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Analysis of Hydrogel/Glass Nitric Oxide Nanoparticles for Therapeutic Management of Hemorrhagic Shock

A Thesis submitted in partial satisfaction of the requirements

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by

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The thesis of Brian Ho is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2014

For the family, friends, mentors and many others who

believed in me

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Abbreviations

HS: Hemorrhagic Shock

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

RBC: Red Blood Cell

MAP: Mean Arterial Pressure

HR: Heart Rate

FCD: Functional Capillary Density

BE: Base Excess

ATP: Adenosine Triphosphate

ppm: Parts per Million

Symbols

µm: Micron

pO_2: Partial Pressure of Oxygen

pCO_2: Partial Pressure of Carbon Dioxide

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ABSTRACT OF THE THESIS

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by

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Professor Pedro Cabrales, Chair

The purpose of this study is to confirm the effectiveness of nitric oxide (NO) encapsulated nanoparticles as a viable therapeutic treatment for hemorrhagic shock (HS) relative to gaseous delivery and to elucidate the optimal nanoparticle concentration for dosing. To gauge its effectiveness, results for the treatment using the NO nanoparticles were compared to results from HS experiments done using gaseous inhalation of NO. All treatment groups used Hextend® as a volume expander. All experiments were performed with a 50% blood volume HS hamster window model and the parameters tracked were as follows: mean arterial pressure (MAP), heart rate (HR), functional capillary density (FCD), blood flow & diameters of arterioles & venules, blood gas parameters (pH, base excess (BE), pO_2, pCO_2), and blood plasma measurements (nitrates, nitrites, s-nitrosothiols).

The results of our experiments show that NO encapsulated nanoparticles, when used with Hextend® in the 50% blood volume HS hamster window model, were effective in treating circulatory deficits brought on by HS. The optimal concentration was found to be at 15 mg/kg hamster weight. In addition, the results elude to the NO nanoparticles being 5-10% more effective than gaseous inhalation of NO for equivalent conditions and parameters. However, more experiments would be necessary to confirm this observation.

I. Introduction: The Problem of Hemorrhagic Shock & Overview of Volume Expanders

1.1 Hemorrhagic Shock Defined

The concept of "shock" applies to a wide variety of physiological and psychological dysfunctions such as Post-Traumatic Shock Disorder. However, hemorrhagic shock (HS), the primary focus of this thesis, is related to the systemic failures that occur after rapid and severe blood loss from the body. HS was first studied in the early 20th century and these early studies were focused around the possible presence of a "shock toxin" that was released by the body in response to a severe perturbation. It wasn't until the late 1930's that Blalock proposed that HS was a result of the peripheral vasculature being unable to sustain perfusion of essential gases for bodily function [1]. Although this is the primary alteration to the body after severe loss of bodily fluid, the body responds by other means as well. Metabolically, HS induces increased glycogen to glucose conversion in an attempt to maintain a steady state of energy within the body that is being used to compensate decreased heat production. In addition, oxygen consumption is increased to maintain homeostasis in a state of perceived hypoxia. The induced hypoxic state leads to further inefficiencies in the usage of metabolic energy conversion leading to the vicious cycle that could ultimately lead to death.

In general, a loss of 25% of fluid circulation volume is medically severe and if fluid loss increases to 50%, it is observed that the amount of red blood cells (RBCs) in circulation will reach critical levels [1]. These measurements are further differentiated

into a multi-class system where Class 1 is a “non-shock” state of 15% blood loss, Class 2 is a loss between 15% and 30% blood volume and begins to result in physiological symptoms such as increased HR and decreased blood pressure. The more severe Class 3 is associated with 30% to 40% loss in blood volume and results in the patient being disoriented state with even more severe HR and blood pressure consequences. Finally Class 4 is associated with blood loss greater than 40% of total blood volume and is generally considered a pre-death event [2]. Class 4 will ultimately result in multi-organ failure from cellular necrosis and apoptosis due to a sustained hypoxic state because of poor vascular perfusion particularly in peripheral tissues and organs. Tissues that would experience the greatest damage from a severe HS state are those that normally have a higher metabolic rate as this would be significantly reduced by the lack of oxygen availability to carry out oxidative metabolism leading to mitochondrial dysfunction. Mitochondrial dysfunction, in addition to depletion of cellular ATP stores, increases the levels of intracellular calcium ions directly resulting in the signaling of cell death pathways. For example, one such tissue system that is severely affected by HS is the central nervous system. The cells associated with the central nervous system can only tolerate the HS induced hypoxia for a short period of 5 to 7 minutes whereas, in contrast, smooth muscle cells can last up to 3 hours before irreversible damage is incurred [2].

In the clinical setting, a variety of symptoms are observed in patients under a state of HS. Physically, as mentioned above, patients appear disoriented and confused with varying degrees based on the severity of the shock state that they are under. In higher classifications of shock such as Class 3 and 4, patients will appear to be in a

comatose mental state of unawareness. Effects on blood pressure and HR are observed in addition to a progressively severe hypothermic state brought on by the inability for the body to maintain homeostasis. Urine production will also be reduced in HS [2]. Laboratory tests on blood gas parameters performed on patients are used to assess the degree of shock will indicate pH and lactate imbalances. A patient's blood pH would be decreased, a state referred to as acidosis, and lactate levels would be severely elevated. Lactate levels can go from 1 to 2 mmol/L at baseline to greater than 5 mmol/L in cases of severe shock. In addition to these systemic blood measurements, a large variety of other parameters are monitored through laboratory tests to assess organ viability during HS. Organs monitored for injury in particular include the liver, pancreas, heart, kidney and brain.

1.2 The Microcirculatory System and Hemorrhagic Shock

The microcirculatory system is a distal network of blood vessels (arterioles, capillaries and venules) that are around or smaller than 100 μm in diameter and responsible for gas exchange as well as nutrient and waste molecule exchange between circulating body fluid and cells in the body [3]. As such, maintenance of blood flow and perfusion in this network is extremely important to the viability of tissue systems and overall patient condition as a consequence. When this system is compromised, it can be conceived that organ damage would occur as an insufficient supply of nutrients and gases are reaching and exchanging in the tissues of the organs.

In conditions of HS, vasoconstriction occurs especially in larger arterioles adjacent to skeletal muscle tissue (usually ranging from 70 to 150 μm in diameter) but

a variation of responses in smaller arterioles have been observed in animal models for smaller arterioles (usually ranging from 7 to 25 μm in diameter). Constriction also tends to be maintained for longer periods of time in the larger arterioles when compared to smaller arterioles [4]. In contrast to arterioles, venular responses are minimal to none. Minor vasoconstriction is sometimes observed in larger venules but overall significant changes are not often seen [5].

With regards to capillaries, blood flow and diameter is not often measured. Instead, FCD is usually the method of quantifying capillary status during a state of HS [3]. This is a measure of how many capillaries containing moving RBCs are present in a given area of tissue. It is observed that the number of capillaries dramatically decreases during a shock state, ranging from roughly 15% to 30%, relative to baseline [5]. This is seen even in our own data where in some cases capillaries are completely shut down in some fields of view. As can be imagined, when the capillaries are no longer functioning, tissue viability is compromised due to the decreased vascular perfusion.

The RBCs themselves are significantly affected during periods of HS. One of the occurrences is the release of NO into the microcirculation with a vasodilatory effect. However, RBCs also experience a decrease in deformity, directly correlated to serum osmolarity, which would negatively affect oxygen delivery [6]. Deformity normally allows the RBC to adapt to the various vessel diameters in the microcirculatory system and in conditions of shock, the cells experience shrinkage and other shape alternations that could serve to be detrimental to its purpose as gas carrier.

Many of the effects that HS has on the microcirculatory system will continue even after the patient is resuscitated. Changes in diameters, RBC shape alternations, leukocyte adherence and blocking of micro vessels often persist for long periods of time even after resuscitation anywhere from 6 hours to many days later [7]. Some of these changes might even increase in intensity due to impairment of ATP production from mitochondrial dysfunction incurred during shock [3]. This leads to the potential problem of designing therapies that not only combat the immediate rescue of the microcirculation during shock but also enduring recovery for extended periods of time after the insult as this may lead to improved patient outcome overall.

Because of the existence of distinct measurable parameters in the microcirculatory system in conditions of HS, these will constitute a group of the measurements obtained. They will serve as one of the primary indicator of the animal's state of shock and subsequent recovery in the experimental component of this thesis.

1.3 Current Treatment Options for Hemorrhagic Shock

Treatment options for patients in a state of HS currently revolve around alleviating the downstream problems brought on by the fluid loss severity to reduce mortality probabilities. This focus consists of treating hypothermia, acidosis, and coagulopathy (brought on by a dilution of factors that inhibit the ability of the body to form blood clots). These three primary consequences of HS are commonly referred to in many papers as the "lethal triad" of symptoms that will ultimately reduce in circulatory system failure, multi-organ failure and finally death.

The first phase of treatment is associated with the containment of the hemorrhage in the shortest time period possible [8]. In the pre-hospital setting, this includes the deployment of tourniquets and pelvic girdles as well as various other packings and dressings to compress the bleeding site and in some cases enhance blood clotting through the use of haemostatic agents. Upon reaching an equipped medical setting, the patient undergoes what is commonly referred to as “damage control surgery”. This is focused around reducing or stopping the loss of bodily fluid by any means possible to restore physiology if not anatomy within the patient. Time is of the essence (it is suggested that this phase should not last more than 2 hours) as systemic parameters would still be in decline during this period and in a severely injured patient, rapid damage control has been directly correlated to decreased patient mortality. Even through surgical techniques have not changed very much throughout the evolution of treatment for severe trauma, the increasing presence and discovery of haemostatic agents have further increased surgical success rates [9]. Angiographic embolization is also used during this first phase if treatment if the hospital is equipped and experienced in conducting the procedure. Angiographic embolization has been correlated with better patient outcome in cases of pelvic injury, liver injury, and splenic injury. The combined surgical intervention and embolization is the groundwork for a treatment protocol referred to as RAPTOR (Resuscitation with Angiography, Percutaneous Techniques and Operative Repair) [10].

The second phase of treatment is referred to as “damage control resuscitation” and is concerned with addressing the consequences brought about by the “lethal triad”,

albeit not always directly. Members of the “lethal triad” are often used as indicators of patient status and decline as well as being focal points for therapeutic treatments.

Hypothermia is the first member of the “lethal triad” and is often the simplest to alleviate in patients with severe trauma. Hypothermia operates as both an indicator and an aggravator of further decline in patient status as it shows that mechanisms governing thermoregulation are failing as well as the direct effect of hypothermia being the dysfunction of vital enzymes in bodily function [8]. To address hypothermia, it is not sufficient to not just prevent heat loss by the use of blankets and air warming devices but it is also required to rewarm the patient through the use of infusion fluids with accelerator-heaters. Despite the fact that hypothermia being used as an indicator and treatment focus, it has been shown that a mild state of hypothermia (bodily temperature falling between 30-34C) for a short period has a protective effect by repressing the generation of reactive oxidation species particularly in the head region reducing brain damage [1].

Acidosis, the second member of the “lethal triad”, is primarily used as an indicator of the state of severity of HS that the patient is under and is often not expressly treated. As previously mentioned, it is measured along with lactate levels in laboratory tests in HS assessment. However, it is of note that acidosis can sometimes be induced as a consequence of resuscitative therapy as the infusion of isotonic saline can develop hyperchloremic metabolic acidosis [8].

The last primary consequence of HS is trauma-induced coagulopathy. Like hypothermia, coagulopathy is both an indicator and treatment focus for a patient with

HS. Coagulopathy is brought about by the release of factors from injured tissues that result in systemic anticoagulation and fibrinolysis [9]. The state of coagulopathy is further aggravated by the previous two members of the "lethal triad" as well as surgical and treatment interventions in the treatment of HS such as incisions to control bleeding and infusions of resuscitative fluid. Addressing coagulopathy involves the use platelet and fibrinogen concentrates in conjunction of massive transfusion to both encourage blood clotting as well as maintain bodily blood volume. The factors in the concentrates also reduce complications resulting from transfusion and increase the trauma patient's overall condition [11]. Antifibrinolytic agents, such as tranexamic acid injections, are also used in the treatment of coagulopathy.

The third phase of treatment, and the most controversial phase as well as the focus of this thesis, is the therapeutic application of fluid resuscitation and the selection fluids in particular that are used. The primary goal of fluid resuscitation, whether it is through a blood transfusion or infusion of blood substitute, is to maintain the fluid volume within the body and increase the patient's mean arterial pressure (MAP). This is then physiologically correlated to increased vascular perfusion that is potentially the root of multi-organ failure and circulatory system failure. However, it is of note that the goal is to not seek to normalize the MAP back to baseline but to tolerate hypotension, also referred to as "permissive hypotension" [8]. Mild hypotension has been shown to improve survival in states of HS and it is therefore suggested that the MAP be maintained around the range of 55 mmHg correlating to a systolic blood pressure maintenance in the range of 85 mmHg [12]. In addition to the observed increased

survivability, limiting fluid resuscitation also has the added effect of reducing haemodilution in the body.

The controversy in fluid resuscitation surrounds the fact that no particular protocol, with associated choice of fluid, shows a consistent advantage over another [9]. The fluids used in resuscitation can be grouped into two categories: crystalloids and colloids. Crystalloids are solutions of water soluable materials such as salts of which hypertonic saline is a commonly used crystalloid in HS resuscitation. Crystalloids are often used when a larger volume of fluid is necessary but often contributes to metabolic acidosis, one of the "lethal triad". They are also often cheaper and easier to obtain than colloids. Colloids, on the other hand, are more effective in terms of rate and persistence of volume expansion but affects fibrinogen polymerization and, in turn, aggravates coagulopathy, another member of the "lethal triad". Blood itself is a colloid but a common blood substitute used in resuscitation therapy is a hydroxyethyl starch solution. This combination of advantages and disadvantages have spurred the research and development of fluids that are composed of both colloidic and crystalloidic properties [8]. Plasma is often mixed into the resuscitation fluid used as it naturally decreases the incidence of coagulopathy resulting from the aggravation from the other members of the "lethal triad". It was found that the ideal ratio for plasma to RBCs is somewhere in between 1:1 and 1:2 [1].

In addition to the fluid used in the resuscitation procedure in general, there is disagreement in how the fluid should be applied in therapeutic treatment. Although immediate and aggressive fluid resuscitation has been common practice for treatment

of HS, it is found that such an intervention aggravates coagulopathy by breaking down partially formed clots and diluting factors essential to blood coagulation [2]. It has also been observed that delayed fluid resuscitation presents modest increases in patient survivability versus immediate fluid resuscitation because of increased hemorrhagic effect, organ damage through perfusion injury, and increased coagulopathy.

1.4 Overview of Resuscitation Fluid and Hextend®

As stated in the previous subsection, in general, there are two types of fluids used in therapeutic response to HS: colloids and crystalloids. In this subsection we will examine an example of each as well as introduce the solution that will be used specifically in this thesis: Hextend® (Hetastarch in Lactated Electrolyte).

1.4.1: Hypertonic Saline

Hypertonic saline is one of the most commonly used fluids in post-HS resuscitative therapy and research. It is a relatively low cost crystalloid especially when comparing to colloid resuscitation fluid. It appears to be effective in restoring vascular volume and, in turn, vascular perfusion and reduces post-injury oedema [13]. In addition it can be associated with reducing the inflammatory response associated with HS as well as cell necrosis by reducing concentrations of necrosis factors in circulation and increasing the relative concentration of anti-inflammatory cytokines. As previously mentioned, one of the controversies associated with fluid resuscitation protocols is that, despite numerous clinical trials and studies, there isn’t a solution that provides a distinctively and statistically significant difference in end patient outcome. However,

evidence suggests that the use of hypertonic saline shows some levels of efficacy in patients with traumatic brain injury and penetrating trauma [14, 15].

1.4.2: Blood and Blood Substitutes

Although not always available to severe trauma patients entering a state of HS especially in the pre-hospital setting, blood transfusion continues to be the prevalent resuscitative therapy in clinical use. In particular, there is a protocol referred to as a "massive transfusion" protocol in response to patients in HS. This protocol refers to the relatively accepted procedure of immediately, and in a sustained fashion, of providing blood and plasma to severely injured patients. The goal of the "massive transfusion" protocol is to attempt to sustain the normal content and volume of whole blood within the body. In addition, it has been observed that increased survival is correlated with increased plasma and platelet to RBC levels. These increased ratios also have the effect of reducing coagulopathy but are at increased risk of septic problems [16]. However it has been suggested that patient outcome is because they survive long enough to get the higher ratio transfusion instead of the high ratio transfusion being the cause of the increased survivability [17]. In general, conclusive statements cannot be made at this time regarding the optimal treatment plan for any one trauma patient due to the varying results from studies.

Blood substitutes have also been examined as a potential therapeutic infusion treatment for HS. However, like the controversy behind blood transfusion protocols, this also has varying results. Gould et al. [18] reported decreased mortality whereas Sloan et al. [19] reported increased mortality relative to a saline control. More research

into the use of blood substitutes would be necessary to examine whether or not it is a suitable therapy for HS patients but at this point, it it's a relatively inconclusive method of treatment.

1.4.3: Hextend® (Hetastarch in Lactated Electrolyte Solution)

The main resuscitation fluid used in the experiments conducted for this thesis is Hextend®. Hextend® is a commercially available colloidial volume expander with electrolyte supplementation manufactured and distributed by Hospira Inc. Hetastarch is 6% hydroxyethyl starch in 0.9% NaCl and Hextend® in particular has been designed to contain physiological levels of calcium and sodium. It also contains slightly lower than physiological levels of potassium and magnesium [20]. Hydroxyethyl starch solutions in general are commonly used in resuscitative therapy in research for HS. However, hydroxyethyl starch solution usage is cautioned and is only recommended in cases of severe hypotension. This is because of the risk of possible side effects regarding decreased patient survivability in septic shock and renal dysfunction [8]. There are also some controversial studies in the use of hydoxyethyl starch solutions in resuscitative therapy in that it can be related to increased levels of coagulopathy [2]. However, as will later be described, we will be using a severe HS model, considered Class 4 under the classification system, with resultant drastic hypotensive effects on blood pressure. This means that the use of a colloidial resuscitative fluid is warranted and recommended.

II. Nitric Oxide Nanoparticle Dose Optimization

In the previous chapter we discussed the problem that HS prevents to trauma patients as well as current treatment options with volume expanders. In this chapter, we will focus on research in the field to improve HS patient outcomes through the development of new therapeutic approaches. Specifically, this thesis focuses on the use of nitric oxide (NO) to preserve the state of a patient's microcirculation and this chapter will analyze the effective dosing of NO as delivered via hydrogel/glass nanoparticles.

2.1 Introduction to Nitric Oxide

Nitric oxide is a free radical that is involved in numerous biological reactions and signaling. This is because NO chemistry is dependent on the immediate environment which, in turn, leads to a multitude of potential different cellular interactions. It is a relatively stable molecule and can diffuse over several microns of distance before reacting in a collision-mediated manner [21]. In this section we will further examine NO and its biological effects in more detail.

2.1.1: Chemical Basis & Biological Relevance

NO in the body was first discovered as an endothelium-derived relaxing factor in rabbit aortae by Furchgott and Zawadski in 1980. The factor wasn't identified as NO until a series of experiments performed in 1977 and 1987 [20]. It is the basis for many reactions in biology due to its relatively unique free-radical structure of a partial double and partial triple bond from an unpaired electron [22].

Because of its high reactivity as a lipophilic gas, its half-life in biological systems is relatively low. It is constantly produced with at varying concentrations in

many biological systems such as the cardiovascular and nervous systems. NO is then free to diffuse across many cellular membranes to initiate signaling cascades. Some examples of these signaling pathways are the soluble guanylyl cyclase-cyclic guanosine monophosphate pathway and S-nitrosylation of cysteine residues on amino acid chains. These pathways are then responsible for many physiological effects such as vasodilation, muscle cell proliferation, leukocyte adhesion and platelet aggregation [23]. The diverse effects of NO cause it to become the focus of research into new therapies in the laboratory especially since there evidence of reduced NO bioavailability and regulation in patients with cardiovascular abnormalities [24].

In the body, NO is generated by NOS in three isoforms depending on location in the body (eNOS, nNOS and iNOS). However, it is noted that all three isoforms exist and are expressed in the cardiovascular system in humans [20]. In addition, it is commonly understood that RBCs act as NO sinks in that they readily take up and inactivate NO in the blood stream by convertible oxyhemglobin to methemoglobin and nitrate [25]. This naturally reduces bioavailability of NO as well as presents a significant challenge for the developments of therapies using NO as a central component. NO is also involved in s-nitrosation of thiol group residues, producing s-nitrosothiols, in proteins for post-translational regulation [26].

For therapeutic application of NO, the goal is to induce vasodilation. NO is used a primary signaling molecule by the body causing soluble guanylate cyclase to form cGMP (cyclic GMP). Increased levels of cAMP subsequently activates pkG (protein kinase G) which then signals the cell to reuptake Ca^{2+} calcium ions. This effectively

relaxes the muscle cells and induces vasodilation by preventing phosphorylation of cellular myocin [27].

2.1.2: Current Medical Applications and Clinical Manipulations of Nitric Oxide in Hemorrhagic Shock

Hypoxia is a direct consequence of shock due to the decreased availability of circulating RBCs in the blood stream. It has been correspondingly observed that nitrite in the body is enzymatically reduced to NO by protein mediation when the body is under a state of hypoxia [28]. This is done to help compensate for the reduced bioavailability of NO due to the fact that NOS is impaired in these conditions. Therefore, there is sufficient reason to supplement the body with additional NO to assist with the shortage of the molecules in circulation with the intention of increasing the beneficial effects such as maintaining blood vessel diameter to maintain blood flow and vascular perfusion to peripheral tissues.

However, it is also good to note that in conditions of shock, there is the possibility of a systemic inflammatory response that contributes to vasodilation. This is detrimental to the body as it increases the duration of the period that the patient remains in shock and potentially decreases survivability. As previously mentioned, NO is associated with vasodilation and indeed it has been found that iNOS activation is at least partially responsible for systemic inflammation. This inflammatory response side-effect associated with NO levels has produced a second school of thought in that there should be inhibition of NO as a therapeutic treatment for shock especially in cases where the patient is in a persisting state of shock [28]. This was explored in a clinical trial through

the use of a general NOS inhibitor L-NMMA in shock patients. However, it was observed that although the patient's blood pressure increased at two hours, there was no effect in overall patient outcome or mortality rates in the long term leading to uncertainty with this approach as an overall strategy [29].

The focus of this thesis is with the former approach with supplementing NO to the body as a therapeutic response to cases of HS. It has been found that NO is strongly associated with homeostasis in a patient's microcirculation [28]. By improving conditions in the microcirculatory system, there is the hope that it would then improve situations connected with multiple organ failure as well as improve a patient's overall vascular perfusion.

2.1.3: Delivery Methods of Nitric Oxide and Advantages of Nanoparticles

Currently, in medical practice, the only accepted delivery method of supplementing NO to the body is through gaseous inhalation. However, NO is only approved for neonatal use (to treat pulmonary hypertension) in the United States. Its other uses, such as a treatment pathway for the same dysfunction in adults, irrespective of delivery method, is an ongoing research effort that remains to be approved despite many laboratory results indicating a beneficial effect.

Some disadvantages of the use of using gaseous inhalation as a method of NO delivery include but are not limited to the high cost inconvenience of pressurized NO tanks, the inability to precisely control and sustain a constant delivery rate as well as the possibility of inadequate whole body diffusion due to rapid uptake by erythrocytes in the blood stream [30]. These shortcomings of inhaled NO gas have spurred research into

other ways of delivering the molecule for therapeutic treatments. One such product of this research movement is the use of a novel hydrogel/glass based nanoparticle to intravenously deliver NO to the body. This thesis is focused upon the continued analysis of this particle's use for treatment of HS.

Generally speaking, this nanoparticle is generated through the use of a sol-gel based precursor with chitosan and polyethylene glycol. Sodium nitrite was used in the creation of the initial hydrogel/glass composite and this was reduced to NO upon exposure to redox reactions with electrons from glucose present in the composite. Upon lyophilization, it becomes a fine powder that retains NO and NO precursors when it is dry and, upon contact with moisture, facilitates a controlled release of NO [31]. These particles are relatively stable when dry and release properties are can be chemically modified by adjusting the molecular length of polyethylene glycol. It was observed that the half-life of the NO nanoparticle as synthesized in Cabrales et al 2010 was approximately 4 hours in circulation [31]. This level of control allows for precise dosing of NO as a therapy for varying degrees of shock as well as convenience and reduced cost of deployment for patients in both hospital and pre-hospital settings.

2.2 Experimental Aims

The goal of this study is to elucidate the optimal concentration of nanoparticles that contain encapsulated NO for the treatment of HS specifically in the hamster model. As will be detailed in the next section, the hamsters will be subjected to 50% blood volume HS followed by a 25% blood volume resuscitation using the volume expander, Hextend®, which has been supplemented with varying doses (1 mg/kg, 10 mg/kg, 15

mg/kg, 20 mg/kg, and 40 mg/kg) of NO nanoparticles. The animals will then be monitored for a period of 90 minutes after resuscitation.

2.3 Detailed Methodology

2.3.1: Significance of a Small Animal Model & Golden Syrian Hamsters

The significance of using a model at all is to have a miniaturized analogous system to the human body and its many functions. To date, most of knowledge accumulated by the scientific community regarding human physiology and pathology has been through the use of animal models. However, it is good to note that the model is not perfect. Genetic variations both between and within species can result in varying responses to the same experimental insult. This of course means that an encouraging outcome for a rodent model might not translate to a valid therapeutic approach in human patients. In studies for HS specifically, animal models are used to collect information on physiological response as well as response to pre-clinical experimental treatment procedures.

In the experiment outlined in these sections, golden Syrian hamsters are used as the chosen rodent model. Although mice can be easier to house, breed and perform genetic variations on, their low blood volume makes them sensitive to HS procedures [32]. Their small size also can lead to handling complications during implementation of both the shock and recovery protocols. Rats are able to overcome these disadvantages due to their increased size, however, it has been found that their immune response during shock can be somewhat different to that in humans [33]. In addition to overcoming challenges presented by both the mouse and rat models (larger in size and have

comparable immune response to humans), hamsters are easy to breed, not as susceptible to spontaneous diseases, comparable circulatory features to humans and have rapid developmental cycles [34].

2.3.2: The Hamster Window Chamber System

A window chamber system is used to be able to visualize the microcirculation. This system was first described for hamsters in 1943 by Algire et al [35] and subsequently developed by Endrich et. al in 1980 [36].

First, the animal is anesthetized with pentobarbital sodium (5 mg/100g body weight) and its hair is removed. The underlying skin sterilized with Betadine solution and the dorsum skin is pulled back followed by application of the aluminum frame. A round area of skin and tissue underneath is removed from a 1.5cm diameter circle on one side of the skin fold. The layer of subcutaneous tissue with intact epidermis is covered by glass coverslip while other intact side stays open to the environment. A PE-10 catheter is then placed into each the jugular vein and carotid artery. The end result is a "window" on the hamster's back that provides a view into the microcirculatory system of the region. Under a microscope, venules, arterioles and capillaries can be clearly visualized and measurements taken through photo or video recordings. Animals are then allowed to recover 2 days prior to experimental procedures. The detailed procedure for creating the window chamber can be found in Colantuoni et. al (1984) [37].

For our experiment, the window will be used to take video recordings of the microcirculatory system. These videos would then be analyzed after completion of the

experiment to obtain parameters relating to venule and arteriole diameters and center-line flow velocities.

2.3.3: Nitric Oxide Nanoparticles Synthesis

The complete procedure for the generation of the NO nanoparticles can be found in Friedman et. al 2008 [38]. In summary, the nanoparticles were generated from a hydrogel/glass composite by combining tetramethylorthosilicate, polyethylene glycol, chitosan, and glucose in a pH 7 0.5M sodium phosphate buffer. Experimental nanoparticles also contained sodium nitrite and NO would be produced in the polymer matrix due to redox reactions with electrons donated from glucose. The matrix would then be lyophilized and the resultant powder would contained trapped NO. Upon contact with aqueous material, the opening of water channels serve to release the trapped NO over controlled time periods based on polyethylene glycol molecular size in the original mixture.

2.3.4: Procedural Description

2.3.4.1: Experimental Setup for Hemorrhagic Shock Implementation and Resuscitation

The following protocol has been adapted from Cabrales (2009) [39]. The experiment was performed in 55-65g male Golden Syrian Hamsters (Charles River Laboratories, Boston, MA). These hamsters were fitted with the dorsal skinfold window chamber as described in the previous section. Other inclusion criteria include: HR > 340 bpm, MAP > 80 mmHg and arterial oxygen partial pressure > 50 mmHg. Examination of the tissue chamber (3-4 days after implantation) must also no exhibit signs of edema or bleeding. All animal handling and care followed the NIH guide for the Care and Use

of Laboratory Animals and the protocol has been approved by the local animal care committee. HS was induced by withdrawing 50% of total blood volume (estimated by 7% of body weight) from the carotid artery catheter within 5 min. One hour after inducing the hemorrhage, animals were resuscitated with 25% of total blood volume of Hextend® (Hospira, Lake Forest, IL, 6% Hetastarch 670 kDA in Lactated Electrolyte Injection) into the carotid artery catheter at a rate of 200 uL/min. Depending on the treatment group, the Hextend® contained diluted nanoparticles at concentrations of 1 mg/kg, 10 mg/kg, 15 mg/kg, or 40 mg/kg of baseline weight. Systemic and microvascular parameters were analyzed at baseline, 50 min post-hemorrhage, and up to 90 min after volume resuscitation.

2.3.4.2: Data Collection Procedures

The following procedures have been adapted from Cabrales (2009) [39]. MAP and HR were continuously monitored (MP 150, Biopac System, Santa Barbara, CA). Hematocrit was measured by centrifuging an arterial blood sample in a heparinized capillary tube. A second capillary tube sample was obtained to analyze for pO_2, pCO_2, base excess (BE) and pH (Blood Chemistry Analyzer 248, Bayer, Norwood, MA). [Hb] was found spectrophotometrically from a drop of blood from the arterial blood sample (B-Hemoglobin, Hemocue, Stockholm, Sweden). Blood plasma was obtained by collecting a sample from the carotid artery and centrifuging at 15,000 rpm for 10 min then removing the supernatant and measured with a NOxanalyzer (ENO-20; Eicom, Kyoto, Japan).

For arteriole/venule and FCD measurements, the unanesthetized animal was placed in a restraining tube with a longitudinal slit for the window chamber. The tube was then fixed to a microscope stage of a transillumination intravital microscope (BX51WI, Olympus, New Hyde Park, NY). Animals were allowed 20 minutes to settle before measurements were taken. Same sites on the window chamber were followed throughout he experiment so comparisons could be made to baseline measurements obtained. Videos were taken of the arterioles/venules at 1000 fps with a 20x magnification water immersion objective. Functional capillaries are defined as segments that have RBC transit of one RBC in a 60 second period. Up to 6 microscopic fields per hamster window were addressed at 40X with a water immersion objective.

2.3.4.3: Data Analysis Procedures

Blood flow velocity was analyzed using an image processing algorithm on the videos that uses 2D cross correlation. Details on the program can be found in Ortiz et. al (2014) [40]. Vessel diameters were measured using the ImageJ utility provided by the National Institute of Health.

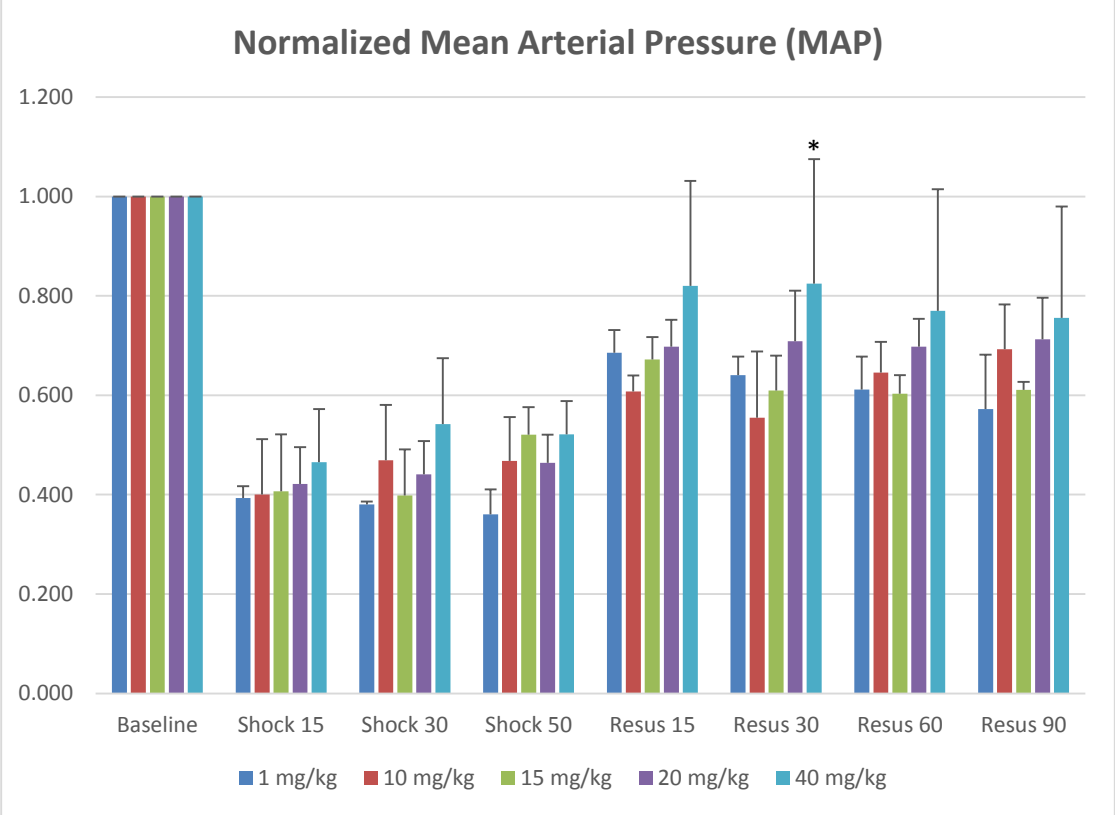
Results are presented mean±standard deviation. Data within each group was analyzed using two-way ANOVA and, when appropriate, post hoc analyses were performed with the Bonferroni post test. With the exception of blood plasma measurements, all data was normalized relative to baseline levels obtained before the experimental procedure. Statistics were calculated using GraphPad Prism 4.03 (GraphPad Software, Inc., San Diego, CA). Changes were considered statistically significant if $P < 0.05$.

2.4: Results

2.4.1: Mean Arterial Pressure and Heart Rate

The majority of hamster specimens had a baseline MAP in the range of 90-110 mmHg. Upon starting hemorrhage, and during the one hour "rest" state post-hemorrhage before resuscitation, MAP was observed to drop dramatically (between 40-60 mmHg). It was noted that in these studies, if the pressure drops below 40 mmHg during this time, mortality would become severely affected. These animals were subsequently removed from the study. When the resuscitation phase of the experiment begins, blood pressure would be increased rapidly due to the infusion of fluid volume into the animal resulting in an immediate spike sometimes reaching up to 80 mmHg. This would then taper off and decrease as time passes to settle between 60-70 mmHg (starting at about 60 minutes post-resuscitation) and remain so for the rest of the experiment.

Graph 1: Normalized Mean Arterial Pressure for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. Recovery of MAP was significant across these timepoints P<0.001. *P<0.05 for 40 mg/kg compared to 10 mg/kg.

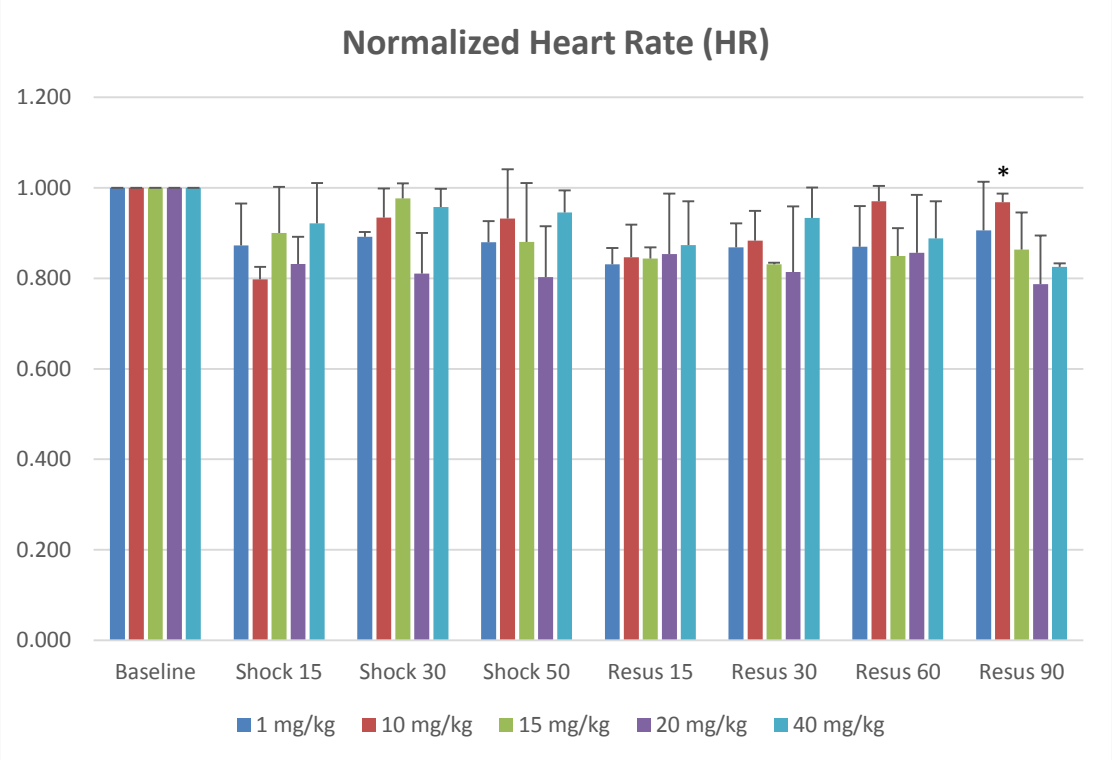


The previously mentioned trends for MAP can be visualized in the graph above (Graph 1). With 50% fluid volume hemorrhage, blood pressure is observed to drop to 40-50% of baseline levels and upon resuscitation, there is an immediate spike due to the increased fluid volume in some cases to 70% of baseline MAP. With regards to the treatment groups, increasing nanoparticle concentration appears to have a direct effect on the animal specimen's MAP resulting in better outcomes with increasing levels of NO delivered. This is especially observed and is significant for time periods immediately after resuscitation (15 minutes to 60 minutes) at which point the effect

appears to taper off irrespective of infused NO concentration. Overall recovery was significant ($P<0.001$).

The other main systemic parameter charted over time was HR. At baseline, hamsters used in this experiment have a HR in the range of approximately 410-450 bpm. It was observed that there was a small drop during shock to approximately 350 bpm where it would only increase slightly upon resuscitation. Overall, as can be seen in the chart below (Graph 2), NO supplemented resuscitation fluid does not appear to have a significant effect on restoring the HR to pre-shock values. It can be noted that higher concentrations of NO tends to have an adverse effect on HR recovery.

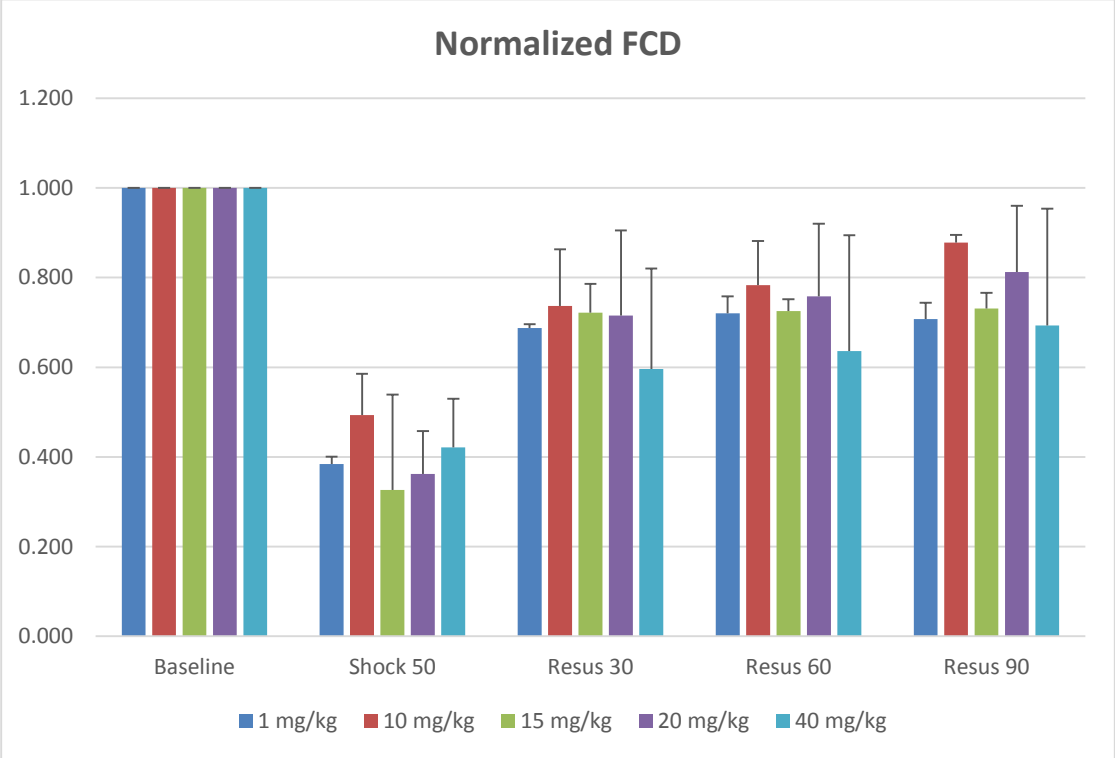
Graph 2: Normalized Heart Rate for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. *P<0.01 for 10 mg/kg compared to 20 mg/kg.



2.4.2: Functional Capillary Density

One of the primary measurements of vascular perfusion is functional capillary density (FCD). FCD is severely affected in conditions of HS due to the drastically decreased available circulating fluid volume. Blood is also rerouted to more essential organ systems resulting in a shutdown of smaller capillaries, especially in peripheral tissues. This is distinctly observed in our experimental data. FCD is severely reduced from baseline and in some cases, functional capillaries are not seen at all in the chosen fields of view where an intricate network with blood flow was observed before shock. In the chart below (Graph 3), it can be seen that the resuscitation procedure has a very significant beneficial effect to restoring the FCD in the periphery ($P<0.001$).

Graph 3: Normalized Functional Capillary Density for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. Recovery of FCD was significant across these timepoints P<0.001.



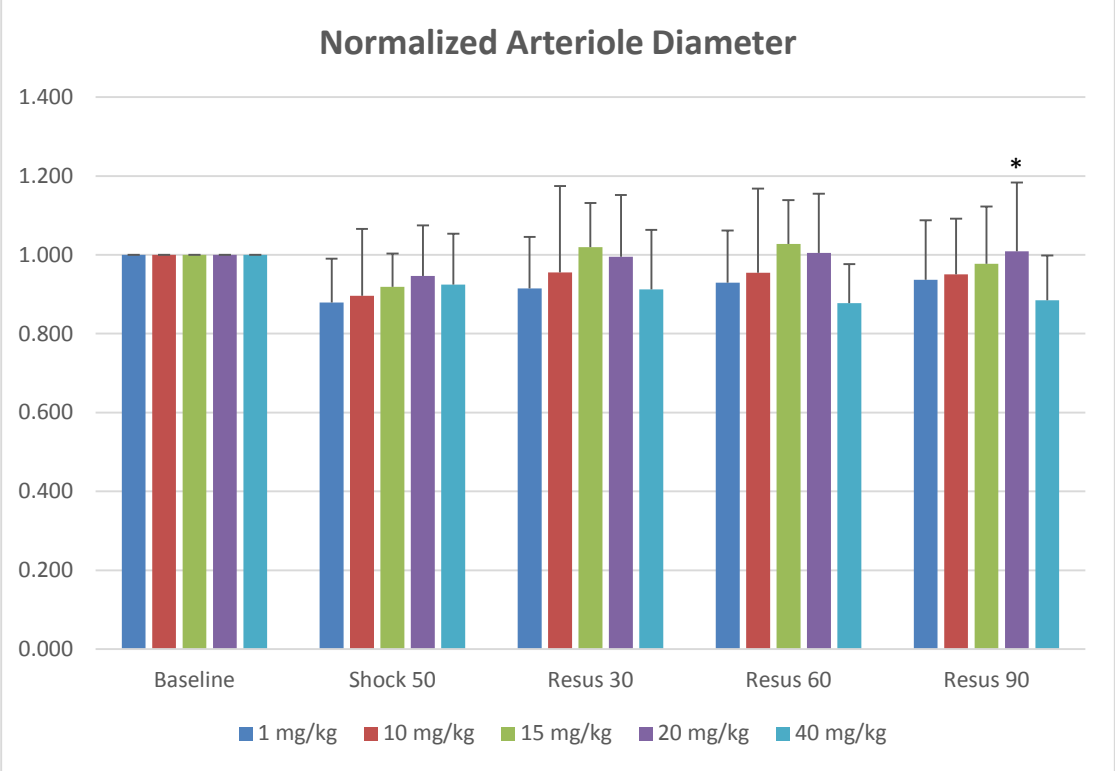
From our data, FCD is typically restored to about 50% of baseline values within 30 minutes after resuscitation. FCD continuously and steadily increases as time passes. At 90 minutes after resuscitation, FCD has recovered to anywhere in the range of 70-85% of baseline values and in some cases even better or complete recovery was observed. Interestingly, increasing the concentration of nitric oxide nanoparticles above 10 mg/kg appears to have a detrimental effect on FCD recovery and it can be seen that averages of 40 mg/kg dosing results in significantly lowered recovery.

2.4.3: Arteriole/Venule Diameter

NO's function as a vasodilator is well accepted. It is also understood that during HS, the body's network of arteries begin to constrict in an attempt to maintain fluid

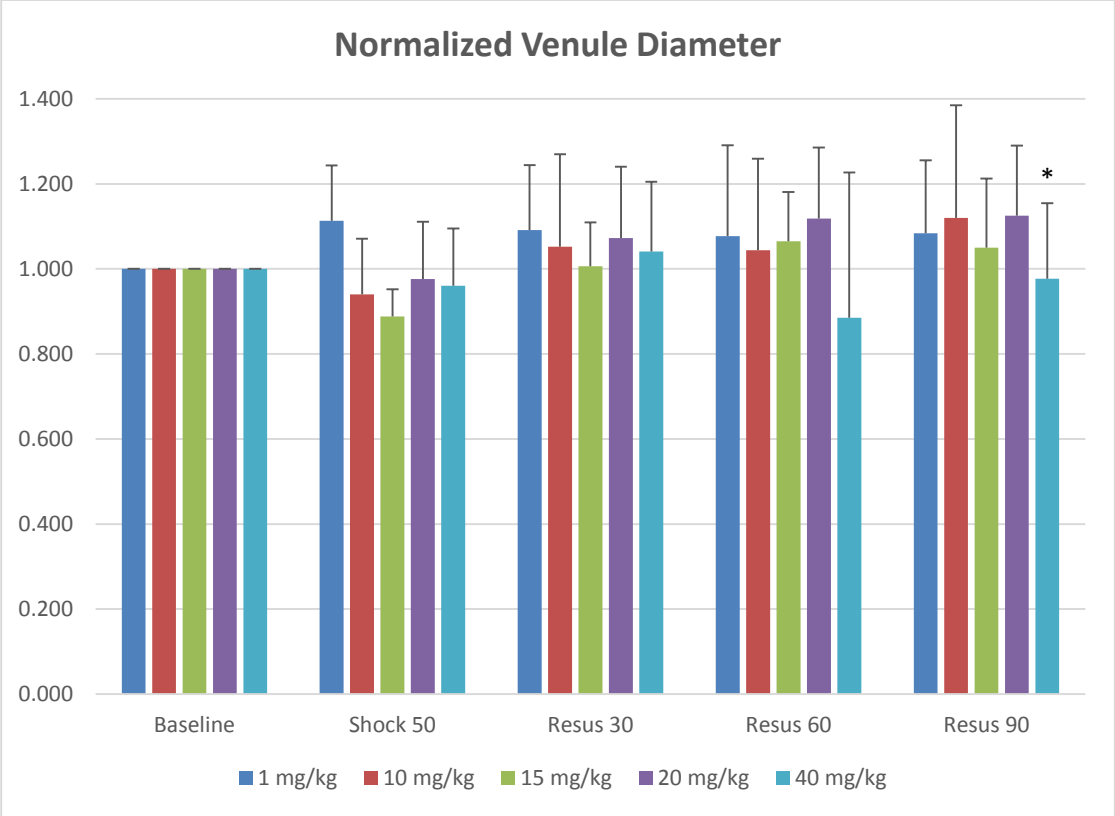
pressure in conditions of reduced fluid volume. In our experiments, both of these points were observed. Arteriole diameter is reduced to in response to the shock state and, recovers, albeit to a small degree, upon resuscitation with NO supplemented fluid. The chart below (Graph 4) portrays the data obtained normalized to baseline arteriole diameter values and there is significant variation between the treatments ($P<0.05$). In shock, arterioles constrict to about 85-90% of their original diameter and upon resuscitation, the diameters are typically restored to values of 95% and greater of baseline values. Overall, this shows that NO is effective at reversing the constriction induced by HS, particularly in the arterioles. It can be seen that a dose of 15-20 mg/kg of NO nanoparticles appears optimal in recovering arteriole diameter. However, like previously observed in the FCD data, high concentrations of NO nanoparticles seem to have an adverse effect and result in little to no recovery at a dose of 40 mg/kg.

Graph 4: Normalized Arteriole Diameters for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. Differences between treatments were significant at these timepoints P<0.05. *P<0.01 for 20 mg/kg compared to 40 mg/kg.



It is known that venules are less affected and do not constrict during HS. This can be seen with our data on venule diameters in the graph below (Graph 5). Slight decreases in diameter were observed but not statistically significant. Differences between treatments were observed to be significant (P<0.01). However, as NO still functions as a vasodilator irrespective of the lack of constriction, venules diameters increased beyond baseline values when the NO supplemented resuscitation fluid was infused. This resulted in increases of 5-10% in venule diameter beyond baseline values.

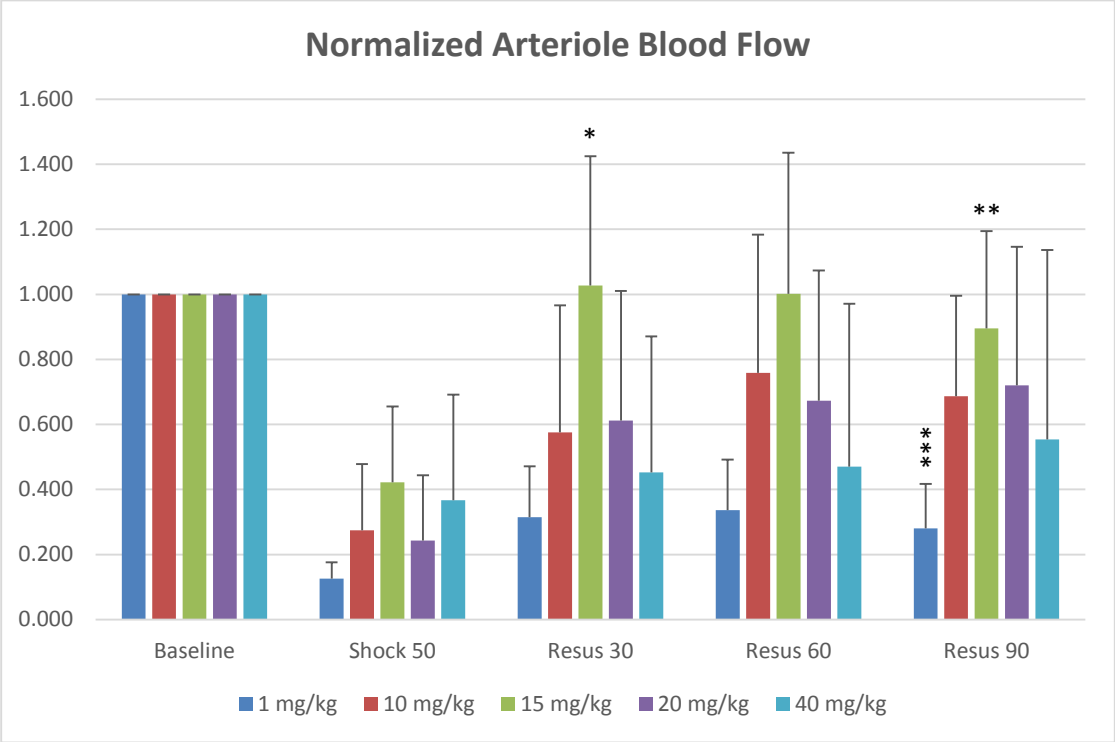
Graph 5: Normalized Venule Diameters for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. Differences between treatments were significant at these timepoints P<0.01. Recovery of venule diameters were significant across these timepoints P<0.001. *P<0.05 for 40 mg/kg compared to 10 mg/kg and P<0.01 for 40 mg/kg compared to 20 mg/kg.



2.4.4: Arteriole/Venule Blood Flow

Although diameters of arterioles and venules only change by 5-10% relative to baseline, if at all, fluid flow within the arterioles and venules is severely affected. It was observed during the experiments that some arterioles and venules would shut down completely resulting in zero observable, and measurable, fluid flow particularly in arterioles and venules with smaller baseline diameter values. Flows in larger arterioles and veins would be severely compromised. These observations can be visualized in the bar graphs presented below (Graph 6).

Graph 6: Normalized Arteriole Blood Flow Velocity for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. Differences between treatments were significant at these timepoints P<0.001. Recovery of arteriole blood flow was significant across these time points P<0.001. *P<0.001 for 15 mg/kg compared to 1 mg/kg and 40 mg/kg; P<0.01 for 15 mg/kg compared to 10 mg/kg and 20 mg/kg. **P<0.001 for 15 mg/kg compared to 1 mg/kg; P<0.05 for 15 mg/kg compared to 40 mg/kg. ***P<0.05 for 1 mg/kg compared to 10 mg/kg; P<0.01 for 1 mg/kg compared to 20 mg/kg.

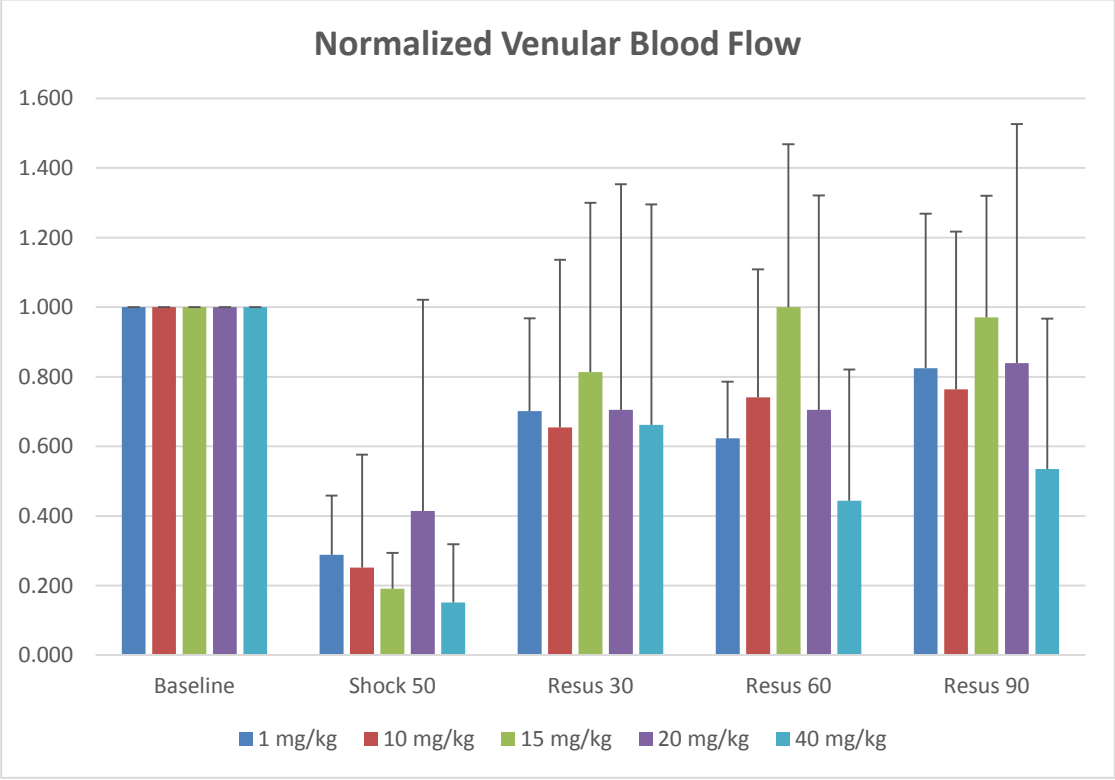


During HS, blood flow velocity in the arterioles becomes drastically decreased (P<0.001). Generally flow values ranged from 0-40% of baseline as a function of observed baseline diameter. Smaller diameters resulted in greater flow reductions and this can be correlated to why FCD measurements show significant reduction. Although more data is needed to draw strong conclusions, from our preliminary data shown here, a 15 mg/kg nanoparticle dose appears to be optimal in arteriole blood flow recovery

($P<0.05$). Again, high doses of nanoparticles, 40 mg/kg, is shown to be ineffective in inducing optimal recovery of blood flow.

Similar observations regarding general recovery can be seen in the venule data ($P<0.001$). A 15 mg/kg dose still appears to be optimal but again more data is needed to statistically confirm this trend. Like in the arterioles, a dose of 40 mg/kg appears to be too high and has reduced effectiveness in restoring venular blood flow velocity.

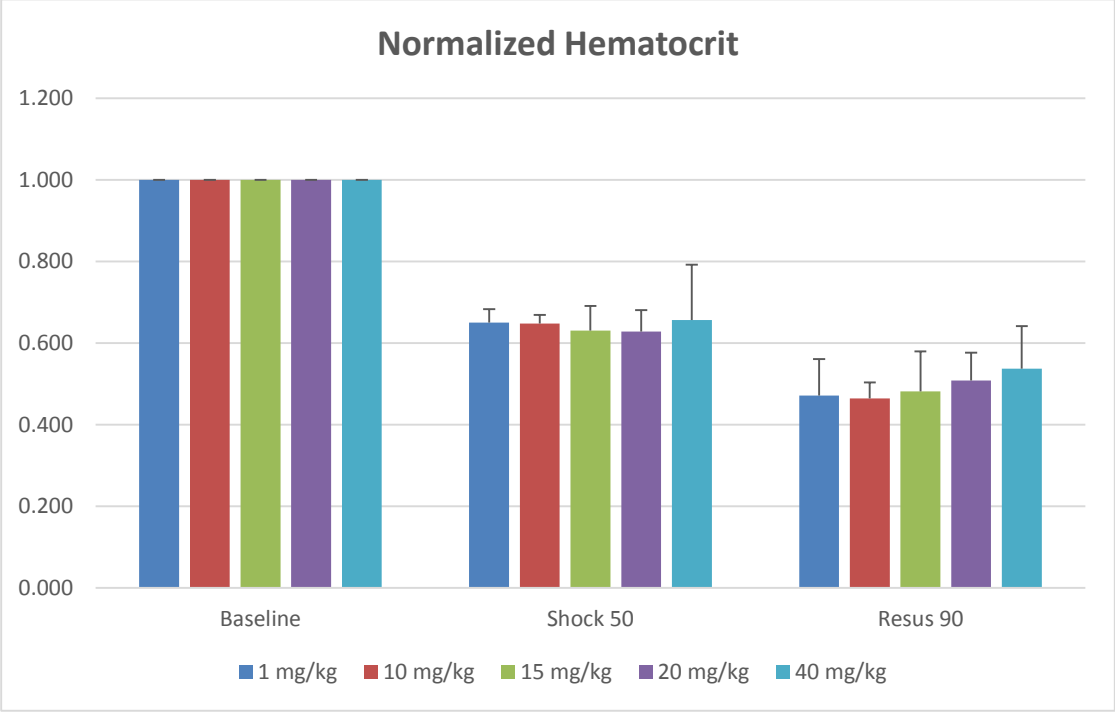
Graph 7: Normalized Venular Blood Flow Velocity for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. Recovery of venular blood flow was significant across these time points $P<0.001$.



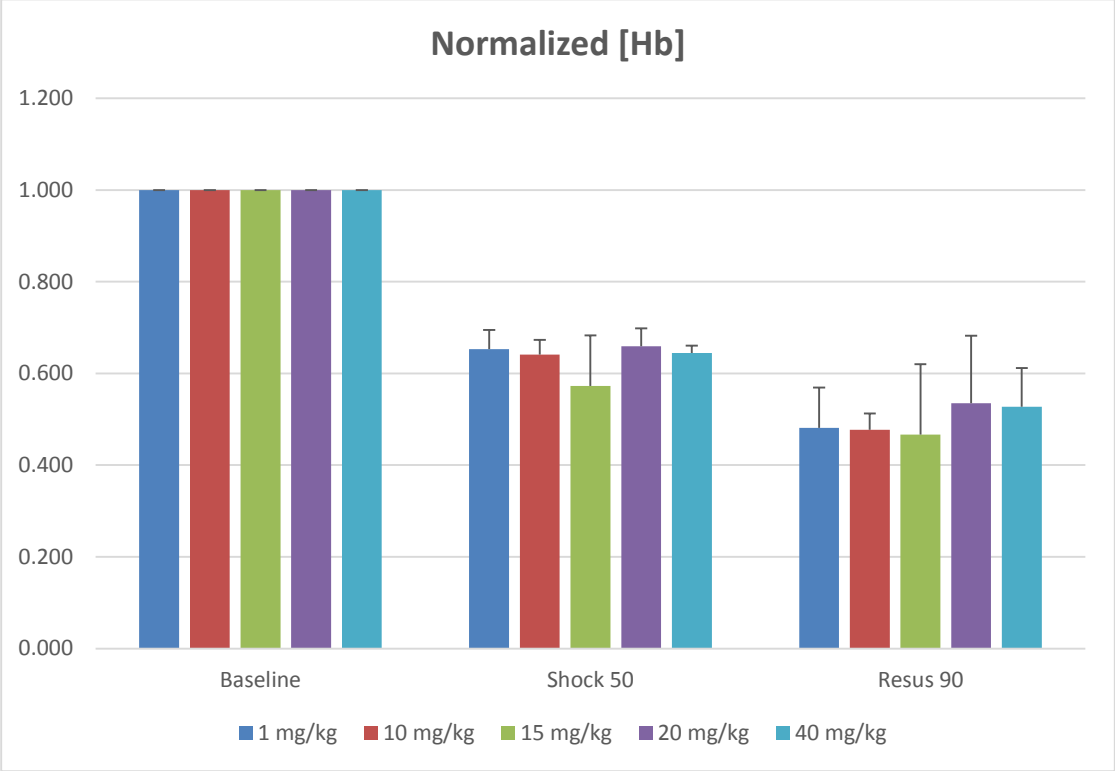
2.4.5: Hematocrit and [Hb]

Hematocrit and [Hb] are common measurements used as an indicator of the presence and severity of HS. They are essentially indirect measurements of RBC quantity in the circulating blood stream relative to other circulating fluids and matter such as blood plasma. In states of HS, hematocrit and concentration of hemoglobin are reduced from baseline by a function of shock severity. It can be seen from the graphs below (Graph 8, Graph 9) that, in our 50% HS experiments, both hematocrit and [Hb] are reduced to 60-65% of baseline values. This translates to a real value drop from approximately 50% hematocrit and 15.0 mmol/L hemoglobin concentration standard to roughly 30% hematocrit and 9.0 mmol/L hemoglobin concentration. However, these parameters are not a target in this experiment as nitric oxide has relatively no effect on hematocrit and hemoglobin levels in the bloodstream on its own. However, since a fluid volume expander is infused in resuscitation, a drop in hematocrit and hemoglobin concentration can be observed due to further hemodilution of the circulating blood fluid ($P<0.001$).

Graph 8: Normalized Hematocrit for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. Statistical Analysis was performed on timepoints Shock 50 and Resus 90. Reduction in hematocrit was significant across these time points P<0.001.



Graph 9: Normalized Hemoglobin Concentrations for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. . Statistical Analysis was performed on timepoints Shock 50 and Resus 90. Hemoglobin concentration reduction was significant across these time points. P<0.001.



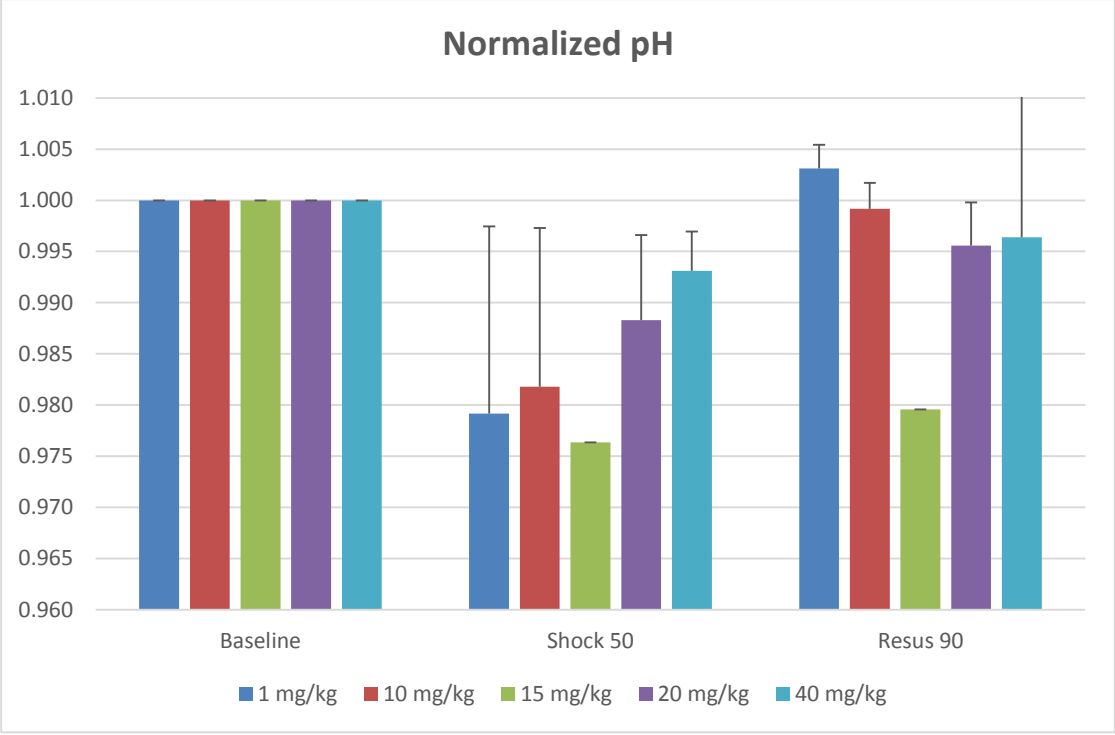
As expected, there is no significant variation between the concentrations of nanoparticles and gaseous delivery method as well as no improvement in hematocrit and hemoglobin concentration levels. It is also as expected in that the normalized hematocrit and hemoglobin levels fall to the same degree indicating that the measurements are essentially equivalent and data output of the experiment is consistent.

2.4.6: Blood Gas Parameters (pH, pO_2, pCO_2, BE)

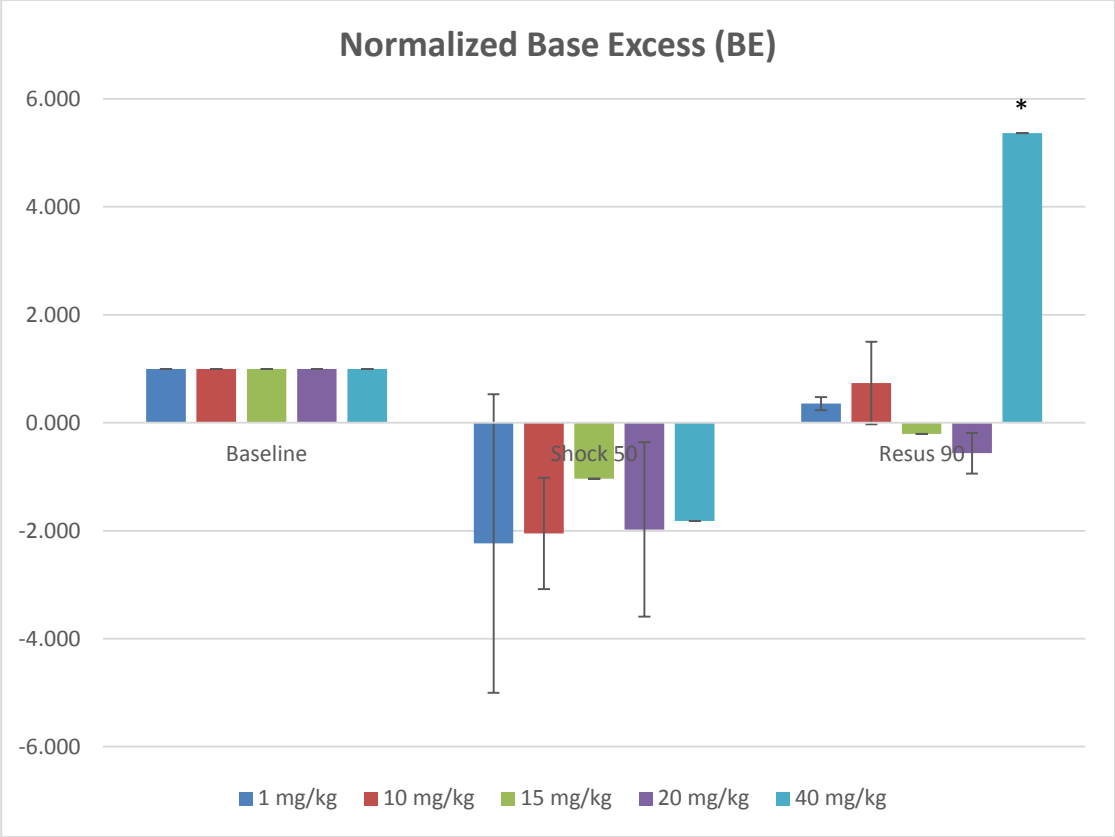
It can be seen that pH drops when HS conditions are imposed (Graph 10). From our data this drop translated to a drop range of 0-2.5% relative to baseline. This is because of inadequate oxygen supply to tissues resulting from decreased vascular

perfusion. Tissues switch to anaerobic metabolism to maintain energy upkeep but acids are produced as a byproduct. Upon restoration of vascular perfusion from resuscitation, pH is rapidly restored ($p<0.05$). Although only 90 minute post-resuscitation data was collected, it can be seen that pH recovery to baseline is complete or near complete (within 0.5%) with some cases becoming more basic than at baseline. It should be noted that more data is required for the 15 mg/kg treatment group. BE is an analogous measure as it measures the level of acidosis occurring.

Graph 10: Normalized pH for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. Statistical Analysis was performed on timepoints Shock 50 and Resus 90. Recovery of pH was significant at these timepoints $P<0.05$.

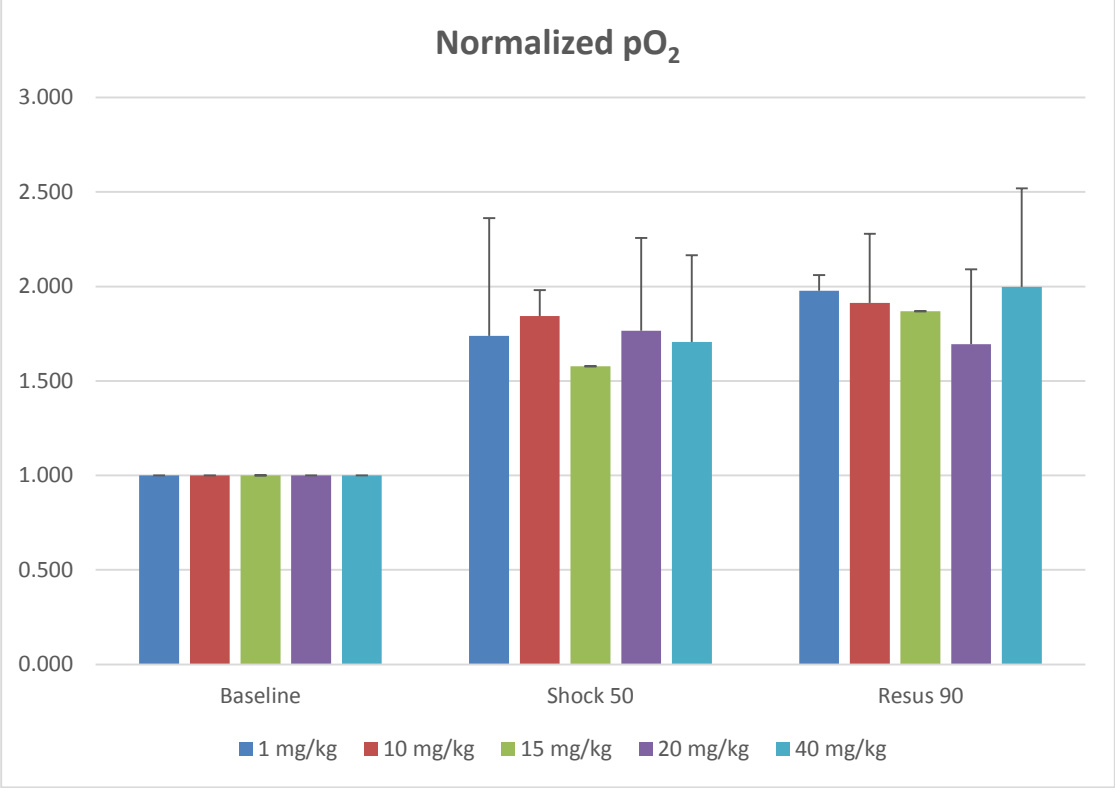


Graph 11: Normalized Base Excess for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. Statistical Analysis was performed on timepoints Shock 50 and Resus 90. Treatment groups were significantly different across these timepoints $P<0.05$. Recovery of BE was significant at these timepoints $P<0.001$. *$P<0.05$ for 40 mg/kg compared to 10 mg/kg and 15 mg/kg; $P<0.01$ for 40 mg/kg compared to 1 mg/kg; $P<0.001$ for 40 mg/kg compared to 20 mg/kg.



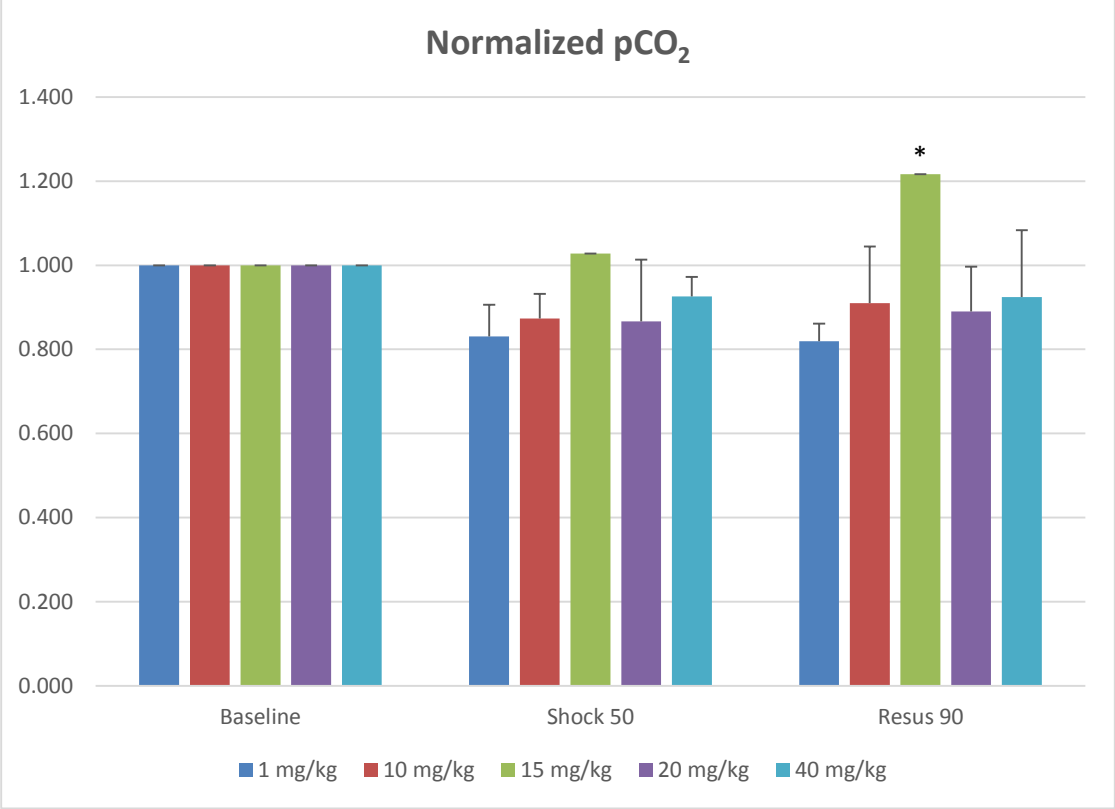
pO_2 is another blood gas parameter that is affected by HS. pO_2 is increased in periods of shock as the body attempts to compensate for lack of oxygen delivery to tissues by increasing respiration. This increase is respiratory rate results in a pO_2 increase of 50-100% of pO_2 in the bloodstream above baseline. However, this is unaffected by our nanoparticle NO treatment due to the fact that respiratory compensation appears to extend beyond the 90 minute post-resuscitation observation period, or at least the effects thereof (Graph 12).

Graph 12: Normalized pO_2 for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. Statistical Analysis was performed on timepoints Shock 50 and Resus 90. Changes not significant.



As the rate of respiration is increased (as seen by increased pO_2), correspondingly, pCO_2 would decrease in the bloodstream. From the chart below (Graph 13), this decrease is approximately to 80-90% of baseline levels and, similar to pO_2 levels, maintains this state and is unaffected by the resuscitation phase of the experiment irrespective of treatment group.

Graph 13: Normalized pCO_2 for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. Statistical Analysis was performed on timepoints Shock 50 and Resus 90. Differences in treatment groups were significant across these time points P<0.01. *P<0.05 for 15 mg/kg compared to 1 mg/kg and 20 mg/kg.

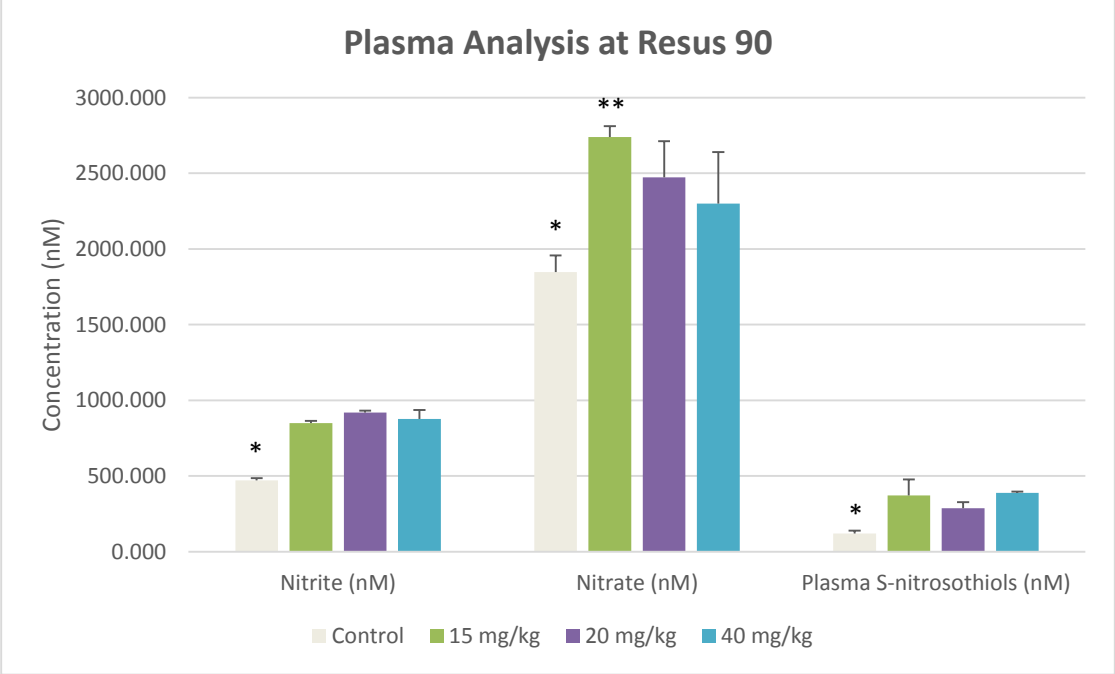


2.4.7: Nitrite, Nitrate and S-nitrosothiols for NO-np vs Control

Blood plasma samples were collected and analyzed for nitrite, nitrate and s-nitrosothiols after completion of the experiment (at 90 minute post-resuscitation). Control animals were animals that were not supplemented with NO as part of treatment. As can be observed (Graph 14), NO supplementation resulted in a significant increase in all three measured parameters (P<0.001). There was an almost two-fold increase in nitrite concentration, a 25-40% increase in nitrate concentration and a two to three-fold increase in s-nitrosothiol concentration. Interestingly, increasing the dose of NO

nanoparticles did not result in a proportional increase in these parameters. Nitrite and s-nitrosothiol levels are relatively level irrespective of the increase in nanoparticle concentration. However, nitrite levels showed a roughly 15% decrease moving from a 15 mg/kg dose to a 40 mg/kg dose.

Graph 14: Blood Plasma Analysis Concentration Data at 90 min post-resuscitation for 1 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg and 40 mg/kg Nitric Oxide Nanoparticle Concentrations. Statisical analysis was run on all 3 parameters. Differences across treatment groups were significant P<0.001. *P<0.001 for Control compared to 15 mg/kg, 20 mg/kg and 40 mg/kg for Nitrate. P<0.01 for Control compared to 15 mg/kg and 40 mg/kg for Nitrite. P<0.001 for Control compared to 20 mg/kg for Nitrite. **P<0.01 for 15 mg/kg compared to 40 mg/kg.



2.5: Discussion of Results for Concentration Optimization

2.5.1: Systemic Parameters

Although a NO nanoparticle supplemented volume expander has relatively no effect on HR, its effect on MAP is profound. Our results show a 20-30% recovery

relative to baseline at a time period of 90 minutes after resuscitation. This is a significant improvement particularly over gaseous inhalation of NO which only produced a roughly 10% recovery relative to baseline. Complete recovery of baseline MAP values were not achieved, nonetheless, the improvement is significant and, as previously stated in Chapter 1, permissive hypotension is deemed favorable to patient outcome.

In addition to MAP, blood gas related measurements also show that these nanoparticles result in better patient outcome. Changes in blood gas parameters are from an indirect effect of the circulating NO. It has been well characterized that in conditions of HS, vascular perfusion is compromised resulting in decreased oxygen gas availability especially to peripheral and non-essential tissues and organs. This forces the cells in these tissue systems to revert to anaerobic metabolic processes to maintain energy production. However, these processes release acidic byproducts that subsequently decrease the pH of circulating bodily fluid in a process termed metabolic acidosis. The increase in acidity can be observed in our charts depicting pH and BE values relative to baseline where there is an immediate drop in both upon initiating the HS protocol. NO assists the recovery of pH and BE values by increasing vascular perfusion and restoring oxygen availability to tissue systems that otherwise would be operating under a severe shortage of oxygen. This effect is able to quickly reverse pH decreases due to metabolic acidosis and restore balance to the body and restore values back close to baseline observed values. Despite this effect on pH, as stated in the results, NO has little effect on pO_2 and pCO_2 levels in the timeframe of this study as respiratory rate compensation

and associated effects initiated by shock appears to extend beyond the 90 minute allotted recovery period.

Blood plasma analysis shows that the supplemented NO is being utilized by the body. Naturally, NO in the body is generated by conversion of nitrates (ingested) to nitrite and then subsequently to NO. In the presence of exogenously supplemented NO, both nitrate and nitrite values are higher as they are no longer being utilized at the same rate for conversion to NO. In addition s-nitrosothiols are a byproduct of NO usage in the body and this increased close to three-fold when NO was supplemented using the nanoparticles relative to control. Our plasma data shows that a concentration of 15 mg/kg has the greatest effect per amount of NO supplied and that increasing beyond this provides little gains.

2.5.2: Microcirculatory Parameters

Improvements in vascular perfusion can be characterized by observing fluid flow and vessel diameters of the microcirculatory system. In the case of functional capillary density (FCD), our data shows that there is roughly a 20% improvement relative to baseline within the first 30 minutes after resuscitation with NO and a volume expander. Unlike MAP which slowly decreases over time after resuscitation, FCD recovery continues to improve and at 90 minutes after resuscitation shows 30% improvement relative to baseline from shock. It was observed that in some cases, FCD would improve completely back to baseline for some fields of view. FCD recovery is especially important as it is indicative of increased vascular perfusion and, consequently, oxygen delivery to peripheral cells and tissues. As previously stated,

acidosis would be reduced and the risk of multi-organ failure arising in the body is alleviated. This, of course, results in better overall patient outcome.

Taking a step back to view larger vasculature, it can be seen that it is not as clear cut as capillaries which are either flowing or non-flowing. It is observed that flow rates are significantly impaired in both arterioles and venules during a state of HS, however, often times it would not completely stop. The parameter of flow within arterioles and venules also demonstrates the effectiveness of NO as a mitigator of HS symptoms and it can be seen from our data that the use of the NO nanoparticles facilitates a recovery of flow velocity in both venules and arterioles. However, due to the great variation in our data, more experiments would need to be performed to obtain statistically significant values. Regardless, this data alludes to the fact that 15 mg/kg might be the optimal dose for the nanoparticles as, on the average, it produced the greatest recovery in our data set despite the variation. It is also seen that 40 mg/kg might be an overdose of NO leading to lower than optimal flow velocity recovery. Again, it is difficult to draw strong conclusions at this point and more studies focusing on flow velocity in particular are necessary.

As previously stated in Chapter 1, venule diameter is generally not affected very much by a state of HS and an exogenous NO supplementation results in diameters that go beyond baseline values as seen in our data. This shows the potent non-selective nature of NO as a molecule and can be seen as an example of why dosing would need to be optimized for therapeutic use. On the other hand, NO is very effective for restoring arteriole diameters that have been constricted as a result of HS. Indeed our data shows

that arteriole diameters are nearly restored to baseline values at 90 minutes post-resuscitation particularly at 15 mg/kg and 20 mg/kg concentrations of NO nanoparticles.

2.5.3: Optimal Dosing

Although it is difficult to judge an optimal dose from our acquired data, the data, arteriole flow in particular, suggests that the dose is approximately at 15 mg/kg (at least for our hamster model). Blood plasma data indicated that there were little to no gains to be had going beyond this concentration. Arteriole diameter and FCD recovery data also suggests that the dose is in the 15 mg/kg to 20 mg/kg range.

III. Effectiveness of Nanoparticles Compared to Gaseous Inhalation of Nitric Oxide

In Chapter 2, we analyzed the effectiveness of NO nanoparticles and determined that the optimum dose is 15 mg/kg in hamsters with severe HS (rapid loss of 50% blood volume). In this chapter, we will compare the efficacy of the nanoparticle delivery method with the accepted gaseous inhalation methodology.

3.1 Background on Nitric Oxide

Nitric oxide is a free radical that is involved in numerous biological reactions and signaling. This is because NO chemistry is dependent on the immediate environment which, in turn, leads to a multitude of potential different cellular interactions. It is a relatively stable molecule and can diffuse over several microns of distance before reacting in a collision-mediated manner [21]. In this section we will further examine NO and its biological effects in more detail.

3.1.1: Chemical Basis and Biological Relevance

NO in the body was first discovered as an endothelium-derived relaxing factor in rabbit aortae by Furchgott and Zawadski in 1980. The factor wasn't identified as NO until a series of experiments performed in 1977 and 1987 [6]. It is the basis for many reactions in biology due to its relatively unique free-radical structure of a partial double and partial triple bond from an unpaired electron [22].

Because of its high reactivity as a lipophilic gas, its half-life in biological systems is relatively low. It is constantly produced with at varying concentrations in many biological systems such as the cardiovascular and nervous systems. NO is then

free to diffuse across many cellular membranes to initiate the corresponding signaling cascades. Some examples of these signaling pathways are the soluble guanylyl cyclase-cyclic guanosine monophosphate pathway and S-nitrosylation of cysteine residues on amino acid chains. These pathways are then responsible for many physiological effects such as vasodilation, muscle cell proliferation, leukocyte adhesion and platelet aggregation [23]. The multiple effects cause NO to become the focus of research into new therapies in the laboratory especially since there evidence of reduced NO bioavailability and regulation in patients with cardiovascular abnormalities [24].

In the body, NO is generated by nitric oxide synthase (NOS) in three isoforms depending on location in the body (eNOS, nNOS and iNOS). However, it is noted that all three isoforms exist and are expressed in the cardiovascular system in humans [20]. It is commonly understood that RBCs act as NO sinks in that they readily take up and inactivate NO in the blood stream by convertible oxyhemglobin to methemoglobin and nitrate [25]. This naturally reduces bioavailability of NO as well as presents a significant challenge for the developments of therapies using NO as a central component. In addition to this mechanism, NO is also involved in s-nitrosation of thiol group residues, producing s-nitrosothiols, in proteins for post-translational regulation [26].

For therapeutic application of NO, the goal is to induce vasodilation. NO is used a primary signaling molecule by the body causing soluble guanylate cyclase to form cGMP (cyclic GMP). Increased levels of cAMP subsequently activates pkG (protein kinase G) which then signals the cell to reuptake Ca2+ calcium ions. This effectively

relaxes the muscle cells and induces vasodilation by preventing phosphorylation of cellular myocin [27].

3.1.2: Medical Application and Clinical Manipulation of Nitric Oxide in Hemorrhagic Shock

Hypoxia is a direct consequence of shock due to the decreased availability of circulating RBCs in the blood stream. It has been correspondingly observed that nitrite in the body is enzymatically reduced to NO by protein mediation when the body is under a state of hypoxia [28]. This is done to help compensate for the reduced bioavailability of NO due to the fact that NOS is impaired in these conditions. Therefore, there is sufficient reason to supplement the body with additional NO to assist with the shortage of the molecules in circulation with the intention of increasing the beneficial effects such as maintaining blood vessel diameter to maintain blood flow and vascular perfusion to peripheral tissues.

The experiments outlined in this chapter will use approach of supplementing NO to the body as a therapeutic response to cases of HS. It has been found that NO is strongly associated with homeostasis in a patient's microcirculation [28]. By improving conditions in the microcirculatory system, there is the hope that it would then improve situations connected with multiple organ failure as well as improve a patient's overall vascular perfusion.

3.1.3: Delivery Methods of Nitric Oxide and Advantages of Nanoparticles

Currently, in medical practice, the only accepted delivery method of supplementing NO to the body is through gaseous inhalation. However, NO is only

approved for neonatal use (to treat pulmonary hypertension) in the United States. Its other uses, such as a treatment pathway for the same dysfunction in adults, irrespective of delivery method, is an ongoing research effort that remains to be approved despite many laboratory results indicating a beneficial effect.

Some disadvantages of the use of using gaseous inhalation as a method of NO delivery include but are not limited to the high cost inconvenience of pressurized NO tanks, the inability to precisely control and sustain a constant delivery rate as well as the possibility of inadequate whole body diffusion due to rapid uptake by erythrocytes in the blood stream [30]. These shortcomings of inhaled NO gas have spurred research into other ways of delivering the molecule for therapeutic treatments. One such product of this research movement is the use of a novel hydrogel/glass based nanoparticle to intravenously deliver NO to the body. The continued analysis of this particle's use for treatment of HS is the purpose of this thesis.

Generally speaking, this nanoparticle is generated through the use of a sol-gel based precursor with chitosan and polyethylene glycol. Sodium nitrite was used in the creation of the initial hydrogel/glass composite and this was reduced to NO upon exposure to redox reactions with electrons from glucose present in the composite. Upon lyophilization, it becomes a fine powder that retains NO and NO precursors when it is dry and, upon contact with moisture, facilitates a controlled release of NO [31]. These particles are relatively stable when dry and release properties are can be chemically modified by adjusting the molecular length of polyethylene glycol. It was observed that the half-life of the NO nanoparticle as synthesized in Cabrales et al 2010 was

approximately 4 hours in circulation [31]. This level of control allows for precise dosing of NO as a therapy for varying degrees of shock as well as convenience and reduced cost of deployment for patients in both hospital and pre-hospital settings.

3.2 Experimental Aims

The purpose the experiment outlined in this chapter is to compare the optimal concentration of NO nanoparticles found in the Chapter 2 (15 mg/kg) to traditional gaseous delivery at 90 ppm for the treatment of HS in the hamster model. Again the hamsters will be subjected to 50% blood volume HS followed by a 25% blood volume resuscitation using the volume expander, Hextend®, which has been supplemented with either 15 mg/kg NO nanoparticles or 90ppm inhaled NO. As in the Chapter 2, the animals will be monitored for 90 minutes post-resuscitation.

3.3 Detailed Methodology

3.3.1: Significance of a Small Animal Model & Golden Syrian Hamsters

The significance of using a model at all is to have a miniaturized analogous system to the human body and its many functions. To date, most of knowledge accumulated by the scientific community regarding human physiology and pathology has been through the use of animal models. However, it is good to note that the model is not perfect. Genetic variations both between and within species can result in varying responses to the same experimental insult. This of course means that an encouraging outcome for a rodent model might not translate to a valid therapeutic approach in human patients. In studies for HS specifically, animal models are used to collect information

on physiological response as well as response to pre-clinical experimental treatment procedures.

In the experiment outlined in this thesis, golden Syrian hamsters are used as the chosen rodent model. Although mice can be easier to house, breed and perform genetic variations on, their low blood volume makes them sensitive to HS procedures [32]. Their small size also can lead to handling complications during implementation of both the shock and recovery protocols. Rats are able to overcome these disadvantages due to their increased size, however, it has been found that their immune response during shock can be somewhat different to that in humans [33]. In addition to overcoming challenges presented by both the mouse and rat models (larger in size and have comparable immune response to humans), hamsters are easy to breed, not as susceptible to spontaneous diseases, comparable circulatory features to humans and have rapid developmental cycles [34].

3.3.2 The Hamster Window Chamber System

For the experiment outlined in this thesis, a window chamber system is used to be able to visualize the microcirculation. This system was first described for hamsters in 1943 by Algire et al [35] and subsequently developed by Endrich et. al in 1980 [36].

First, the animal is anesthetized with pentobarbital sodium (5 mg/100g body weight) and its hair is removed. The underlying skin sterilized with Betadine solution and the dorsum skin is pulled back followed by application of the aluminum frame. A round area of skin and tissue underneath is removed from a 1.5cm diameter circle on one side of the skin fold. The layer of subcutaneous tissue with intact epidermis is

covered by glass coverslip while other intact side stays open to the environment. A PE-10 catheter is then placed into each the jugular vein and carotid artery. The end result is a "window" on the hamster's back that provides a view into the microcirculatory system of the region. Under a microscope, venules, arterioles and capillaries can be clearly visualized and measurements taken through photo or video recordings. Animals are then allowed to recover 2 days prior to experimental procedures. The detailed procedure for creating the window chamber can be found in Colantuoni et. al (1984) [37].

For our experiment, the window will be used to take video recordings of the microcirculatory system. These videos would then be analyzed after completion of the experiment to obtain parameters relating to venule and arteriole diameters and center-line flow velocities.

3.3.3: Nitric Oxide Nanoparticles Synthesis

The complete procedure for the generation of the NO nanoparticles can be found in Friedman et. al 2008 [38]. In summary, the nanoparticles were generated from a hydrogel/glass composite by combining tetramethylorthosilicate, polyethylene glycol, chitosan, and glucose in a pH 7 0.5M sodium phosphate buffer. Experimental nanoparticles also contained sodium nitrite and NO would be produced in the polymer matrix due to redox reactions with electrons donated from glucose. The matrix would then be lyophilized and the resultant powder would contained trapped NO. Upon contact with aqueous material, the opening of water channels serve to release the trapped NO over controlled time periods based on polyethylene glycol molecular size in the original mixture.

3.3.4: Procedural Description

3.3.4.1: Experimental Setup for Hemorrhagic Shock Implementation and Resuscitation

The following protocol has been adapted from Cabrales (2009) [39]. The experiment was performed in 55-65g male Golden Syrian Hamsters (Charles River Laboratories, Boston, MA). These hamsters were fitted with the dorsal skinfold window chamber as described in the previous section. Other inclusion criteria include: HR > 340 bpm, MAP > 80 mmHg and arterial oxygen partial pressure > 50 mmHg. Examination of the tissue chamber (3-4 days after implantation) must also no exhibit signs of edema or bleeding. All animal handling and care followed the NIH guide for the Care and Use of Laboratory Animals and the protocol has been approved by the local animal care committee. HS was induced by withdrawing 50% of total blood volume (estimated by 7% of body weight) from the carotid artery catheter within 5 min. One hour after inducing the hemorrhage, animals were resuscitated with 25% of total blood volume of Hextend® (Hospira, Lake Forest, IL, 6% Hetastarch 670 kDA in Lactated Electrolyte Injection) into the carotid artery catheter at a rate of 200 uL/min. Depending on the treatment group, the Hextend® contained diluted nanoparticles at a concentration of 15 mg/kg of baseline weight or allowed to inhale a 90 ppm concentration of gaseous NO mixed with 78.3% Nitrogen and 21.7% Oxygen. Systemic and microvascular parameters were analyzed at baseline, 50 min post-hemorrhage, and up to 90 min after volume resuscitation.

3.3.4.2: Data Collection Procedures

The following procedures have been adapted from Cabrales (2009) [39]. MAP and HR were continuously monitored (MP 150, Biopac System, Santa Barbara, CA). Hematocrit was measured by centrifuging an arterial blood sample in a heparinized capillary tube. A second capillary tube sample was obtained to analyze for pO_2, pCO_2, base excess (BE) and pH (Blood Chemistry Analyzer 248, Bayer, Norwood, MA). [Hb] was found spectrophotometrically from a drop of blood from the arterial blood sample (B-Hemoglobin, Hemocue, Stockholm, Sweden). Blood plasma was obtained by collecting a sample from the carotid artery and centrifuging at 15,000 rpm for 10 min then removing the supernatant and measured with a NOxanalyzer (ENO-20; Eicom, Kyoto, Japan).

For arteriole/venule and FCD measurements, the unanesthetized animal was placed in a restraining tube with a longitudinal slit for the window chamber. The tube was then fixed to a microscope stage of a transillumination intravital microscope (BX51WI, Olympus, New Hyde Park, NY). Animals were allowed 20 minutes to settle before measurements were taken. Same sites on the window chamber were followed throughout he experiment so comparisons could be made to baseline measurements obtained. Videos were taken of the arterioles/venules at 1000 fps with a 20x magnification water immersion objective. Functional capillaries are defined as segments that have RBC transit of one RBC in a 60 second period. Up to 6 microscopic fields were addressed at 40X with a water immersion objective.

3.3.4.3: Data Analysis Procedures

Blood flow velocity was analyzed using an image processing algorithm on the videos that uses 2D cross correlation. Details on the program can be found in Ortiz et. al (2014) [40]. Vessel diameters were measured using the ImageJ utility provided by NIH.

Results are presented mean±standard deviation. Data within each group was analyzed using two-way ANOVA and, when appropriate, post hoc analyses were performed with the Bonferroni post test. With the exception of blood plasma measurements, all data was normalized relative to baseline levels obtained before the experimental procedure. Statistics were calculated using GraphPad Prism 4.03 (GraphPad Software, Inc., San Diego, CA). Changes were considered statistically significant if $P < 0.05$.

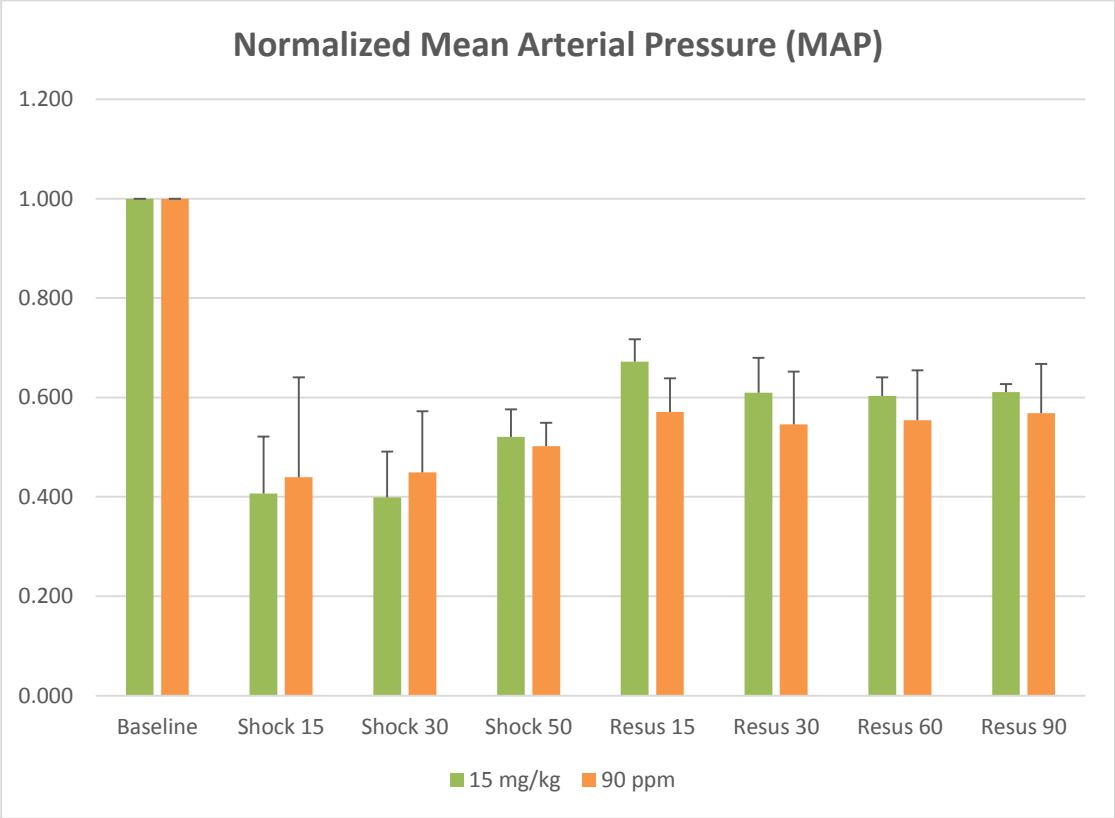
3.4: Results

3.4.1: Mean Arterial Pressure and Heart Rate

The majority of hamster specimens had a baseline MAP in the range of 90-110 mmHg. Upon starting hemorrhage, and during the one hour "rest" state post-hemorrhage before resuscitation, MAP was observed to drop dramatically (between 40-60 mmHg). It was noted that in these studies, if the pressure drops below 40 mmHg during this time, mortality would become severely affected. These animals were subsequently removed from the study. When the resuscitation phase of the experiment begins, blood pressure would be increased rapidly due to the infusion of fluid volume into the animal resulting in an immediate spike sometimes reaching up to 80 mmHg. This would then taper off and decrease as time passes to settle between 60-70 mmHg

(starting at about 60 minutes post-resuscitation) and remain so for the rest of the experiment.

Graph 15: Normalized Mean Arterial Pressure for 15 mg/kg Nanoparticle Concentration vs 90 ppm Gaseous Inhalation. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. Changes not significant.

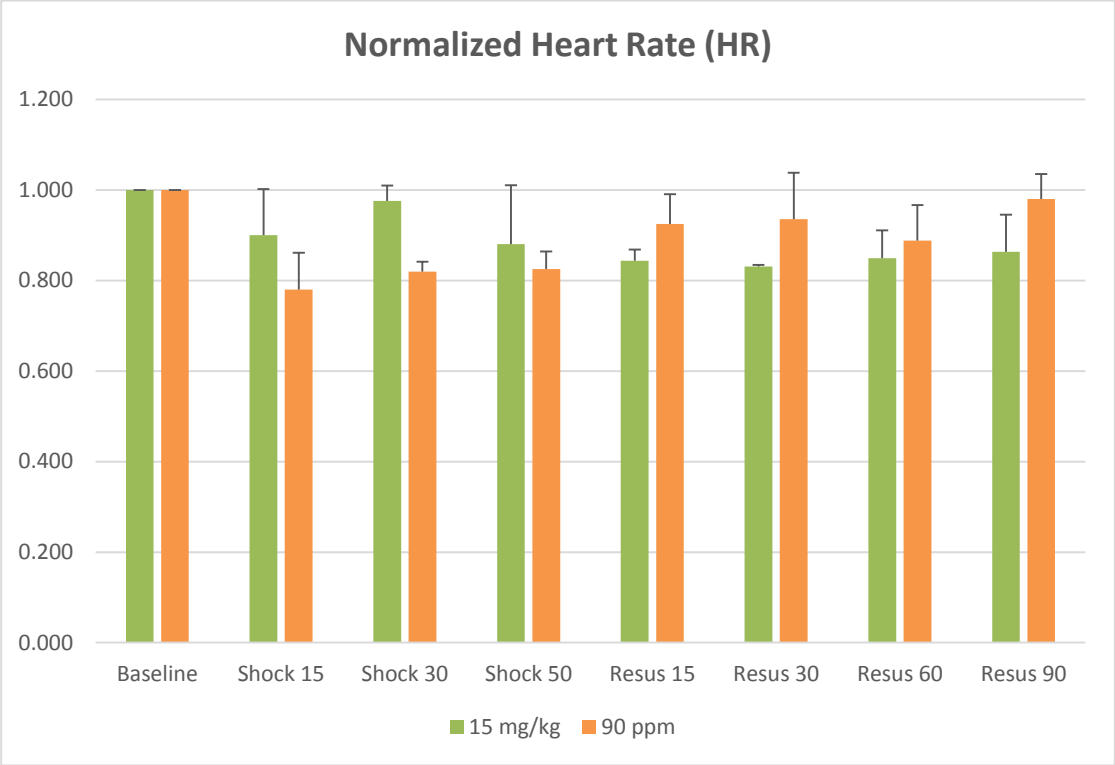


The previously mentioned trends for MAP can be visualized in the graph above (Graph 15). With 50% fluid volume hemorrhage, blood pressure is observed to drop to 40-50% of baseline levels and upon resuscitation, there is an immediate spike due to the increased fluid volume, in some cases to 70% of baseline MAP. Gaseous delivery of NO is generally not as effective, by roughly a factor of 5%, in restoring MAP post-

hemorrhage when compared to the nanoparticle delivery method. More data will be needed to increase statistical power.

The other main systemic parameter charted over time was HR. At baseline, hamsters used in this experiment have a HR in the range of approximately 410-450 bpm. It was observed that there was a small drop during shock to approximately 350 bpm where it would only increase slightly upon resuscitation. Overall, as can be seen in the chart below (Graph 16), NO nanoparticle supplemented resuscitation fluid does not appear to have a significant effect on restoring the HR to pre-shock values. Interestingly, gaseous NO has an increased effect over the nanoparticles in restoring HR post-HS by a factor of approximately 10%.

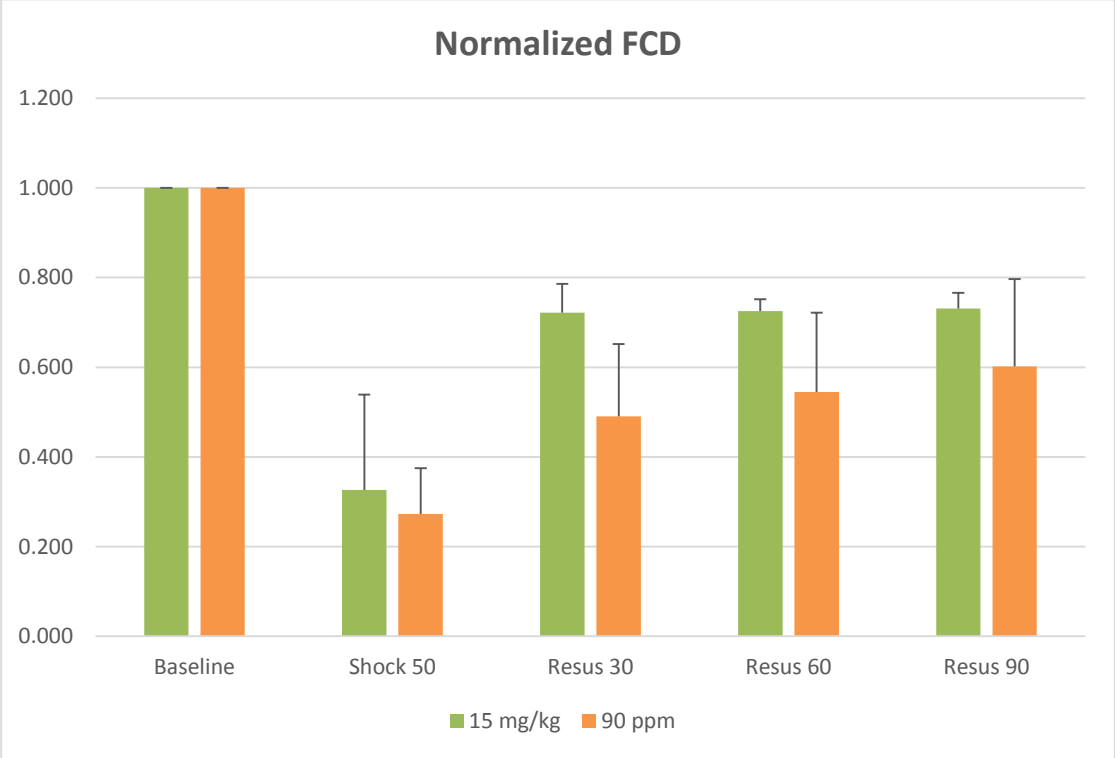
Graph 16: Normalized Heart Rate for 15 mg/kg Nanoparticle Concentration vs 90 ppm Gaseous Inhalation. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. Changes not significant.



3.4.2: Functional Capillary Density

One of the primary measurements of vascular perfusion is functional capillary density (FCD). FCD is severely affected in conditions of HS due to the drastically decreased available circulating fluid volume. Blood is also rerouted to more essential organ systems resulting in a shutdown of smaller capillaries, especially in peripheral tissues. This is distinctly observed in our experimental data. FCD is severely reduced from baseline and in some cases, functional capillaries are not seen at all in the chosen fields of view where an intricate network with blood flow was observed before shock. In the chart below (Graph 17), it can be seen that the resuscitation procedure has a very significant beneficial effect to restoring the FCD in the periphery ($P<0.001$).

Graph 17: Normalized Functional Capillary Density for 15 mg/kg Nanoparticle Concentration vs 90 ppm Gaseous Inhalation. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. Recovery of FCD was significant at these timepoints P<0.001.



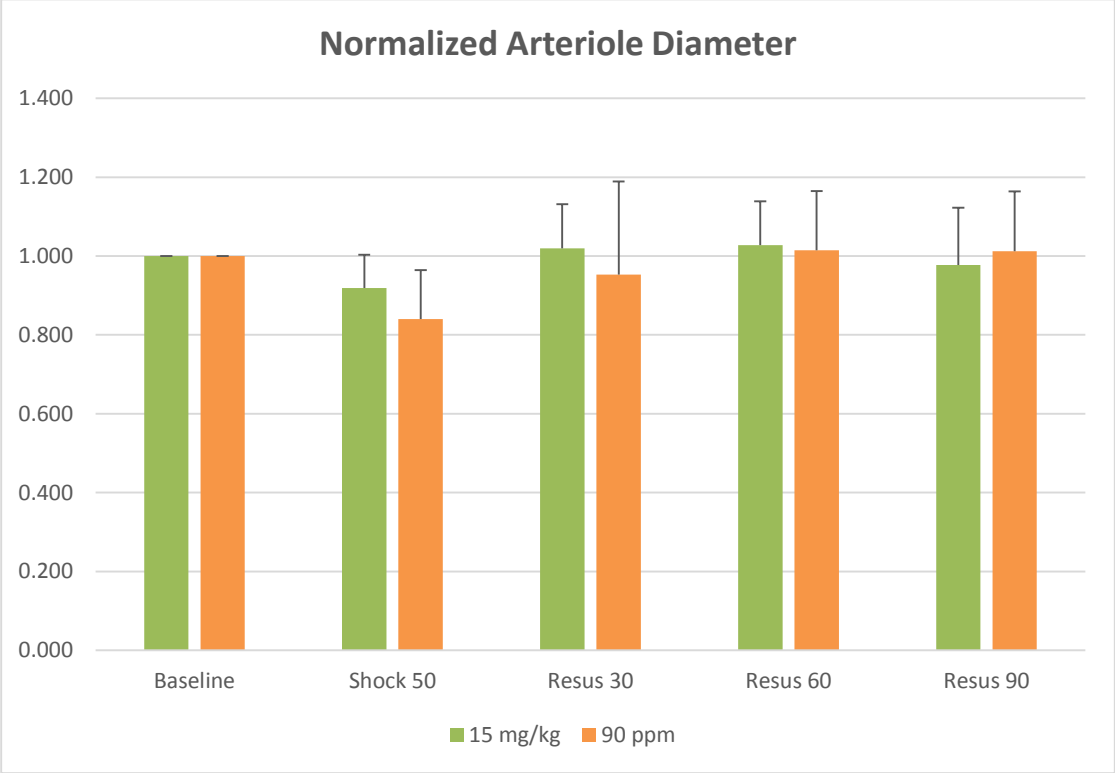
From our data, FCD is typically restored to about 50-70% of baseline values within 30 minutes after resuscitation. It was observed that FCD recovery is relatively maintained as time passes and even at 90 minutes after resuscitation it doesn't taper off (or in the case of gaseous inhalation, FCD recovery improves). Despite the positive trend over time, gaseous delivery of NO in conjunction with resuscitation fluid does not appear to have as strong of an effect on FCD relative to nanoparticles by a factor of roughly 10% relative to baseline.

3.4.3: Arteriole/Venule Diameter

NO's function as a vasodilator is well accepted. It is also understood that during HS, the body's network of arteries begin to constrict in an attempt to maintain fluid

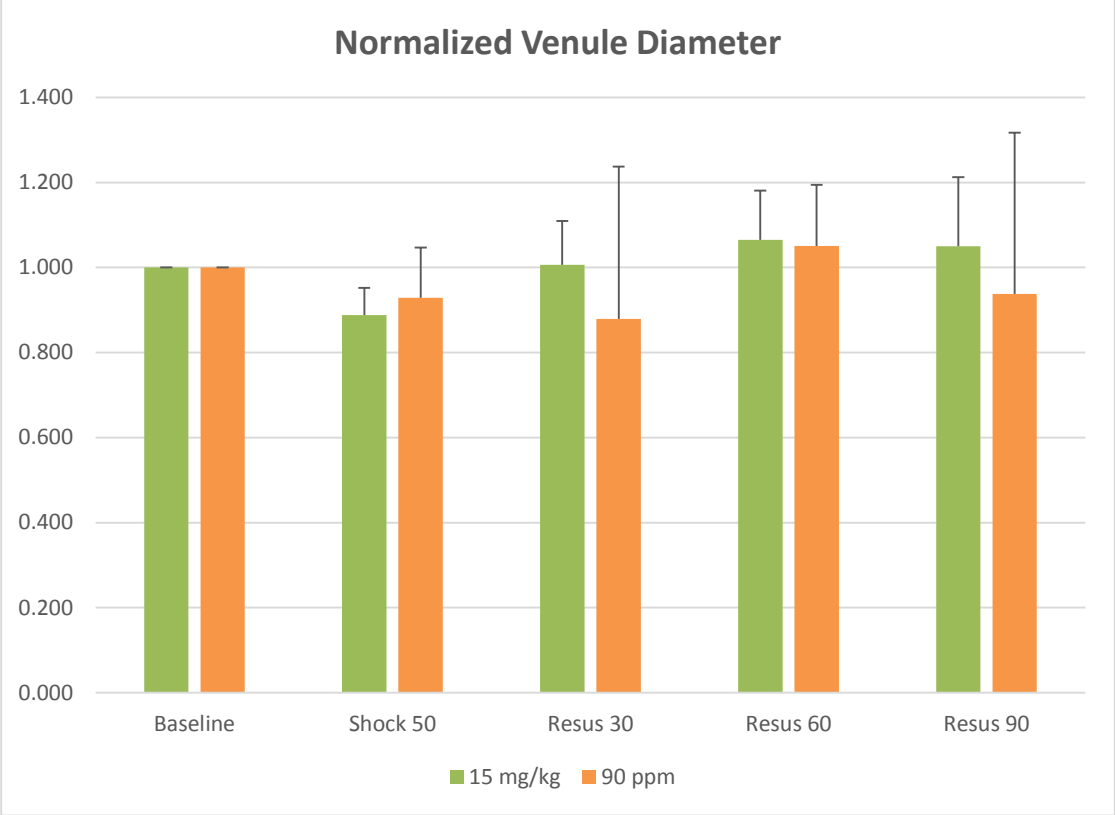
pressure in conditions of reduced fluid volume. In our experiments, both of these points were observed. Arteriole diameter is reduced to in response to the shock state and, recovers, albeit to a small degree, upon resuscitation with NO supplemented fluid. The chart below (Graph 18) portrays the data obtained normalized to baseline arteriole diameter values. In shock, arterioles constrict to about 85-90% of their original diameter and upon resuscitation, the diameters are typically restored to values of 95% and greater with respect to baseline values ($P<0.05$). Overall, this shows that NO at a dose of 15 mg/kg is effective at reversing the constriction induced by HS, particularly in the arterioles. Gaseous delivery appears to have a delayed effect but at longer periods of time is also as successful as nanoparticle delivery in recovering arteriole diameter as a statistical difference was not found between the two treatments.

Graph 18: Normalized Arteriole Diameter for 15 mg/kg Nanoparticle Concentration vs 90 ppm Gaseous Inhalation. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. Recovery of arteriole diameter was significant across these timepoints $P<0.05$.



It is known that venules are not really affected and do not constrict during HS. This can be seen with our data on venule diameters in the graph below (Graph 19). Slight decreases in diameter were observed but not statistically significant. However, as NO still functions as a vasodilator irrespective of the lack of constriction, venules diameters increased beyond baseline values when the NO supplemented resuscitation fluid was infused. This resulted in increases of 5-10% in venule diameter beyond baseline values. Gaseous NO and the nanoparticle encapsulated NO performs equivalently (no statistical difference found).

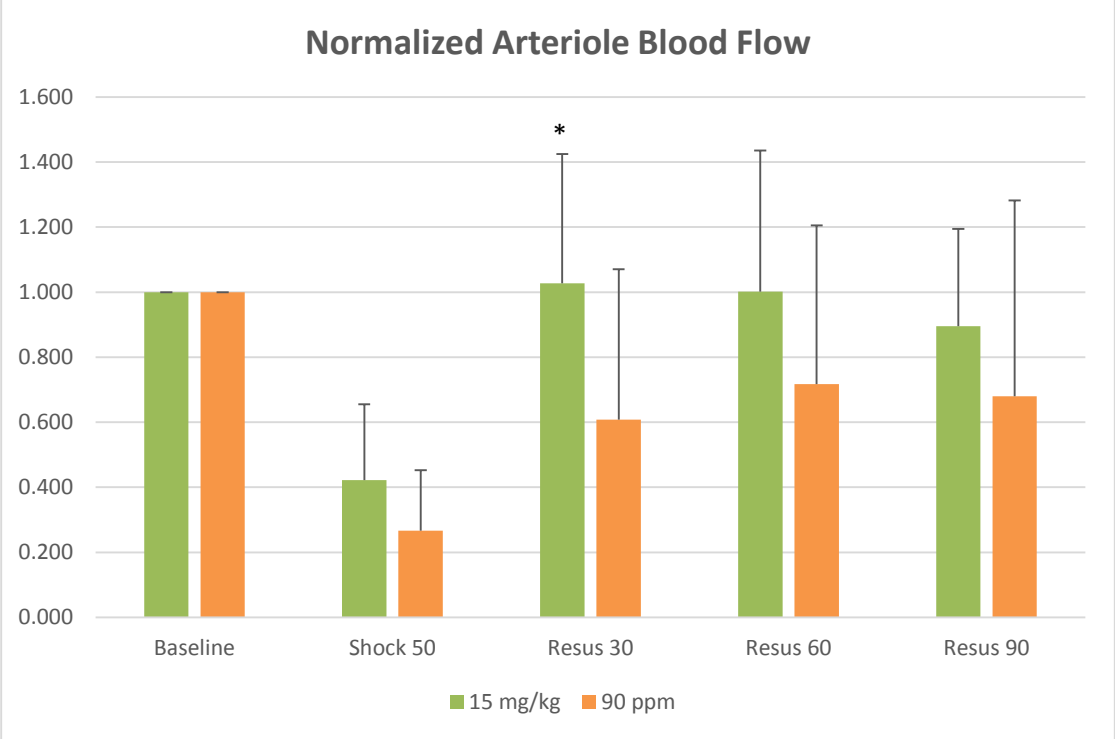
Graph 19: Normalized Venule Diameter for 15 mg/kg Nanoparticle Concentration vs 90 ppm Gaseous Inhalation. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. Changes not significant.



3.4.4: Arteriole/Venule Blood Flow

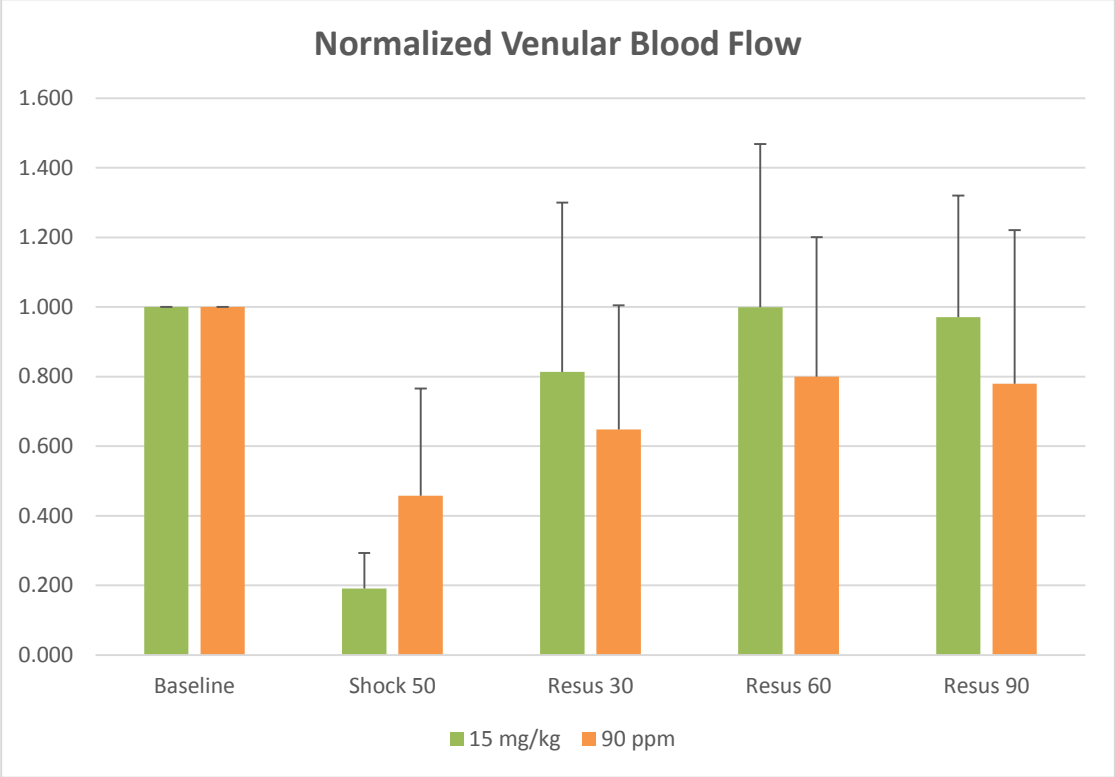
Although diameters of arterioles and venules may only change by 5-10% relative to baseline, if at all, fluid flow within the arterioles and venules is severely affected. It was observed during the experiments that some arterioles and venules would shut down completely resulting in zero observable, and measurable, fluid flow particularly in arterioles and venules with smaller baseline diameter values. Flows in larger arterioles and veins would be severely compromised. These observations can be visualized in the bar graphs presented below (Graph 20).

Graph 20: Normalized Arteriole Blood Flow Velocity for 15 mg/kg Nanoparticle Concentration vs 90 ppm Gaseous Inhalation. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. Difference between treatments was significant across these time points P<0.01. Arteriole blood flow recovery was significant at these time points P<0.001. *P<0.05 for 15 mg/kg compared to 90 ppm.



During HS, blood flow velocity in the arterioles becomes drastically decreased. Generally flow values ranged from 0-40% of baseline as a function of observed baseline diameter. Smaller diameters resulted in greater flow reductions and this can be correlated to why FCD measurements show significant reduction. Recovery of blood flow through the use of NO supplementation is significant (P<0.001). Gaseous delivery of NO is not as effective, by as much as 20-40% relative to baseline, as the optimal nanoparticle concentration for restoring and preserving arteriole blood flow velocity. A similar trend can be seen in the venules as well although the difference between gaseous and nanoparticle NO delivery is less pronounced.

Graph 21: Normalized Venular Blood Flow Velocity for 15 mg/kg Nanoparticle Concentration vs 90 ppm Gaseous Inhalation. Statistical Analysis was performed on timepoints Shock 50, Resus 30 and Resus 90. Recovery of venular flow was significant at these timepoints P<0.001.

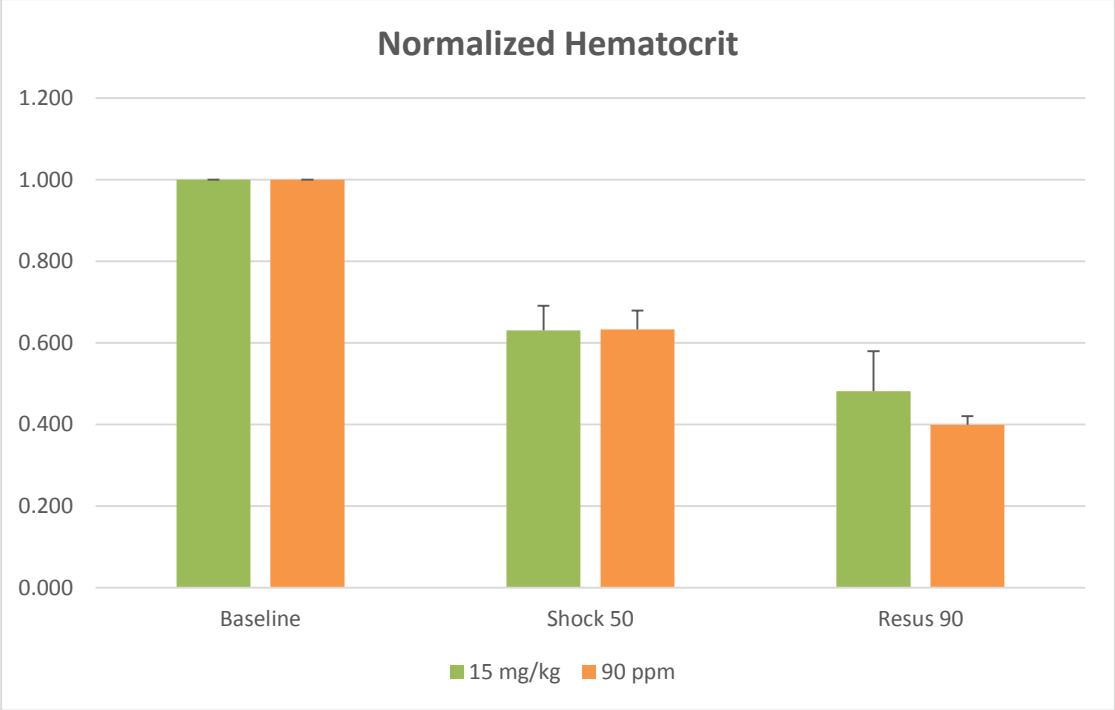


3.4.5: Hematocrit and [Hb]

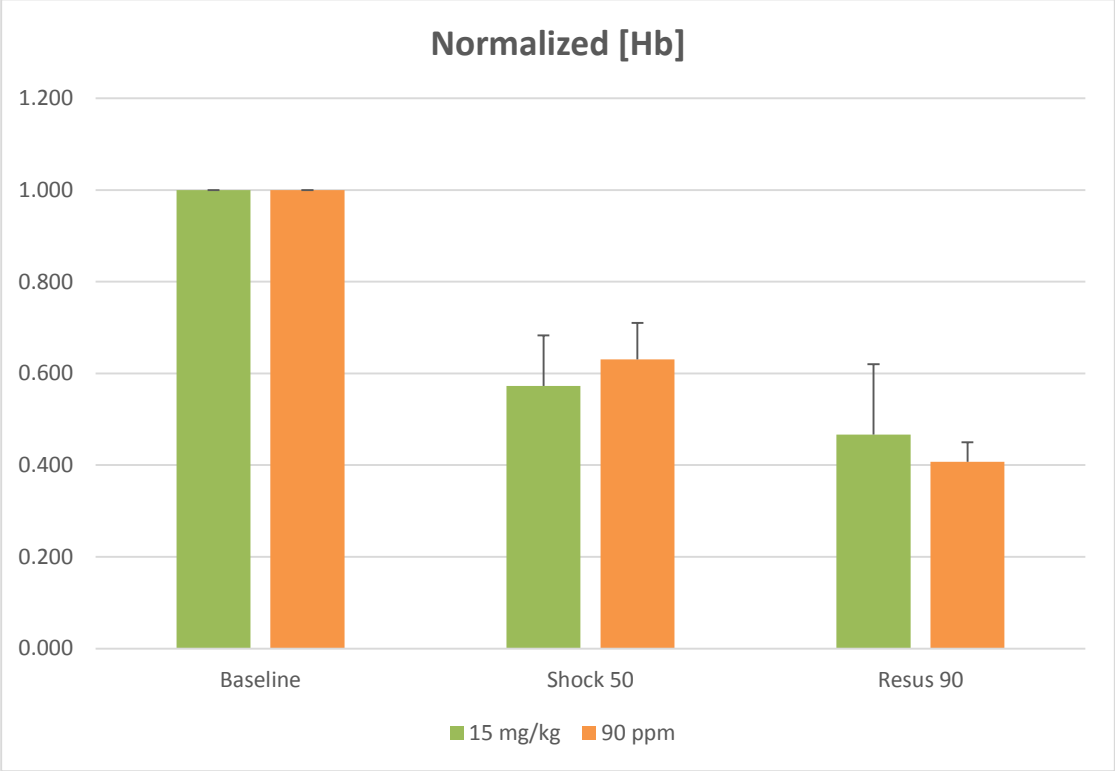
Hematocrit and [Hb] are common measurements used as an indicator of the presence and severity of HS. They are essentially indirect measurements of red blood cell quantity in the circulating blood stream relative to other circulating fluids and matter such as blood plasma. In states of HS, hematocrit and concentration of hemoglobin are reduced from baseline by a function of shock severity. It can be seen from the graphs below (Graph 22, Graph 23) that, in our 50% HS experiments, both hematocrit and [Hb] are reduced to 60-65% of baseline values. This translates to a real value drop from approximately 50% hematocrit and 15.0 mmol/L hemoglobin concentration standard to

roughly 30% hematocrit and 9.0 mmol/L hemoglobin concentration. However, these parameters are not a target in this experiment as NO has relatively no effect on hematocrit and hemoglobin levels in the bloodstream on its own. However, since a fluid is infused in resuscitation, a drop in hematocrit and hemoglobin concentration can be observed due to further hemodilution of the circulating blood fluid.

Graph 22: Normalized Hematocrit for 15 mg/kg Nanoparticle Concentration vs 90 ppm Gaseous Inhalation. Statistical Analysis was performed on timepoints Shock 50 and Resus 90. Decrease of hematocrit was significant across these timepoints P<0.001.



Graph 23: Normalized Hemoglobin Concentration for 15 mg/kg Nanoparticle Concentration vs 90 ppm Gaseous Inhalation. Statistical Analysis was performed on timepoints Shock 50 and Resus 90. Decrease of hemoglobin concentration was significant across these timepoints $P<0.01$.



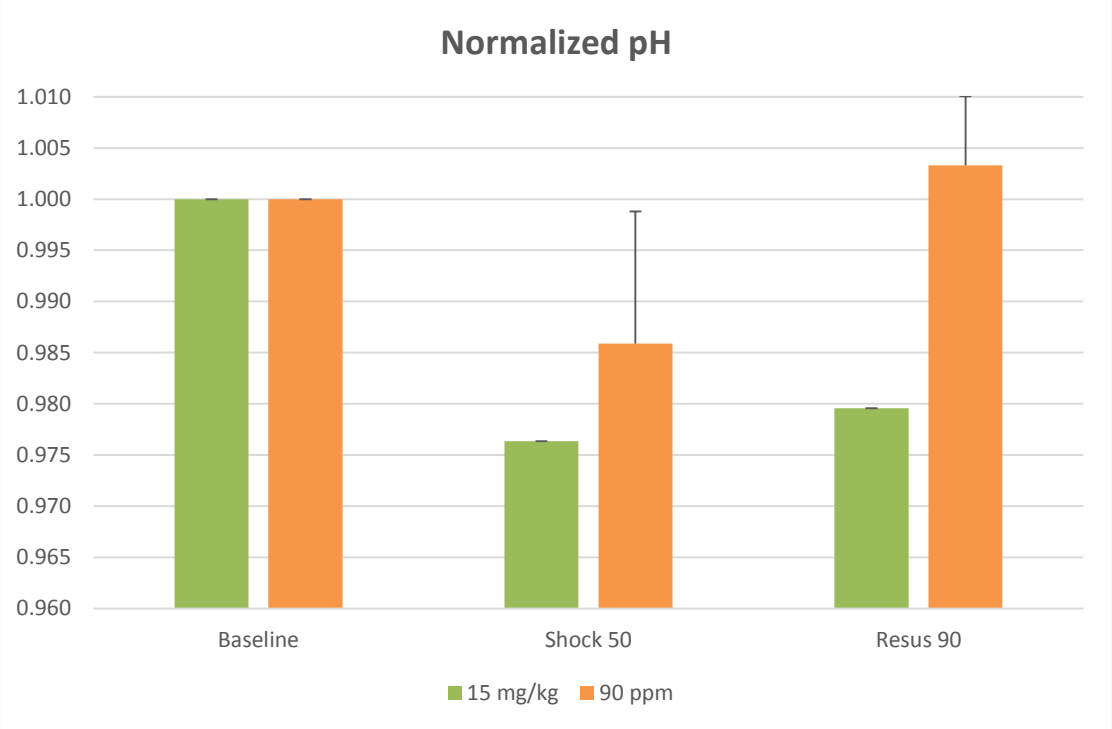
As expected, there is no significant variation between the nanoparticles and gaseous delivery method as well as no improvement in hematocrit and hemoglobin concentration levels. It is also as expected in that the normalized hematocrit and hemoglobin levels fall to the same degree indicating that the measurements are essentially equivalent and data output of the experiment is consistent.

3.4.6: Blood Gas Parameters (pH, pO_2, pCO_2, BE)

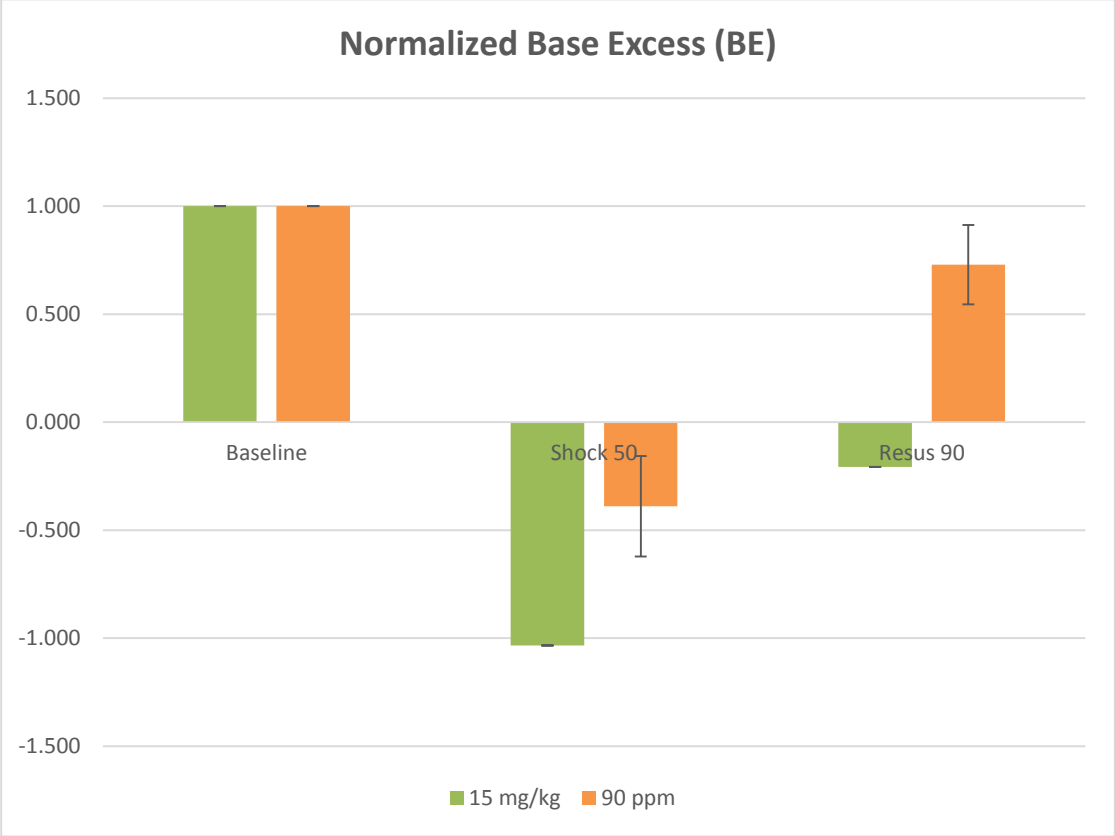
It can be seen that pH drops when HS conditions are imposed. From our data this drop translated to a drop range of 0-2.5% relative to baseline. This is because of inadequate oxygen supply to tissues resulting from decreased vascular perfusion.

Tissues switch to anaerobic metabolism to maintain energy upkeep but acids are produced as a byproduct. Upon restoration of vascular perfusion from resuscitation, pH is rapidly restored. Although only 90 minute post-resuscitation data was collected, it can be seen that pH recovery to baseline is complete or near complete (within 0.5%) with some cases becoming more basic than at baseline (Graph 24). BE is an analogous measure as it measures the level of acidosis occurring (Graph 25). Currently, due to insufficient data, gaseous delivery appears to have a greater recovery than nanoparticle delivery but more experiments are necessary to confirm.

Graph 24: Normalized pH for 15 mg/kg Nanoparticle Concentration vs 90 ppm Gaseous Inhalation. Statistical Analysis was performed on timepoints Shock 50 and Resus 90. Changes not significant.

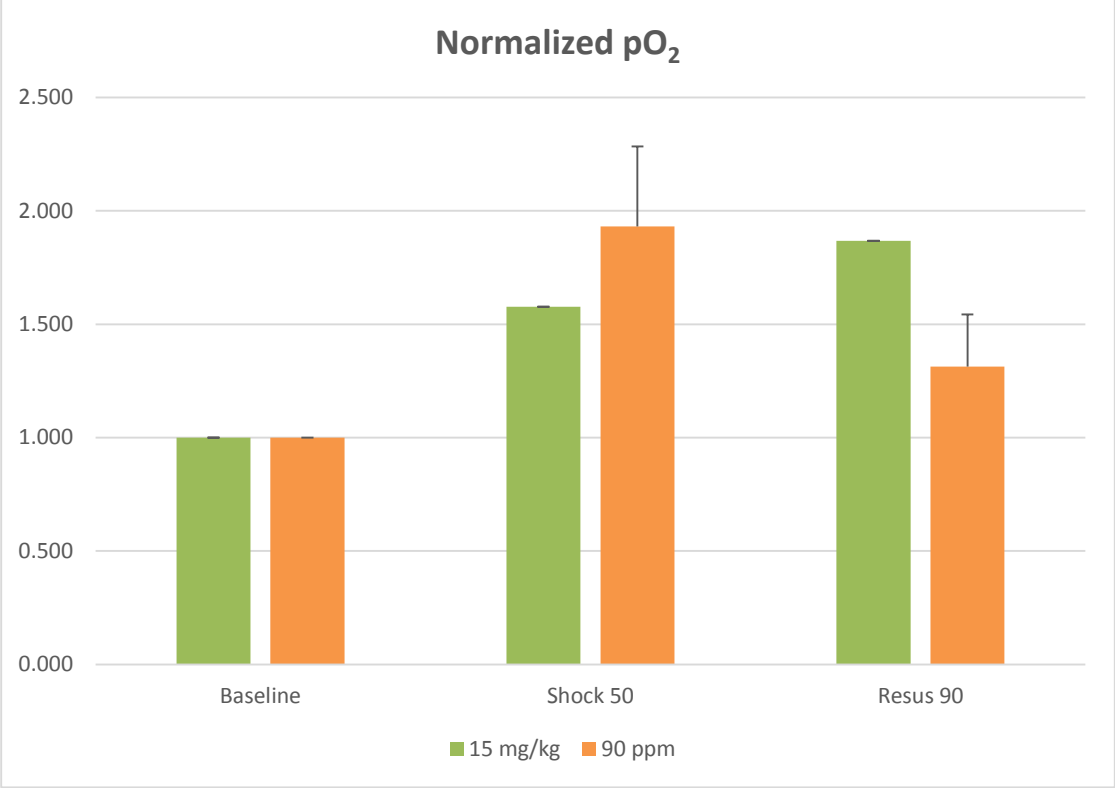


Graph 25: Normalized Base Excess for 15 mg/kg Nanoparticle Concentration vs 90 ppm Gaseous Inhalation. Statistical Analysis was performed on timepoints Shock 50 and Resus 90. Differences between treatment groups were significant across these timepoints $P<0.05$. Increase in BE was significant at these time points $P<0.05$.



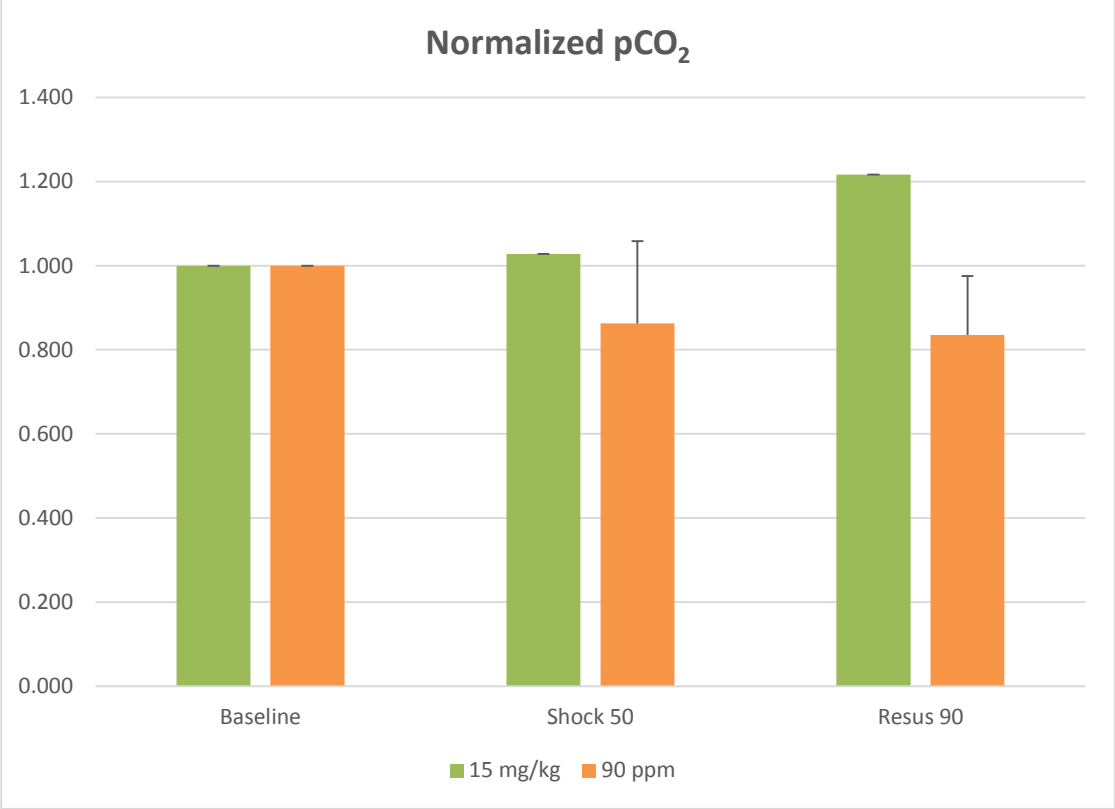
pO_2 is another blood gas parameter that is affected by HS. pO_2 is increased in periods of shock as the body attempts to compensate for lack of oxygen delivery to tissues by increasing respiration. This increase is respiratory rate results in a pO_2 increase of 50-100% of pO_2 in the bloodstream above baseline. However, this is unaffected by our nanoparticle NO treatment due to the fact that respiratory compensation extends beyond the 90 minute post-resuscitation observation period. In the case of gaseous delivery, pO_2 is lower as the composition of O_2 that the animal specimen breathes is decreased due to the presence of gaseous NO in the inhalation gas mixture.

Graph 26: Normalized pO_2 for 15 mg/kg Nanoparticle Concentration vs 90 ppm Gaseous Inhalation. Statistical Analysis was performed on timepoints Shock 50 and Resus 90. Changes not significant.



As the rate of respiration is increased (as seen by increased pO_2), correspondingly, pCO_2 would decrease slightly in the bloodstream. From the chart below (Graph 27), this decrease is approximately to 80-90% of baseline levels and, similar to pO_2 levels, maintains this state and is unaffected by the resuscitation phase of the experiment irrespective of treatment group. Again, nanoparticle delivery data is incomplete and more would need to confirm the upward trend observed.

Graph 27: Normalized pCO_2 for 15 mg/kg Nanoparticle Concentration vs 90 ppm Gaseous Inhalation. Statistical Analysis was performed on timepoints Shock 50 and Resus 90. Changes not significant.



3.5: Discussion

3.5.1: Systemic Parameters

Although a NO nanoparticle supplemented volume expander has relatively no effect on HR, its effect on MAP is profound. Our results show a 10% recovery relative to baseline at a time period of 90 minutes after resuscitation. This is a significant improvement particularly over gaseous inhalation of NO which only produced a roughly 5% recovery relative to baseline. Complete recovery of baseline MAP values were not achieved, nonetheless, the improvement is significant and, as previously stated in Chapter 1, permissive hypotension is deemed favorable to patient outcome.

3.5.2: Microcirculatory Parameters

Improvements in vascular perfusion can be characterized by observing fluid flow and vessel diameters of the microcirculatory system. In the case of functional capillary density (FCD), our data shows that there is roughly a 40% improvement relative to baseline within the first 30 minutes after resuscitation with NO and a volume expander. Unlike MAP which slowly decreases over time after resuscitation, FCD recovery is maintained. It was observed that in some cases, FCD would improve completely back to baseline for some fields of view. Gaseous NO performs at a relatively equivalent level to the nanoparticles with regards to this parameter. It only falls behind at a maximum of less than 10%, relative to baseline, compared to the optimum concentration of nanoparticles at 90 minutes post-resuscitation. FCD recovery is especially important as it is indicative of increased vascular perfusion and, consequently, oxygen delivery to peripheral cells and tissues. As previously stated, acidosis would be reduced and the risk of multi-organ failure arising in the body is alleviated. This, of course, results in better overall patient outcome.

Taking a step back to view larger vasculature, it can be seen that it is not as clear cut as capillaries which are either flowing or non-flowing. It is observed that flow rates are significantly impaired in both arterioles and venules during a state of HS, however, often times it would not completely stop. The parameter of flow velocity within arterioles and venules also demonstrates the effectiveness of NO as a mitigator of HS symptoms. It can be seen from our data that the use of the NO nanoparticles at 15 mg/kg facilitates a near complete recovery of flow velocity in both venules and arterioles.

However, due to the great variation in our data, more experiments would need to be performed to obtain more statistically significant values. A firm conclusion can also not be drawn at this point as to whether there is an advantage to using the NO nanoparticles over traditional gaseous delivery, however, it can be seen that it at least is equivalent in performance for all parameters measured in this thesis.

As previously stated in Chapter 1, venule diameter is generally not affected very much by a state of HS and an exogenous NO supplementation results in diameters that go beyond baseline values as seen in our data. This shows the potent non-selective nature of NO as a molecule and can be seen as an example of why dosing would need to be optimized for therapeutic use. On the other hand, NO is very effective for restoring arteriole diameters that have been constricted as a result of HS. Indeed our data shows that arteriole diameters are nearly restored to baseline values at 90 minutes post-resuscitation particularly at a concentration of 15 mg/kg of NO nanoparticles. As generally is the case, gaseous delivery of NO appears to have an equivalent effect to the optimal nanoparticle concentrations, resulting in near complete recovery at 90 minutes.

IV. Final Words & Future Directions

The primary objective of this thesis was to prove that hydrogel/glass NO nanoparticles improve conditions overall and provides a better outcome than traditional gaseous delivery for HS patients. We were also looking to discover the optimal dosing of this new system of NO delivery. This study found that NO nanoparticles are at least equivalent to gaseous delivery in performance (and exceeds gaseous delivery with regards to blood flow rates) in assisting patient recovery from HS when coupled with an accepted volume expander solution. This conclusion is supported by assessment of multiple parameters both systemic and otherwise. Such parameters include MAP, HR, FCD, arterial/venule diameter/blood flow, and various blood gas parameters. In addition, the resulting data also allude to the fact that the optimal dose of the NO nanoparticles is 15 mg/kg in a volume expander solution for severe HS patients. However, additional experiments will be necessary to confirm this dosing.

One of the main overarching questions is whether the nanoparticles present a better alternative to gaseous delivery for the therapeutic treatment of HS in real world situations beyond the controlled settings in the laboratory. As previously stated, the combined data presented in this thesis suggests only minor gains, if any at all, for moving from gaseous delivery to nanoparticles. These gains are particularly pronounced when considering the recovery of the microcirculation. However, nanoparticles present in a clear advantage when non-patient outcome parameters are considered such as ease of storage, ease of deployment, and overall cost. Large gas tanks of NO can be prohibitively expensive and difficult to provide to patients in pre-hospital settings. In

addition, they take up significantly more space than a small container of dry powder form nanoparticles. Another advantage that the nanoparticle provides is the ability to chemically control release rates of NO. Although more research would be needed to take advantage of this last fact, all things combined, NO nanoparticles represent an improved method of NO delivery over gaseous inhalation.

Throughout this thesis, it has been alluded that more experiments are necessary to reach more statistically significant conclusions both for dose optimization and for comparison of delivery methods. In addition to this, however, survival studies would need to be performed to test the long term effectiveness of NO in improving patient mortality. At the time of this writing, one experiment has been performed with a NO nanoparticle concentration of 20 mg/kg. Although observable animal physical condition was not optimal, the animal survived the full duration of the experiment of 8 days. Parameters measured at 8 days produced results that indicated near complete or complete recovery. Again, more data would be needed to draw conclusions on effectiveness of this treatment in the long term as well as to design and conduct studies at the optimal 15 mg/kg dosage. It would also be beneficial for more research to be conducted on optimizing the nanoparticle in terms of both release rates and possibly implementing intelligent targeting by conjugating antigens/antibodies to the particle surface.

Appendix

Baseline Value Tables

Table 1: Mean Arterial Pressure, Heart Rate, and Functional Capillary Density Baseline Ranges; n-value in parenthesis.

	1 mg/kg	10 mg/kg	15 mg/kg	20 mg/kg	40 mg/kg	90 ppm
MAP (mmHg)	109 +/- 11 (2)	118 +/- 13 (4)	130 +/- 7 (2)	116 +/- 13 (8)	116 +/- 10 (5)	115 +/- 7 (7)
HR (bpm)	430 +/- 8 (2)	422 +/- 25 (4)	426 +/- 8 (2)	431 +/- 39 (8)	422 +/- 24 (5)	439 +/- 21 (7)
FCD (per animal)	83 +/- 11 (2)	70 +/- 11 (4)	60 +/- 37 (2)	78 +/- 16 (8)	91 +/- 28 (5)	88 +/- 25 (7)

Table 2: Arteriole/Venule & Diameter/Blood Flow Baseline Ranges; n-value in parenthesis.

	1 mg/kg	10 mg/kg	15 mg/kg	20 mg/kg	40 mg/kg	90 ppm
A-Diameter (µm)	69.3 +/- 19.2 (10)	51.5 +/- 26.5 (20)	60.6 +/- 14.8 (10)	42.4 +/- 22.7 (39)	53.8 +/- 28.7 (25)	59.3 +/- 17.4 (33)
A-Flow (nL/s)	16.7 +/- 10.0 (10)	7.5 +/- 9.7 (19)	4.4 +/- 2.9 (10)	7.8 +/- 7.1 (35)	9.3 +/- 10.6 (24)	5.4 +/- 5.6 (30)
V-Diameter (µm)	67.2 +/- 26.5 (10)	50.6 +/- 30.5 (20)	59.1 +/- 17.1 (10)	40.9 +/- 20.8 (40)	43.0 +/- 22.1 (25)	70.2 +/- 27.3 (33)
V-Flow (nL/s)	4.0+/-2.6 (9)	2.6 +/- 2.5 (17)	1.3 +/- 1.1 (10)	2.0 +/- 2.5 (24)	2.0 +/- 2.1 (20)	2.4 +/- 2.6 (21)

Table 3: pH, pO_2, pCO_2, BE Baseline Ranges; n-value in parenthesis.

	1 mg/kg	10 mg/kg	15 mg/kg	20 mg/kg	40 mg/kg	90 ppm
pH	7.360 +/- 0.019 (2)	7.391 +/- 0.033 (4)	7.442 +/- 0.000* (1)	7.374 +/- 0.034 (8)	7.368 +/- 0.028 (4)	7.388 +/- 0.040 (5)
pO_2 (mmHg)	57.1 +/- 2.3 (2)	58.9 +/- 5.8 (4)	59.9 +/- 0.0* (1)	58.4 +/- 9.0 (8)	53.0 +/- 9.6 (4)	58.3 +/- 6.1 (5)
pCO_2 (mmHg)	58.1 +/- 6.9 (2)	52.7 +/- 4.7 (4)	46.2 +/- 0.0* (1)	53.2 +/- 5.2 (6)	54.8 +/- 3.0 (3)	52.3 +/- 4.3 (5)
BE (mmol/L)	4.7 +/- 1.4 (2)	4.9 +/- 3.1 (2)	5.8 +/- 0.0* (1)	3.8 +/- 2.1 (8)	4.0 +/- 2.0 (1)	6.1 +/- 1.2 (2)

Table 4: Hematocrit and Hemoglobin Concentration Baseline Ranges; n-value in parenthesis.

	1 mg/kg	10 mg/kg	15 mg/kg	20 mg/kg	40 mg/kg	90 ppm
Hct (%)	46 +/- 4 (2)	50 +/- 2 (5)	50 +/- 1 (2)	47 +/- 5 (8)	47 +/- 4 (3)	50 +/- 2 (7)
[Hb] (HB/uL)	14.3 +/- 0.7 (2)	15.0 +/- 0.8 (5)	16.2 +/- 2.1 (2)	14.0 +/- 1.1 (8)	15.0 +/- 0.9 (4)	15.6 +/- 0.6 (7)

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