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Effects of Hemoglobin-Based Oxygen Carriers on the Vasoactivity of the Spinotrapezius Muscle of the Rat

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EFFECTS OF HEMOGLOBIN-BASED OXYGEN CARRIERS ON
VASOACTIVITY IN THE
SPINOTRAPEZIUS MUSCLE OF THE RAT

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science at the Medical College of Virginia Campus
Virginia Commonwealth University

By

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B.S. and B.A., University of Virginia 2006

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ABSTRACT

EFFECTS OF HEMOGLOBIN-BASED OXYGEN CARRIERS ON VASOACTIVITY IN THE SPINOTRAPEZIUS MUSCLE OF THE RAT

By Pete Dennis Meliagros

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2008

Advisor: Roland N. Pittman, Ph.D.
Department of Physiology and Biophysics

Hemoglobin-based oxygen carriers (HBOCs) offer a safe, more plentiful and long term alternate to blood banks. However, they have been found to increase blood pressure which can be attributed to an increase in total peripheral resistance (TPR). Lumenal nitric oxide (NO) scavenging by these HBOCs seems to be responsible for this hypertensive effect. In addition, it is believed that hemoglobin (Hb) tetramers and dimers may extravasate and consume additional nitric oxide in the perivascular and interstitial space. The purpose of the present study was to elucidate the role of NO scavenging and to confirm extravasation as a contributor to HBOC vasopressor effects in the spinotrapezius muscle. The present study investigated the vessel reactivity and mean arterial pressure response to three HBOCs: HBOC 201, HBOC 205 MW 400, and HBOC 205 MW 600. These varied in molecular weight (MW) and percentage of tetramers and dimers. It was found that larger polymers of HBOC showed no significant decrease in vasoactivity. Although larger polymers are less likely to extravasate, the remaining tetramers and dimers seem sufficient to contribute to the observed vasoactivity. Using NaNO₂, a NO donor, in conjunction with the HBOCs almost completely abolished this
hypertensive effect at higher concentrations. Further examination utilizing a nitric oxide synthase (NOS) inhibitor to mimic the HBOC vasopressor effects demonstrated that lower concentrations of NaNO₂ were able to abolish the hypertensive effect. *In vitro* studies only further supported these results by demonstrating that NO consumption increases with HBOC dose. HBOC labeled with TRITC showed conclusive evidence that extravasation also plays a role in NO scavenging, even when minimal amounts of tetramers and dimers are present. In conclusion, the present study offers strong evidence that NO scavenging is responsible for the observed vasopressor effects. It also offers evidence supporting the theory that HBOC extravasation may be contributing to these vasopressor effects as well.
INTRODUCTION

The cardiovascular system is responsible for maintaining several homeostatic functions. In addition to being a component of arterial blood pressure and body temperature, it also delivers regulatory hormones and oxygen throughout the body. The cardiovascular system consists of a branching system of blood vessels that allow adjustments to altered physiologic states such as hemorrhage and exercise.

Blood is the medium of the cardiovascular system that carries these homeostatic factors in blood vessels throughout the body. Blood consists of four components: plasma, erythrocytes, leukocytes, and thrombocytes. Blood consists of approximately 55% plasma by volume, which functions to suspend the erythrocytes and leukocytes. The erythrocytes, or red blood cells (RBC), consist of approximately 40-45% of the blood volume and function to carry oxygen and nutrients to tissues and carbon dioxide and waste products away from tissues. The leukocytes, or white blood cells, consist of less than 0.1% of the blood volume and play a role in the immune system. Thrombocytes, or platelets, consist of less than 0.1% of the blood volume and are involved in blood clotting.

Oxygen delivery to tissues is important because it enables cells to produce energy more efficiently by means of oxidative phosphorylation. Oxygen transport predominately occurs in the microcirculation. The microcirculation begins with the arterioles which have a thin sleeve of smooth muscle to regulate blood flow. The arterioles give rise to multiple capillaries that have thin walls which allow simple diffusion of small solutes and gases. The capillaries then converge into venules. The increase in oxygen consumption in the microcirculation, predominantly in the capillaries,
is due to the lower velocity of blood and the larger surface area-to-volume ratio found in these smaller vessels.

In pathological states that exhibit hemorrhage, the traditional treatments involve resuscitation with colloid or crystalloid solutions. Although these solutions are effective and highly available, they are not ideal for resuscitation. These solutions replenish the blood volume and therefore increase cardiac output, but are not sufficient to oxygenate tissues under severe blood loss. Extended use of these solutions may result in severe tissue decay. Blood is currently the ideal resuscitation fluid, but it has many disadvantages. These include the cost of acquiring and screening the blood, its short storage life of 42 days, and the possibility of transmitting diseases. Perfluorocarbons (PFCs) and hemoglobin-based oxygen carriers (HBOCs) are two types of blood substitutes that are currently being tested as possible alternative means of resuscitation. PFCs are synthetic molecules in which a linear or cyclic carbon backbone is substituted with fluorine. PFCs are emulsified in a particular solution (58% perfluoroctyl bromide and 2% perfluorodecyl bromide) that improves its flow in the circulation. PFCs deliver oxygen and carbon dioxide; however, they exhibit a linear relationship between oxygen carrying capacity and partial pressure of oxygen. Therefore, they require high inspiratory oxygen concentrations to deliver sufficient oxygen to tissues. The present study focuses on the use of hemoglobin-based oxygen carrier as blood substitutes.

**HBOC Background**

With the limited blood supply and concerns regarding disease transmission, as well as transfusion reactions, there has been much progress investigating the use of HBOCs. These solutions offer universal compatibility, freedom of disease transmission,
and long storage life. HBOCs are derived from modified human or animal hemoglobin and from recombinant human hemoglobin (Pawson et al., 2007). Regardless of its efficacy and potential, certain undesirable side effects impede HBOC use clinically. HBOCs are known to increase mean arterial pressure (MAP) due to systemic vasoconstriction, cause some gastrointestinal discomfort and loss of motility, and induce kidney failure. Hypertension and gastrointestinal discomfort are assuredly due to the influence of HBOCs on smooth muscle tone. Kidney damage is caused by tetramer dissociation into dimers which cause oxidative stress (Chang et al., 2001). Structural modifications have been devised to reduce these effects and to prolong HBOC half-life. These modifications include covalent intramolecular links, cross-linked hemoglobin (Hb) linked with polyalkylene oxides, covalently linked macromolecules (such as dextran) to Hb, polymerization of Hb tetramers, or microencapsulated Hb solutions. Currently, HBOC use has yet to be approved clinically in humans because vasoconstriction and hypertension are severe side effects that persist.

**Theories of HBOC Side Effects**

There are two competing theories that purport to account for the observed vasoconstriction and vasopressor effects. One theory attributes the systemic vasoconstriction to changes in concentration of vasoactive molecules such as endothelins and NO. The second theory suggests that vasoconstriction is due to an overcompensating autoregulatory response to excessive O₂ delivery caused by the more efficient extracellular Hb molecules (Winslow, 2000).

An advantage of HBOCs is their ability to transport oxygen efficiently to tissues. Some believe that the low oxygen affinity of many HBOCs may in fact increase the
oxygen levels in regulatory arterioles and therefore induce an autoregulatory response in which vasoconstriction increases vascular resistance and reduces flow (Winslow, 2000). There has been much debate in the literature over this theory. Rolhf et al (Rolhf et al., 1998) conducted studies comparing cross-linked Hb tetramers that differed in oxygen affinity and cooperativity. They deduced a direct inverse correlation between oxygen affinity and vasopressor response. HBOCs that induced sustained MAP increases exhibited the lowest oxygen affinity whereas less vasoactive HBOCs exhibited the highest oxygen affinity (Rohlf et al., 1998). On the contrary, Olson et al. (Olson et al., 2003) also conducted studies using cross-linked tetramers that demonstrated that there is no significant decrease in vasoconstriction or MAP response when the oxygen affinity of HBOCs is increased. Instead, they found that there was a strong correlation between the rise in total peripheral resistance (TPR) and the rate of NO interaction with hemoglobin.

Endothelins are a family of peptides which comprises endothelin-1, endothelin-2, and endothelin-3. Endothelin-1 is a potent vasoconstrictor generated mainly in endothelial cells. Some of its hypertensive effects can be attributed to its direct influence on the renin-angiotensin-aldosterone system. ET-1 also interacts with two receptor types, the ET_A and ET_B. ET_A receptors are localized in the smooth muscle cells while ET_B receptors are located on endothelial cells and smooth muscle cells. Activation of ET_A receptors by ET-1 induces a slow and long acting vasoconstriction. ET-1 and ET-3 equally interact with ET_B receptors inducing a transient increase in NO production resulting in vasodilation (Agapitou and Haynes, 2002). Some studies demonstrated that ET_A receptor antagonists substantially inhibited the pressor activity elicited by diaspirin crosslinked Hb (Rioux et al., 1999, Gulati et al. 1994). However, some studies have
found conflicting results using endothelin converting enzyme inhibitors. These studies found no effect on the pressor effects elicited by diaspirin crosslinked Hb (Schultz et al., 1993, Gulati et al., 1995).

The most widely accepted explanation for vasoconstriction is NO scavenging. NO is a known EDRF (endothelial-derived relaxing factor) and therefore changes in NO have predictable effects on vascular smooth muscle tone. NO interacts with deoxy-Hb with picomolar affinity and reacts irreversibly with oxyhemoglobin to form nitrate (NO$_3^-$) and MetHb (Rohlfs et al., 1998). These reactions occur at bimolecular rate constants of 30 –50 µM$^{-1}$s$^{-1}$ at 37 °C (Eich et al., 1996). The rate of these reactions could account for the rapid rise in TPR. It has also been suggested that oxygen-linked S-nitrosylation reactions of Hb may also regulate vascular tone (Jia et al., 1996). This occurs by S-nitrosothiols interacting with the cysteine 93 residue in the Hb beta subunit. However, the rate of this reaction seems to be too slow to account for the observed immediate increases in MAP and vasoconstriction.

NO interactions with Hb would lead one to believe that red blood cells (RBCs) should be the ‘black holes’ of the vascular system. These interactions have been found to be diffusion limited in RBCs. Azarov et al. conducted studies that showed NO interacting with intact RBCs was slower by a factor of ~1,000 compared with free Hb (Azarov et al., 2005). Therefore, free Hb can scavenge NO and therefore greatly affect vascular tone. In addition, it is believed that a fraction of HBOCs may extravasate and scavenge NO in the perivascular space. Sampei et al. (Sampei et al., 2005) conducted studies comparing the effects of HBOCs on tight endothelial junctions found in cranial pial arterioles to the less permeable vessels found in skeletal and ventricular muscle.
They concluded that HBOCs do not scavenge sufficient NO in the plasma to significantly affect baseline tone in vascular beds with tight endothelial junctions, but do produce substantial constriction in vascular beds with porous endothelium (Sapmei et al., 2005). Other studies also support this theory. Sikai et al. (Sakai et al., 2000) found that larger polymer HBOCs resulted in less vasoconstriction and hypertension presumably due to less extravasation (Sakai et al., 2000).

Further studies focusing on different HBOCs have provided more evidence supporting the NO scavenging hypothesis. Pawson et al. (Pawson et al., 2007) demonstrated that the non-specific NO synthase inhibitor L-NAME induced similar vasoactivity to that of HBOC 201. When simultaneously administered, there was no further enhancement of constriction (Pawson et al., 2007). Many studies have used recombinant Hb that varies in P50 and NO reactivity to test the two competing theories of the HBOC-induced pressor effect. A correlation was observed between the rate of NO reactivity, but not P50 (Doherty et al., 1998, Olson et al., 2003). These data support the NO scavenging hypothesis.

There have been some studies that indicate that NO scavenging may not be responsible for the pressor effects. Fitzpatrick et al. collected arterial NO concentration samples that did not seem to significantly change with administration of HBOC 201 (Fitzpatrick et al., 2004). However, they also measured an increase in the metHb level which suggests that NO was interacting with HBOC 201. A non-significant decrease in NO levels was observed, which may be attributed to HBOC 201 interacting with NO with a compensatory increase in NO output. Recently, Tsai et al. (Tsai et al., 2006) utilized NO electrodes to measure NO directly in the perivascular space in response to a variety
of HBOCs. Their results indicate a decrease in NO in the perivascular space in response to all HBOCs, however, the largest MW HBOC tested did not induce vasoconstriction. They suggested that factors such as molecular dimension, retention time, and gas-carrying properties are more likely to regulate vascular tone (Tsai et al., 2006).

**Biochemistry of NO**

*Nitric Oxide Properties and Roles*

NO is a unique signaling molecule because of its various chemical properties. It is perhaps these chemical properties that allow it to have such diverse physiological functions in the human body. These functions may be attributed to its ability to act as either a Lewis acid or Lewis base. It can also act as either an oxidizing or reducing agent due to its intermediate oxidation state. Its short half-life of only a few seconds and its ability to freely diffuse through tissues also makes it an ideal messenger (Ignarro, 1990, Lane et al., 2002).

The physiological functions of NO have only recently been better defined. With respect to the present study, NO has been found to activate soluble guanylate cyclase by heme-dependent mechanisms. The NO-heme moiety is responsible for enzyme activation (Ignarro, 1990). This activation increases cGMP which in turn activates a kinase cascade that decreases intracellular Ca\(^{2+}\). This sequence attenuates contractile tone and contributes to vasodilation (Lane et al., 2002). NO has also been found to be important in immunosuppression. NO is released by macrophages and neutrophils when necessary. The rapid oxidation of NO to both nitrate and nitrite is associated with the killing of phagocytized microorganisms at higher concentrations of NO. Additionally, this local release of NO has been found to partake in an inflammatory response by
inhibiting platelet aggregation and improving local blood flow. The latter mechanisms are less relevant with respect to the present study, but show how the diverse chemical properties of this signaling molecule correspond to its diverse physiological functions.

*Nitric Oxide Synthesis and Regulation*

The enzyme that is responsible for the synthesis of NO is NO synthase (NOS). Three isoforms of NOS have been identified. nNOS (NOS1) and eNOS (NOS3) are Ca\(^{2+}\)-dependent forms whose expression can be either constitutive or regulated. nNOS is expressed in neurons and eNOS is expressed in endothelial cells, cardiac myocytes, and blood platelets. The third isoform is commonly known as iNOS (NOS2). Its expression is regulated by cytokines.

Co-translational modifications render eNOS membrane-bound unlike the cytoplasmic nNOS and iNOS isoforms. The localization of eNOS with the membrane of endothelial cells makes it a prime candidate for further review. eNOS activity is regulated at the transcriptional and post translational level. Transcription of eNOS has been found to be influenced by many factors including shear stress, sterol regulatory elements, estrogen-responsive elements, cAMP-responsive elements, erythropoietin, hypoxia, and NO itself. Post-translational regulation includes eNOS palmitoylation and serine phosphorylation. These regulate the stability, localization, and activity of eNOS. The Ca\(^{2+}\)-dependency of eNOS also permits many hormones to affect its activity such as bradykinin, VEGF, and histamine (Gosink et al., 1993, Papapetropoulos et al., 1997, Kelm et al., 1993).

The regulation of NOS is complex, while the production of NO is more straightforward. eNOS (and nNOS) contains binding sites for heme and calmodulin
which are both essential for enzyme activation (Govers et al., 2001). Upon Ca\(^{2+}\)-bound calmodulin interacting with eNOS, NADPH donates electrons at the reductase domain. These electrons are subsequently shuttled through the calmodulin-binding domain toward the heme-containing eNOS oxygenase domain, which results in the formation of citrulline and NO (Abu-Soud et al., 1993).

**Nitric Oxide Sources**

There are various known sources of NO that may contribute to vascular tone. The vascular endothelium is believed to be the predominant source of NO. Endothelial cells express basal levels of NO and therefore can help maintain homeostasis based upon the NOS regulation discussed previously. nNOS produces NO in neurons which have been found to innervate the vasculature to some degree in rats (Toda et al., 2003). Kavdia and Popel demonstrated that NO release by nNOS would significantly affect available smooth muscle NO (Kavdia and Popel, 2004). Myocytes contain eNOS which suggests they too may be involved in localized vascular reactivity, but there has yet to be any conclusive evidence to support this. There has been some debate over whether nitrites and S-nitrosothiols (SNO) also act as NO reserves. SNO plasma levels have been recorded in the 10 nM range (Cosby et al., 2003). SNO production has been determined to occur in albumin and Hb. In albumin, the copper and sometimes heme can facilitate S-nitrosylation by NO. Hb can react with NO and nitrite to produce SNO-Hb. Hb most likely out-competes albumin for NO, but this outcome is dependent on the rate and amount of NO that is made available. It has been found that at higher levels of NO, Hb breaks NO down into metHb and nitrate (Stamler, 2004). Plasma nitrite levels range from 150-1000 nM. This vast pool for NO has been proposed to be converted by either
enzymatic reduction or nonenzymatic disproportionation. Cosby et al. found that nitrite levels decrease upon NOS inhibition while others have also found that nitrite is consumed during exercise (Cosby et al., 2003). Only deoxyHb converts nitrite to NO (and met Hb). These observations seem to make nitrite an ideal source of NO in a hypoxic environment.

In summary, there are many NO sources available. Endothelial, neuronal, and muscular NO production engender paracrine and autocrine effects on the vasculature. The stability of SNO and nitrite allow NO to engender endocrine effects as well. The combined complexity that all these factors have on the vasculature is just beginning to be understood.

**Nitric Oxide Metabolism**

Endogenous NO has a biological half-life of 3-5 seconds. The majority of NO is oxidized to NO$_2^-$, A small portion is converted to NO$_3^-$. The metabolism of NO is also inversely related to superoxide anion concentration. *In vivo*, the superoxide anion concentrations are minimal due to the high activities of superoxide dismutase. NO may also react with Hb in RBCs to form MetHb and biologically inactive nitrate; however, this reaction is diffusion-limited (Buerk, 2007). Any free Hb from lysed RBCs is more accessible to degrade NO. The ability of NO to diffuse easily through tissues and its oxygen-catalyzed inactivation are primarily responsible for NO metabolism (Ignarro, 1990).

**Purpose of the Present Study**

The purpose of the present study was to investigate and compare the vasoactive properties of three different HBOCs: HBOC 201, HBOC 205 MW 400, and HBOC 205...
The vasoactive properties were based on observations of mean arterial pressure and vessel diameters in the spinotrapezius muscle of the rat. The present study also set out to elaborate and quantify the interactions of HBOCs with NO, as well as the degree of HBOC extravasation.
MATERIALS AND METHODS

Anesthesia and Animal Preparation

The experiments were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and were conducted on 10-wk-old 200-275 g male Sprague-Dawley rats (Harlan, Indianapolis, IN, N = 67). Rats were housed in a vivarium under controlled conditions (temperature 20-23 °C, humidity 40-60 %, 12/12 hour light/dark cycle). Free access to food and water were maintained under the supervision of a certified animal technician.

Prior to experimentation, rats were anesthetized with an intraperitoneal injection of a ketamine and acepromazine mixture (75 mg/kg of ketamine and 2.5 mg/kg of acepromazine, Henry Schein, Melville, NY). A continuous (0.254 ml/hr) intravenous infusion of Alfaxan (Alfaxalone 10 mg/ml, Abbeyvet Export LTD, North Yorkshire, England) via the left cannulated jugular vein was utilized to maintain anesthesia for the remainder of the experiment. The animal was placed on a homeothermic blanket set at 40 °C to maintain body temperature at 37 °C. Upon completion of the experiment, the animal was euthanized with Euthasol (pentobarbital 390 mg/ml and phenytoin 50 mg/ml, Delmarva Laboratories Inc., Midlothian, VA).

Surgical Procedures

A tracheostomy was utilized to insure a patent airway. A small midline incision was made in the anterior neck over the trachea. The subcutaneous tissue was dissected and the sternohyoid muscle was split longitudinally by blunt dissection to expose the trachea. The trachea was isolated, incised, and cannulated with PE 240 tubing. The skin incision was then extended inferiorly and to the left in order to expose the jugular vein as
it passed over the clavicle. The jugular vein was cannulated with PE 10 tubing for continuous (0.254 cc/hr) infusion of Alfaxan. Maintenance of a surgical plane of anesthesia was observed via absence of a toe pinch reflex and stable vital signs (mean arterial pressure and respiratory rate). The left carotid artery was then exposed in the crease between the sternohyoid muscle and the mastoid portion of the sternocephalic muscle. The left carotid artery was cannulated with PE 50 tubing to allow continuous MAP measurements and collection of arterial blood samples. The left femoral vein was cannulated with PE 10 tubing for infusion of experimental agents. Patency of vascular cannulae was maintained with either a heparin lock (20 units of heparin/ml of 0.9 % normal saline) or continuous infusion.

The left spinotrapezius muscle was utilized for microcirculatory observations using surgical preparations described by Gray (Gray, 1973). A midline incision was made on the dorsal side at the level of the lumbar vertebrae and extended superiorly to the scapula. The subcutaneous connective tissue was carefully dissected to avoid trauma and bleeding of the underlying spinotrapezius muscle. Bleeding was prevented or minimized by using a low-temperature cautery device (Gemini RS-300, Roboz Surgical Instrument CO., Rockville, MD). The muscle was kept moist with frequent applications of phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO; pH 7.4). The muscle was separated from the surrounding deeper tissues and sutured around the periphery with 5-0 silk fastened at ~ 1 cm intervals. These sutures allowed for the muscle to be surgically manipulated and firmly attached to the custom-made thermostable microscope platform for microscopic observation as described by Golub and Pittman (Golub and Pittman, 2003) and modified by Lowman (Lowman, 2004). This method of preparation
leaves the supplying nerve and vasculature intact. The muscle tissue was moistened with PBS and covered with plastic film (Saran, Dow Corning Midland, MI) to minimize desiccation and atmospheric gas exchange.

**Measurements**

Mean arterial blood pressure (MAP) was measured through the carotid artery catheter which was connected to a CyQ 103/301 display system (CyberSense Inc., Nicholasville, KY). The system gave a continuous reading of MAP, which was recorded every 5 minutes and averaged for all periods in the study.

Blood sampling (2 x 55 µl) was utilized to determine hematocrit, arterial blood gas measurements (ABL 705, Radiometer America Inc., Westlake, OH), and Hb/oxygenation status (OSM3 Hexoximeter, Radiometer, Copenhagen, DK).

Microscopic measurements were made using an Axioplan-2 microscope (Zeiss, Thornwood, NY) equipped with Achroplan objectives). The microscope was connected to a CCD camera (Cool Snap cf, Roper Scientific, Tucson, AZ) and a computer (Dell Dimension 8250, Dell), which was used for acquiring images of the microcirculation. Diameter measurements were made directly over the image generated. A 100-watt halogen lamp was used to transilluminate the muscle preparation. A 10X objective was used to obtain images for determination of the internal diameter of microvessels.

**Solutions**

*Pharmacological Agents*

Experiments were conducted to examine the vasoactivity of HBOC 201, HBOC 205 MW 400, and HBOC 205 MW 600 (kindly provided by Biopure Corp., Cambridge, MA). A plasma HBOC dose range of 0.01, 0.1, 1.0, 10, 100 µM was utilized to assess
vasoactivity. Doses were dependent on plasma volume. The physicochemical properties of Hb products were characterized by THb, oxyHb, MetHb, oxyHb, osmolality, MW, and oxygen affinity.

\[ \text{N}ω-\text{Nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma-Aldrich Corporation, St. Louis, MO) was utilized to mimic HBOC vasoactivity. A stock solution of 200 mg of L-NAME in 10 ml of 0.9 % normal saline was made. An intravenous bolus of 5 mg/kg was given to achieve the desired vasoactivity.} \]

A dose range of NaNO\(_2\) (10, 100, 1000, 3000 \(\mu\)M) was utilized to compensate for increased vasoconstriction and MAP (Sigma-Aldrich Corporation, St. Louis, MO). Doses were dependent on blood volume.

*Conjugated TRITC-HBOC Solution*

Tetramethyl-rhodamine-isothiocyanate (TRITC, Sigma-Aldrich Corporation, St. Louis, MO) was utilized to tag HBOC 201 and HBOC 205 MW 600. Two ml of HBOC were combined with 0.5 ml of concentrated carbonate-bicarbonate buffer (prepared according to directions; pH = 9.4, C-3041, Sigma-Aldrich, St. Louis, MO) and 5 mg TRITC (with 1 drop DMSO added, Research Organics Inc., Cleveland, OH). The solution was deoxygenated by bubbling with nitrogen gas. The solution was then sealed with Parafilm, covered with aluminum foil for light protection, and stirred for 2 hours.

The mixture was then dialyzed to remove excess unbound TRITC. The labeled HBOC was sealed in Spectra/Por 4 molecular porous membrane tubing (diameter 16 mm, Spectrum Laboratories, Rancho Dominguez, CA; MWCO = 12-14 kDa) and placed into a beaker containing 10.75 g Polyvinylpyrrolidone (PVP, Aldrich Chemical Co.,
Milwaukee, WI; MW = 55 kDa) dissolved in 125 ml PBS. The beaker containing the dialysis tubing was continuously stirred for 18 hours in the absence of light.

Subsequently, the mixture was transferred from the dialysis bag into a 15 ml cone tube and centrifuged at 4000 rpm for 4 minutes in a high speed microcentrifuge (Jouan, Winchester, VA). The clear supernatant was then transferred in 0.5 ml aliquots into Click-Snap PCR tubes and placed in a 2-5°C refrigerator for later use.

**Calculations**

Blood volume was determined as 6 % of the body weight (Costanzo, 2006). Plasma Volume was determined as blood volume times (1- Hct).

**Experimental Protocols**

*In Vivo*

Prior to experimental collections for all studies, blood gas measurements, Hb/oxygen saturation, and hematocrit were recorded via blood sampling. A 30-minute equilibration period was allowed to normalize the animal prior to blood sampling. Baseline vessel diameter and MAP measurements were recorded prior to experimental infusions. During experimental periods, diameter and MAP measurements were recorded manually every 5 minutes. The initial experimental infusion was marked as time 0. Experimental agents were infused via the femoral vein at 0.01 ml/s. After the experiment, blood gas measurements (Appendix VI) and Hb/oxygen saturation were again recorded.

*Vasoactivity Response to Topload HBOC 201, HBOC 205 MW 400, HBOC 205 MW 600 Infusion*
Sprague-Dawley rats (N = 6, N=6, N=6) received a HBOC 201, 205 MW 400, and 205 MW 600 topload, respectively, (0.01, 0.1, 1.0, 10.0, 100.0 µM) systemically via the left jugular vein. Vessel diameter and MAP were noted pre-treatment (i.e., baseline), and subsequently every 5 minutes post-treatment for 70 minutes. Volume top-loading with 5.9 % human serum albumin was used to test whether increases in plasma volume had any effect on vessel diameter and MAP (N=7).

Effect of NaNO₂ on Vessel Diameter and MAP in the Presence of HBOC 201 and HBOC 205 MW 600 Infusion

A first group (N=6) received HBOC 201 (100 µM) at time 0. At t = 15 min, a dose response of NaNO₂ (10, 100, 1000 µM) was infused intravenously via the right femoral vein. This protocol was repeated with HBOC 205 MW 600 (N=6). HBOC 201 and 205 MW 600 were infused in the absence of NaNO₂ to test their vasoactive longevity (N=2, N=2, respectively). In all groups, vessel diameter and MAP were noted pre-treatment, and subsequently every 5 minutes post-treatment for 65 minutes.

Effect of NaNO₂ on Vessel Diameter and MAP in the Absence of HBOC 201 and HBOC 205 MW 600 Infusion

Sprague-Dawley rats (N=6) received a series of NaNO₂ doses (10, 100, 1000 µm, estimated plasma concentration) at t=0 via the right femoral vein. Vessel diameter and MAP were noted pre-treatment, and subsequently every 5 minutes post-treatment for 65 minutes.

Effect of Concomitant NaNO₂ and HBOC 205 MW 600 Infusion on Vessel Diameter and MAP

Rats received a bolus dose of 100 µM HBOC 205 MW 600 and 3000 µM NaNO₂ at time 0 (N=5). Vessel diameter and MAP were noted pre-treatment, and subsequently every 5 minutes post-treatment for 55 minutes.
**Effect of NaNO₂ on Vessel Diameter and MAP in Presence of L-NAME**

Sprague-Dawley rats (N=6) received an intravenous bolus of L-NAME (5 mg/kg). After a 25 minute period to assure L-NAME’s maximal effect, a sequence of doses of NaNO₂ (10, 100, 1000, 3000 µM, estimated plasma concentration) was infused intravenously. L-NAME was infused in the absence of NaNO₂ to test its longevity (N=2). Vessel diameter and MAP were noted pre-treatment, and subsequently every 5 minutes post-treatment for 85 minutes.

**HBOC 201 and HBOC 205 MW 600 Extravasation**

Before and after experimentation, a site consisting of arterioles and venules was photographed using IP Lab Software (Scanalytics, Inc., Fairfax, VA) (N=3, N=3, respectively). HBOC 201 and 205 MW 600 were labeled with TRITC and infused at time 0 (50 µM). Images were recorded using a green filter every second for 2 minutes using IP Lab software. Subsequently, images were recorded every minute for 5 minutes and then every 5 minutes for 20 minutes.

**In Vitro**

**HBOC 201 and HBOC 205 MW 600 NO Consumption**

An Amino 700 NO electrode (Innovation Instrument Inc. Tampa, FL) was utilized to determine NO consumption rates of HBOC 201 and HBOC 205 MW 600. The NO electrode was submerged in deionized H₂O overnight and then polarized in PBS for two hours at +0.9 mV. Calibration of the electrode was then conducted using nitrogen, 650 nM NO (392 ppm), and 1300 nM NO (818 ppm). The chamber NO concentration was measured with the NO electrode and based on a linear calibration curve. The calibration and experiment were run at room temperature (27 °C).
A multi-port measurement chamber (World Precision Instruments, Inc. Sarasota, FL) was filled with 1 mL fluid volume. NO and HBOC concentrations were calculated to mimic *in vivo* studies (NO: 650 nM, HBOC: 0.1, 1.0, 10, 100 µM). Data were collected using a Keithley picoammeter (Keithley Instruments Inc. Cleveland, OH) with Excel software.

Three trials were run for each HBOC tested. The first step was conducted using a 650 nM NO solution. This solution was then utilized to test the NO consumption of HBOC 201 and HBOC 205 MW 600 (0.1, 1.0, 10, 100 µM). The chamber was cleaned and refilled with the 650 nM NO solution for each subsequent dose of HBOC.

**Statistical Analysis**

Control and experimental groups were analyzed and compared using one-way ANOVA statistics and paired t-tests. Data are reported as mean ± standard error (SE) and P < 0.05 was regarded as statistically significant.
RESULTS

Vasoactivity Response to Topload HBOC 201, HBOC 205 MW 400, HBOC 205 MW 600 Infusion

First, a topload of 5.9 % albumin was utilized to verify that volume expansion was not significantly affecting MAP and vessel diameters (Figure 1). As depicted in Figure 2A, the topload infusion of HBOC 201 (0.01, 0.1, 1.0, 10.0, 100.0 µM) increased MAP significantly at the higher doses of 10 and 100 µM from 96 ± 6 to 117 ± 3 and 135 ± 5 mmHg, respectively (P < 0.01, P < 0.001). This was equivalent to a 22.4 ± 5.9 and 42.5 ± 10.2 % increase, respectively (Figure 3A). In Figure 2B, the topload infusion of HBOC 201 decreased vessel diameters at 100 µM from 122 ± 5 to 104 ± 5, 70 ± 2 to 60 ± 3, and 34 ± 2 to 28 ± 1 µm (P < 0.05, P < 0.01, P < 0.05, respectively). This was equivalent to a 13.9 ± 3.4, 14.3 ± 4.5, and 15.8 ± 4.3 %, respectively (Figure 3B, C, D).

As depicted in Figure 4A, the topload infusion of HBOC 205 MW 400 (0.01, 0.1, 1.0, 10.0, 100.0 µM) increased MAP significantly at higher doses of 10 and 100 µM from 105 ± 4 to 132 ± 6 and 142 ± 7 mmHg, respectively (P < 0.01, P < 0.001, respectively). This was equivalent to a 25.4 ± 3 and 34.8 ± 4.9 % increase, respectively (Figure 5A). In Figure 4B, at 10 µM, vessel diameters decreased from 124 ± 4 to 105 ± 8 69 ± 3 to 61 ± 4, and 40 ± 1 to 35 ± 3 µm (P < 0.05). This was equivalent to a 14.8 ± 6.3, 8.5 ± 3.9, and 6.7 ± 3.8 % decrease (Figure 5B, C, D). At 100 µM, vessel diameters decreased further to 88 ± 5, 69 ± 3 to 56 ± 4 and 40 ± 1 to 32 ± 2 µm (P < 0.001, P < 0.01, P < 0.01, respectively). This was equivalent to a 28 ± 5.1, 18.6 ± 3.6, and 20.0 ± 5.0 % decrease (Figure 5B, C, D).
Figure 1. Response of MAP and Arteriolar Diameter to HSA (N=7). A topload infusion of human serum albumin (HSA) did not alter MAP (A) or arteriolar diameter (B) by means of volume expansion.
Figure 2. Response of MAP and arteriolar diameter to HBOC 201 (N=6). A) MAP significantly increased at 10.0 µM and 100 µM concentrations of HBOC 201. B) Large, intermediate, and small arteriolar diameters significantly decreased in response to HBOC 201 at 100 µM (*P < 0.05, **P < 0.01, ***P < 0.001).
Figure 3. % Change in MAP and arteriolar diameter in response to HBOC 201 (N=6). A) There was a significant % change in MAP at 1.0, 10, and 100 µM (P < 0.05). B) There was a significant % change in large vessel diameter at 100 µM (P < 0.05). C) There was a significant % change in intermediate vessel diameter at 10 and 100 µM. D) There was a significant % change in small vessel diameter at 100 µM. (*P < 0.05, **P < 0.01, ***P < 0.001).
Figure 4. Response of MAP and arteriolar diameter to HBOC 205 MW 400 (N=6). A) MAP significantly increased at 10.0 µM and 100 µM concentrations of HBOC 205 MW 600. B) Large, intermediate, and small arteriolar diameters significantly decreased in response to HBOC 201 at 100 µM (*P < 0.05, **P < 0.01, ***P < 0.001).
Figure 5. % Change in MAP and arteriolar diameter in response to HBOC 205 MW 400. Response of MAP and arteriolar diameter to HBOC 205 MW 400 (N=6). 
A) MAP significantly increased at 1.0, 10.0 and 100 µM concentrations of HBOC 205 MW 400. 
B, C, D) Large and intermediate arteriolar diameters significantly decreased in response to HBOC 201 at 10 and 100 µM. Small arteriolar diameter significantly decreased in response to HBOC 201 at 0.1, 1.0, 10, and 100 µM. (*P < 0.05, **P < 0.01, ***P < 0.001).
As depicted in Figure 6A, the topload infusion of HBOC 205 MW 600 (0.01, 0.1, 1.0, 10.0, 100.0 µM) increased MAP significantly at higher doses of 10 and 100 µM from 92 ± 5.3 to 121 ± 4.4 and 138 ± 4.9 mmHg, respectively (P < 0.01, P < 0.001). This was equivalent to a 31.6 ± 3.4 and a 50 ± 4.2 % increase in vessel diameter, respectively (Figure 7A). In Figure 6B, the topload infusion of HBOC 205 MW 600 decreased arteriolar diameters at 100 µM from 122 ± 6.5 to 106 ± 6.6, 70 ± 2.4 to 64 ± 3.2, and 36 ± 1.5 to 27 ± 2.3 (P < 0.05, P < 0.05, P < 0.01). This was equivalent to a 10.5 ± 4.6, 8.9 ± 3.9, and 25.7 ± 6.5 % decrease, respectively (Figure 7B).

**Effect of NaNO\textsubscript{2} on Vessel Diameter, MAP, and Blood Constituents in the Presence and Absence of HBOC 201, HBOC 205 MW 600, and L-NAME Infusion**

In the presence of HBOC 201 (100 µM), a sequence of NaNO\textsubscript{2} doses (10, 100, 1000 µM) reduced MAP at 1000 µM from 143 ± 5 to 115 ± 7.9 mmHg (Figure 8A, P < 0.01). This dose did not induce complete compensation to baseline MAP of 99 ± 3 mmHg. Arteriolar diameters increased slightly, although not significantly, from 112 ± 5, 61 ± 3, and 33 ± 1 µm at 1000 µM (Figure 8B).

In the presence of HBOC 205 MW 600, a sequence of NaNO\textsubscript{2} doses (10, 100, 1000, and 3000 µM) at 100 µM HBOC reduced MAP from 139 ± 3 to 130 ± 4 mmHg (Figure 9A, P < 0.05). At 1000 µM, NaNO\textsubscript{2} reduced MAP to 120 ± 3 mmHg (P < 0.001). At 3000 µM, NaNO\textsubscript{2} reduced MAP back to baseline levels of 103 ± 3 mmHg (P < 0.001). Large arteriolar diameters increased from 105 ± 7 to 122 ± 2 µm at 3000 µM of NaNO\textsubscript{2} (P < 0.05). Intermediate and small arterioles returned to baseline diameters increasing from 56 ± 5 to 70 ± 4 and 30 ± 2 to 35 ± 3 µm at 3000 µM NaNO\textsubscript{2} (Figure 9B).
Figure 6. Response of MAP and arteriolar diameter to HBOC 205 MW 600 (N=6). A) MAP significantly increased at 10.0 µM and 100 µM concentrations of HBOC 205 MW 600. B) Large, intermediate, and small arteriolar diameters significantly decreased in response to HBOC 201 at 100 µM (*P < 0.05, **P < 0.01, ***P < 0.001).
Figure 7. % Change in MAP and Arteriole Diameter in Response to HBOC 205 MW 600 (N=6). A) MAP significantly increased at 1.0, 10.0 uM and 100 uM concentrations of HBOC 205 MW 600. B) Large and intermediate arteriole diameters significantly decreased in response to HBOC 201 at 10 and 100 uM. Small arteriole diameters significantly decreased in response to HBOC 201 at 1.0, 10, 100 uM. (*P<.05, **P<.01, ***P<.001).
Figure 8. Response of MAP and arteriolar diameter to HBOC 201 and NaNO\textsubscript{2} (N=6). A) HBOC 201 significantly increased MAP. MAP significantly decreased at 1000 µM NaNO\textsubscript{2}. B) HBOC 201 significantly decreased arteriolar diameters. Arteriolar diameters increased slightly at 1000 µM NaNO\textsubscript{2}. (*P < 0.05, **P < 0.01, ***P < 0.001).
Figure 9. Response of MAP and arteriolar diameter to HBOC 205 MW 600 and NaNO$_2$ (N=6). A) MAP increased significantly in response to HBOC 205 MW 600 (P < .05) and returned to baseline upon administration of 3000 µM NaNO$_2$. B) HBOC 205 MW 600 significantly decreased arteriolar diameters. Large arteriolar diameter increased significantly at 3000 µM NaNO$_2$. Intermediate and small arteriolar diameters returned back to baseline at 3000 µM NaNO$_2$. (*P < 0.05, **P < 0.01, ***P < 0.001).
In the absence of NaNO₂, HBOC 201 and HBOC 205 MW 600 maintained an elevated MAP and constricted arterioles for the entirety of the experiment (see Figure 10 and 11). In the absence of HBOC, a sequence of doses of NaNO₂ (10, 100, 1000, 3000 µM) reduced MAP at 1000 and 3000 µM from 90 ± 3 to 83 ± 3 and 75 ± 4 mmHg, respectively (Figure 12A, P < 0.05). Vessel diameters increased slightly at 3000 µM NaNO₂ in larger and intermediate arterioles, although not significantly. Smaller arteriolar diameter increased from 39 ± 1 to 50 ± 7 µm (Figure 12B, P < 0.05). This was equivalent to a 28 ± 17 % increase (Figure 13D).

Table 1 shows the initial and final OSM3 values for control and experimental groups. In the absence of NaNO₂, HBOC 201, HBOC 205 MW 400, and HBOC 205 MW 600 significantly increased the % metHb from 0.52 ± 0.18 to 0.83 ± 0.18, 0.25 ± 0.04 to 0.4 ± 0.07, and 0.47 ± 0.11 to 0.75 ± 0.14, respectively (P < 0.05). In the presence of HBOC 201, NaNO₂ further increased the % metHb from 0.4 ± 0.1 to 0.95 ± 0.38 % (P < 0.05). In the presence of HBOC 205 MW 400, NaNO₂ further increased the % metHb from 0.54 ± 0.17 to 1.13 ± 0.23 % (P < 0.05). In the presence of L-NAME, NaNO₂ further increased the % metHb from 0.24 ± 0.04 to 1.26 ± 0.08 % (P < 0.05). In the presence of a NaNO₂ alone, the % metHb increased from 0.28 ± 0.18 to 1.15 ± 0.27 % (P < 0.05).

Rats infused with HBOCs showed an increase in total Hb concentration (Table 2). HBOC 201 significantly increased total Hb concentration from 13.38 ± 0.65 to 15.7 ± 0.46 (P < 0.05). HBOC 205 MW 400 increased total Hb concentration from 13 ± 0.5 to 14.3 ± 0.62 (P < 0.05). HBOC 205 MW 600 increased total Hb concentration from 14.4 ± 0.3 to 14.9 ± 0.25.
Figure 10. HBOC 201 Longevity (N=2).  A) A 100 µM HBOC 201 infusion maintained an elevated MAP for 65 minutes.  B) A 100 µM HBOC 201 infusion maintained constricted large and intermediate arteriolar diameters for 65 minutes.
Figure 11. HBOC 205 MW 600 Longevity (N=2).  A) A 100 µM infusion of HBOC 205 MW 600 maintained an elevated MAP for 65 minutes (*P < 0.05).  B) A 100 µM HBOC 205 MW 600 infusion maintained constricted large, intermediate, and small arteriolar diameters for 65 minutes.
Figure 12. Response of MAP and arteriolar diameter to NaNO₂ (N=6). A) A 1000 and 3000 µM NaNO₂ dose significantly decreased MAP. B) Arteriolar diameters increased slightly with increasing concentrations of NaNO₂, although only small arteriolar diameter increased significantly at 3000 µM NaNO₂. (*P < 0.05).
Figure 13. % Change in MAP and arteriolar diameter in response to NaNO₂ (N=6). A) MAP significantly decreased at 10.0 and 100 µM concentrations of NaNO₂. B) Large arteriolar diameter significantly increased in response to 1000 and 3000 µM NaNO₂. Small arteriolar diameter significantly increased in response to 100, 1000, and 3000 µM NaNO₂. (*P < 0.05, **P < 0.01).
Table 1. Initial and Final OSM3 values. (*P < 0.05)

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In the presence of L-NAME, MAP increased gradually to $132 \pm 6$ mmHg ($P < 0.001$). This was equivalent to a $37.5 \pm 3\%$ change. Vessel diameters decreased from $110 \pm 5$, $71 \pm 1$, and $42 \pm 1$ to $81 \pm 6$, $62 \pm 2$, and $34 \pm 1$, respectively (Figure 14A, $P < 0.01$, $P < 0.001$, $P < 0.001$). This was equivalent to a $23 \pm 6$, $13 \pm 4$, and $20 \pm 4\%$ decrease (Figure 15A). A NaNO$_2$ dose of 100 µM returned MAP back to baseline. A 1000 µM dose of NaNO$_2$ was required to return large arteriolar diameter back to baseline. A 100 µM dose of NaNO$_2$ was required to return small arteriolar diameter back to baseline, and a 10 µM dose of NaNO$_2$ was required to return intermediate arteriolar diameter back to baseline (Figure 14B). Administration of L-NAME in the absence of NaNO$_2$ induced a sustained hypertensive response (Figure 16, $P < 0.05$).

*Effect of Concomitant infusion of NaNO$_2$ and HBOC 205 MW 600 on arteriolar diameter and MAP*

Concomitant infusion of NaNO$_2$ and HBOC 205 MW 600 did not significantly alter MAP from baseline of $98 \pm 6$ mmHg. Arteriolar diameters also did not exhibit any changes from baseline of $114 \pm 2$, $68 \pm 1$, and $37 \pm 1$ µm (Figure 17).
Figure 14. Response of MAP and arteriolar diameter to L-NAME and NaNO₂ (N=6). A) L-NAME gradually increased MAP (P < 0.001). MAP significantly decreased at 10 µM NaNO₂ and returned to baseline at 100 µM. MAP further decreased at 1000 and 3000 µM NaNO₂ (P < 0.01, P < 0.001). B) L-NAME gradually decreased arteriole diameters (P < 0.01, P < 0.001, P < 0.001). Large arteriole diameters increased back to baseline at 3000 µM NaNO₂. Intermediate arteriole diameters increased back to baseline at 10 µM and small arteriole diameters increased back to baseline at 1000 µM. (*P < 0.05, **P < 0.01, ***P < 0.001).
Figure 15. % Change in MAP and arteriolar diameter in response to L-NAME and NaNO₂ (N=6). A) L-NAME gradually increased MAP. MAP returned to baseline at 100 and 1000 µM NaNO₂ and decreased further at 3000 µM NaNO₂. B) L-NAME gradually decreased arteriolar diameters. Large arterioles significantly increased in diameter at 10, 100, and 1000 µM NaNO₂. Large arterioles returned to baseline at 3000 µM NaNO₂. Intermediate arteriolar diameter returned to baseline at 10 µM NaNO₂. Small arterioles returned to baseline diameter at 100 µM NaNO₂. (*P < 0.05, **P < 0.01, ***P < 0.001).
Figure 16. L-NAME Longevity (N=2). A) L-NAME maintained an elevated MAP for 85 minutes. B) L-NAME maintained constricted arterioles for 85 minutes. (*P < 0.05).
Figure 17. Response of MAP and arteriolar diameter to concomitant HBOC 205 MW 600 and NaNO\textsubscript{2} (N=5). A and B) Concomitant infusion of HBOC 205 MW 600 and NaNO\textsubscript{2} did not significantly change MAP or arteriolar diameter.
**HBOC 201 and HBOC 205 MW 600 Extravasation**

Figures 18 and 19 show the fluorescence of the TRITC-HBOC 201 at 0”, 10”, 20”, 40”,”60”, 80”, 100”, 3’,4’,5’,10’, and 15’. Fluorescently-labeled HBOC 201 flowed through the arterioles and venules seconds after infusion and then was progressively concentrated at the vessel wall and slowly extravasated into the interstitium. The yellow rectangle in Figure 19 shows a cross section of both the arteriole and venule used to quantify the fluorescent intensity at each time point. Figure 20 shows the intensity of this section at each time point. It is evident that the fluorescent intensity increased the most in the vessel wall and surrounding interstitium. Figure 21 isolates three locations: the lumen, vessel wall, and interstitium. After infusion of the TRITC-HBOC 201, the lumen of both arterioles and venules exhibited the fastest increase in intensity while the interstitium exhibited the slowest. The luminal intensity was eventually surpassed by the vessel wall and interstitium intensity approximately between 80-100 seconds. In comparison to the venule, the intensity was highest in the arteriole in each location.

Figure 22 and 23 show the fluorescence of the TRITC-HBOC 205 MW 600 at 0”, 10”, 20”, 40”, 60”, 80”, 100”, 3’,4’,5’,10’ and 15’. Labeled HBOC 205 MW 600 flowed through the arterioles and venules seconds after infusion and then was progressively concentrated in the vessel wall and slowly extravasating into the interstitium. The yellow rectangle in Figure 23 defines the measurement region for both the arteriole and venule used to quantify the fluorescent intensity at each time point. Figure 24 shows the intensity of this region at each time point. It is evident that the largest increase in fluorescent intensity occurred near the vessel wall and the surrounding interstitium. Figure 25 isolates three locations: the lumen, vessel wall, and interstitium. After infusion of the TRITC-HBOC 205 MW 600, the lumen of both arterioles and venules exhibited the fastest increase in intensity, while the interstitium exhibited the slowest.
The luminal intensity was eventually surpassed by the intensity near the vessel wall and in the interstitium approximately 80-100 seconds following infusion of the labeled HBOC. In comparison to the venule, the intensity was highest in the arteriole at each location.

Effect of HBOC 201 and HBOC 205 MW 600 on NO Consumption

The present in vitro study demonstrated that NO interacts with different HBOCs. Similar concentrations (0.1, 1, 10, 100 µM) to those used in vivo were utilized in this study to determine the effect of HBOCs on the rate of NO consumption. HBOC 201 and HBOC 205 MW 600 were found to scavenge NO at similar rates for similar doses. NO was found to diffuse from the chamber at a slow rate of 0.239 ± 0.003 nM/s. The introduction of HBOC 201 and HBOC 205 MW 600 into the chamber at a concentration of .1 µM increased the rate of NO consumption significantly to 1.708 ± 0.104 and 3.059 ± 0.153 nM/s, respectively (P < 0.05). Increasing the HBOC’s concentration to 1 µM further increased NO consumption significantly to 5.765 ± 0.409 and 6.573 ± 0.329 nM/s respectively (P < 0.05). An increasing trend was seen with the subsequent doses of HBOC, however these increases were not significant (see Figure 26).
Figure 18. Fluorescent Images of TRITC-HBOC 201 Infusion.
Figure 19. Fluorescent Images of TRITC-HBOC 201 Infusion.
Figure 20. Fluorescent Profile Analysis of Arteriole and Venule in Response to HBOC 201. The fluorescence intensity initially increased in the lumen, but the fluorescence intensity in the vessel wall and interstitium surpassed the luminal intensity as time progressed. Vertical lines denote location of wall-lumen interface.
Figure 21. Regional Fluorescent Intensity Versus Time. A) Fluorescent intensity in the arteriole increased at the fastest rate in the lumen, but eventually was surpassed by the fluorescent intensity in the vessel wall and interstitium between 40-60 seconds. B) Fluorescent intensity in the venule also increased at the fastest rate in the lumen, but eventually was surpassed by the fluorescent intensity in the vessel wall between 40-60 seconds and interstitium between 100-120 seconds.
Figure 22. Fluorescent Images of HBOC 205 MW 600 Infusion.
Figure 23. Fluorescent Images of HBOC 205 MW 600 Infusion.
Figure 24. Profile Analysis in Response to TRITC-HBOC 205 MW 600 Infusion. The fluorescence intensity initially increased in the lumen, but the fluorescence intensity in the vessel wall and interstitium surpassed the luminal intensity as time progressed.
Figure 25. Regional Intensity Versus Time. A) Fluorescent intensity in the arteriole increased at the fastest rate in the lumen, but eventually was surpassed by the fluorescent intensity in the vessel wall and interstitium between 40-60 seconds. B) Fluorescent intensity in the venule also increased at the fastest rate in the lumen, but eventually was surpassed by the fluorescent intensity in the vessel wall between 60-80 seconds and in the interstitium between 2 and 3 minutes.
Figure 26. *In vitro* NO Consumption Rate for HBOC 201 and HBOC 205 MW 600. HBOC 201 and 205 MW 600 significantly increased NO consumption at .1 uM 1, 10, and plateau at 100 uM. (**P<.001) concentrations. NO consumption further increased and reached a
DISCUSSION

There have been many studies investigating the vasopressor effects that HBOCs engender. However, the mechanisms behind these effects are controversial. Some experimenters support the hypothesis that the vasoconstriction is due to an overcompensating autoregulatory response due to the excessive O$_2$ delivery caused by the increased availability of oxygen from cell-free Hb (Winslow, 2000, Rolhfs et al., 1998). However, evidence supporting the NO scavenging hypothesis seems to be growing. This study offers evidence that refutes the autoregulatory response hypothesis and explores certain mechanisms of NO scavenging.

**HBOC Molecular Weight**

Previous studies have found that increasing the average molecular weight of HBOCs reduces the vasopressor effects (Olson et al., 2003). This is presumably due to a decrease in extravasation. Other studies have found that there is no response to HBOCs in vessels with tight endothelial junctions, further supporting extravasation as a contributor to the hypertensive effect (Tsai et al., 2006). The present study suggests that increasing molecular weight is beneficial up to a point, however the percentage of tetramers and dimers is more indicative of vasopressor effects. All three HBOCs that were tested showed similar effects on MAP and vessel diameters at similar concentrations (10 and 100 µM). HBOC 201, HBOC 205 MW 400, and HBOC 205 MW 600 contain approximately 2, 0.32, and 0.6 % tetramers and dimers, respectively, while the percentage of molecules greater than 500 kD is 10.6, 24, and 61 %, respectively. Previous studies conducted in this laboratory demonstrated that HBOC 301 induces similar increases in MAP and vasoconstriction at lower concentrations (1 µM). HBOC
301 contains approximately 20% dimers and tetramers while only 10% of the molecules are greater than 500 kD. This increased sensitivity to HBOC 301 and the similar response observed among the HBOCs presently studied indicate that the percentage of tetramers and dimers have a higher correlation with the observed vasopressor effects.

**Oxygen Delivery and the Autoregulatory Response**

There still remain some advocates of the autoregulatory response theory. This theory proposes that excessive O$_2$ delivery by extracellular Hb causes vasoconstriction. Gregory et al. (Gregory et al., 2006) conducted studies testing HBOCs with different oxygen affinities. They found no correlation between MAP and oxygen affinity. The present study also provides evidence that does not support this hypothesis. Vasopressor effects were observed at low concentrations of HBOC (10 and 100 µM), too low to provide much of an increase in oxygen delivery. Plasma HBOC concentrations needed to supply significant oxygen delivery are known to be in the mM range. Therefore, this study concludes that HBOC increases MAP and vasoconstriction by means other than the autoregulatory mechanism.

**NO Scavenging**

Most experiments deriving conclusions of NO scavenging have been based on the inhibition of NOS. Administration of L-NAME, a NOS inhibitor, resulted in vasopressor effects similar to those observed following administration of HBOCs. This effect was more gradual than the immediate effects of HBOC because NOS inhibition does not immediately eliminate NO. Administration of HBOC and L-NAME did not enhance the observed vasopressor effects (Rees et al., 1990). These studies suggest that these agents induce such effects by means of the same mechanism. The present study examined the
NO donor NaNO₂ in order to further test the NO scavenging hypothesis. A dose range of NaNO₂ was utilized, but only at a 3000 µM concentration did NaNO₂ completely abolish the hypertensive effects brought on by HBOC 205 MW 600. A 3000 µM dose of NaNO₂ in the absence of HBOC did not result in an increase in vessel diameter that was equivalent to the compensation seen with HBOC 205 MW 600. This is presumably due to a vasodilatory limit of the microcirculation in the spinotrapezius muscle. To better examine the possibility of NO scavenging, L-NAME was utilized to closely mimic the effects of the HBOCs. When L-NAME and a high enough dose of NaNO₂ were co-administered, complete compensation was achieved at much lower concentrations of NaNO₂. These results show that HBOCs must be interacting with the NO donated by NaNO₂.

A significant increase in metHb was observed upon administration of HBOC 201, HBOC 205 MW 400, and HBOC 205 MW 600. A larger increase was observed upon administration of NaNO₂ in conjunction with HBOCs. These increases in metHb provide further evidence supporting HBOC/NO interactions.

*In vitro* studies were conducted to offer more evidence supporting the NO scavenging hypothesis. These studies also allowed a quantification of the NO consumption rate of HBOC 201 and HBOC 205 MW 600. The consumption rates were found to be similar and progressively increased with increases in HBOC concentration. Theses findings coincide with the *in vivo* studies that demonstrated a progressive increase in the vasopressor response.
Tissue Specific Responses

There have been many studies focusing on the vessel reactivity in response to particular HBOCs. Some studies have found greater constriction in smaller vessels, while others have found greater constriction in larger vessels, however, the amount of constriction seems to be dependent on location. For example, Pawson et al. (Pawson et al., 2007) found a greater response to cell-free Hb in the rat aorta versus the mesentery. With respect to the spinotrapezius muscle, the present study shows an equivalent decrease in vessel diameters ranging from small, intermediate, and large vessels which correlate to parallel increases in MAP. This variation is influenced by the permeability of particular vessels to HBOCs (Tsai et al., 2006), but may also be due to tissue-specific mechanisms of maintaining vascular tone. Some tissues may exhibit differences in NO production, perhaps due to fewer NOS enzymes in endothelial cells and/or possibly less neuronal innervation. Particular tissues also utilize other vasoactive substances such as endothelin and acetylcholine (Clark and Fuch, 1997). For example, it has been demonstrated in the mesentery, particularly in smaller vessels, that other EDRFs predominantly contribute to vascular tone (Feletou and Vanhoutt, 2006).

This variation observed throughout the systemic system in response to HBOC administration seems to provide evidence against the autoregulatory response theory. Excessive oxygen delivery would be equally distributed to all tissues, however not all tissues respond similarly. Future studies should focus on how individual tissues maintain vascular tone. This area of focus may further the understanding of how HBOCs produce vasopressor effects.
**HBOC Extravasation**

Many experiments have found that the vasopressor effects observed were influenced by HBOC molecular weight. Experiments comparing microcirculatory tissues with tight endothelial junctions versus those with porous endothelia also demonstrated a correlation between vasopressor effects and HBOC molecular weight. These findings indirectly show that extravasation of HBOC may contribute to vasopressor effects. Some scientists claim that the time scale of extravasation could not be entirely responsible for vasopressor effects (Fitzpatrick et al., 2004). The present study found that increases in total blood Hb concentration after administration of HBOC were not equivalent to the amount of Hb administered. This may be due to excretion through the kidney of smaller molecules, but may also be due to extravasation.

The present study focused more directly on this theory by tagging HBOC 201 and HBOC 205 MW 600 with TRITC. These HBOCs are similar except that HBOC 201 has a smaller average molecular weight and has more tetramers and dimers. Even so, both HBOC 201 and HBOC 205 MW 600 produced similar responses. The TRITC labeled HBOC 201 and 205 MW 600 reached the microcirculation of the spinotrapezius muscle within seconds. This corresponded to the immediate increase in MAP and vasoconstriction observed upon HBOC administration. It is difficult to determine exactly when extravasation begins, however it definitely occurs between 0 and 40 seconds for both HBOC 201 and HBOC 205 MW 600. An increase in fluorescence is observed in the walls of the arterioles and venules, although this increase is less pronounced in the venules. After a few minutes, the extravasation seems to surpass the vessel walls and enter the interstitium, again with a lower fluorescence surrounding the venules. The
immediate vasopressor effects cannot unequivocally be explained by extravasation, but are more likely due to the direct scavenging of NO from the luminal side of the vessel. However, extravasation into the vessel walls and interstitium is evident and any endogenous NO in these areas will be scavenged. This could contribute to the longevity of symptoms observed upon HBOC administration.

Conclusions

The present study focused on elucidating the mechanisms behind the vasopressor effects observed upon HBOC administration. The data presented offer strong evidence that NO scavenging is a key mechanism involved in vasoconstriction in the spinotrapezius muscle in small, intermediate, and large arterioles. These results do not offer any evidence of similar effects in microcirculatory beds of other tissues; however, it is hypothesized that tissues regulated by vasoactive substances other than NO may not respond similarly.

The present study directly demonstrates for the first time that even trace amounts of tetramers and dimers extravasate. These molecules most likely contribute to the longevity of the HBOC effects, but may be responsible for the immediate vasopressor effects as well. Future studies should focus on pure HBOCs that contain no tetramers or dimers to further examine the effects of HBOC extravasation.

Although the present study offers strong evidence to support the NO scavenging hypothesis, it does not investigate the interactions of HBOC with other EDRF’s. Future studies can focus to elucidate the interactions of other EDRFs with HBOCs. This study does offer evidence that refutes the autoregulatory response hypothesis. Minimal concentrations of HBOC which do not carry significant levels of oxygen to tissues were
found to induce vasopressor effects in the spinotrapezius. These results demonstrate that there is a different mechanism responsible for vasoconstriction at low concentrations of HBOC. At higher concentrations the autoregulatory response cannot be disregarded as a possible contributor to vasoconstriction. Future studies involving HBOCs with different P50 values at higher concentrations could further examine the role of the autoregulatory response in vasoconstriction.

In conclusion, the data presented in this study offer a means of improvement of current HBOCs being tested. Further purification of the HBOCs and concomitant infusion with NO donors may completely abolish any hypertensive side effects, while maintaining the efficacy of the HBOC. With this better understanding of the functionality of these HBOCs, daily clinical use of such blood substitutes may be just over the horizon.
REFERENCES


Clark SG and Fuchs LC. Role of Nitric oxide and Ca\textsuperscript{++}-Dependent K\textsuperscript{+} channels in mediating heterogeneous microvascular responses to acetylcholine in different vascular beds. Journ. Of Pharm. and Exp. Therap. 282: 1473-1479, 1997.


## APPENDIX

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**Appendix I.** Initial and final ABL values.
VITA

Pete Dennis Meliagros was born on August 15th, 1984, in Smithtown, New York. He graduated from Mathews High School in 2002. He graduated from the University of Virginia with a Bachelor of Science in Biology, a Bachelor of Arts in Economics, and a minor in Astronomy in 2006. Pete began the Master of Physiology program at Virginia Commonwealth University in 2006. He was awarded the Poland award for his outstanding first year graduate performance. He completed his Master of Science in May 2008.