 ± 37.9
<b>IL-18</b>	138.9 ± 20.6	133.2 ± 38.0	91.7 ± 28.1	169.7 ± 75.9
<b>IFN<math>\gamma</math></b>	54.8 ± 54.8	117.4 ± 42.3	ND	186.6 ± 186.6
<b>MIP-1<math>\alpha</math></b>	25.2 ± 2.0	26.1 ± 3.4	20.9 ± 3.7	28.2 ± 8.9
<b>MCP-1</b>	N/A	N/A	N/A	N/A
<b>TNF<math>\alpha</math></b>	14.9 ± 1.4	16.0± 2.7	12.6 ± 2.9	18.4 ± 6.2
<b>RANTES</b>	799.4 ± 192.7	683.1 ± 126.8	720.7 ± 26.8	662.9 ± 130.3



**Table 5.7:** Serum cytokine concentrations at baseline (0h) and 1, 3, 6, 24, 48, 72, and 168 hours following IT instillation of citrate capped 20 nm AgNP. N/A indicates a cytokine was not examined at a time point. ND indicates a cytokine fell below detection range. Values are represented as a mean of concentration (pg/mL) at each time point  $\pm$  SEM, n =4.

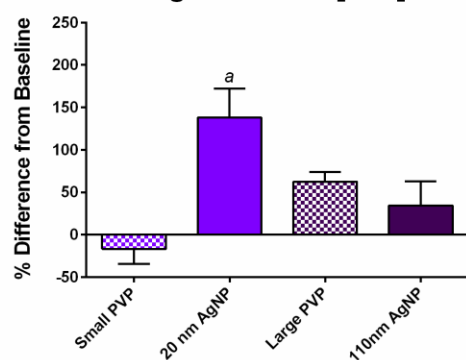
<b>20 nm AgNP/C itrate</b>	<b>0h</b>	<b>+3h</b>	<b>+24h</b>	<b>+168h</b>
<b>G-CSF</b>	32.8 ± 11.2	55.9 ± 23.6	51.7 ± 16.5	42.8 ± 25.3
<b>GM-CSF</b>	ND	ND	3.835 ± 3.835	ND
<b>IL-1<math>\beta</math></b>	3.5 ± 1.5	12.6 ± 9.2	8.0 ± 4.3	4.1 ± 2.4
<b>IL-2</b>	107.5 ± 32.1	169.7 ± 52.3	181.2 ± 32.7	114.0 ± 26.5
<b>IL-5</b>	229.3 ± 77.5	331.8 ± 62.4	374.3 ± 32.0	284.2 ± 36.6
<b>IL-6</b>	114.3 ± 68.1	351.8 ± 214.6	444.5 ± 346.9	93.0 ± 53.7
<b>IL-10</b>	26.3 ± 8.8	39.9 ± 16.7	54.5 ± 16.2	27.0 ± 10.5
<b>IL-13</b>	10.45 ± 4.0	19.09 ± 7.5	23.65 ± 16.5	0.8 ± 0.8
<b>IL-17</b>	62.44 ± 20.9	86.35 ± 27.5	98.89 ± 17.8	63.2 ± 17.2
<b>IL-18</b>	107.0 ± 36.1	151.3 ± 55.8	184.7 ± 43.0	108.7 ± 27.7
<b>IFN<math>\gamma</math></b>	34.2 ± 34.2	75.2 ± 59.6	140.7 ± 140.7	7.7 ± 7.7
<b>MIP-1<math>\alpha</math></b>	18.7 ± 6.3	26.5 ± 6.3	29.3 ± 3.4	19.6 ± 4.1
<b>MCP-1</b>	N/A	N/A	N/A	N/A
<b>TNF<math>\alpha</math></b>	11.72 ± 3.0	18.8 ± 5.2	18.77 ± 3.6	13.01 ± 2.7
<b>RANTES</b>	828.1 ± 153.2	807.8 ± 27.4	746.1 ± 92.1	652.8 ± 88.5

**Table 5.8:** Serum cytokine concentrations at baseline (0h) and 1, 3, 6, 24, 48, 72, and 168 hours following IT instillation of citrate capped 110 nm AgNP. N/A indicates a cytokine was not examines at a time point. ND indicates a cytokine fell below detection range. Values are represented as a mean of concentration (pg/mL) at each time point  $\pm$  SEM, n= 4

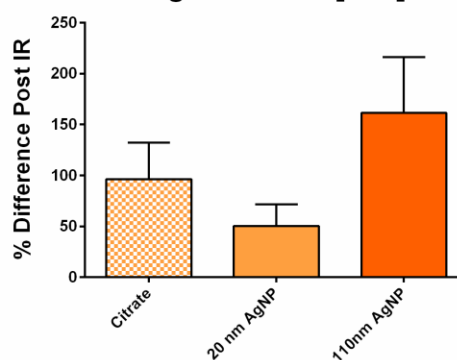
<b>110 nm AgNP/Citrate</b>	<b>0h</b>	<b>+3h</b>	<b>+24h</b>	<b>+168h</b>
<b>G-CSF</b>	212.3 ± 55.8	43.5 ± 7.5	11.1 ± 3.9	53.0 ± 38.2
<b>GM-CSF</b>	3.1 ± 3.1	1.2 ± 1.2	ND	ND
<b>IL-1β</b>	14.3 ± 14.3	7.6 ± 3.8	12.1 ± 8.7	11.6 ± 11.6
<b>IL-2</b>	239.4 ± 46.7	225.0 ± 57.5	259.2 ± 42.9	126.7 ± 60.7
<b>IL-5</b>	372.3 ± 48.7	361.0 ± 56.7	385.7 ± 33.1	249.6 ± 75.4
<b>IL-6</b>	685.5 ± 305.6	636.8 ± 355.2	508.6 ± 286.5	495.4 ± 286.0
<b>IL-10</b>	70.2 ± 27.1	75.9 ± 26.8	74.1 ± 16.0	43.5 ± 26.7
<b>IL-13</b>	22.0 ± 10.4	22.6 ± 13.1	17.2 ± 10.2	21.5 ± 13.6
<b>IL-17</b>	102.2 ± 19.3	94.0 ± 22.0	108.3 ± 17.4	53.4 ± 31.7
<b>IL-18</b>	209.5 ± 49.1	218.0 ± 63.1	212.8 ± 40.9	119.9 ± 69.8
<b>IFNγ</b>	25.4 ± 25.4	45.0 ± 28.9	ND	102.0 ± 102.0
<b>MIP-1α</b>	39.01 ± 7.6	37.4 ± 8.2	40.2 ± 4.5	23.1 ± 8.9
<b>MCP-1</b>	N/A	N/A	N/A	N/A
<b>TNFα</b>	23.2 ± 4.4	21.1 ± 5.6	23.7 ± 4.5	12.4 ± 5.5
<b>RANTES</b>	1262 ± 253.1	1179 ± 215.9	1192 ± 116.1	829.3 ± 229.8

**Figure 5.1:** Serum cytokine concentrations changes from baseline to 7 day post exposure I/R injury. 20 nm PVP capped AgNP elicited a greater inflammatory response following I/R injury when compared to vehicle than Citrate capped AgNP. *a* denotes statistical significance from vehicle  $p < 0.05$  by t-test,  $n = 4$ . No differences exist within the Citrate capped groups or vehicle.

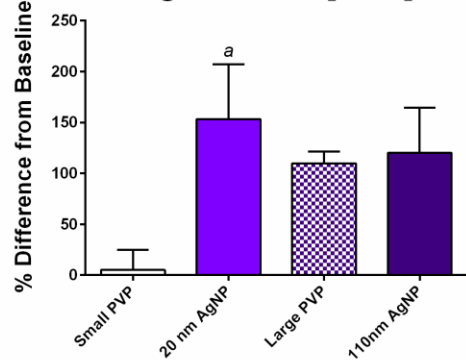
**A. Changes in PVP Capped AgNP Serum [IL-2]**



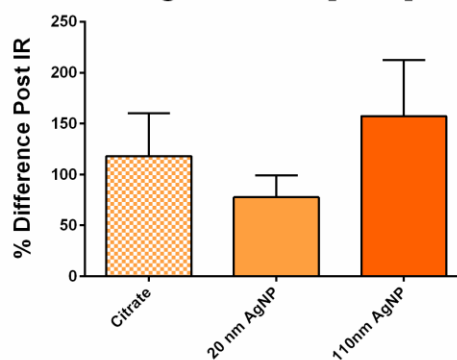
**B. Changes in Citrate Capped AgNP Serum [IL-2]**



**C. Changes in PVP Capped AgNP Serum [IL-18]**

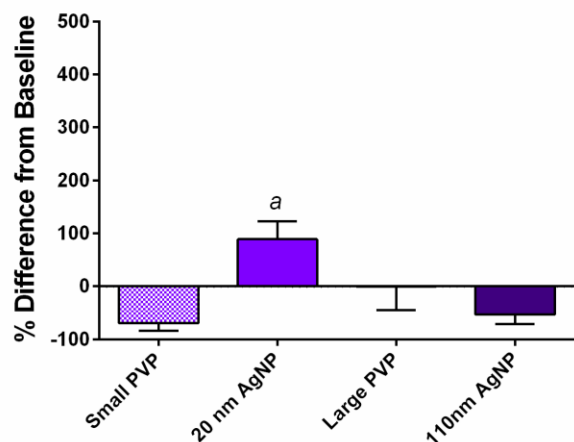


**D. Changes in Citrate Capped AgNP Serum [IL-18]**

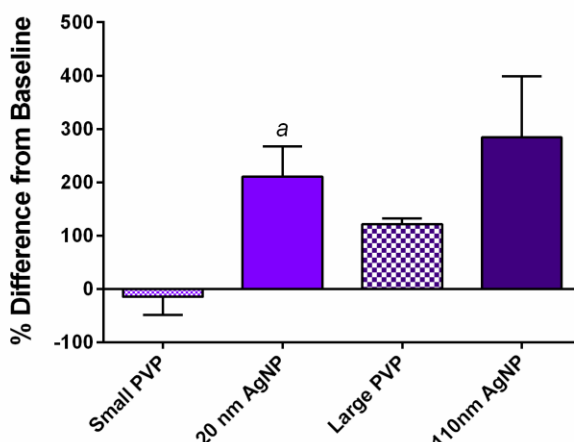


**Figure 5.2:** Changes in serum IL-6 concentrations from baseline following I/R injury at 1 and 7 days after IT instillation. When I/R injury is induced 24 hours or 7 days following IT instillation, exposure to 20 nm PVP capped AgNP results in the greatest release of inflammatory cytokines compared to vehicle (A) which persists at 7 days (B). No differences were detected in citrate capped AgNP at 24 hours (C) or 7 days (D). Overall, strength of inflammatory response varies depending on particle size from 1 to 7 days in PVP capped AgNP while citrate capping results in a greater inflammatory response to I/R injury from 1 to 7 days after exposure. a denotes statistical significance from vehicle  $p < 0.05$  by t-test,  $n = 4$ .

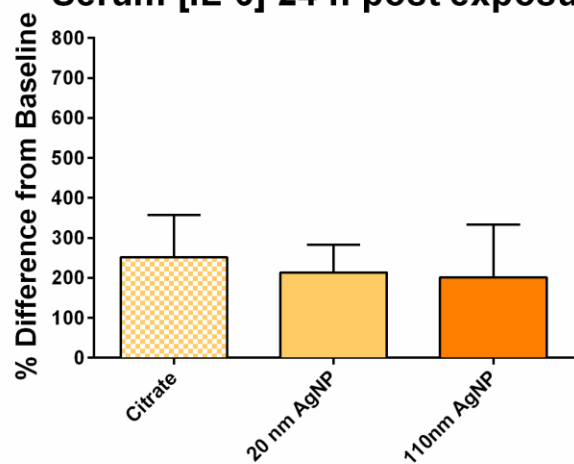
**A. Changes in PVP Capped Serum [IL-6] 24 h post exposure**



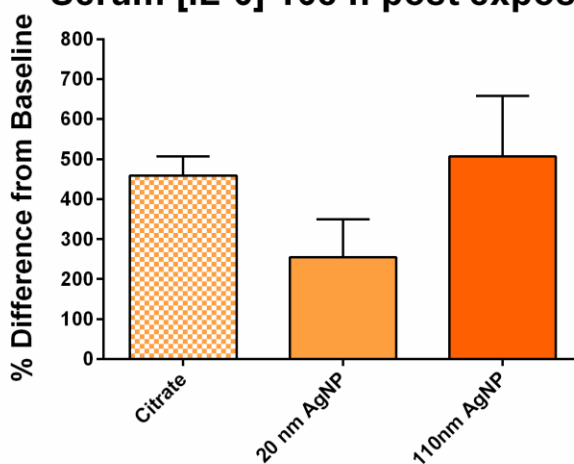
**B. Changes in PVP Capped AgNP Serum [IL-6] 168 h post exposure**



**C. Changes in Citrate Capped AgNP Serum [IL-6] 24 h post exposure**



**D. Changes in Citrate Capped AgNP Serum [IL-6] 168 h post exposure**





### 5.3 DISCUSSION

There has been much investigation into the role of pulmonary exposure to various forms of PM and the ability to induce an inflammatory response (79, 110, 129). However, the mechanisms through which AgNPs generate inflammatory responses are less than clear. We hypothesized that IT instillation of AgNP would result in a systemic inflammatory response measured by circulating cytokines and chemokines. IT exposure to AgNP does not generate a strong inflammatory response compared to naïve or vehicle groups. However, there is literature to suggest that exposure to PM alone, particularly engineered nano particles, is insufficient to induce a strong inflammatory response (54). It may be that exposure to engineered nanomaterials such as AgNP result in changes to inflammatory set points whereby an initial exposure to AgNP results in minor a release of inflammatory mediators such as cytokines and chemokines but a secondary insult results in an exaggerated inflammatory response compared to vehicle control (95). Our model utilized cardiac ischemia reperfusion as our secondary injury whereby we examined the impact of particle exposure on the inflammatory response to ischemia reperfusion injury. Induction of ischemia reperfusion injury resulted in increases of several circulating cytokines including IL-2, IL-6, and IL-18 that was exaggerated when compared to the response with naïve or vehicle alone treatment groups.

The observed particle response appears to be largely dependent on size and capping agent. Exposure to 20 nm AgNP consistently induces a greater increase in selected pro inflammatory cytokines compared to vehicle control than did the 110 nm AgNP exposure. These increases were only ascribed to the PVP capped AgNP group. Interestingly, PVP capping of 110 nm AgNP appeared to reduce circulating levels of IL-6 compared to control vehicle and 20 nm AgNP at 24 hours post exposure. This differential response may be further evidence of a size-capping agent dependent inflammatory response. Our data revealed that

24 hours following IT instillation of AgNP elicited a greater inflammatory response in 20 nm PVP capped AgNP than 20 nm citrate following I/R injury as reflected by greater levels of selected pro-inflammatory cytokines found in the serum. Intratracheal exposure to citrate capped AgNP demonstrated no difference in response compared to its vehicle control. The differential response to various size particle and capping agents may be related to the formation of protein coronas (108) which reflect differing biological interactions with the capping agents. The formation of unique protein coronas based on the capping material may influence the inflammatory response to AgNP by modulating the way nanomaterials interact with cells or tissues. Independent of the formation of protein coronas, the capping agents may also influence nanoparticle interactions at biological interfaces (27). One study of gold nanoparticles revealed different capping agents have the ability to elicit differential effects on both *in vitro* and *in vivo* toxicity (30). The results of this study allow us to suggest that different capping agents may have the ability to change the manner in which capped AgNP may impact toxicity.

The final aspect of the inflammatory response to AgNP is the duration of response. The increase in circulating cytokines (i.e. IL-6) to PVP capped 20 nm AgNP is diminished following I/R injury at 7 days post exposure compared to 24 hours. In contrast, 7 days post exposure 110 nm PVP capped AgNP had a greater levels of circulating IL-6 in response to I/R injury than at 24 hours. Citrate capped AgNP resulted in no differences between control vehicle and 20 or 110 nm AgNP following I/R injury at 24 hours post IT instillation. Citrate capped AgNP also resulted in diminished levels of IL-6 at 7 days following I/R injury compared to 24 hours. These observations may indicate a time-dependent aspect of particle size-capping interactions.

## **CHAPTER 6**

### **INTEGRATED DISCUSSION AND CONCLUSION**

#### **6.1 DISCUSSION**

The rapidly expanding uses of engineered nanomaterials have raised questions regarding the safety of exposure to such particles. To date, there is little consensus regarding the safety of engineered nanomaterials or the mechanisms by which they may induce a toxic response. This thesis evaluated the impact of pulmonary exposures to silver nanoparticles (AgNP) on myocardial ischemia reperfusion (I/R) injury. We hypothesized that intratracheal (IT) instillation of AgNP would result expansion of I/R injury. This hypothesis was based on the interpretation of effects of previously investigated particulate matter and nanomaterials including but not limited to: diesel (71), C<sub>60</sub> fullerene (14), and multi-wall carbon nanotubes (MWCNT) (91). Currently, it is thought that pulmonary exposure to PM and engineered nanomaterials can lead to cardiovascular disease and dysfunction through multiple pathways (63, 13). One proposed pathway involves PM directly stimulating sensory fibers within the lung stimulating pulmonary-neural reflex arcs that can alter the sympathetic or parasympathetic balance in the heart leading to changes in heart rate, heart rate variability, cardiac conduction and repolarization, arrhythmogenesis, and blood pressure (13, 63, 66). Activation of pulmonary-neural reflex arcs can also lead to increased release of cytokines which may be augmented by PM induced decreases in vagal tone inhibiting neuronal regulation of inflammation (75). A second proposed pathway involves particulate induced oxidative stress in the lungs leading to inflammatory responses including the release of cytokines (58, 111), which leads to systemic inflammation and cardiovascular dysfunction (63). We examined several endpoints of AgNP toxicity: 1) autonomic and cardiac electrical dysfunction as measured by HR, PR interval, and QT interval, 2) systemic inflammation as measured by serum cytokine levels, 3) exacerbation of myocardial I/R injury following AgNP exposure. A final element of toxicity unique to AgNP is

the direct impact of ionic silver on cardiovascular toxicity, which was evaluated by examining the expansion of cardiac I/R injury following instillation of several concentrations of silver ions. The proposed mechanisms by which pulmonary exposure to AgNP elicits cardiovascular toxicity and exacerbates cardiac I/R injury as well as how our aims relate are summarized in figure 6.1. We determined that exposure to AgNP results in expansion of myocardial infarction following a course of coronary artery occlusion and subsequent reperfusion occurring 24 hours following IT instillation as well as 7 days following instillation. Despite demonstrating that AgNP have the ability to expand I/R injury the mechanisms through which AgNP may influence the expansion of I/R injury remains unclear.

The balance of autonomic control in the heart is crucial for normal cardiac function. Changes in parasympathetic and sympathetic tone may underlie cardiac disease. Furthermore, dysregulation of autonomic balance has been associated with increased mortality from cardiovascular disease (CVD) (88). In order to evaluate the effect of AgNP on electrical activity in the heart as a measure of autonomic balance we measured heart rate (HR), PR interval, and QT interval. Heart rate has long been used as a measure of autonomic balance as well as being an independent risk for cardiovascular mortality (88). Sympathetic and parasympathetic innervation of the sinoatrial node control HR through a balance of excitatory and inhibitory inputs; this balance can be modulated by psychosocial stressors (23) as well as various toxicants, such as PM (24, 113, 133). Our study demonstrated that IT instillation of AgNP did not result in significant changes in heart rate. Alterations in QT interval may be an important indicator of cardiotoxicity (7, 45) whereby QT prolongation can lead to increased arrhythmogenesis and sudden cardiac death (2, 56). We also report that exposure to AgNP did not significantly impact electrical activity in terms of ventricular depolarization and repolarization times as measured by QT interval. Despite finding no differences in HR and QT

interval following exposure to AgNP, our analysis did uncover differences in PR interval during reperfusion in AgNP exposed SD rats, compared to vehicle. The PR interval reflects the time it takes for an impulse to be conducted from the atria to the ventricles via the atrioventricular node (89). The duration of the PR interval in large part is dictated by a balance of autonomic tone whereby a shift towards parasympathetic or sympathetic dominance can increase or decrease PR interval, respectively. Our data suggests that exposure to 20 nm AgNP may influence vagal tone following a period of myocardial ischemia, and that the capping agent may differentially impact autonomic tone. In light of the differential responses to AgNP capping agents, selection of appropriate capping agents may be crucial to avoid unwanted cardiovascular toxicity associated with pulmonary exposure to AgNP. Several caveats must be considered when interpreting our electrocardiogram (ECG) data. First, only ECGs from surgeries 7 days post exposure were evaluated for HR, PR, and QT. It may be possible that 7 days following exposure any changes in autonomic balance or electrical activity induced by AgNP may have resolved. Pulmonary exposure to fine PM has been associated with changes in ventricular repolarization occurring with 3-4 hours of exposure (60). Earlier time points should be evaluated for changes in autonomic function and electrical activity. Second, all ECG recordings were performed under conditions of general anesthesia with ketamine/xylazine which may have influenced autonomic tone at the time of surgery and may blunt autonomic and electrical responses to AgNP (116).

We initially hypothesized that IT instillation of AgNP would result in a systemic inflammatory response, which could then drive the expansion of I/R injury. In order to assess the inflammatory response we quantified serum levels of several cytokines and chemokines which are known to be associated with either particulate matter (PM) exposure or CVD. Serially collected serum samples revealed no temporal relationship between IT instillation of

AgNP and circulating levels of cytokines. Interestingly, even though there was no increase in circulating cytokines prior to I/R injury, exposure to AgNP lead to increased presence of several inflammatory cytokines (i.e., IL-2, IL-6, IL-18) following I/R injury compared to pre-IT instillation levels . Although, I/R injury is associated with increases in inflammatory cytokines such as IL-6 (14, 139) and IL-18 (30, 61, 92) the extent of cardiac I/R injury does not correlate to changes in circulating cytokines. Although not directly related to cardiac I/R injury, IL-2 may mediate organ specific proinflammatory mechanisms through control of T-cell differentiation and control of Th2 cytokine response (48, 50). The lack of correlation between circulating cytokines and the extent of I/R injury suggest that the observed cytokine response is not being driven by I/R injury. Furthermore, the increases in serum IL-2, IL-6, and IL-18 are only observed in rats exposed to 20nm AgNP, which may indicate an interaction between particle size and capping agent drives post I/R injury inflammation. This finding further allows us to suggest an alternate hypothesis; engineered nanomaterials may not directly induce an inflammatory response but exacerbate the inflammatory response to a secondary insult, such as inoculation with lipopolysaccharide (LPS) (49). Previous studies investigating the role of exposure to engineered nanomaterials and the development of pulmonary fibrosis in rats demonstrated that exposure to nanomaterials alone were insufficient to induce fibrosis, however inoculation with LPS following MWCNT exposure was able to induce fibrotic changes greater than LPS or MWCNT alone (25). This may evidence the ability for AgNP to exacerbate inflammatory responses within I/R injury and ultimately expand myocardial I/R injury. Although we were unable to demonstrate a strong inflammatory response to instillation of AgNP in serum samples, analysis of cytokines localized to tissues of interest, such as myocardium, may be a more appropriate target of investigation. Overall, the limited changes in circulating cytokine concentrations post exposure to AgNP appear to result from

sensitization of the immune system in response to a secondary insult are largely correlated with capping agents and particle size. IT instillation of 20 nm PVP capped AgNP induced a greater rise in concentrations of select cytokines (i.e., IL-2, IL-6, IL-18) compared to vehicle than citrate capped AgNP. IT instillation of both PVP and citrate capped AgNP 110 nm particles resulted in no significant differences from vehicle controls. These results allow us to suggest that particle size is an important factor when evaluating the toxicity associated with exposure to AgNP.

The contribution of ionic silver ( $\text{Ag}^+$ ) dissociation to cardiovascular toxicity cannot be overlooked, especially regarding AgNP. Silver has long been known to have innate toxicological properties. However, biological responses to ionic silver vary widely by cell or tissue type as well as species according to a review by Chernousova et al. (26). The direct cardiovascular toxicity associated with exposure to ionic silver has been the subject of little investigation, however oral ingestion of silver nitrate or silver chloride was associated with left ventricular hypertrophy following long term exposure to silver (83). However, this thesis did reveal a concentration dependent expansion of I/R injury following exposure to 0.1 mg/mL and 1 mg/mL concentrations of silver acetate (AgAc). The direct mechanisms of silver ion cardiotoxicity are unclear; however oxidative stress has been demonstrated to increase following exposure to ionic silver in cell culture (28). Silver ions can induce oxidative stress via multiple mechanisms including direct catalyzation of superoxide radicals from  $\text{O}_2$  (28), or by interacting with thiol groups of respiratory chain enzymes (69, 4). A rise in oxidative stress resulting from exposure to AgNP may explain how a secondary insult (e.g., I/R, LPS) manifests as an exaggerated inflammatory response without an initial rise in inflammatory markers (i.e., cytokines) following instillation (5). Given  $\text{Ag}^+$  ionic dissociation from nanomaterials of between 1 and 30 percent (9, 74, 112) and the relationship of concentrations of AgAc to I/R injury (fig

3.5) we believe that free silver ions play a contributing role in the toxicological end points examined. However, ionic dissolution is unlikely to be the sole source of toxicity following exposure to AgNP because only the highest concentration of AgAc resulted in an I/R injury roughly equivalent to 20 nm AgNP; 110 nm AgNPs were able to induce expansion of I/R injury equivalent with 20 nm AgNP at 24 hours despite slower ionic dissolution rates due to smaller surface area.

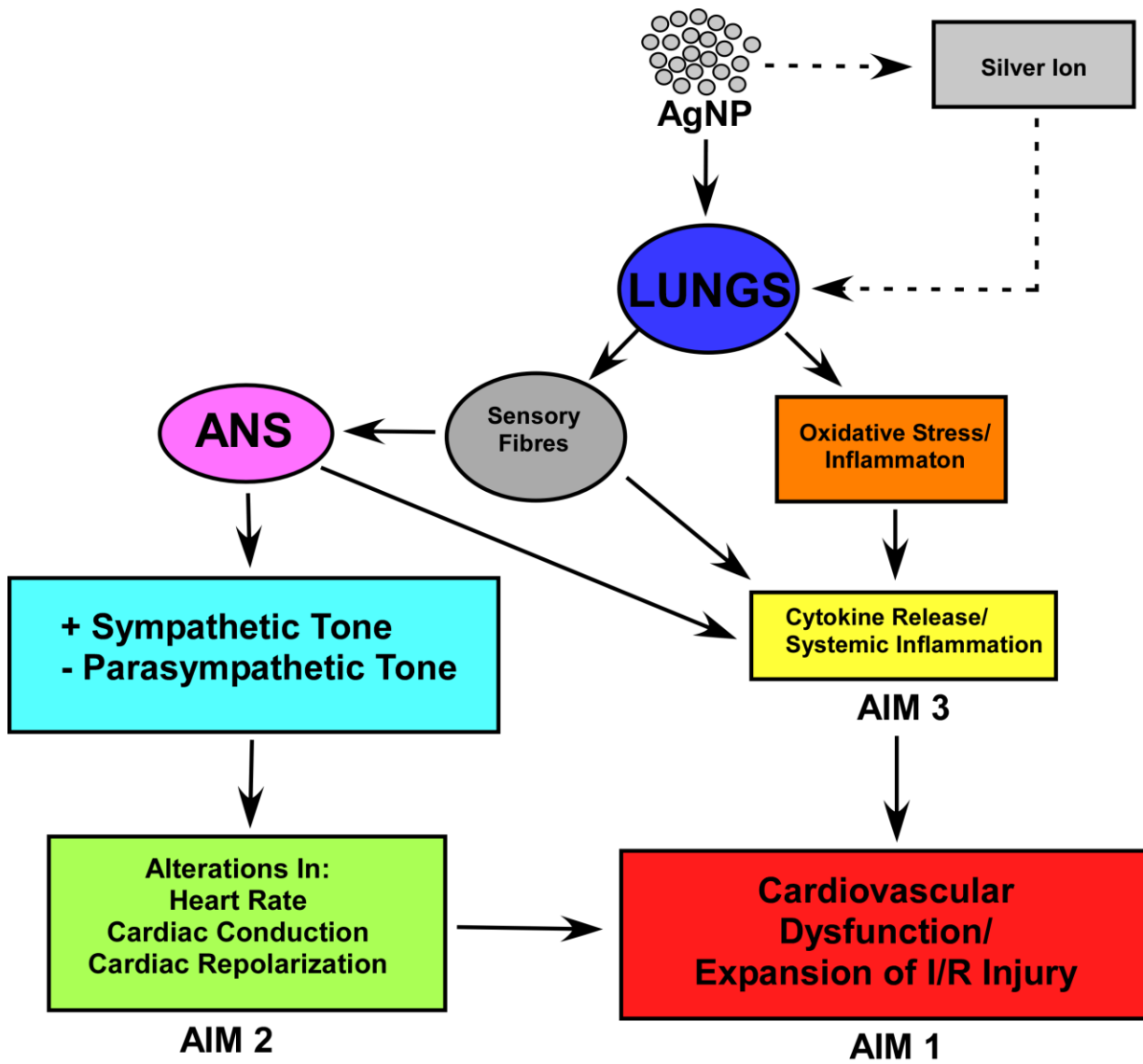
A fundamental question regarding the toxicity of AgNP is whether or not differences in particle number or mass dosing influences our toxicological end points. Traditionally toxicology underscores to the classical adage, “the dose makes the toxin” but that can be expressed either in either mass or surface area/ particle number. Given the different sizes of AgNP but an equivalent mass delivered in these studies it is reasonable to assume that there are higher numbers of particles in a fixed 200  $\mu$ L sample of 20 nm AgNP than 110 nm AgNP. However, we demonstrated that there is no significant difference in I/R injury 24 hours following IT instillation of AgNPs. At 24 hours it seems that particle number is not strongly associated with the extent of I/R injury. Furthermore, 7 days following IT exposure a differential response is seen between the citrate capped (Fig 3.4) and PVP (Fig 3.3) capped AgNP, suggestive of influence of capping agent on the cardiac I/R injury response. It is possible that the cardiac I/R endpoint may simply be insensitive to particle number induced toxicity. Results from the cytokine concentrations may indicate that surface area of AgNP along with capping agent play the more prominent roles in inducing cardiovascular toxicity. Increased surface area may lead to increased ionic dissociation as well as availability for interaction at biological interfaces (102). The capping agents of AgNP may further modulate the way particles interact with aforementioned biological interfaces leading to increased toxicity (30). The influence of



capping agent on cardiovascular toxicity may result from differences in surface charge, which can impact particle adsorption affinity for cellular surfaces (11) as well as cellular uptake (41).

Although it does not appear citrate capped or 110 nm AgNP have a strong influence on systemic inflammatory response or myocardial electrical activity, there is a strong effect on the expansion of I/R injury. We suggest that AgNP mediated systemic inflammation or electrical dysfunctions are not the only mechanisms contributing to expansion of I/R injury. Potential alternative mechanisms of AgNP toxicity include induction of oxidative stress (28, 74), or vascular dysfunction (12, 18, 77) which lead to an expansion of I/R injury 24 hours and 7 days following IT exposure to AgNP. Furthermore cardiovascular toxicity of AgNP likely stem from a complex interaction of surface area and capping agents which modulate ionic dissolution and nanoparticle interactions with biological interfaces *in vivo*.

**Figure 6.1:** Proposed mechanisms by which pulmonary exposure to AgNP induces cardiovascular toxicity. Pulmonary exposure to AgNP results in cardiovascular toxicity by activation of sensory fibers that initiate an inflammatory cascade and cytokine release, as well as feedback to the autonomic nervous system altering the balance of sympathetic and parasympathetic tone altering: heart rate, cardiac conduction, and cardiac repolarization. Additionally, AgNP induction of oxidative stress within the lungs may lead to systemic cytokine release and exacerbate cardiac ischemia reperfusion injury.



## 6.2 CONCLUSION

The ubiquitous use of engineered nanomaterials makes understanding the potential toxicological outcomes of exposure to such materials chief public health concern. We investigated the cardiovascular toxicity associated with pulmonary exposure to AgNP. In order to evaluate the cardiovascular toxicity associated with exposure to AgNP we evaluated measures of electrical and autonomic dysfunction, as well as investigated the impact of AgNP on cardiac I/R injury. In an attempt to elucidate mechanisms of AgNP toxicity we also evaluated the inflammatory response to AgNP which we hypothesized to drive expansion of I/R injury. Our data reveals that IT instillation of AgNP results in expansion of cardiac I/R injury 24 hours and 7 days compared to naïve and vehicle controls. Furthermore, IT instillation of AgNP did not significantly impact HR or QT interval, but did impact PR interval during reperfusion. Instillation of AgNP did not elicit a strong inflammatory response as measured by circulating cytokines, but I/R injury following IT instillation did result in a greater inflammatory response in PVP capped 20nm AgNP compared to vehicle controls. The cardiovascular toxicity of AgNP seems to be dependent on several factors including: particle size, capping agent, and ionic dissolution. Future investigations should focus on uncovering the mechanisms that underlie the cardiovascular toxicity associated with exposure to AgNP. Additionally, determining the persistence of cardiac I/R injury beyond the 7 day time point may be important in understanding the potential public health impact of pulmonary exposures to AgNP.

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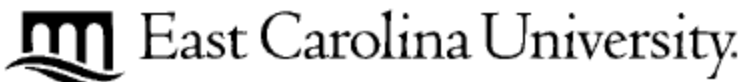
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## APPENDIX A: IACUC APPROVAL LETTERS FOR ANIMAL USE PROTOCOLS



**Animal Care and  
Use Committee**

212 Ed Warren Life  
Sciences Building  
East Carolina University  
Greenville, NC 27834

252-744-2436 office  
252-744-2355 fax

November 9, 2011

Chris Wingard, Ph.D.  
Department of Physiology  
Brody 6N-98  
ECU Brody School of Medicine

Dear Dr. Wingard:

The Amendment to your Animal Use Protocol entitled, "Cardiovascular and Cytokine Changes Following RTIs C60 and MWCNT Administration to Non-Pregnant, Pregnant, and Lactating Rats", (AUP #Q300) was reviewed by this institution's Animal Care and Use Committee on 11/9/11. The following action was taken by the Committee:

"Approved as amended"

**\*\*Please contact Dale Aycock prior to any hazard use**

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

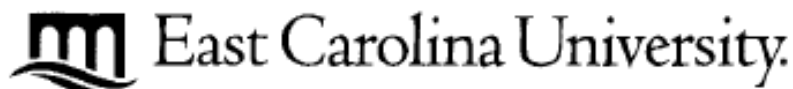
Sincerely yours,

A handwritten signature in black ink, appearing to read 'S. E. Gordon'.

Scott E. Gordon, Ph.D.  
Chairman, Animal Care and Use Committee

SEG/jd

enclosure



**Animal Care and  
Use Committee**

212 Ed Warren Life  
Sciences Building  
East Carolina University  
Greenville, NC 27834

252-744-2436 office  
252-744-2355 fax

February 15, 2012

Chris Wingard, Ph.D.  
Department of Physiology  
Brody 6N-98  
ECU Brody School of Medicine

Dear Dr. Wingard:

The Amendment to your Animal Use Protocol entitled, "Cardiovascular and Cytokine Changes Following RTIs C60 and MWCNT Administration to Non-Pregnant, Pregnant and Lactating Rats", (AUP #Q300) was reviewed by this institution's Animal Care and Use Committee on 2/15/12. The following action was taken by the Committee:

"Approved as amended"

**\*\*Please contact Dale Aycock prior to any hazard use**

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours

A handwritten signature in black ink, appearing to read 'Scott E. Gordon'.

Scott E. Gordon, Ph.D.  
Chairman, Animal Care and Use Committee

SEG/jd

enclosure



**Animal Care and  
Use Committee**

212 Ed Warren Life  
Sciences Building  
East Carolina University  
Greenville, NC 27834

252-744-2436 office  
252-744-2355 fax

February 24, 2014

Christopher Wingard, Ph.D.  
Department of Physiology  
Brody 6N-98  
ECU Brody School of Medicine

Dear Dr. Wingard:

Your Animal Use Protocol entitled, "Cardiovascular and Cytokine Changes Following Silver and Carbon Based Nanoparticle Administration to Non-Pregnant and Pregnant Rats" (AUP #Q325) was reviewed by this institution's Animal Care and Use Committee on 2/24/14. The following action was taken by the Committee:

"Approved as submitted"

**\*Please contact Dale Aycock at 744-2997 prior to hazard use\***

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

A handwritten signature in black ink, appearing to read 'S. B. McRae'.

Susan McRae, Ph.D.  
Chair, Animal Care and Use Committee

SM/jd

Enclosure