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Erythrocyte-Associated Transients in Capillary PO2 in the Rat Spinotrapezius Muscle During Hemodilution with Hespan and a Hemoglobin-Based Oxygen Carrier

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ERYTHROCYTE-ASSOCIATED TRANSIENTS IN CAPILLARY PO_2 IN THE RAT SPINOTRAPEZIUS MUSCLE DURING HEMODILUTION WITH HESPAN AND A HEMOGLOBIN-BASED OXYGEN CARRIER

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2,3-DPG	2,3-diphosphoglycerate
A	cross-sectional area
A	amplitude of the fast post-excitation transient
α	oxygen solubility coefficient
ANH	acute normovolemic hemodilution
B	baseline offset
β	proportionality factor
C_{Hb}	oxygen binding capacity of hemoglobin
cm	centimeter
D	free diffusion coefficient for a particular molecule
Da	Dalton
°C	degrees Celsius
δ	half width of the PO_2 distribution
ΔPO_2	partial pressure difference
Δx	diffusion distance
dl	deciliter
EATs	erythrocyte-associated transients
EDRF	endothelial derived relaxing factor
FCD	functional capillary density
FFT	fast Fourier transform

g	gram
HB1	hemoglobin-based oxygen carrier value at hemodilution Step 1
HB2	hemoglobin-based oxygen carrier value at hemodilution Step 2
HBOC	hemoglobin-based oxygen carrier
[Hb]	total hemoglobin concentration
$[Hb]_{plasma}$	plasma hemoglobin concentration
$[Hb]_{RBC}$	concentration of hemoglobin in a single red blood cell
HbO_2	oxyhemoglobin
Hct	systemic hematocrit
HD1	hemodilution Step 1
HD2	hemodilution Step 2
H_F	final systemic hematocrit
H_I	initial systemic hematocrit
HS1	Hespan value at hemodilution Step 1
HS2	Hespan value at hemodilution Step 2
Hz	hertz
I(0)	amplitude of the phosphorescence signal at t = 0
I(t)	magnitude of the phosphorescence signal
JO_2^D	quantity of oxygen moved per unit time
k_0	rate constant
kD	kilo Dalton

kg	kilogram
kHz	kilohertz
k_q	quenching coefficient
k_q	second-order tae constant for phosphorescence decay
LT	light transmission
M	mean PO_2
MAP	mean arterial pressure
MCHC	mean corpuscular hemoglobin concentration
metHb	met-hemoglobin
µl	microliter
µm	micrometer
µs	microseconds
mg	milligram
ml	milliliter
mM	milimolar
mmHg	milimeters of mercury
min	minute
N	number of animals
n	number of capillaries
nJ	nanojoules
NLA	nitro-L-arginine
nm	nanometer

NO	nitric oxide
NO_3^-	nitrate
NOD	nitric oxide dioxygenation
O_2	oxygen
PA	phosphorescence amplitude
P_aCO_2	systemic arterial partial pressure of carbon dioxide
P_aO_2	Systemic arterial partial pressure of oxygen
PCO_2	partial pressure of carbon dioxide
P_cO_2	mean capillary partial pressure of oxygen
Pd	palladium
Pd-MTCPP	meso-tetra-(4-carboxyphenyl)-porphyrin
PE-10	size 10 polyethylene tubing
PE-20	size 20 polyethylene tubing
PE-240	size 240 polyethylene tubing
PE-50	size 50 polyethylene tubing
PE-90	size 90 polyethylene tubing
PEG	polyethylene glycol
PFCs	perfluorocarbons
PG	plasma gap
PMT	photomultiplier tube
PO_2	partial pressure of oxygen
PQM	phosphorescence quenching microscopy

QO_2^{CONV}	convective flow of oxygen
QO_2^{DIFF}	diffuse flow of oxygen
RBC	red blood cell
S_aO_2	arterial oxygen saturation
SFH	stroma-free hemoglobin
SO_2	fractional oxygen saturation of red blood cell hemoglobin
SR_{RBC}	red blood cell supply rate
t	time from the beginning of phosphorescence decay
T	lifetime of the fast post-excitation transient
τ	phosphorescence lifetime
τ_o	lifetime of phosphorescence decay in the absence of O_2
V_B	effective blood volume associated with hemodilution
V_{RBC}	volume of a single red blood cell
W_B	body weight of the animal

ABSTRACT

ERYTHROCYTE-ASSOCIATED TRANSIENTS IN CAPILLARY PO_2 IN THE RAT SPINOTRAPEZIUS MUSCLE DURING HEMODILUTION WITH HESPAN AND A HEMOGLOBIN-BASED OXYGEN CARRIER

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Hemoglobin-based oxygen carriers for use as transfusion fluids have emerged as a leading technology directed at stemming shortages of a safe blood supply and providing a readily available resuscitation fluid in various trauma situations. The purpose of this investigation was to determine the effects of isovolemic hemodilution with Hespan and a hemoglobin-based oxygen carrier (HBOC) on erythrocyte-associated transients (EATs) in capillary PO_2. The particulate nature of blood flow in capillaries, when observed from a stationary observation point, results in fluctuations of PO_2 as alternating red blood cells and plasma gaps move through the detection region. Therefore, through experimental methods which provided the necessary temporal and spatial resolution required to make such measurements, EATs can be observed and corresponding PO_2 fluctuations can be determined. The spinotrapezius muscle in sixteen Sprague-Dawley rats was exteriorized for intravital microscopy measurements in capillaries. Capillary PO_2 was measured using

Pd-porphyrin phosphorescence quenching microcopy. The hemodiluents used in isovolemic hemodilution included Hespan, a non-oxygen carrying plasma expander, and Oxyglobin®, a HBOC. Two isovolemic hemodilution steps were performed, reducing the systemic hematocrit to an average of 27.5% after the first step and 13.5% after the second step. Results showed that erythrocyte-associated transients in PO_2 can be observed in the rat spinotrapezius with significant differences occurring between red blood cell and plasma gap PO_2 under control conditions, isovolemic hemodilution with Oxyglobin after step one, and isovolemic hemodilution with Hespan after step two. This study concludes that EATs are observable and PO_2 transients relating to EATs can be measured in the rat spinotrapezius muscle. Furthermore, it can be concluded that the HBOC Oxyglobin caused a decrease in erythrocyte-associated capillary PO_2 transients, as well as a general decrease in capillary PO_2. In addition, this study concludes that erythrocyte-associated capillary PO_2 transients can best be observed under control conditions and after step two of isovolemic hemodilution with Hespan.

INTRODUCTION

The Blood and Circulation

Oxygen is a fundamental component required to sustain life of most cells in the body of all mammals. The metabolic need for oxygen of cells in tissues requires the transport of oxygen to those sites. Mammals appear to have evolved in accordance with the principles of symmorphosis (i.e., a state of structural design corresponding to functional needs resulting from regulated morphogenesis), possessing tissues, blood, and a circulatory structure to carry out these essential functions of life (Weibel 1984).

Blood Composition

Circulating blood volume accounts for approximately 7% of body weight. About 55% of this volume is plasma and the other 45% is composed of blood cells. Dissolved in the plasma is a complex solution of gases, salts, proteins, carbohydrates, and lipids (Berne and Levy 1998). In the blood cell component there are approximately between 7,000 and 12,000 white cells, between 150,000 and 400,000 platelets, and between 4.5 and 5.5 million red blood cells in a volume of a cubic millimeter of blood (Nucci and Abuchowski 1998). These two components perform multiple tasks including: the transport of nutrients, oxygen, carbon dioxide and waste products; the delivery of hormones, leukocytes, and platelets to their sites of action; and the distributions of water, other solutes, and heat (Berne and Levy 1998; Nucci and Abuchowski 1998).

Red Blood Cells and Hemoglobin

Red blood cells, or erythrocytes, are nonnucleated, terminally differentiated cells that possess an average lifetime of 120 days. They are flexible, biconcave disks with an average diameter of 7 μm (Berne and Levy 1998). Erythrocytes are hollow vesicles filled with molecules called hemoglobin that have the capacity to carry oxygen. Hemoglobin turns such a hollow vesicle into a discrete oxygen transporting package, with hemoglobin occupying approximately a quarter of each cell's internal volume (about $3 \cdot 10^{-11}$ g/cell) (Weibel 1984).

Hemoglobin (MW = 66,000 Da) is a protein tetramer with two α and two β polypeptide chains. Each polypeptide chain has an iron-containing heme group which is used to reversibly bind oxygen. Consequently, four molecules of oxygen can bind to each hemoglobin molecule (Weibel 1984; Chang 1999).

As individual oxygen molecules bind to a subunit of the hemoglobin molecule, its configuration is altered, allowing for increased binding affinity of each subsequent oxygen molecule, until all four binding sites are occupied (i.e., positive cooperativity). This cooperativity results in a sigmoidally shaped binding curve that can undergo shifts influenced by local factors thereby affecting the oxygen affinity of hemoglobin. These factors include PCO_2, pH, temperature, and 2,3-diphosphoglycerate (2,3-DPG) concentration (Berne and Levy 1998; Costanzo 1998).

Oxygen is transported in the blood in two forms: 1) dissolved in the plasma and 2) bound to hemoglobin. Dissolved oxygen comprises approximately 2% of the total oxygen content of blood and is inadequate to supply the demands of the tissues. The

remaining 98% of the oxygen is reversibly bound to hemoglobin, so that the oxygen content of blood is principally determined by the oxygen binding capacity and the concentration of hemoglobin (Costanzo 1998).

In the lungs, there is a reciprocal exchange of carbon dioxide and oxygen, at different sites, as hemoglobin molecules flow through the pulmonary capillaries adjacent to alveolar gas. Carbon dioxide, a waste product of cellular respiration, is unloaded and oxygen binding to the heme groups of the hemoglobin takes place. From there, oxygen is delivered to cells for utilization as the final electron acceptor in oxidative phosphorylation (Weibel 1984; Costanzo 1998).

The Circulation and Oxygen Transport in Blood Vessels

William Harvey (1578-1657) was the first person to accurately describe the overall workings of the circulatory system. Prior to Harvey, there existed multiple explanations as to the purpose, source and directional flow of blood, none of them considering a unidirectional circuit. Galenic physiology taught that there were two types of blood, the venous and the arterial, each with distinct pathways, independent sources, and functions. Harvey established that the blood must constantly move in a circuit through the large vessels stemming from the heart and then to the lungs and tissue and back to the heart again (Porter 1998). What he could not understand then was the complex system involving vascular networks and oxygen delivery.

In general, blood is propelled from the heart through the arteries (main distribution vessels) to capillaries (primary exchange vessels) and then into veins

(collecting vessels) which lead back to the heart (Weibel 1984). This is not much more information than Harvey gleaned from his studies. What he could not see then and what has become the location and focus of many studies in the circulatory system today is the microcirculation. The multiple networks are comprised of branching arterioles and still finer networks of capillaries, vessels whose walls are comprised of only a single layer of endothelial cells and whose lumen permits red blood cells to pass only in single file (Berne and Levy 1998; Costanzo 1998).

Capillary structure is maximized for the local diffusion of oxygen down a gradient into the mitochondrial sinks created by oxidative phosphorylation (Weibel 1984). Their walls are thin and they exhibit the greatest surface-area-to-volume ratio in the microcirculation (Costanzo 1998). In addition, red blood cell velocity is low and gas diffusion distance is minimized by the close proximity of capillaries to surrounding tissue cells (Pittman 1995).

Convection and Diffusion

Oxygen is transported via convection (i.e., the movement of oxygen in blood by bulk flow) and diffusion (i.e., molecular movement of oxygen in the fluid or gas phase down partial pressure gradient) (Weibel 1984; Pittman 2000). Convective flow of oxygen can be expressed by the following (Pittman 1995):

Equation 1

$$QO_2^{CONV} = C_{Hb}[Hb]_{RBC}V_{RBC}SR_{RBC}SO_2$$

where C_{Hb} is the oxygen binding capacity of hemoglobin (i.e. the maximum amount of oxygen that is able to bind to hemoglobin per unit volume of blood (Costanzo 1998), $[Hb]_{RBC}$ is the concentration of hemoglobin in a single red blood cell, V_{RBC} is the volume of a single red blood cell, SR_{RBC} is the red blood cell supply rate (cells/s), and SO_2 is the fractional oxygen saturation of the red blood cell hemoglobin.

Diffusive flow of oxygen (QO_2^{DIFF}) can be determined by the application of Fick's Principle, a statement of the conservation of mass, by the following:

Equation 2

$$QO_2^{DIFF} = QO_2^{CONV} \text{ (upstream)} - QO_2^{CONV} \text{ (downstream)}$$

where QO_2^{CONV} is convective flow of oxygen (Pittman 1995; Pittman 2000). Alternatively, capillary diffusion can be described more directly as the radial diffusive flux of oxygen as:

Equation 3

$$JO_2^{D} = - D\alpha A \Delta PO_2/\Delta x$$

where JO_2^{D} is the quantity of oxygen moved per unit time, D is the free diffusion coefficient for a particular molecule, α is the oxygen solubility coefficient, A is the cross-sectional area, and $\Delta PO_2/\Delta x$ is the partial pressure gradient (i.e., ΔPO_2 is the partial pressure difference of oxygen and Δx is the diffusion distance) (Berne and Levy 1998; Pittman 2000). The ability for diffusion to function as the primary mechanism in the exchange of gases, substances, and waste products between the capillaries and the tissue

cells for the entire body, despite large diffusion distances of 80 μm compared to 0.5 μm in pulmonary capillaries, is due to the large capillary surface area and capillary recruitment (Berne and Levy 1998).

Artificial Oxygen Carriers

Historical Background

The desire to find safe and effective blood substitutes dates back to the mid-16th century to the early 17th century (Scott, Kucik et al. 1997; Creteur, Sibbald et al. 2000). During this time Christopher Wren had been attempting to introduce medicinal liquors into the bloodstream, believing that the alcoholic spirits could substitute for the vital "spirit" of the blood (Porter 1998; Creteur, Sibbald et al. 2000). Jean-Baptiste Denis performed the first documented successful human-to-human blood transfusion in 1667 (Nucci and Abuchowski 1998).

Modern scientific approaches to replace blood began to be successful in the early 1900s with a better understanding of allogeneic blood transfusion and the function of red blood cells (Kim and Greenburg 2004). More recently, concerns of long term storage, supply limitations, and avoidance of disease transmission have fueled the need to develop blood substitute possibilities. Numerous studies performed throughout the early to mid-1900s focused attention on infusing hemoglobin solutions for purposes of trauma resuscitation to help restore oxygen carrying capacity lost through hemorrhagic shock (Creteur, Sibbald et al. 2000). Although these investigations resulted in an unexplained

pressor response, altered renal function, and hemoglobinuria, among others, the stage was set for the development of artificial oxygen carriers (Scott, Kucik et al. 1997; Creteur, Sibbald et al. 2000).

Major Approaches to Artificial Oxygen Carriers

Under conditions of moderate blood loss where oxygen delivery is not seriously compromised, intravascular blood volume can be replaced with non-oxygen carrying plasma expanders. Plasma expanders come in two forms, either as a crystalloid (e.g., isotonic saline, Ringer's lactate) or as a colloid (e.g., human serum albumin, dextran, hetastarch) (Kim and Greenburg 2004). Under conditions of severe blood loss, where oxygen transport capacity has been reduced below critical levels, an oxygen carrier must be infused to sustain normal cellular function.

The purpose of artificial oxygen carriers is to act solely as an oxygen-carrying, volume-replacement solution (Scott, Kucik et al. 1997). The ideal product should have all of the important properties of red blood cell transfusions including: 1) high oxygen carrying capacity; 2) transport of oxygen at normal physiological oxygen tensions; 3) desirable elimination characteristics; 4) low incidence of side effects when properly screened and administered (Dietz, Joyner et al. 1996). In addition, undesirable properties should be excluded such as: 1) relatively short shelf-life; 2) antigenicity testing; 3) transfusion reactions; 4) dependence on a limited donor pool; 5) infectious disease transmission; 6) immune system suppression (Dietz, Joyner et al. 1996; Scott, Kucik et al. 1997; Ketcham and Cairns 1999).

Although such a product has been sought for several centuries, it was not until the 1980s and significant investment by the United States military that commercial development of such a product began (Winslow 2000). Since then, two major approaches to artificial oxygen carriers have emerged: bio-artificial carriers and synthetic carriers. Of these, hemoglobin-based oxygen carriers (HBOCs) and perfluorocarbon emulsions (PFCs) are the most promising candidates (Spahn 2000; Kim and Greenburg 2004).

Perfluorocarbons

PFCs are synthetic aromatic carbon-fluorine compounds characterized by a high gas-dissolving capacity, low viscosity, and biological inertness. These compounds are emulsified in solution with an agent (58% perfluorooctyl bromide and 2% perluorodecyl bromide) that allows its dispersal in the blood (Nucci and Abuchowski 1998; Spahn and Pasch 2001). PFCs, unlike hemoglobin molecules, do not bind oxygen, but rather they increase the solubility of oxygen in the plasma (Chang 1999; Ketcham and Cairns 1999). The oxygen capacity of PFCs is linearly related to PO_2, making efficacy of such a solution dependent on the amount of oxygen available from inspired air (Goodnough, Scott et al. 1998).

Hemoglobin-based Oxygen Carriers

Currently, HBOCs are developed from stroma-free hemoglobin (SFH). SFH is derived from human or animal (typically bovine) red blood cells that have been lysed and the stroma removed through chemical methods, centrifugation, and/or filtration (Haney,

Buehler et al. 2000). However, unmodified SFH has a high oxygen affinity, elevated intravascular oncotic pressure, and is rapidly cleared through the kidneys (Ketcham and Cairns 1999; Winslow 2000). Furthermore, the tendency for the hemoglobin tetramer to dissociate into dimers often results in renal toxicity. For these reasons, chemical or genetic modifications need to be made to create the desirable oxygen offloading characteristics, extend the intravascular half-life, and curtail renal toxicity (Kim and Greenburg 2004).

Hemoglobin-based Oxygen Carriers

Modifications of the Hemoglobin Molecule

The high oxygen affinity and subsequent inability of unmodified HBOCs to efficiently deliver oxygen at tissue sites is due to the loss of 2,3-DPG during the purification process (Kim and Greenburg 2004). Normally, 2,3-DPG has a specific binding cleft within the tetrameric hemoglobin molecule which stabilizes the structure in the deoxy form and promotes positive cooperativity. Without 2,3-DPG oxygen binds to hemoglobin in the lungs, but will not be unloaded in the tissue (Nucci and Abuchowski 1998; Haney, Buehler et al. 2000). An alternative approach to avoid high oxygen affinity is to utilize low oxygen affinity bovine hemoglobin as a starting material. Contrary to human hemoglobin, the affinity of bovine hemoglobin for oxygen is not dependent on 2,3-DPG and can be modified to achieve the desired oxygen affinity and circulation time (Lee, Atsumi et al. 1989)

A variety of chemical modifications can be made to SFH to adjust the oxygen affinity to one resembling human encapsulated hemoglobin and to prevent other undesirable side effects. One method is to bind chemical subgroups to SFH to act in place of 2,3-DPG (Poly-SFH-P) (Ketcham and Cairns 1999). Another method is to cross-link the dimers of the hemoglobin tetramer through covalent bonds either intramolecularly or intermolecularly (commercial examples include diaspirin cross-linked hemoglobin and rHb1.1). Polymerization is another technique used to link tetramers together into longer chains (commercial examples include Poly-SFH-P and Oxyglobin), increasing the overall size of the structure and reducing the possibility of renal filtration and consequent renal damage (Nucci and Abuchowski 1998; Chang 1999; Ketcham and Cairns 1999). Conjugating hemoglobin with a polymer such as polyethylene glycol (PEG) also increases the size of the structure, reduces renal damage, and protects the molecule from renal excretion (Nucci and Abuchowski 1998; Kim and Greenburg 2004). More recently, recombinant human hemoglobin is produced by genetically engineered *Escherichia coli* bacteria, where the subunits are fused together to prevent tetramer disassociation (Chang 1999). Lastly, human or animal SFH can be encapsulated in a phospholipid vesicle, resulting in hemoglobin concentrations and reversible oxygen binding similar to that of human blood (Kim and Greenburg 2004).

Physiological Effects of HBOCs

A number of physiological side effects and negative attributes have been observed with HBOCs, despite successful modifications. Animals that have received hemoglobin

solutions maintain normal oxygen consumption and carbon dioxide production, as well as cardiac output, yet there are increases in mean arterial pressure, pulmonary artery pressure, and systemic vascular resistance (Dietz, Joyner et al. 1996). The hypertensive effects are caused by vasoconstriction, with studies observing up to 35% increases in diastolic and systolic pressures and 45% increases in systemic and pulmonary vascular resistance (Goodnough, Scott et al. 1998). Mechanisms of the vasopressor effect have been attributed to nitric oxide (NO) "scavenging" by hemoglobin, excess oxygen delivery to peripheral tissues, direct effects on peripheral nerves, the oxidative properties of hemoglobin (Sanders, Ackers et al. 1996), or endothelin release (Gulati, Sharma et al. 1996). However, most investigators have focused on NO interactions with hemoglobin.

NO, once referred to as endothelial derived relaxing factor (EDRF), normally induces vasodilation, thereby reducing blood pressure by the relaxation of vascular smooth muscle. With the addition of SFH, NO is believed to interact with bound oxygen in a process called NO dioxygenation (NOD) resulting in met-hemoglobin (metHb) and nitrate (NO_3^-). This process occurs rapidly and in vessels creates a diffusion gradient as the hemoglobin molecules stream along vessel walls acting as an NO "sink" reducing NO diffusion to smooth muscle receptors (Olson, Foley et al. 2004). This is known as NO scavenging. Studies have also suggested unpolymerized hemoglobin molecules may penetrate into the interstitial space (extravasation), resulting in a more direct interference with normal vasomotor activity (Ketcham and Cairns 1999; Spahn 2000; Olson, Foley et al. 2004).

NO scavenging has also been found to cause aggregation of platelets (Ketcham and Cairns 1999). The usual antiplatelet role of NO is removed due to scavenging by hemoglobin, resulting in increased platelet aggregation at sites of vascular injury (Ketcham and Cairns 1999; Creteur, Sibbald et al. 2000). However, hemodilution up to 50% of blood volume replacement with a HBOC should not significantly impair normal coagulation mechanisms (Kim and Greenburg 2004). Other contraindications of note include gastrointestinal distress and neurotoxicity (Ketcham and Cairns 1999; Kim and Greenburg 2004).

Another function of NO to consider is its ability to regulate respiration (Sarti, Giuffre et al. 2003). NO reversibly inhibits cytochrome *c* oxidase, the terminal electron acceptor in the mitochondrial respiration chain, by competing with oxygen (Cleeter, Cooper et al. 1994; Schweizer and Richter 1994). Nanomolar concentrations of NO have been found to reversibly inhibit cytochrome *c* oxidase which inhibits normal mitochondrial function causing oxygen consumption to decrease (Brown and Cooper 1994). Conversely, it has been demonstrated that the suppression of NO synthesis and therefore a decreased presence of NO available to act on the mitochondrial respiratory chain resulted in large increases in oxygen consumption (Shen, Hintze et al. 1995).

Possible Indications for HBOCs

The clinical applications of HBOCs are varied, but much focus is placed on utilization in the surgical setting, trauma medicine, and military applications. Purposed applications include use in situations of the following: shock/trauma, hemodilution,

perioperative use, perfusion of ischemic organs, septic shock, organ transplantation, and tumor therapy (Dietz, Joyner et al. 1996; Creteur, Sibbald et al. 2000). In addition, clinical application of HBOCs can be found in stroke, sickle cell crises, anemia, and patients with multiple red cell surface antibodies (Dietz, Joyner et al. 1996; Chang 1999). HBOCs are ideal for military and trauma settings as a result of long shelf life (> 1 year) at room temperature, avoidance of the need to cross-match patients, lack of adverse transfusion reactions, and minimized risk of disease transmission (Goodnough, Scott et al. 1998).

Hemodilution

Hemodilution refers to the increase in plasma volume resulting in a reduced concentration of red blood cells in the blood (hematocrit). Isovolemic hemodilution is the process of withdrawal of a specified volume of blood and replacement with an equal volume of fluid, either crystalloids, colloids, or artificial oxygen carrier. Hemodilutions up to 50% of the red blood cell mass and down to a hematocrit of 9% is well tolerated in physiological systems and the microcirculation (Habler and Messmer 2000; Tsai and Intaglietta 2001). The clinical significance of this procedure is that it can significantly reduce blood transfusion requirements during surgery and can be used in the treatment of acute ischemic stroke and peripheral arterial occlusive disease, among others (Spahn, Leone et al. 1994).

Acute normovolemic hemodilution (ANH) is often carried out in the perioperative setting. During ANH, a patient's blood is removed immediately before or during surgery and replaced with another fluid (Fakhry and Sheldon 1995; Scott, Kucik et al. 1997).

Both isovolemic hemodilution and ANH, at mild to moderate levels, result in an increase in cardiac output and blood flow velocity through the decrease of blood viscosity. These physiological alterations constitute a compensatory mechanism for the decrease in arterial oxygen content, so that oxygen delivery is maintained (Spahn, Leone et al. 1994; Tsai and Intaglietta 2001).

Erythrocyte-associated Transients in PO_2

Blood flows in large vessels as a homogeneous mixture where the components, plasma and red blood cells, cannot be easily distinguished. This trend continues as the bulk flow of blood moves from arteries to arterioles and finally into capillaries. It is at the capillary level that the individual red blood cells can be easily discerned from the plasma spaces around them. The phrase "particulate (or discrete) nature of blood flow" has been used to describe this phenomenon in capillaries.

In recognition of this phenomenon, in 1977 Hellums proposed the term "erythrocyte-associated transients" (EATs) (Hellums 1977; Hellums, Nair et al. 1996) which refers to the fluctuations of PO_2 in a capillary caused by the alternate passage of individual red blood cells and plasma gaps. Since then, many mathematical models have been developed to explore the implications of these transients (Federspiel and Sarelius

1984; Federspiel and Popel 1986; Groebe and Thews 1989; Popel 1989; Wang and Popel 1993; Hellums, Nair et al. 1996; Kisliakov 1996; Eggleton, Roy et al. 1998).

These transients are a manifestation of the PO_2 gradients between the plasma and red blood cells with varying amplitudes dependent on cell spacing, shape, and orientation. The PO_2 gradient is also affected by the diffusion gradient that exists for oxygen outside of the capillary. In many mathematical models, intracapillary resistance has been shown to be a sizable contribution to overall resistance to oxygen transport. This stems, presumably, from the low solubility of oxygen in the plasma, which hinders diffusive oxygen movement down its gradient from the red blood cell through the surrounding plasma sheath and plasma gaps into the tissue (Golub and Pittman 2005; Pittman 2005).

Purpose of the Present Study

In this study I set out to observe erythrocyte-associated transients in capillary PO_2 in the rat spinotrapezius muscle under conditions of isovolemic hemodilution with a non-oxygen carrying plasma expander, Hespan, and a hemoglobin-based oxygen carrier, Oxyglobin. When using Hespan as the hemodiluent, one might expect cell spacing in capillaries to increase and intracapillary resistance to oxygen offloading to also increase as hematocrit and total hemoglobin concentration decreased with isovolemic hemodilution. A direct consequence of this would be more pronounced transients of EATs and therefore a greater difference between red blood cell PO_2 and plasma PO_2.

Conversely, when using an artificial oxygen carrier as the hemodiluent under similar conditions of isovolemic hemodilution, one might expect an increase in cell spacing but a decrease in intracapillary resistance to oxygen offloading as a result of an increased contribution of an oxygen carrier to the plasma. This, in turn, would result in a reduction in EAT transients.

The purpose of the present study is to apply a detection method previously used for EATs in the rat mesentery to a skeletal muscle model to answer the following questions: 1) What happens to EATs with the addition of a non-oxygen carrying plasma expander, like Hespan, under isovolemic hemodilution?; 2) What happens to EATs with the addition of an oxygen-carrying plasma expander such as a HBOC under isovolemic hemodilution?

MATERIALS AND METHODS

Anesthesia and Animal Preparation

Sixteen female Spraque-Dawley rats (Harlan, Indianapolis, IN; weight of 241.8 (SD 14.7) grams, age of 83.4 (SD 6.3) days were housed in plastic isolation containers with continual ventilation in a climate-controlled room kept at 20-23 °C with a continuous 12 hr:12 hr light-dark cycle. Laboratory chow and water were provided ad libitum under the supervision of a certified animal care technician.

Due to technical limitations, it was not possible to obtain capillary PO_2 values from all animals for which systemic data were obtained. Table 1 catalogs the number of animals in the groups where systemic and capillary data were acquired.

Initial anesthesia with a combination of Acepromazine (2.5 mg/kg) and Ketamine (75 mg/kg) was administered via intraperitoneal (i.p.) injection. A continuous infusion of Saffan (Alphaxolone 9 mg/ml and Alphadolone 3 mg/ml, Schering-Plough Animal Health, Hertfordshire, England) was used for supplemental anesthetic maintenance administered intravenously (i.v.) via the cannulated right jugular vein using an infusion pump (Harvard Apparatus Co., Inc., Dover, Mass.) set at an average of .00188 (SD .001) ml/min. Upon conclusion of experimentation, the animals were euthanized by Euthasol (Pentobarbital 390 mg/ml and Phenytoin 50 mg/ml, Delmarva Laboratories, Inc., Midlothian, VA) administration of 0.4 ml/kg.

Table 1. Number of animals and capillaries used for systemic and PO_2 determination.

	Control	Step 1	Step 2
Hespan	8/2/26	8/5/26	5/5/53
HBOC	8/2/20	8/5/26	5/5/50

Entries in the Table occur in the format: X/Y/Z where X = Number of animals with systemic data; Y = Number of animals with capillary data; Z = Number of capillaries analyzed from the data set.

Hair between the ventral clavicles and mentum, ventral right groin, and dorsal regions was removed by electric shears, followed by a depilatory cream (Nair, Church and Dwight Co., Inc., Princeton, NJ). Afterward, the animal was placed in the supine position on a metal surgical platform with temperature regulation via automated electronic controls for cannulations and muscle exteriorization.

Tracheal and Vascular Cannulations

First, to ensure a patent airway and spontaneous room-air breathing, the trachea was cannulated using a 4 cm length of polyethylene tubing (PE-240, Clay Adams, Parsippany, NJ). Subsequent cannulation of the right jugular vein with PE-90 tubing and the left common carotid artery with PE-50 tubing allowed for continuous i.v. administration of Saffan and arterial pressure monitoring, respectively. Further cannulations of the left femoral vein and artery were performed using PE-20 and PE-10 tubing (Clay Adams, Parsippany, NJ), respectively, under a stereo microscope (Nikon SMZ660, Melville, NY). The femoral artery was used for the withdrawal of blood during the hemodilution procedure, blood sampling for arterial blood gas measurements (ABL 705, Radiometer America Inc., Westlake, OH), and hemoglobin measurements (Radiometer OSM3, Radiometer, Crawley, West Sussex, UK). The femoral vein was utilized for Hespan or HBOC infusion and albumin-bound Pd-porphyrin phosphorescence probe injection. Heparinized saline (20 units of heparin/ml of normal saline) or continuous infusion ensured patency of all vascular lines.

Spinotrapezius Preparation

The use of intravital microscopy techniques require the surgical exteriorization of the spinotrapezius muscle first described by Gray (Gray 1973). A modification of Gray's technique was used in which a dorsal midline incision was made from the lumbar to the cervical region, exposing the underlying superficial connective tissue. The tissue was removed cautiously, taking care not to damage the thin strap muscle on the left lateral border running parallel to the spinotrapezius muscle. A pair of curved scissors was used to separate the muscle from the underlying muscle layers starting at the left lateral border towards the muscle insertion point on the scapular spine and continuing towards the distal end of thoracolumbar origination. 5-0 silk was sutured every 1 cm along the strap muscle as the muscle was separated to ensure minimal trauma to the spinotrapezius and to facilitate surgical and experimental muscle manipulation. The right lateral border was excised from the vertebral axis, liberating the muscle except from its scapular attachment, and likewise sutured. A low-temperature cautery device (Gemini RS-300, Roboz Surgical Instrument Co., Rockville, MD) was used to staunch any vascular bleeding during the exteriorization procedure and the tissue was kept moist with frequent application of normal saline. After exteriorization of the spinotrapezius the animal was placed on a thermostatic animal platform (Golub and Pittman 2003).

Phosphorescence Intravital Microscope

The preparation was observed with an Ortholux microscope (Leitz, Germany) configured for epi-illumination through a 100X/1.30 objective (Leitz, Germany).

Immersion oil was placed between the objective and the Saran covering the spinotrapezius muscle.

Phosphorescence excitation was achieved with a TTL-modulated DPSS 532 nm laser (GMS1-038-15T, Lasermate Corp., Pomona, CA) which delivered 60 nJ for a duration of 10 μs to a 0.9 μm diameter point at 100 Hz. The laser light was reflected by a dichroic mirror (565 DCLP, Chroma Technology Corp., Rockingham, VT) into the objective lens, then focused onto the mid-plane of a capillary with an adjustable correction lens between the laser and the microscope. Off-axis alignment of the laser was used to eliminate reflection of laser light from the various optical surfaces. A photomultiplier tube (PMT; R3896 with high voltage socket HC123-01, Hamamatsu Corp., Bridgewater, NJ) detected the emitted phosphorescence that was collected by the 100X objective. A sliding mirror allowed for either video imaging or phosphorescence light collection with the PMT. A CCD camera (WAT-902B, Watec Co., Ltd., Japan) and a 5.6" monochrome LCD monitor (45M056, Imaging Solutions, Inc., Korea) where used for video imaging and indirect capillary observation.

The signal from the PMT was directed to a modified amplifier (OP37EP, Analog Devices, Norwood, MA) designed to be a current-to-voltage converter outfitted with a precision analog switch (ADG419BN, Norwood, MA) allowing it to be disabled during the laser pulse generation of 10 μs duration at 100 Hz. Therefore, each phosphorescence decay curve was recorded from the end of the 10 μs excitation pulse until practical zero was reached, a requirement for 12-bit resolution. The signal was directed from the current-to-voltage converter to a 12-bit analog-to-digital converter (PC-MIO-16E-4,

National Instruments, Austin, TX). The digital data were stored on a Dell PC (Round Rock, TX).

Data acquisition was controlled by the "DualGetCurve" program written in LabVIEW (National Instruments, TX) and allowed sampling at 200 kHz with 400 data points per curve and 100 curves per measurement sequence. Origin 6.1 (OriginLab, Northampton, MA) was used to analyze acquired data.

The laser light passing through the capillary was detected with a T-5 photodiode (Intor, Socorro, NM) integrated with a 532 nm laser line filter. The signal collected from the diode was converted to a voltage with Amplifier B and directed to the second channel of the analog-to-digital converter and stored in the PC. Both the phosphorescence and light transmission signals were monitored with an oscilloscope (72-3060, Tenma, Springboro, OH).

Selection of a capillary for measurements, and positioning the focal plane of the laser was carried out under transillumination using an OG-570 filter (Edmund Optics, Barrington, NJ). During periods of capillary PO_2 and light transmission (LT) measurement, background light sources were minimized to reduce any effect of stray light. Figure 1 is a schematic diagram of the phosphorescence quenching microscope setup described above.

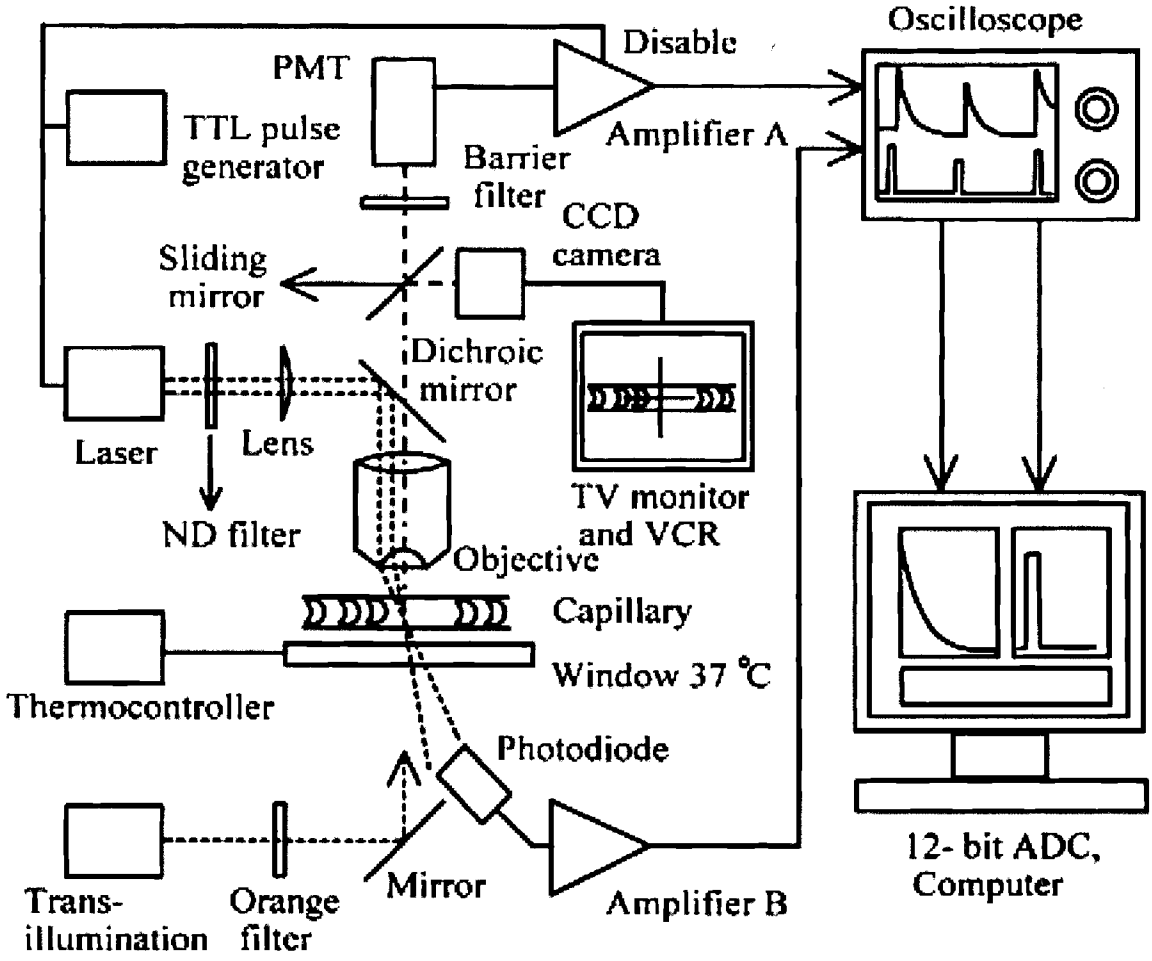


Figure 1. Representative Phosphorescence Intravital Microscope Setup

Solution and Probe Preparations

Preparation of Albumin-bound Pd-porphyrin Phosphorescent Probe

Preparation of 20 ml of albumin-bound phosphorescence probe solution (10 mg/ml) required binding of 200 mg palladium meso-tetra-(4-carboxyphenyl)-porphyrin (Pd-MTCPP; Oxygen Enterprises, Philadelphia, PA) to 1.32 g bovine serum albumin (BSA; Fraction V, Sigma, St. Louis, MO). Over a period of approximately 30 min the Pd-MTCPP was added to a 1 mM BSA solution and stored overnight. This solution was dialyzed in 1 mM polyvinylpyrrolidine solution (PVP; Sigma-Aldrich, Inc.) and Spectra/Por-4 membranes to eliminate any unbound Pd-MTCPP from solution. After 18 hr the solution was removed from the dialysis membranes and filtered through a sterilization filter unit (Nalgene, Rochester, NY). Aliquots of 0.5 ml of the final probe solution were placed in microcentrifuge tubes, flash frozen in liquid nitrogen, and promptly stored at -80 °C.

Properties and Preparation of Oxyglobin®

Oxyglobin® (also known as HBOC-301), a bovine hemoglobin glutamer (Biopure, Cambridge, Mass.), is a 7.8 pH solution containing 13 g/dl polymerized bovine hemoglobin in a modified lactated Ringer's solution with an osmolality of 300 mOsm/kg. The P_{50} for Oxyglobin is 35 mmHg. Less than 5% of the hemoglobin polymer solution is comprised of unstabilized tetramers, approximately 50% has a molecular weight between 65 and 130 kD with no more than 10% having a molecular weight >500 kD. Oxyglobin

can be stored at room temperature or refrigerated at 2-30 °C for up to 36 months. Plasma half-life is estimated to range between 18 and 43 hours for dosages of 10-30 ml/kg in dogs.

Heparinized Saline

Heparinized saline was necessary to assure patency of cannulas with intravascular placement. The solution was prepared using 5 ml heparin solution (100 units/ml; Heparin Lock, BD, Franklin Lakes, NJ) per 20 ml 0.9% NaCl normal saline solution. Only 1 ml syringes were used for each cannulated vessel.

Hespan

Hespan (Abbott Laboratories, Chicago, IL) belongs to the class of drugs called plasma volume expanders and consists of 6% hetastarch in a 0.9% sodium chloride solution. It is a nonpyrogenic, sterile fluid isosmotic with blood (310 mOsm/l) and has no significant oxygen-carrying capacity.

PO_2 Measurements

Capillary Selection and Probe Administration

Before the partial pressure of oxygen (PO_2) was measured, each capillary considered had to meet the following specified selection criteria: 1) sufficient light transmission (LT) with evident red blood cell (RBC) fluctuations as observed on the

oscilloscope, 2) continuous RBC flow, 3) surface capillary, and 4) three-dimensional capillary isolation from other microvessels, arterioles, and venules. After capillary selection, PO_2 was determined in each capillary using the phosphorescence quenching method for intravital microscopy (Zheng, Golub et al. 1996). Measurements were made in the centerline of the capillary in a region of high epi-illumination and visual contrast.

The phosphor probe (30 mg/kg) was administered via the right femoral vein prior to taking measurements. Approximately one minute of equilibration time was allowed before initiation of PO_2 measurements.

Phosphorescence Quenching Microscopy (PQM)

PO_2 determination using PQM is based on O_2-dependent quenching of phosphorescence from photoexcited Pd-porphyrin. The Stern-Volmer equation defines the relationship between phosphorescence lifetime (τ) and PO_2 as follows:

Equation 4

$$1/\tau = 1/\tau_o + k_q PO_2$$

where τ_o (546 μs) is the lifetime of phosphorescence decay in the absence of O_2 and k_q is the quenching coefficient ($3.06 \cdot 10^{-4}$ $\mu s^{-1} mmHg^{-1}$). Phosphorescence decay curves were analyzed using a non-linear curve fitting the Rectangular distribution model in Origin 6.1 software (Microcal Software Inc., Northampton, MA) as developed by Golub et al. (Golub, Popel et al. 1997):

Equation 5

$$I(t) = I(0)exp[-(k_0 + k_qM)t]\cdot sinh(k_q\delta t)/(k_q\delta t) + Aexp(-t/T) + B$$

where t (μs) is the time from the beginning of phosphorescence decay, $I(t)$ (volt) is the magnitude of the phosphorescence signal, $I(0)$ (volt) is the amplitude of the phosphorescence signal at t = 0, M (mmHg) is the mean PO_2, δ (mmHg) is the half width of the PO_2 distribution, T (μs) is the lifetime of the fast post-excitation transient, A (volt) is the amplitude of the fast post-excitation transient and B (volt) is the baseline offset. Constants $k_0 = 18.3 \cdot 10^{-4}$ μs^{-1} and $k_q = 3.06 \cdot 10^{-4}$ $\mu s^{-1} mmHg^{-1}$ were determined by Zheng et al. (Zheng, Golub et al. 1996). A representative plot, Figure 2, illustrates this relationship.

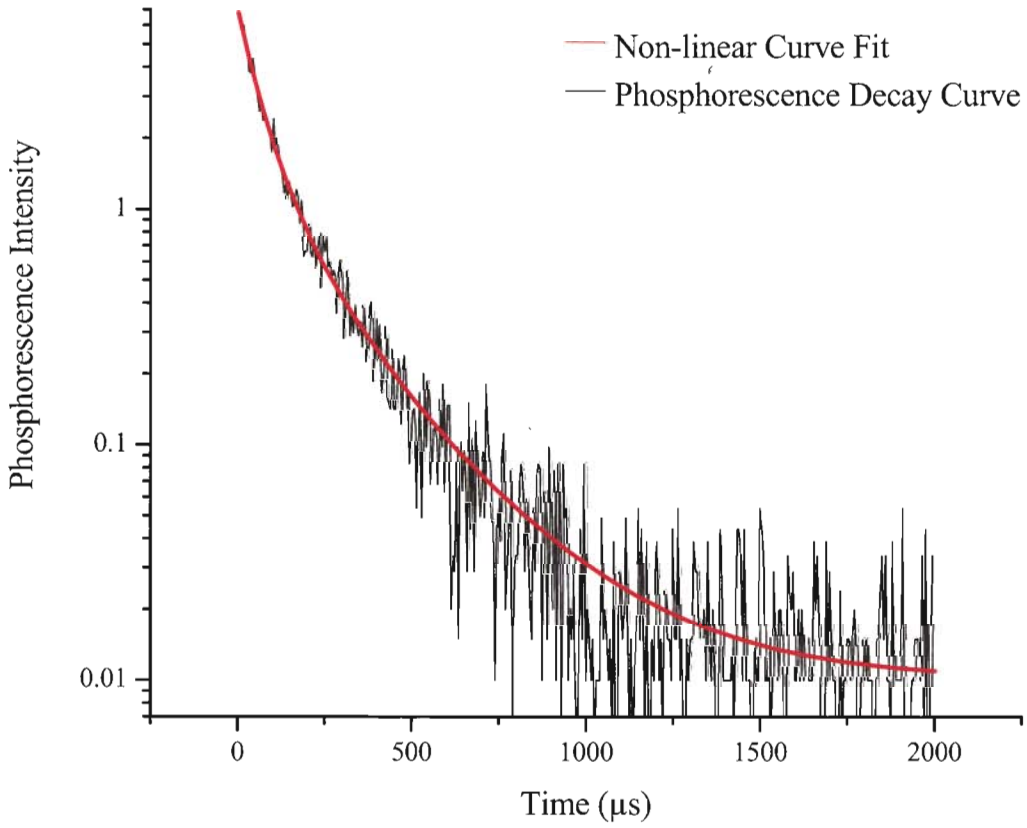


Figure 2. Representative phosphorescence decay curve.

The phosphorescence decay curve shown on a logarithmic scale is composed of 400 connected data points taken at 200 kHz over 2000 μs. The decay curve was analyzed using a non-linear curve fitting the Rectangular distribution model and is shown here by the continuous line fit.

Light Transmission, Phosphorescence Amplitude Measurements, and PO_2

In order to detect fluctuations of PO_2 in the detection volume associated with the presence of RBCs or plasma gaps, light transmission (LT) and phosphorescence amplitude (PA) were measured.

LT was measured via photodiode registration of the intensity of the laser light passing through a capillary and the data collected by the "DualGetCurve" program. Maximum PA was determined from analyzing the beginning of the decay curve after the end of the excitation pulse. The maximum amplitude of the phosphorescent signal is proportional to the amount of plasma present in the detection volume, because the probe is homogeneously dissolved in plasma and does not enter RBCs. This was demonstrated using densitometric scanning and phosphorescence imaging of the Pd-porphyrin probe location that detected the presence or absence of RBCs (Golub and Pittman 2005).

Upon analysis, the maximum amplitude of the phosphorescence decay curve (PA) and the light transmittance pulses were averaged using the three highest points at the peak of each signal. Noise from LT, PA, and PO_2 values was reduced by filtering the signal with a fast Fourier transform filter (FFT; Origin 6.1, OriginLab, Northampton, MA) with 50 Hz and 20 Hz cutoff frequency filtering.

Although 100 individual PO_2 measurements were made over the 1 s time course, a system was derived to choose which capillaries and PO_2 measurements to calculate across the 1 s data collection interval. Golub and Pittman (2005) made two conclusions

that provided selection parameters for PO_2 calculation: 1) high positive correlations between PA and LT predicted the presence of EATs; 2) PA and PO_2 had a more significant negative correlation than LT and PO_2 indicating PA is a more consistent predictor of whether or not a RBC is in the detection region. With these parameters in mind, capillary selection was based on the highest correlation coefficients for each condition. Furthermore, capillary PO_2 for red blood cells and plasma gaps was determined using the ten lowest PA values and the ten highest PA values, respectively, from the 100 collected data points. This corresponds to the idea that high PA should indicate the presence of plasma in the detection volume and low PA should indicate the presence of a RBC in the detection volume.

Of the total 319 capillaries that were measured, 201 capillaries were chosen to determine PO_2 values corresponding to the combined 20 high and low PA values. Each of the PO_2 calculations using Origin 6.1 (OriginLab, Northampton, MA) took approximately two minutes.

Mean Arterial Pressure

Mean arterial pressure (MAP) was continuously monitored from the right common carotid artery using the CyQ 103/301 acquisition and display system (CyberSense Inc., Nicholasville, KY). Specific measures of MAP were recorded under control conditions and after a 10 min stabilization period subsequent to each hemodilution step.

Hemodilution Procedure

Isovolemic hemodilution was accomplished by the exchange of a given volume of blood with an equal volume of fluid (e.g., colloid, crystalloid, artificial oxygen carrier), resulting in decreased hematocrit, while maintaining a constant total blood volume. During this study, a two step withdrawal/infusion process was utilized with each step taking approximately forty-five minutes to complete. Additionally, each of these steps was reached by dividing the hemodilution volumes into four discrete withdrawal/infusion volumes.

Isovolemic hemodilution was achieved through the withdrawal of blood from the right femoral artery followed by infusing the hemodiluent into the right femoral vein. The hematocrit of each animal was systematically lowered through two hemodilution steps: from 39.5 ± 2.7 % (Control, mean ± SD) to hematocrit of 27.5 ± 2.8 % (Step 1, mean ● SD for both hemodiluents) and then to a hematocrit of 13.5 ± 2.1 % (Step 2, mean ± SD for both hemodiluents).

In using this "discrete" method of hemodilution the effective blood volume associated with hemodilution, V_B, was initially determined by:

Equation 6

$$V_B = \beta \cdot W_B$$

where β is a proportionality factor in ml/g and W_B is the body weight of the animal in grams. A proportionality factor of 0.06 ml/g was used for these experiments.

Determination of initial systemic hematocrit, H_I, and final systemic hematocrit, H_F, was achieved through a centrifuged 75 µl sample collected from the right femoral arterial line in a heparinized microhematocrit capillary tube (Scientific Products, McGaw Park, IL). Assessments of systemic hematocrit were performed at control conditions and after hemodilution Steps 1 and 2.

The target value of H_F after each hemodilution step was defined by the following equation:

Equation 7

$$H_F = (1 - (V_{HD}/V_B)) \cdot H_I$$

where V_{HD} is the hemodilution volume. This volume of blood is withdrawn and replaced with an equal volume of hemodiluent. Therefore, the volume of blood that needs to be removed and then replaced is:

Equation 8

$$V_{HD} = V_B \cdot (1 - (H_F/H_I))$$

PO_2 measurements were made during control conditions (N = 16) and after hemodilution Step 2 in ten animals, five for each Hespan and Oxyglobin conditions. PO_2 measurements were also made after the intermediate hemodilution Step 1, eight for each Hespan and Oxyglobin conditions.

Statistics

Data within these experiments are shown as mean ± standard deviation in tables and mean ± error in figures. One Way Analysis of Variance (ANOVA) was conducted with the software JMP version 4 (SAS Institute Inc., Cary NC) to assess statistical significance for the means of each experimental condition. Statistical significance between PO_2 values in the different hemodilution steps was determined using Tukey's Multiple Comparison test. Additional testing of ΔPO_2 values in the different hemodilution steps was accomplished with a Student's t-test. Linear regression statistics were also calculated using JMP version 4. Statistical significance was taken to be $p < 0.05$.

RESULTS

Phosphorescence Amplitude, Light Transmission and PO_2

An example of simultaneous one second recordings containing 100 consecutive points for measurements of PO_2, PA, and LT are shown in Figure 3 for a single capillary after Step 1 of isovolemic hemodilution with Hespan. The PA and LT signals changed in parallel while there were inverse temporal variations of PO_2 that occurred in comparison to the PA and LT signals. This is demonstrated quantitatively in Figure 4 and Figure 5 which yielded the following correlation coefficients: 0.905 for PA versus LT, -0.354 for PO_2 versus LT, and -0.244 for PO_2 versus PA. Similar PA and LT modulations were observed in other capillaries (n = 101). In capillaries where all PO_2 measurements (100 data points generating 100 distinct PO_2 measurements) were analyzed (n = 5), similar modulations in comparison to the single time course presented in Figure 3 were also observed.

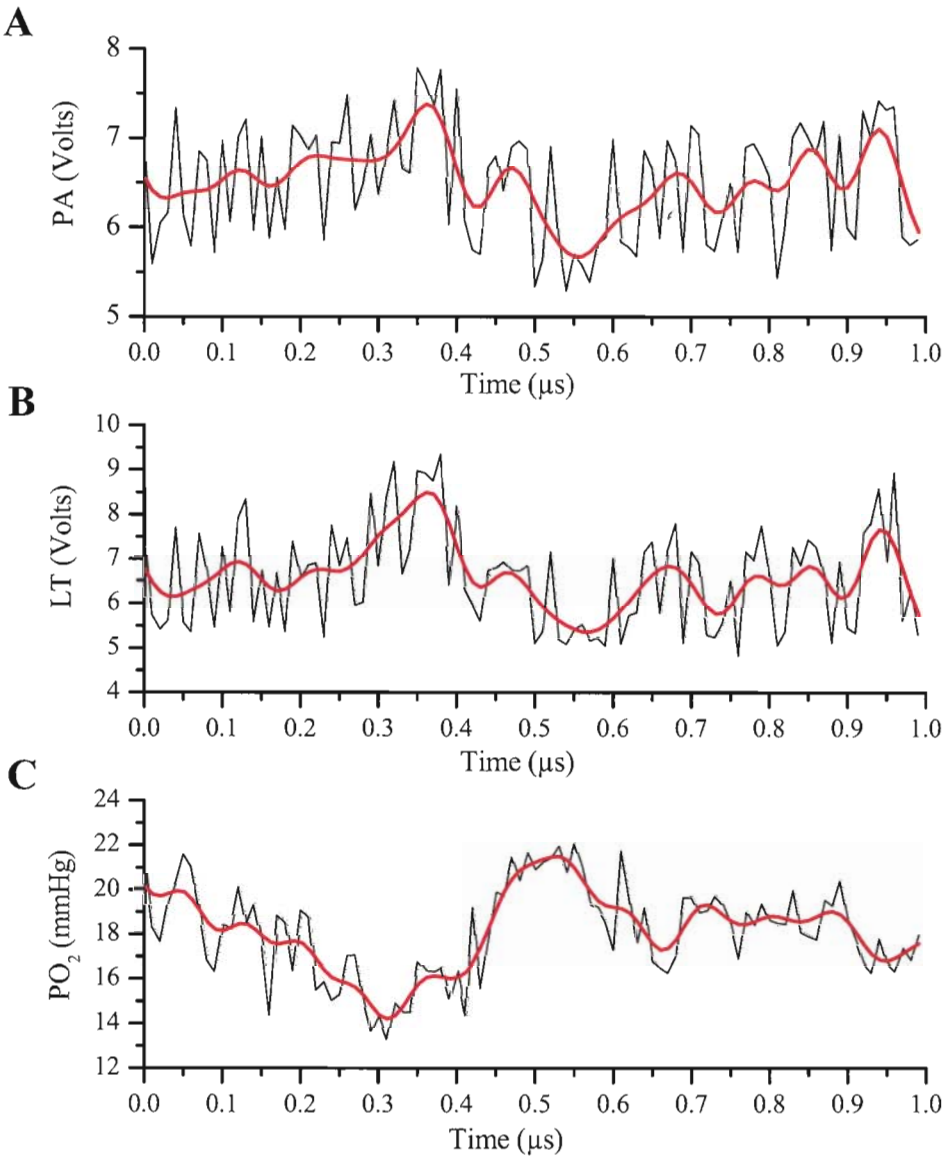


Figure 3. Example of PO2, light transmission, and phosphorescence amplitude versus time.

Tracings represented by the non-smoothed lines represent simultaneous one second recordings containing 100 consecutive data points taken from a stationary observation point in a capillary. Smoothed line represents the data using a 50-20 Hz fast Fourier transform (FFT). See text for further explanation.

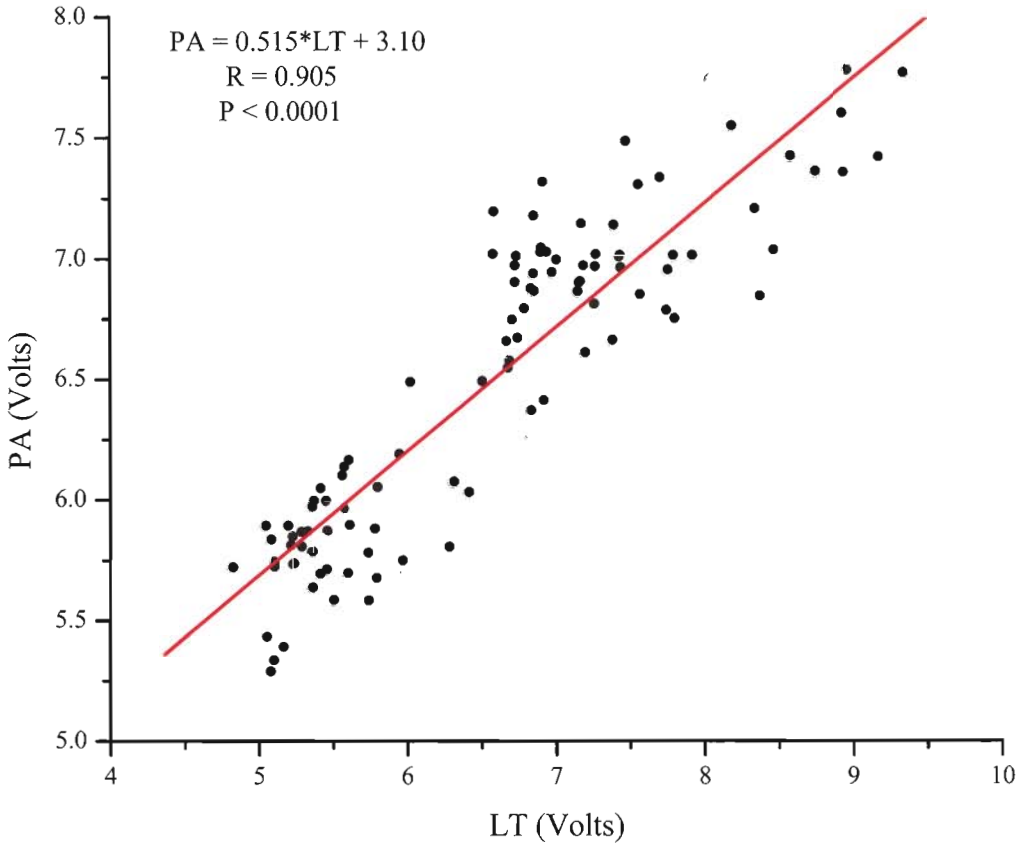
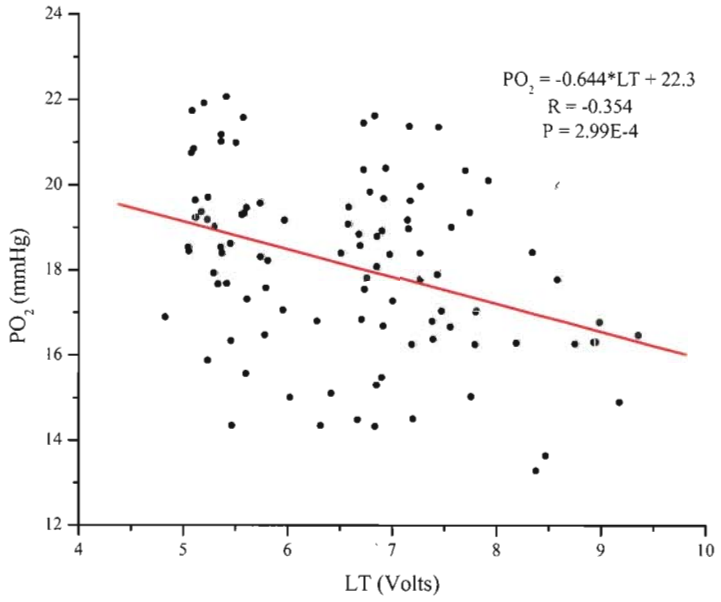


Figure 4. Plot of phosphorescence amplitude versus light transmission.

PA and LT data are from a simultaneous one second recording containing 100 consecutive data points taken from a stationary observation point in a capillary and plotted here against each other. See text for further explanation.

A



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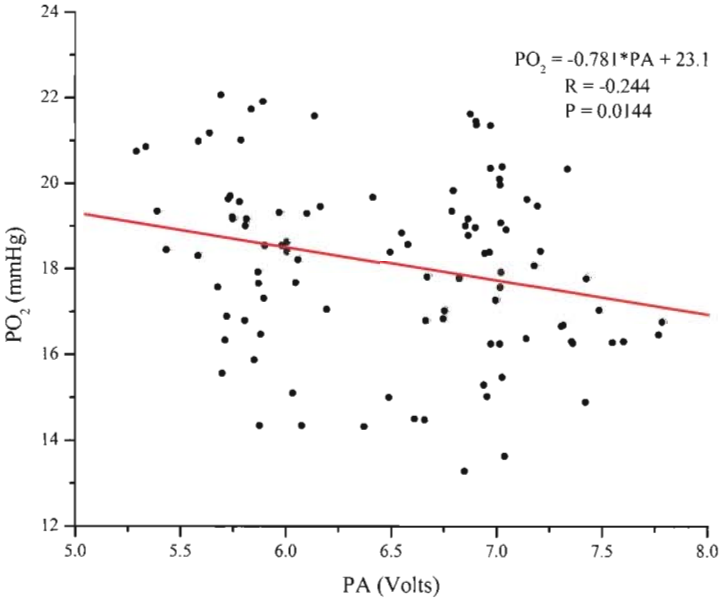


Figure 5. Plots of partial pressure of oxygen versus phosphorescence amplitude and light transmission.

Effects of Hemodilution with Hespan and Oxyglobin on Systemic Variables

Mean Arterial Pressure and Hematocrit

Systemic hematocrit was reduced in two steps to 26.3 ± 2.5 % (Step 1) and then to 14.6 ± 2.3 % (Step 2) through isovolemic hemodilution with Hespan. In a separate group of animals, systemic hematocrit was reduced to 28.6 ± 2.6 % (Step 1) and then to 12.3 ± 1.1 % (Step 2) through isovolemic hemodilution with Oxyglobin. A Control value of 39.5 ± 2.7 % was used for comparisons with both Hespan and Oxyglobin groups. As hematocrit was reduced using Oxyglobin as the hemodilution fluid, the mean arterial pressure increased from 102.9 ± 10.2 mmHg (Control) to 124.4 ± 21.5 mmHg (Step 1) and even further to 135.8 ± 17.7 mmHg (Step 2). Mean arterial pressure increased when Hespan was used as the hemodilution fluid from 102.9 ± 10.2 mmHg (Control) to 105.1 ± 16.9 mmHg (Step 1) and decreased after Step 2 to 97.4 ± 15.6 mmHg. The values for systemic hematocrit and mean arterial pressure for Control, Step 1, and Step 2 for both Hespan and Oxyglobin are presented in Table 2 and in graphic form in Figure 6.

One Way Analysis of Variance yielded statistically significant differences between Control, Step 1, and Step 2. Tukey's Multiple Comparisons test revealed significant differences with mean arterial pressure values between Control and Steps 1 and 2 in the Oxyglobin group. Tukey's test also revealed significant differences of Oxyglobin Step 2 to both Hespan Steps 1 and 2, while Step 1 of hemodilution with Oxyglobin was significant to Hespan Step 1.

Table 2. Mean (± standard deviation) systemic data for all animals.

	Control (N=16)	Hespan HD1 (N=8)	Oxyglobin HD1 (N=8)	Hespan HD2 (N=5)	Oxyglobin HD2 (N=5)
MAP	102.9 ± 10.2	105.1 ± 16.9	124.4 ± 21.5	97.4 ± 15.6	135.8 ± 17.7
P_aO_2	93.0 ± 9.9	73.5 ± 11.7	57.8 ± 21.0	79.1 ± 13.1	75.3 ± 9.1
S_aO_2	92.9 ± 2.5	90.1 ± 5.5	79.2 ± 11.0	91.4 ± 6.3	77.4 ± 3.8
P_aCO_2	51.7 ± 16.3	39.7 ± 5.5	28.6 ± 5.4	31.1 ± 2.4	29.9 ± 5.3
pH	7.31 ± 0.05	7.37 ± 0.04	7.50 ± 0.08	7.43 ± 0.04	7.53 ± 0.06
[Hb]	13.7 ± 0.7	10.1 ± 2.1	12.6 ± 1.1	5.1 ± 0.8	11.3 ± 0.5
Hct	39.5 ± 2.7	26.3 ± 2.5	28.6 ± 2.6	14.6 ± 2.3	12.3 ± 1.1
MCHC	34.7 ± 2.4	35.1 ± 3.6	34.5 ± 1.3	35.1 ± 3.6	34.5 ± 1.3
$[Hb]_{RBC}$	13.7 ± 0.7	9.5 ± 1.6	9.9 ± 1.2	5.2 ± 0.9	4.3 ± 0.3
$[Hb]_{plasma}$	0 ± 0	0 ± 0	3.7 ± 1.7	0 ± 0	8.0 ± 0.4

MAP = Mean arterial pressure (mmHg); P_aO_2 = Systemic arterial partial pressure of oxygen (mmHg); S_aO_2 = Arterial oxygen saturation (%); P_aCO_2 = Systemic arterial partial pressure of carbon dioxide (mmHg); pH_a = Systemic arterial pH; [Hb] = Total hemoglobin concentration (g/dl); Hct = Systemic hematocrit (%); MCHC = Mean corpuscular hemoglobin concentration (g/dl); $[Hb]_{RBC}$ = Red blood cell hemoglobin concentration (g/dl); $[Hb]_{plasma}$ = Plasma hemoglobin concentration (g/dl); HD1 = Hemodilution Step 1; HD2 = Hemodilution Step 2; N = Number of animals.

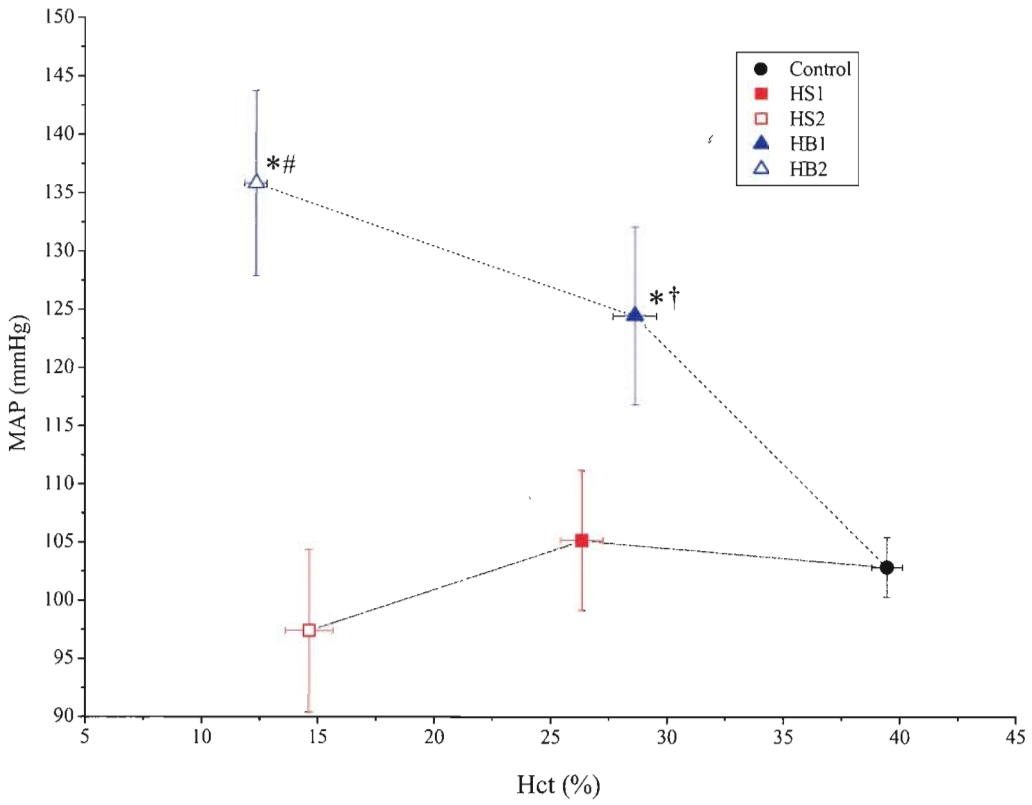


Figure 6. Plot of mean arterial pressure versus hematocrit for hemodilution with Hespan and Oxyglobin.

The filled circle represents the Control value, the filled and open triangles represent the HBOC Oxyglobin values at Step 1 (HB1) and Step 2 (HB2), respectively, and the filled and open squares represent Hespan values at Step 1 (HS1) and Step 2 (HS2), respectively. Standard error bars are shown.

* = Significantly different from Control ($p<0.05$)

= Significantly different from both Hespan Steps 1 and 2 ($p<0.05$)

† = Significantly different from Hespan Step 2 ($p<0.05$)

Mean Arterial Pressure and Plasma Hemoglobin

The plasma hemoglobin concentration increased from an assumed value of 0 g/dl (Control) to 3.7 ± 1.7 g/dl in Step 1 and then to 8.0 ± 0.4 g/dl in Step 2 for hemodilution with Oxyglobin. As plasma hemoglobin concentration increased, the mean arterial pressure increased from 102.9 ± 10.2 mmHg (Control) to 124.4 ± 21.5 mmHg after Step1 and then to 135.8 ± 17.7 mmHg after Step 2 of the isovolemic hemodilution procedure with Oxyglobin. Step 1 and Step 2 for Hespan are not shown because Hespan makes no contribution to plasma hemoglobin concentrations. Linear regression statistics revealed a correlation of 0.662 and p value of < 0.0001. These results are presented in Table 3 and summarized in graphic form in Figure 7.

Effects of Hemodilution with Hespan and Oxyglobin on PO_2 in Capillaries

Red Blood Cell PO_2 and Comparisons with Hematocrit, Total Hemoglobin Concentration, and Plasma Hemoglobin Concentration

Results for the subset of data in which PO_2 was measured in capillaries are contained in Table 3 and Table 4.

Red Blood Cell PO_2 and Hematocrit

Red blood cell PO_2 decreased from 54.9 ± 14.6 mmHg (Control) to 36.7 ± 18.4 mmHg (Step 1) then to 22.2 ± 2.5 mmHg (Step 2) as the hematocrit was reduced from 38.8 ± 1.9 % (Control) to 23.8 ± 2.5 % (Step 1) then to 14.6 ± 2.3 % (Step 2) through isovolemic hemodilution with Hespan. Hemodilution with Oxyglobin exhibited

Table 3. Mean (± standard deviation) systemic data for animal sub-group.

	Control (N=4)	Hespan HD1 (N=2)	Oxyglobin HD1 (N=2)	Hespan HD2 (N=5)	Oxyglobin HD2 (N=5)
Capillary n	46	26	26	53	50
MAP	99.8 ± 14.8	115.8 ± 20.9	121.7 ± 27.2	97.4 ± 15.6	135.8 ± 17.7
P_aO_2	97.0 ± 12.2	78.8 ± 7.8	66.9 ± 40.0	79.1 ± 13.1	75.3 ± 9.1
S_aO_2	93.6 ± 4.2	92.0 ± 1.3	81.7 ± 14.8	91.4 ± 6.3	77.4 ± 3.8
P_aCO_2	45.1 ± 3.5	42.8 ± 4.4	23.0 ± 4.1	31.1 ± 2.4	29.9 ± 5.3
pH	7.31 ± 0.00	7.34 ± 0.08	7.56 ± 0.11	7.43 ± 0.04	7.53 ± 0.06
[Hb]	13.5 ± 0.6	10.8 ± 4.7	13.1 ± 0.4	5.1 ± 0.8	11.3 ± 0.5
Hct	38.8 ± 1.9	23.8 ± 2.5	28.1 ± 1.5	14.6 ± 2.3	12.3 ± 1.1
MCHC	34.9 ± 1.3	33.9 ± -	33.0 ± 0.8	35.7 ± 4.2	34.8 ± 1.2
$[Hb]_{RBC}$	13.5 ± 0.6	8.6 ± -	9.3 ± 0.7	5.2 ± 0.9	4.3 ± 0.3
$[Hb]_{plasma}$	0 ± 0	0 ± 0	5.3 ± 1.5	0 ± 0	8.0 ± 0.4

MAP = Mean arterial pressure (mmHg); P_aO_2 = Systemic arterial partial pressure of oxygen (mmHg); S_aO_2 = Arterial oxygen saturation (%); P_aCO_2 = Systemic arterial partial pressure of carbon dioxide (mmHg); pH_a = Systemic arterial pH; [Hb] = Total hemoglobin concentration (g/dl); Hct = Experimental animal systemic hematocrit (%); MCHC = Mean corpuscular hemoglobin concentration (g/dl); $[Hb]_{RBC}$ = Red blood cell hemoglobin concentration (g/dl); $[Hb]_{plasma}$ = Plasma hemoglobin concentration (g/dl); N = Number of animals; n = Number of capillaries; HD1 = Hemodilution Step 1; HD2 = Hemodilution Step 2.

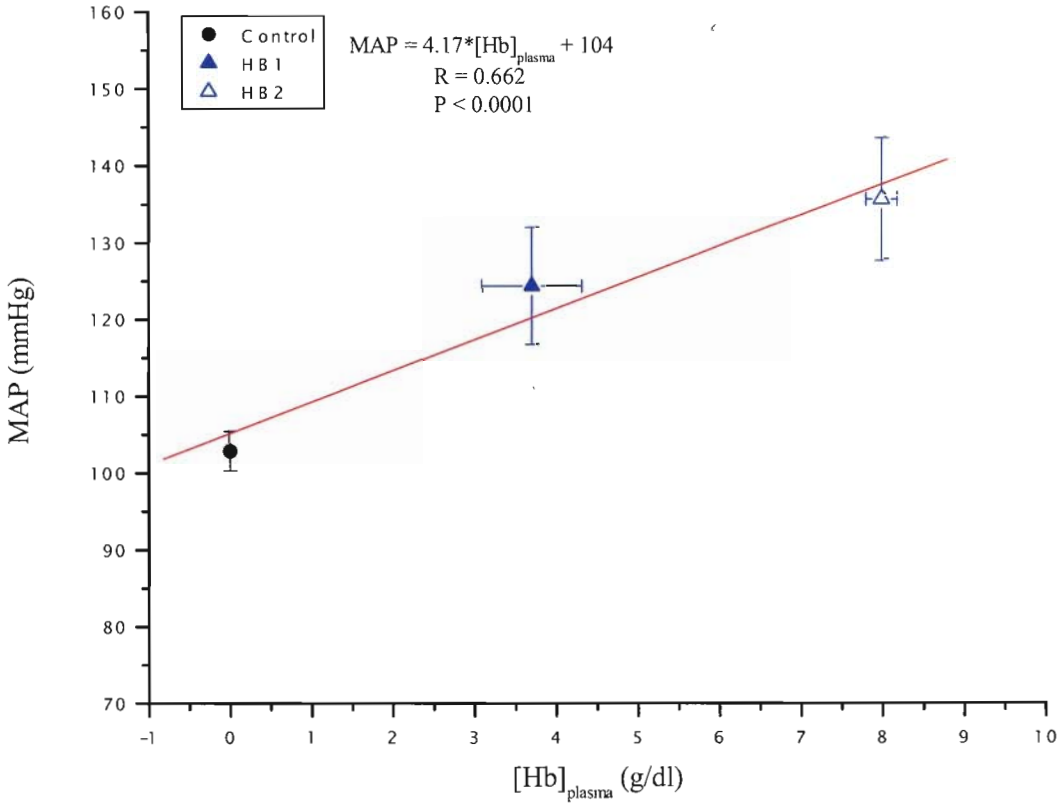


Figure 7. Plot of plasma hemoglobin concentration versus mean arterial pressure for hemodilution with Oxyglobin.

HB1 = HBOC Oxyglobin Step 1, HB2 = HBOC Oxyglobin Step 2. Standard error bars are shown.

Table 4. Mean (± standard deviation) partial pressure of oxygen data for animal sub-group.

	Control (N=4)	Hespan HD1 (N=2)	Oxyglobin HD1 (N=2)	Hespan HD2 (N=5)	Oxyglobin HD2 (N=5)
n	46	26	26	53	50
PG PO_2	45.1 ± 12.9	35.2‡ ± 18.0	32.8‡ ± 10.0	20.9‡ ± 10.3	11.6‡ ± 11.0
RBC PO_2	54.9 ± 14.6	36.7‡ ± 18.4	34.4‡ ± 10.1	22.2‡ ± 10.6	11.6‡ ± 11.0
ΔPO_2	9.7** ± 9.1	1.6 ± 5.4	1.6* ± 2.6	1.3** ± 2.0	0.0 ± 1.1
P_cO_2	50.0 ± 14.5	36.0 ± 18.0	33.6 ± 9.9	21.5 ± 10.4	11.6 ± 11.0

N = Number of animals; n = Number of capillaries; HD1 = Hemodilution Step 1; HD2 = Hemodilution Step 2; PG PO_2 = Plasma gap partial pressure of oxygen; RBC PO_2 = Red blood cell partial pressure of oxygen; ΔPO_2 = Difference between RBC PO_2 and PG PO_2; P_cO_2 = Mean capillary PO_2.

‡ = Significantly different from Control ($p<0.05$)

* = Significantly different from zero ($p<0.05$)

** = Significantly different from zero ($p<0.0001$)

a decrease in red blood cell PO_2 from 54.9 ± 14.6 mmHg (Control) to 34.4 ± 10.1 mmHg (Step 1) then to 11.6 ± 11.0 mmHg (Step 2) as the hematocrit was reduced from 38.8 ± 1.9 % (Control) to 28.1 ± 1.5 % (Step 1) and then to 12.3 ± 1.1 % (Step 2). A graphic presentation of these data is found in Figure 8.

A Bivariate Linear Fit Model with subsequent Analysis of Variance revealed a statistically significant difference between the linear regressions of Hespan and Oxyglobin for red blood cell PO_2 versus hematocrit.

Red Blood Cell PO_2 and Total Hemoglobin Concentration

Red blood cell PO_2 decreased from 54.9 ± 14.6 mmHg at a total hemoglobin concentration of 13.5 ± 0.6 g/dl to 36.7 ± 18.4 mmHg at a total hemoglobin concentration of 10.8 ± 4.7 g/dl after Step 1, and then to 22.2 ± 10.6 mmHg at a total hemoglobin concentration of 5.1 ± 0.8 g/dl after Step 2 of Hespan hemodilution. Red blood cell PO_2 sustained a steeper decline when using Oxyglobin as the hemodilution fluid beginning with the same Control values as stated previously to a PO_2 of 34.4 ± 10.1 mmHg at a total hemoglobin concentration of 13.1 ± 0.4 g/dl after Step 1 and then to a PO_2 of 11.6 ± 11.0 mmHg at a total hemoglobin concentration of 11.3 ± 0.5 g/dl after Step 2. A graphic presentation of these data is found in Figure 9.

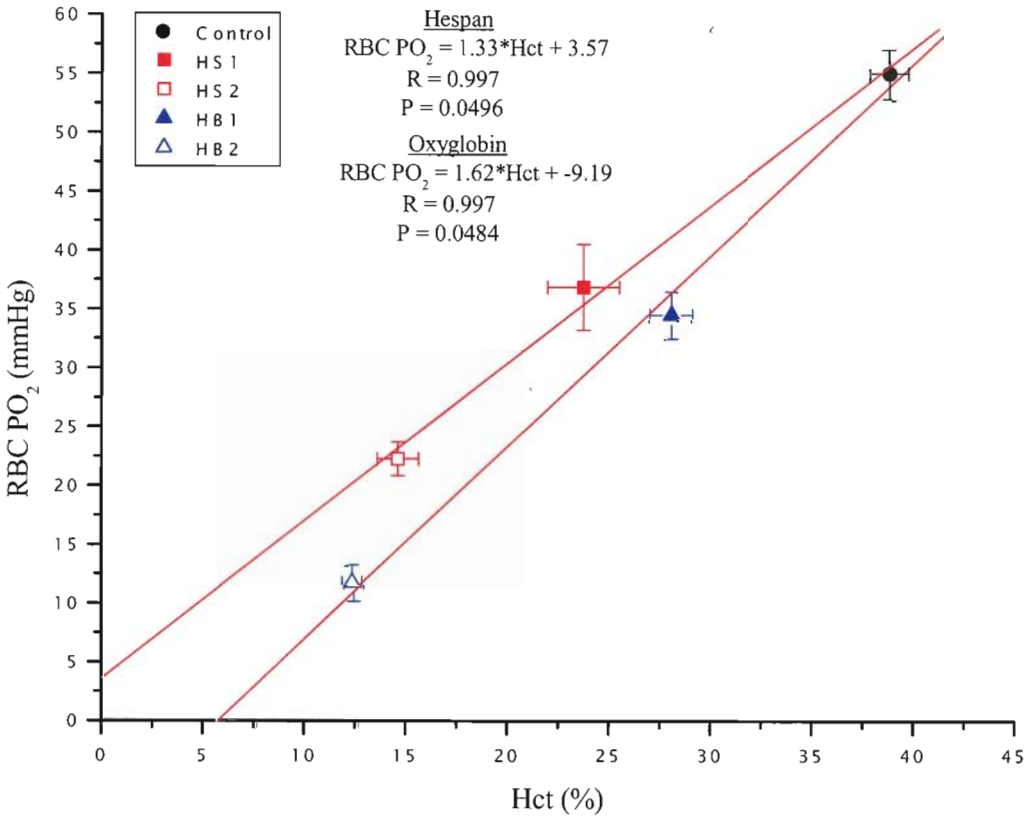
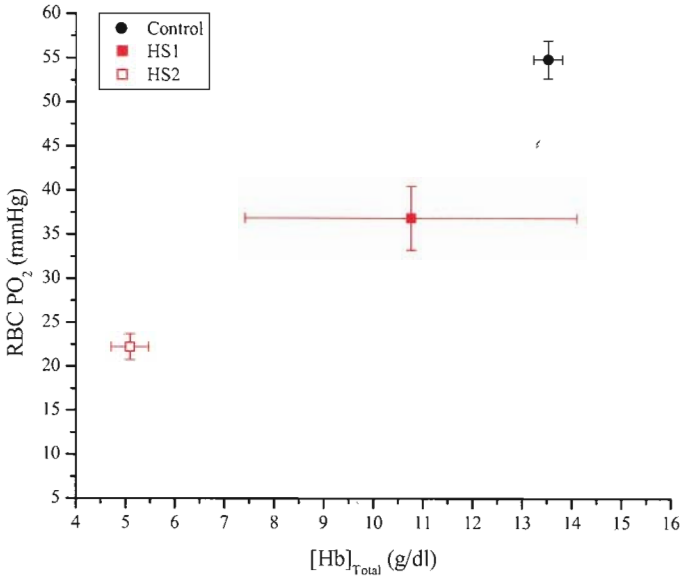


Figure 8. Plot of red blood cell partial pressure of oxygen versus hematocrit for hemodilution with Hespan and Oxyglobin.

HS1 = Hespan Step 1, HS2 = Hespan Step 2, HB1 = HBOC Oxyglobin Step 1, HB2 = HBOC Oxyglobin Step 2. Standard error bars are shown. Linear regression lines are shown for Hespan and Oxyglobin as hematocrit was reduced.

A



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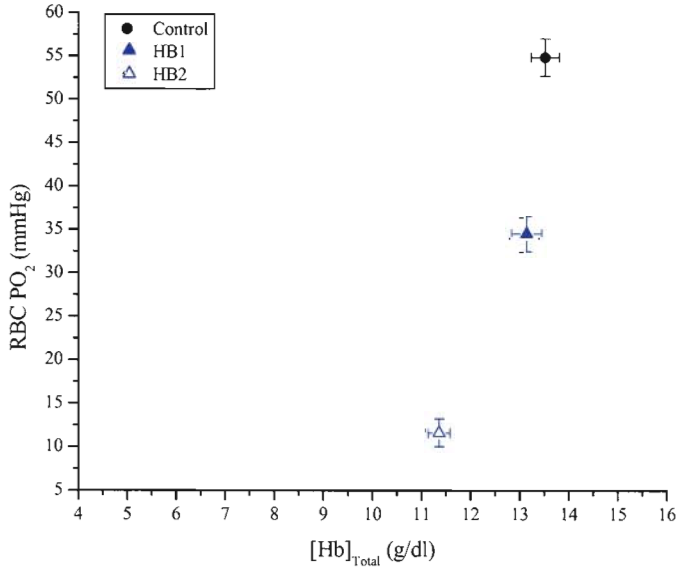


Figure 9. Plots of red blood cell partial pressure of oxygen versus total hemoglobin for Hespan and Oxyglobin.

HS1 = Hespan Step 1, HS2 = Hespan Step 2, HB1 = HBOC Oxyglobin Step 1, HB2 = HBOC Oxyglobin Step 2. Standard error bars are shown.

Red Blood Cell PO_2 and Plasma Hemoglobin Concentration

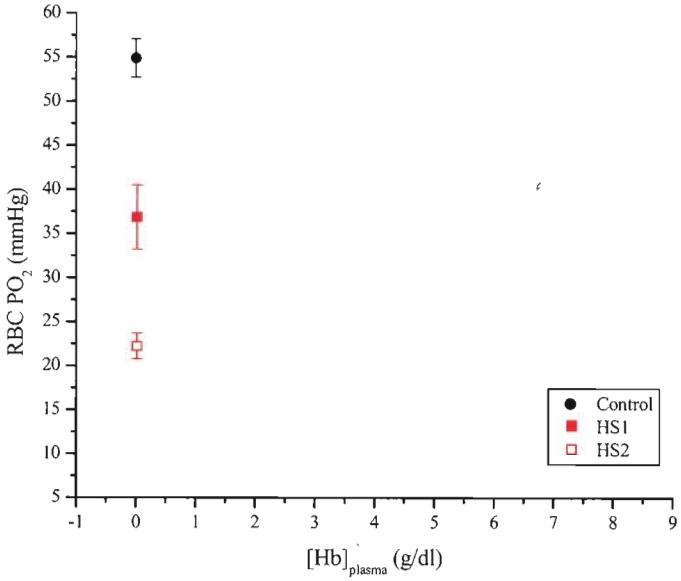
Figure 10A graphically presents red blood cell PO_2 at a plasma hemoglobin concentration of 0 g/dl for Control and hemodilution Steps 1 and 2 for Hespan. During isovolemic hemodilution with Hespan, the red blood cell PO_2 decreased from 54.9 ± 14.6 mmHg (Control) to 36.7 ± 18.4 mmHg (Step1) and then to 22.2 ± 10.6 mmHg after Step 2. Figure 10B depicts the decrease of red blood cell PO_2 with increasing plasma hemoglobin concentration for hemodilution with Oxyglobin. In this case, red blood cell PO_2 decreases from 54.9 ± 14.6 mmHg (Control) to 34.4 ± 10.1 mmHg (Step 1) and then to 11.6 ± 11.0 mmHg (Step 2).

Plasma Gap PO_2 and Comparisons with Hematocrit, Total Hemoglobin Concentration, and Plasma Hemoglobin Concentration

Plasma Gap PO_2 and Hematocrit

With a decrease in hematocrit from 38.3 ● 1.9 % (Control) to 23.8 ± 2.5 % (Step 1) and then to 14.6 ± 2.3 % after Step 2 of Hespan hemodilution, the plasma gap PO_2 decreased from 45.1 ± 12.9 mmHg (Control) to 35.2 ± 18.0 mmHg (Step 1) and then to 20.9 ± 10.3 mmHg (Step 2). Oxyglobin plasma gap PO_2 values decreased to 32.8 ± 10.0 mmHg with a hematocrit of 28.1 ± 1.5 % after Step 1 to a plasma gap PO_2 of 11.6 ± 11.0 mmHg and hematocrit of 12.3 ± 1.1 % after Step 2. Hematocrit can be found in Table 3 (p. 42) and plasma gap PO_2 can be found in Table 4 (p. 44). These data are also presented in graphic form in Figure 11.

A



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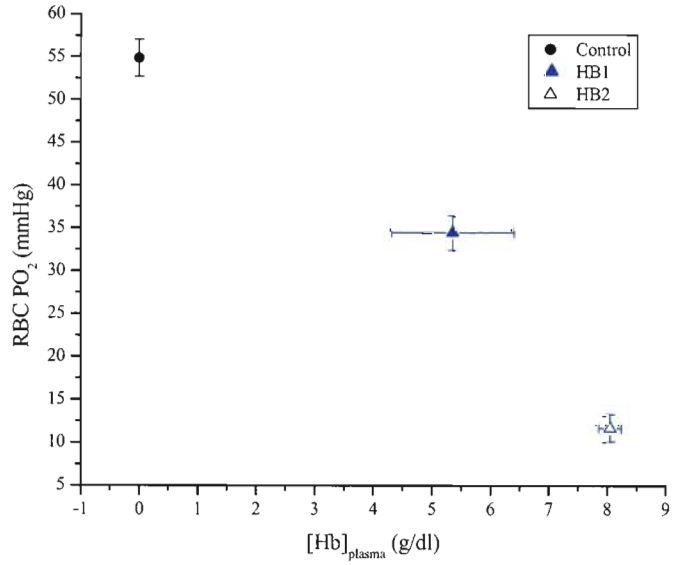


Figure 10. Plots of red blood cell partial pressure of oxygen versus plasma hemoglobin for Hespan and Oxyglobin.

HS1 = Hespan Step 1, HS2 = Hespan Step 2, HB1 = HBOC Oxyglobin Step 1, HB2 = HBOC Oxyglobin Step 2. Standard error bars are shown.

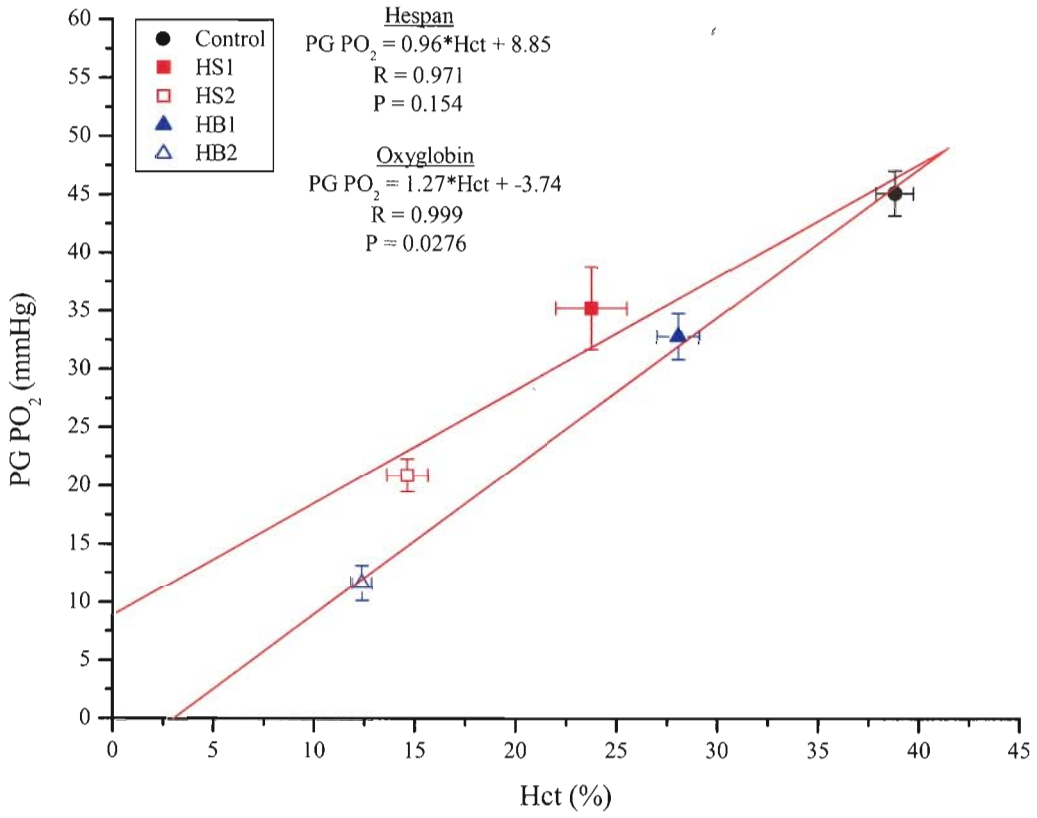


Figure 11. Plot of plasma gap partial pressure of oxygen versus hematocrit for hemodilution with Hespan and Oxyglobin.

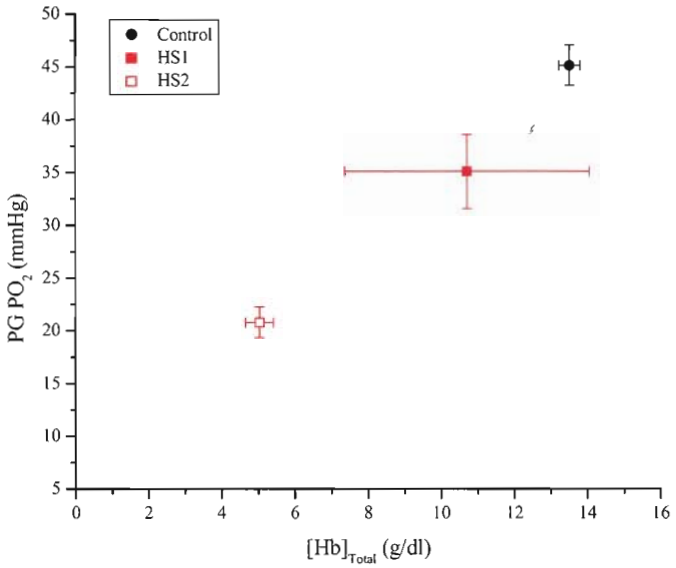
HS1 = Hespan Step 1, HS2 = Hespan Step 2, HB1 = HBOC Oxyglobin Step 1, HB2 = HBOC Oxyglobin Step 2. Standard error bars are shown. Linear regression lines are shown for Hespan and Oxyglobin as hematocrit was reduced.

A Bivariate Linear Fit Model with subsequent Analysis of Variance revealed a statistically significant difference between the linear regressions of Hespan and Oxyglobin for plasma gap PO_2 versus hematocrit.

Plasma Gap PO_2 and Total Hemoglobin Concentration

As the total hemoglobin concentration decreased from 13.5 ± 0.6 g/dl (Control) to 10.8 ± 4.7 g/dl (Step1) and then to 5.1 ± 0.8 g/dl (Step 2), the plasma gap PO_2 decreased from 45.1 ± 12.9 mmHg (Control) to 35.2 ± 18.0 mmHg (Step 1) and then to 20.9 ± 10.3 mmHg (Step 2) when using Hespan as the hemodilution fluid. In the case of Oxyglobin, there was a decrease in plasma gap PO_2 from 45.1 ± 12.9 mmHg (Control) to 32.8 ● 10.0 mmHg (Step1) and then to 11.6 ± 1.2 mmHg after Step 2 hemodilution over a change in total hemoglobin concentration from 13.5 ± 0.6 g/dl (Control) to 13.1 ± 0.4 g/dl (Step 1) and then to 11.3 ± 0.5 g/dl (Step 2). Total hemoglobin concentrations can be found in Table 3 (p. 42) and plasma gap PO_2 can be found in Table 4 (p. 44). These data are also presented in graphic form in Figure 12.

A



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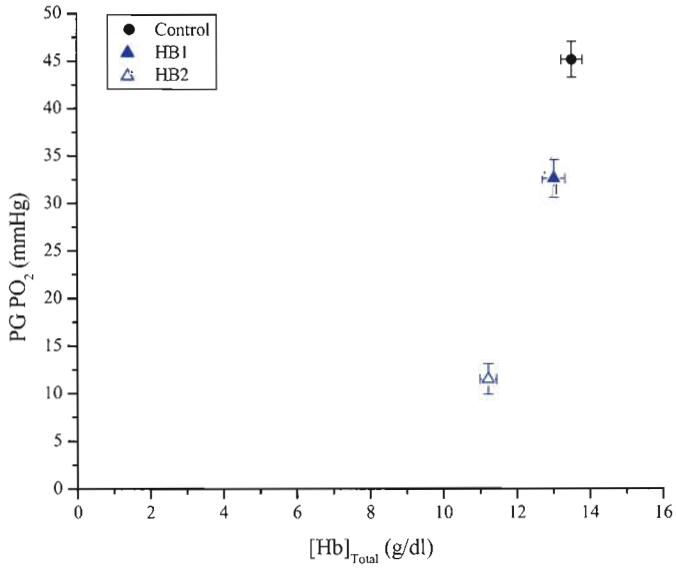


Figure 12. plots of plasma gap partial pressure of oxygen versus total hemoglobin for Hespan and Oxyglobin.

HS1 = Hespan Step 1, HS2 = Hespan Step 2, HB1 = HBOC Oxyglobin Step 1, HB2 = HBOC Oxyglobin Step 2. Standard error bars are shown.

Plasma Gap PO_2 and Plasma Hemoglobin Concentration

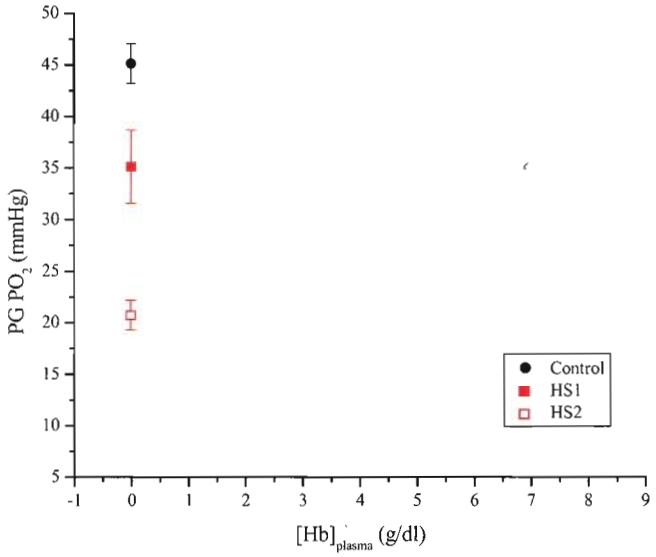
Control plasma gap PO_2 was 45.1 ± 12.9 mmHg at an assumed plasma hemoglobin concentration of 0 g/dl. Plasma gap PO_2 for Hespan Steps 1 and 2 decreased from Control to 35.2 ± 18.0 mmHg and 20.9 ± 10.3 mmHg, respectively, also at plasma hemoglobin concentrations of 0 g/dl. Using Oxyglobin as the hemodilution fluid, plasma hemoglobin concentrations increased from 5.3 ± 1.5 g/dl (Step 1) to 8.0 ± 0.4 g/dl (Step 2) and plasma gap PO_2 decreased to 32.8 ± 10.0 mmHg (Step 1) and then to 11.6 ± 1.2 mmHg (Step 2). Plasma hemoglobin concentrations can be found in Table 3 (p. 42) and plasma gap PO_2 can be found in Table 4 (p. 44). A graphic representation of these data is found in Figure 13.

Red Blood Cell – Plasma Gap PO_2 Difference (ΔPO_2) and Comparisons with Hematocrit, Total Hemoglobin Concentration, Plasma Hemoglobin Concentration, and Red Blood Cell PO_2

ΔPO_2 and Hematocrit

Using Hespan as the hemodilution fluid, hematocrit decreased from 38.8 ± 1.9 % (Control) to 23.8 ± 2.5 % (Step 1) and then to 14.6 ± 2.3 % (Step 2) while ΔPO_2 decreased from 9.7 ± 9.1 mmHg (Control) to 6.2 ± 9.2 mmHg (Step 1) and then to 1.3 ± 2.0 mmHg (Step 2). Using Oxyglobin as the hemodilution fluid, hematocrit decreased from 28.1 ± 1.5 % (Step 1) to 12.3 ± 1.1 % (Step 2), while ΔPO_2 decreased to 1.6 ± 2.6 mmHg (Step 1) and then to 0.0 ± 1.1 mmHg (Step 2) from 9.7 ± 9.1 mmHg (Control). Hematocrit can be found in Table 3 (p. 42) and ΔPO_2 can be found in Table 4 (p. 44). These data are also presented in graphic form in Figure 14.

A



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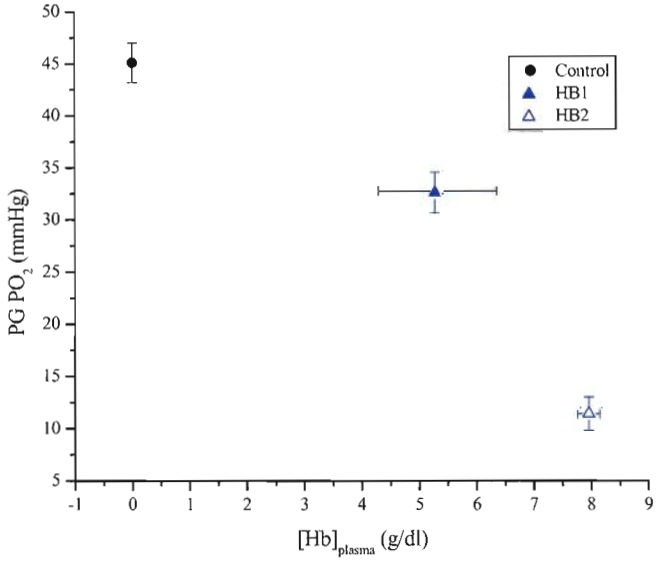
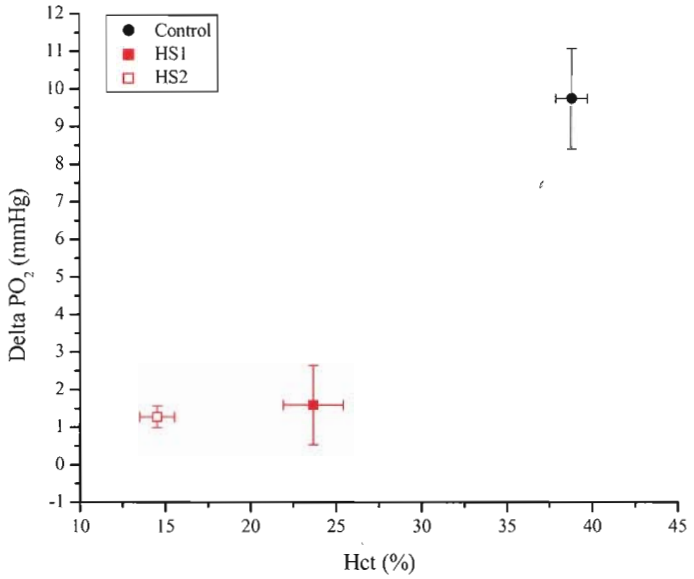


Figure 13. Plots of plasma partial pressure of oxygen versus plasma hemoglobin for Hespan and Oxyglobin.

HS1 = Hespan Step 1, HS2 = Hespan Step 2, HB1 = HBOC Oxyglobin Step 1, HB2 = HBOC Oxyglobin Step 2. Standard error bars are shown.

A



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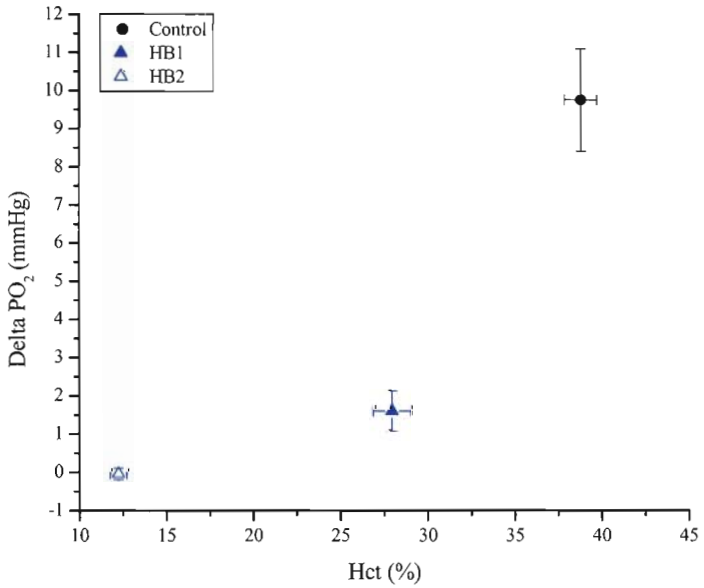

Figure 14. ΔPO_2 versus hematocrit.

HS1 = Hespan Step 1, HS2 = Hespan Step 2, HB1 = HBOC Oxyglobin Step 1, HB2 = HBOC Oxyglobin Step 2. Standard error bars are shown.

ΔPO_2 and Total Hemoglobin Concentration

The total hemoglobin concentration was decreased during isovolemic hemodilution with Hespan from 13.5 ± 0.6 g/dl (Control) to 10.8 ± 4.7 g/dl (Step 1) and then to 5.1 ± 0.8 g/dl (Step 2). During this time the ΔPO_2 decreased from 9.7 ± 9.1 mmHg (Control) to 1.6 ± 5.4 mmHg (Step 1) and then to 1.3 ± 2.0 mmHg (Step 2). The total hemoglobin concentration decreased during isovolemic hemodilution with Oxyglobin from 13.5 ± 0.6 g/dl (Control) to 13.1 ● 0.4 g/dl (Step 1) and then to 11.3 ± 0.5 g/dl (Step 2). The ΔPO_2 decreased from 9.7 ± 9.1 mmHg (Control) to 1.6 ± 2.6 mmHg (Step 1) and then to 0.0 ± 0.8 mmHg (Step 2) using Oxyglobin as the hemodilution fluid. Total hemoglobin concentration can be found in Table 3 (p. 42) and Δ PO_2 can be found in Table 4 (p. 44). These data are also presented in graphic form in Figure 15.

ΔPO_2 and Plasma Hemoglobin Concentration

ΔPO_2 decreased from 9.7 ± 9.1 mmHg (Control) at a plasma hemoglobin concentration of 0 g/dl to 1.6 ± 5.4 mmHg (Step 1) and then to 1.3 ± 2.0 mmHg (Step 2) with corresponding plasma hemoglobin concentrations of 0 g/dl (Steps 1 and 2) for Hespan isovolemic hemodilution. For Oxyglobin, ΔPO_2 decreased to 1.6 ± 2.6 mmHg (Step 1) and then to 0.0 ± 1.1 mmHg (Step 2) from Control with corresponding plasma hemoglobin concentrations of 5.3 ± 1.5 g/dl (Step 1) and 8.0 ± 0.4 g/dl (Step 2). Plasma hemoglobin concentration can be found in Table 3 (p. 42) and ΔPO_2 can be found in Table 4 (p. 44). These data are also presented in graphic form in Figure 16.

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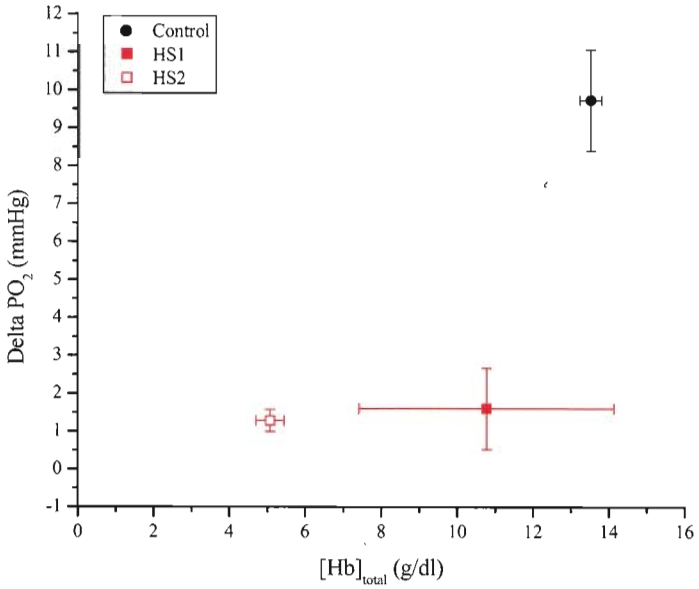

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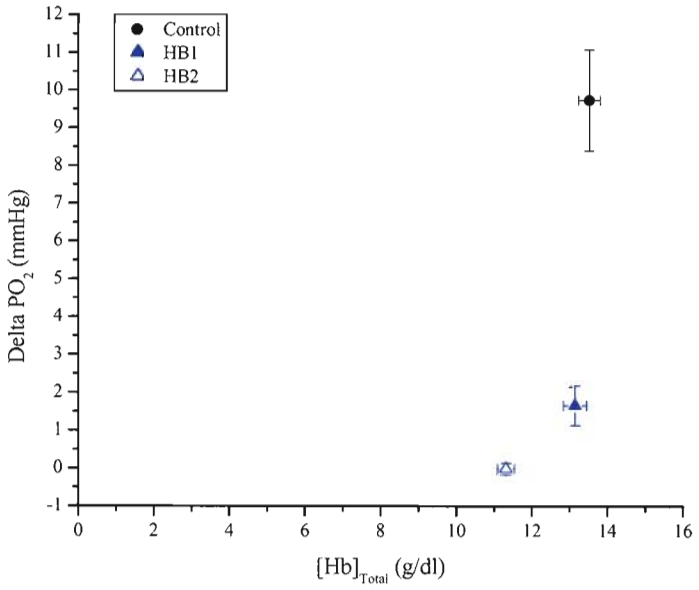


Figure 15. ΔPO_2 versus total hemoglobin concentration.

HS1 = Hespan Step 1, HS2 = Hespan Step 2, HB1 = HBOC Oxyglobin Step 1, HB2 = HBOC Oxyglobin Step 2. Standard error bars are show

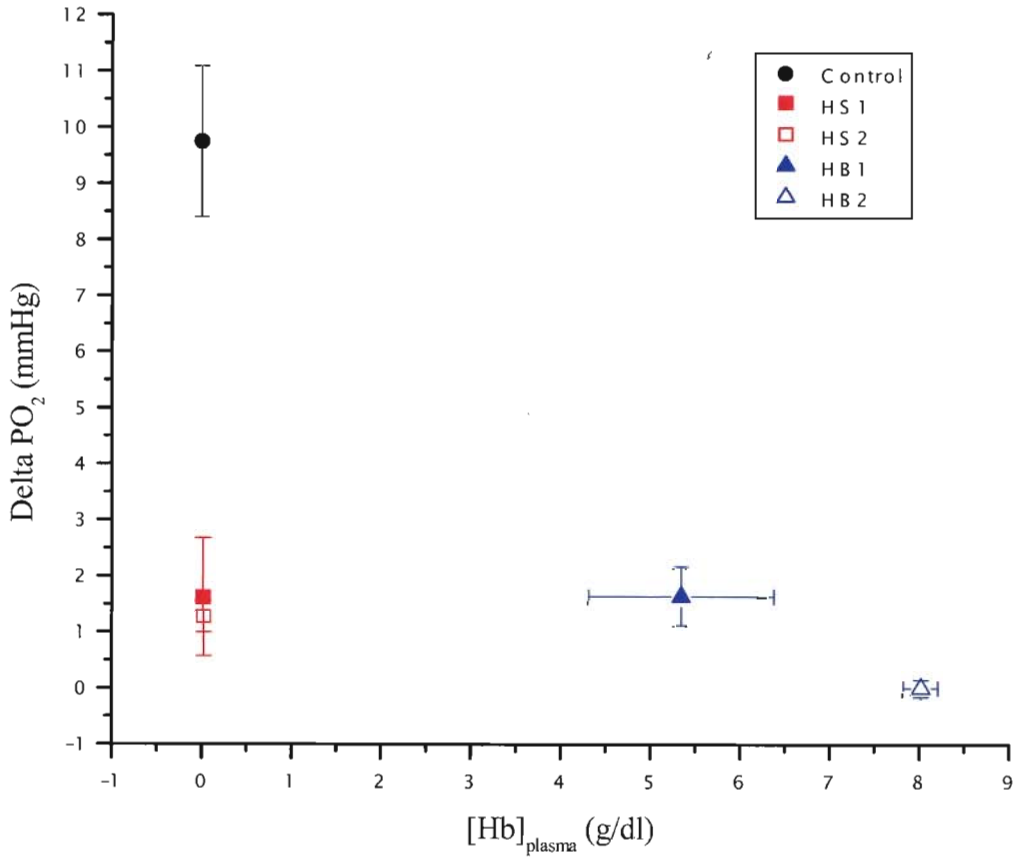


Figure 16. ΔPO_2 versus plasma hemoglobin concentration.

HS1 = Hespan Step 1, HS2 = Hespan Step 2, HB1 = HBOC Oxyglobin Step 1, HB2 = HBOC Oxyglobin Step 2. Standard error bars are shown.

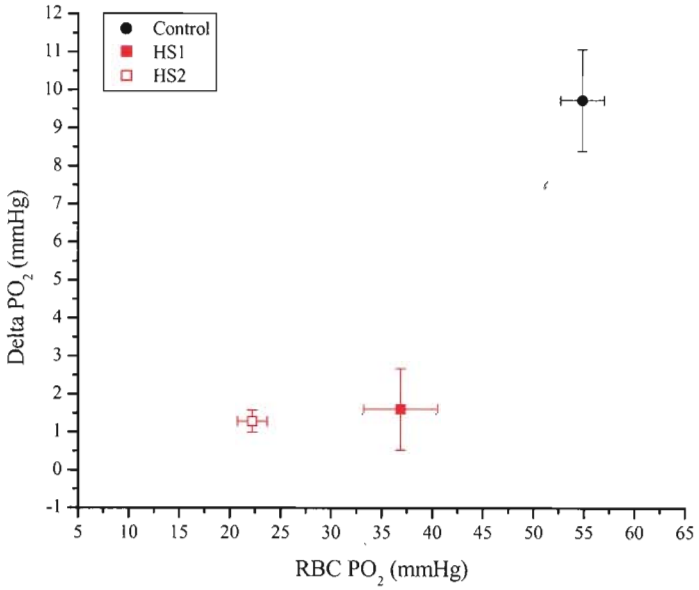
ΔPO_2 and Red Blood Cell PO_2

Red blood cell PO_2 and ΔPO_2 were highest at the Control values of 54.9 ± 14.6 mmHg and 9.7 ± 9.1 mmHg, respectively. Red blood cell PO_2 and ΔPO_2 decreased to 36.7 ± 18.4 mmHg and 1.6 ± 5.4 mmHg, respectively, after Step 1 of Hespan hemodilution. Red blood cell PO_2 and ΔPO_2 decreased to 22.2 ± 10.6 mmHg and 1.3 ± 2.0 mmHg, respectively, after Step 2 of Hespan hemodilution. With Oxyglobin as the hemodilution fluid, red blood cell PO_2 and ΔPO_2 decreased from Control to 34.4 ± 10.1 mmHg and 1.6 ± 2.6 mmHg, respectively, after Step 1 and then to a red blood cell PO_2 of 11.6 ± 11.0 mmHg and ΔPO_2 of 0.0 ± 1.1 mmHg after Step 2. Red blood cell PO_2 and ΔPO_2 can be found in Table 4 (p. 44). These data are also presented in graphic form in Figure 17.

Results of Statistical Analysis

A One Way Analysis of Variance revealed that all values for Hespan and Oxyglobin for both Steps 1 and 2 were significantly different from Control for plasma gap PO_2 and red blood cell PO_2 with all p-values less than 0.05. In addition, for both plasma gap PO_2 and red blood cell PO_2, Tukey's Multiple Comparisons test revealed significant differences between all values except among Hespan and Oxyglobin Step 1. Students t-tests determined significant differences between plasma gap PO_2 and red blood cell PO_2 (ΔPO_2) in the Control group ($p<0.0001$), hemodilution Step 1 with Oxyglobin ($p<0.05$), and hemodilution Step 2 with Hespan ($p<0.0001$).

A



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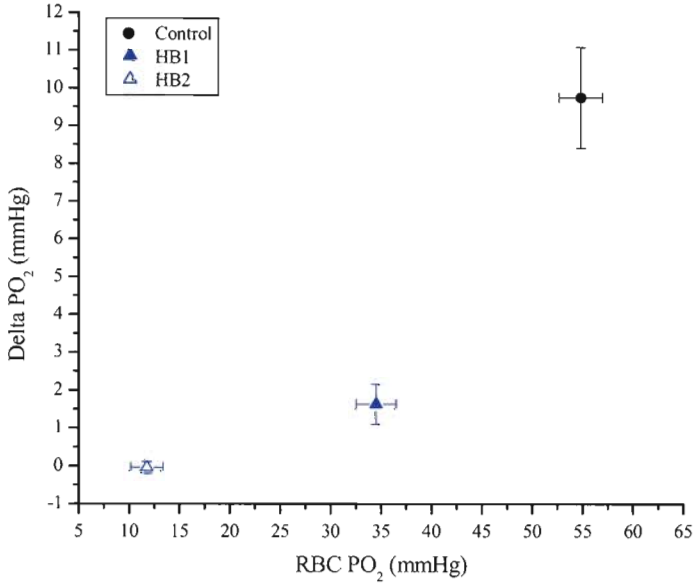

Figure 17. ΔPO_2 versus red blood cell partial pressure of oxygen.

HS1 = Hespan Step 1, HS2 = Hespan Step 2, HB1 = HBOC Oxyglobin Step 1, HB2 = HBOC Oxyglobin Step 2. Standard error bars are shown.

DISCUSSION

Theoretical models have predicted the existence of intracapillary PO_2 gradients that take into account the discrete nature of blood flow in capillaries. Hellums was the first to do so in 1977 (Hellums 1977). The first experimental evidence to support these predictions determined that plasma PO_2 gradients do exist in the form of erythrocyte-associated transients or EATs (Golub and Pittman 2005). The purpose of the present study was to observe EATs in skeletal muscle capillaries. Furthermore, the purpose extended towards describing the nature of EATs under isovolemic hemodilution conditions with Hespan and a hemoglobin-based oxygen carrier. By employing the Pd-porphyrin phosphorescence quenching method (PQM) along with measuring light transmission, it was possible to determine the relative presence or absence of red blood cells in the detection volume over a one second time course.

There were three major findings from this study and multiple minor findings that will be discussed in this section. The first major finding is that EATs can be measured in skeletal muscle using prior detection methods as were applied to the rat mesentery (Golub and Pittman 2005). Secondly, the magnitude of EATs depends on capillary PO_2 values. Thirdly, the magnitude of EATs is dependent on hematocrit and plasma hemoglobin concentration and that these values vary depending on the hemodilution fluid used.

Prior Documentation on Erythrocyte-Associated Transients, Hemodilution and Hemoglobin-Based Oxygen Carriers

The present study is unique for several reasons. Although the description of intracapillary PO_2 heterogeneity (Hellums 1977; Homer, Weathersby et al. 1981) allowed a framework for mathematical modeling which predicted the presence of PO_2 gradients between RBCs and plasma (Federspiel and Sarelius 1984; Federspiel and Popel 1986; Hellums, Nair et al. 1996), Golub and Pittman (2005) collected the first experimental data from capillaries in the rat mesentery, demonstrating the existence of EATs. The present investigation used the rat spinotrapezius muscle and made measurements under hemodilution conditions with two hemodiluents for the first time.

Phosphorescence Amplitude and Light Transmission

Laser excitation of the Pd-porphyrin phosphorescent probe confined to the plasma allowed the measurement of plasma PO_2 transients and the determination of phosphorescence amplitude (PA). The discrete nature of blood flow was also revealed by the simultaneous measurement of light transmission (LT) through the capillary. As a result of the intrinsic nature of red blood cells (RBC) to absorb light, the signal would decrease in the presence of a RBC and increase once the RBC moved out of the detection region. The signal for PA would correspondingly increase, reflecting the presence of plasma measured by the amount of light emitted by the excited phosphor probe in the detection region, and decrease when an RBC was present (i.e., less plasma).

Ideally, PA and LT amplitude fluctuations would correspond exactly. Although these two independent variables were sometimes highly correlated, this was not always the case. Golub and Pittman (2005) found that, due to the complex refraction of transmitted light by a RBC with random size, shape, and orientation and possible misalignment of the photodiode used to measure LT under the tissue, PA was a better indicator of the presence of plasma or RBCs. Other factors that might contribute to variability between these two factors in skeletal muscle are muscle thickness, refraction of transmitted light by muscle tissue, and the presence of multiple microvessels along the incident light beam.

The expectation for the isovolemic hemodilution protocol with Hespan was that LT and PA signal tracings would be more pronounced with troughs and peaks becoming more widely spaced. The expectation for the isovolemic hemodilution protocol with Oxyglobin was that PA signal tracings would show a decreased distinction between troughs and peaks, resulting from the increased plasma hemoglobin concentration.

Neither of these expectations was observed in reviewing LT and PA signal tracings from Control and Steps 1 and 2 for hemodilution with either Hespan or Oxyglobin. In fact, the relative spacing of plasma gaps and RBCs remained relatively unchanged from Control to hemodilution conditions. This could be explained in part by the observation of capillary networks during experimentation where RBCs did not appear to be distributed uniformly in all capillaries; rather, some capillary networks became plasmatic while other capillaries maintained a relatively normal RBC flow.

It is also interesting to note that in multiple capillaries the PA signal population appeared to be bimodal. One possible explanation for this is that the two separate populations represent values corresponding to RBCs and plasma gaps (Figure 18). This explanation corresponds to previously presented ideas that the LT and PA signals should be higher when the detection volume is comprised of plasma and lower when a RBC is present.

Effects of Hemodilution on Systemic Parameters

Summary of Experimental Results for Mean Arterial Pressure and Hematocrit

Hemodilution with Hespan resulted in an overall decrease in mean arterial pressure as the hematocrit was reduced. For Hespan, mean arterial pressure went from 102.9 ± 10.2 mmHg (Control) to 105.1 ± 16.9 mmHg (Step 1) and decreased after Step 2 to 97.4 ± 15.6 mmHg. In the case of hemodilution with Oxyglobin, a progressive increase in mean arterial pressure occurred as the hematocrit was reduced. For Oxyglobin, mean arterial pressure went from 102.9 ± 10.2 mmHg (Control) to 124.4 ± 21.5 mmHg (Step 1) and even further to 135.8 ± 17.7 mmHg (Step 2). The infusion of Oxyglobin caused an immediate and statistically significant increase in mean arterial pressure following hemodilution Step 1. After Step 2 hemodilution there was a sustained increase in mean arterial pressure, yet the difference between MAP for Step 1 and Step 2 was no longer significant. The means for both Oxyglobin Steps 1 and 2 were significantly different from the MAP for Hespan Step 2.

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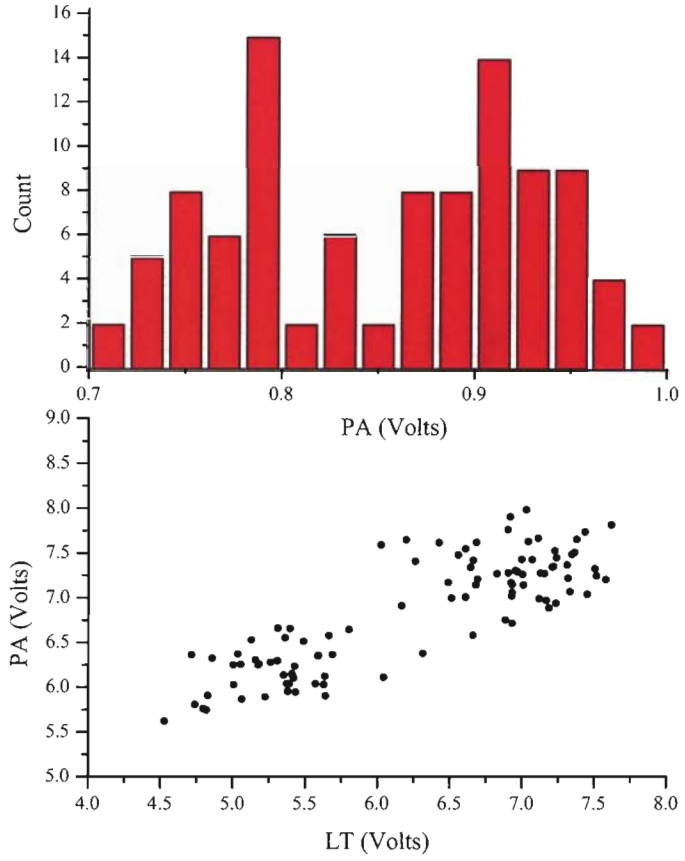

Figure 18A. Plots of Bimodal PA Signal

B

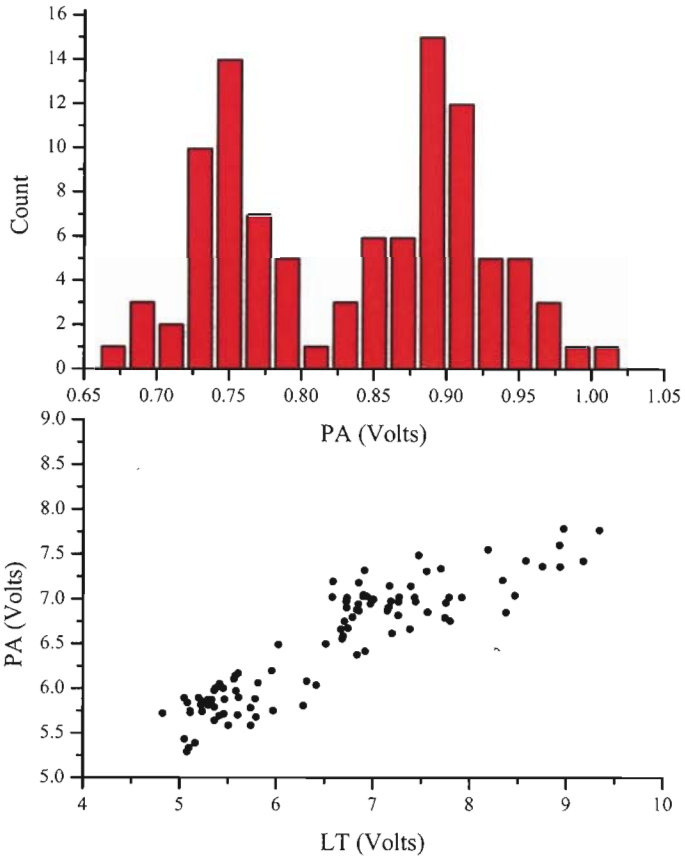

Figure 18B. Plots of Bimodal PA Signal

Explanation of Variation in Results

In several of the Hespan experiments leading to Step 1 measurements and in several of the Oxyglobin experiments leading to Step 2 measurements, technical difficulties regarding the withdrawal/infusion protocol caused an overshoot in hemodilution. In both animals of the Hespan Step 2 group, hematocrit was substantially lower than the target hematocrit of 30% (22% and 25%) and, in three of five animals for the Oxyglobin Step 2 group, hematocrit was lower than the target hematocrit of 15% (11%, 12%, and 12%). There was also an unexplained rise in mean arterial pressure for hemodilution Step 1 using Hespan as the hemodilution fluid (see Figure 6, p. 40).

Summary of Experimental Results for Mean Arterial Pressure and Plasma Hemoglobin Concentration

Isovolemic hemodilution with Oxyglobin caused an increase in plasma hemoglobin concentration as expected from the assumed value of 0 g/dl (Control) to 3.7 - ± 1.7 g/dl in Step 1 and then to 8.0 ± 0.4 g/dl in Step 2. The results demonstrate that a rise in free hemoglobin concentration correlated well with a rise in mean arterial pressure.

Results and Comparisons in Prior Investigations for Mean Arterial Pressure

The mean arterial pressure obtained through hemodilution with Hespan and Oxyglobin are consistent with the results of previous studies. A number of studies have described a decrease in mean arterial pressure during isovolemic hemodilution with a non-oxygen-carrying plasma expander, apparently due to the consequential decrease in

blood viscosity (Krieter, Hagen et al. 1997; Tsai, Friesenecker et al. 1998; Tsai and Intaglietta 2001). The pressor effect of hemoglobin-based oxygen carriers is also well documented. There are primarily two competing theories as to the mechanism of the hypertensive effect of HBOCs, either that nitric oxide (NO) scavenging occurs in the vessel lumen and/or in the extravascular space or that there is excessive O_2 delivery. The former is the most widely accepted interpretation. Several studies describe in detail the mechanism by which extracellular oxy-hemoglobin (HbO_2) reacts at a high rate with NO in the vessel lumen. Extravasation of HbO_2 into the blood vessel wall also causes a reaction to occur with NO in the extravascular space, thereby hampering normal NO signaling that occurs between the endothelium and the smooth muscle (Shen, Hintze et al. 1995; Sarti, Giuffre et al. 2003; Olson, Foley et al. 2004).

Effects of Hemodilution with Hespan and Oxyglobin on Capillary PO_2

Summary of Experimental Results for Red Blood Cell PO_2

Hemodilution with Hespan resulted in a linear decrease in RBC PO_2 as the hematocrit was systematically decreased through the two hemodilution steps (see Figure 17). Hemodilution with Oxyglobin caused a similar linear decrease in RBC PO_2. A statistically significant difference was found when comparing the two linear regressions using a Bivariate Linear Fit Model with subsequent Analysis of Variance indicating that Hespan over the entire course of hemodilution was able to maintain RBC PO_2 better than

Oxyglobin. The linearity of both Hespan and Oxyglobin data suggest that RBC PO_2 is dependent on hematocrit.

Hemodilution with Hespan resulted in the expected decrease in total hemoglobin concentration and concurrent decrease in RBC PO_2 to 22.2 ± 10.6 mmHg. Using Oxyglobin as the hemodiluent the total hemoglobin concentration was kept above 11 g/dl, but the RBC PO_2 sustained a much steeper decline with a PO_2 of 11.6 ± 11.0 mmHg in Step 2.

Plasma hemoglobin concentrations increased from 5.2 ± 1.5 g/dl (Step 1) and then to 8.0 ± 0.4 g/dl as expected, contributing significantly to the total hemoglobin concentrations of 13.1 ± 0.4 g/dl (Step 1) and 11.3 ± 0.5 g/dl (Step 2). RBC PO_2, despite the contribution of free hemoglobin to the plasma, did not benefit from its increased oxygen carrying capacity.

RBC PO2 decreases in capillaries amid the decrease in systemic hematocrit and hemoglobin concentrations because there are fewer red blood cells to deliver oxygen and maintain tissue consumption levels. Therefore, more oxygen off loading occurs in arterioles before the red blood cells reach the capillaries resulting in the low RBC PO_2. This process might be facilitated by the addition of cell-free hemoglobin.

Overall, all RBC PO_2 values for the hemodilution Steps 1 and 2 for Oxyglobin and Hespan were determined to be statistically significant from Control values using a One Way Analysis of Variance. Tukey's Multiple Comparisons test revealed that all other values are significant when compared to each other except Step 1 values.

Summary of Experimental Results for Plasma Gap PO_2

Hemodilution with Hespan resulted in a linear decrease in PG PO_2 as hematocrit was systematically decreased through the hemodilution steps. Hemodilution with Oxyglobin caused a similar linear decrease in PG PO_2. Although the linear correlation for the Hespan values is 0.971 it is not statistically significant ($p = 0.15$). Linear regression statistics indicate a similarly high linear correlation for Control and Oxyglobin Steps 1 and 2 ($R = 0.999$) that is statistically significant ($p = 0.027$). When comparing the linear regressions using a Bivariate Linear Fit Model with subsequent Analysis of Variance, a significant difference was revealed indicating that Hespan, over the entire course of hemodilution, yielded a higher PG PO_2 than Oxyglobin. The high linear correlation of both Hespan and Oxyglobin data indicates that most of the variation of PG PO_2 can be accounted for the hematocrit.

PG PO_2 versus total hemoglobin concentration and plasma hemoglobin closely parallel those of the RBC PO_2 plots. One note of interest is that PG PO_2 was 32.8 ± 10.0 mmHg (Step 1) and 11.6 ± 11.0 mmHg (Step 2) when using Oxyglobin as the hemodiluent. Using Hespan as the hemodiluent resulted in PG PO_2 values of 35.2 ± 18.0 mmHg (Step 1) and 20.9 ± 10.3 mmHg (Step 2).

One Way Analysis of Variance determined that the PG PO_2 values of hemodilution Steps 1 and 2 for Oxyglobin and Hespan are statistically significant compared to Control values. Tukey's Multiple Comparisons test revealed that the Step 1 values for Oxyglobin and Hespan are not significantly different, while there is a significant difference between Oxyglobin Step 2 and Hespan Step 2. PG PO_2 was

expected to be higher for the Oxyglobin groups considering the contribution of cell-free hemoglobin in the plasma.

Summary of Experimental Results for ΔPO_2

As hematocrit was systematically reduced during hemodilution using Hespan and Oxyglobin, ΔPO_2 was dramatically reduced after Step 1. ΔPO_2 for Steps 1 and 2 of the hemodilution protocol with Hespan were approximately the same (1.6 ± 5.4 mmHg and 1.3 ± 2.0 mmHg, respectively), while ΔPO_2 for Steps 1 and 2 using Oxyglobin as the hemodiluent varied slightly (1.6 ± 2.6 mmHg and 0.0 ± 1.1 mmHg, respectively).

When considering the dependence of ΔPO_2 on total hemoglobin concentration, there was approximately an 8 mmHg change in ΔPO_2 going from a total hemoglobin concentration of 13.5 ± 0.6 g/dl (Control) to 10.8 ± 4.7 g/dl (Step 1), when using Hespan as the hemodiluent. This was only a small and non-significant change in ΔPO_2 between hemodilution Steps 1 and 2 using Hespan. Using Oxyglobin as the hemodilution fluid, total hemoglobin concentration varied only slightly, decreasing from 13.5 ± 0.6 g/dl (Control) to 11.3 ± 0.5 g/dl (Step 2), and ΔPO_2 decreased to 0.0 ± 1.1 mmHg.

Consideration of the plasma hemoglobin concentration provides additional insight into the variation of ΔPO_2 with total hemoglobin concentration. Hemodilution with Oxyglobin adds extracellular hemoglobin molecules which causes total hemoglobin concentration to remain stable during hemodilution. As more cell-free hemoglobin was added to the plasma from 0 g/dl (Control) to 5.3 ± 1.5 g/dl (Step 1) and then to 8.0 ± 0.4

g/dl (Step 2), the difference in PO_2 between RBCs and plasma gaps virtually disappeared, as expected.

Student's t-tests determined that significant differences exist between plasma gap PO_2 and red blood cell PO_2 (ΔPO_2) in the following: 1) Control group ($p<0.0001$) with a ΔPO_2 of 9.7 ± 9.1 mmHg; 2) Oxyglobin hemodilution Step 1 ($p<0.05$) with a ΔPO_2 of 1.6 ± 2.6 mmHg; 3) Hespan hemodilution Step 2 ($p<0.0001$) with a ΔPO_2 of 1.3 ± 2.0 mmHg.

Interpretation of Results and Comparison with Prior Investigations

The components of total hemoglobin concentration of the blood, namely the concentration of hemoglobin contained within RBCs and that contained in the plasma were calculated from the following equation:

Equation 9

$$[Hb]_{Total} = [Hb]_{RBC}H_f + [Hb]_{plasma} \cdot (1 - H_f)$$

where $[Hb]_{Total}$ is the total hemoglobin concentration in the blood, $[Hb]_{RBC}$ is the hemoglobin concentration of a single RBC, H_f is the final hematocrit, and $[Hb]_{plasma}$ is the hemoglobin concentration in the plasma.

Hemodilution with Hespan caused almost a three-fold reduction in total hemoglobin concentration, dropping below the transfusion trigger for packed red blood cells normally recognized at 7 g/dl (Intaglietta 1999; Tsai and Intaglietta 2001) after Step 2 ($[Hb]_{plasma} = 5.1 \pm 0.8$ g/dl). This explains the significant decrease in RBC and mean

capillary PO_2 (Table 4). The addition of extracellular hemoglobin molecules in the form of Oxyglobin increased plasma hemoglobin concentration, keeping total hemoglobin concentration close to the Control value. Although total hemoglobin concentration was stabilized by the addition of cell-free hemoglobin, it did not improve the overall delivery of oxygen to the capillaries as evident in the mean capillary PO_2. Equalization of PO_2 between the RBCs and plasma gaps was observed after the final hemodilution step with Oxyglobin resulting in a ΔPO_2 of practically 0 mmHg. Although this result was expected, if not so dramatically, Oxyglobin did not have a stabilizing effect on mean capillary PO_2; rather, it caused a decrease to approximately one-half of the Hespan Step 2 capillary PO_2 value. Prior investigations in our laboratory using measurements taken at the arteriolar and venular ends of capillaries to determine capillary PO_2 (Miller 2000; Tait 2000), have shown that capillary PO_2 decreases with systematic reduction of hematocrit when using Hespan as a hemodiluent. However, when using different HBOCs as the hemodiluent, mean capillary PO_2 remained at stable levels throughout the systematic reduction in hematocrit. This led to the conclusion that total hemoglobin concentration is a major determinant of capillary PO_2. This is contrary to the results obtained with Oxyglobin in this study.

Although MAP increased and total hemoglobin concentration was well maintained with the addition of Oxyglobin, PO_2 in the capillaries was dramatically reduced. Oxygen-carrying plasma expanders should reduce intracapillary resistance, facilitating oxygen offloading into nearby tissue, and should also increase the oxygen carrying capacity in the blood maintaining near to normal values as mentioned above.

Tsai (Tsai 2001) reported abnormally low tissue oxygenation and functional capillary density (FCD), a measurement of the number of red blood cell perfused capillaries as a measure of oxygen supply, with Oxyglobin during low hemoglobin content conditions (<50%). Perhaps this can help to explain the abnormally low mean capillary PO_2 observed in this case.

Another facet to the explanation of abnormally low mean capillary PO_2 that is seen with this particular HBOC might be due to NO scavenging. Evidence from multiple studies demonstrate that NO reversibly inhibits cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain (Brown and Cooper 1994; Cleeter, Cooper et al. 1994; Schweizer and Richter 1994; Sarti, Giuffre et al. 2003). In fact, nanomolar concentrations of NO cause the inhibition of cytochrome *c* oxidase which inhibits normal mitochondrial function causing oxygen consumption to decrease (Brown and Cooper 1994). One particular study found that the inhibition of NO synthesis with nitro-L-arginine (NLA, 10^{-4} mol/L) caused the opposite to occur, consumption of oxygen increased in the tissue by 55±9% (Shen, Hintze et al. 1995).

Putting this into the context of hemodilution with a HBOC, NO scavenging that occurs intravascularly in combination with HBOC extravasation into the interstitial space between the vascular endothelium and smooth muscle could potentially scavenge enough NO to mimic the effects of NLA. Considering this facet of NO activity along with reduced FCD and vasoconstriction, capillaries observed in the current study may have uniquely experienced a drastic reduction of oxygen supply and a concurrent increase in

demand resulting in very low mean capillary PO_2 at a reduced hematocrit when using Oxyglobin as the hemodilution fluid.

The present study is the first to combine a hemodilution procedure with EAT measurements in skeletal muscle. A prior investigation from this laboratory determined PO_2 in single capillaries, taking into account heterogeneity in PO_2, indicating the presence of PO_2 gradients within the plasma (Zheng, Golub et al. 1996). More recent experimental data from this laboratory, using the rat mesentery, concluded the existence and measurability of PO_2 gradients within the plasma gaps between RBCs (Golub and Pittman 2005). The present study has made the determination that the magnitude of the EATs depends on the capillary PO_2 values. Table 4 shows the general trend of a corresponding decrease in capillary PO_2 and ΔPO_2 for the one second observation period. Golub and Pittman (2005) found that EATs in mesenteric capillaries were less prominent in capillaries with high average PO_2 and more prominent in capillaries with low PO_2. Results from this study found that the largest ΔPO_2 with statistical significance occurred in the Control group, which had the highest capillary PO_2. Other statistically significant differences in ΔPO_2 were revealed in Oxyglobin Step 1 and Hespan Step 2. Though statistically significant, the latter condition was hypothesized to have the largest ΔPO_2 because of an expected increase in spacing between RBCs when hematocrit is reduced which in turn increasing intracapillary resistance to oxygen delivery resulting in an increase in EAT transients.

For normal cell separation distance (about 10 μm) and oxygen content of RBCs, the magnitudes of EATs in capillaries of resting tissue have predicted values of 10 mmHg

(Wang and Popel 1993; Kisliakov 1996), consistent with the Control ΔPO_2 from this investigation. Another study calculated oxygen PO_2 gradients in peripheral capillaries (Lawson and Forster 1967). Calculating the PO_2 difference between red cells and plasma under numerous conditions, they hypothesized that under normal resting conditions ΔPO_2 should be 0.2 mmHg and under anemic conditions ($[Hb]_{Total}$ = 3.7 g/dl) ΔPO_2 should be 1.1 mmHg. These calculations were not completely congruent with the data collected in this study. Under Control conditions (considered normal resting) ΔPO_2 was highest at 9.7 ± 9.1 mmHg and under similar anemic conditions ($[Hb]_{Total}$ = 5.1 ± 0.8 g/dl, Hespan Step 2) ΔPO_2 1.3 ± 2.0 mmHg. Many other mathematical calculations have predicted a much higher ΔPO_2 than what has been observed either by this study or by Golub and Pittman (Homer, Weathersby et al. 1981; Wang and Popel 1993; Golub and Pittman 2005).

Finally, it was suggested by Golub and Pittman (2005) that intracapillary resistance would decrease with the addition of an oxygen-carrying blood substitute, thereby damping the modulation of EATs. This is consistent with the present study which found no statistical significance in ΔPO_2 for hemodilution Step 2 with Oxyglobin, where the final hematocrit was one-third what it was initially and total hemoglobin deviated only slightly from the Control value.

Summary and Conclusion

Hemodilution with Oxyglobin resulted in a significant increase in mean arterial pressure as hematocrit was reduced and resulted in a significant contribution to total

hemoglobin concentration. Conversely, hemodilution with Hespan did not significantly alter mean arterial pressure and resulted in a dramatic decrease in total hemoglobin concentration, below the transfusion trigger after Step 2.

Considering EATs, it was determined that the magnitude of EAT modulation decreased with decreased capillary PO_2. Furthermore, it was determined that the magnitude of EAT modulation was related to hematocrit and hemoglobin concentration and that these varied depending on the hemodiluent used. With the systematic reduction of hematocrit, both hemodiluents caused a rapid decline in RBC and PG PO_2. At the hemodilution levels used in this study, Hespan demonstrated the ability to maintain PO_2 at higher levels when compared to Oxyglobin. Although Oxyglobin maintained total hemoglobin concentration near normal, with a significant contribution to the plasma hemoglobin concentration, it did not increase the oxygen delivery of blood in the capillaries but did cause EAT modulation to minimize, reducing the PO_2 gradient between the plasma and red blood cells. Hespan had no effect on hemoglobin concentration.

ΔPO_2 was determined to be statistically significant in three of the five experimental conditions including Control, Oxyglobin Step 1, and Hespan Step 2. The largest numerical ΔPO_2 occurred under Control conditions. Furthermore, the addition of Oxyglobin increased plasma hemoglobin concentration, keeping total hemoglobin concentration well above the "transfusion trigger", while causing the virtual abolishment of a PO_2 gradient between RBCs and plasma gaps. In conclusion, using PQM and the

previous experimental parameters of Golub and Pittman (2005), EATs in capillary PO_2 have been observed experimentally in the rat spinotrapezius muscle.

Recommendations for Future Studies

Parameters used in mathematical models, such as lineal density of RBCs, RBC spacing, RBC velocity, and RBC shape (Federspiel and Sarelius 1984; Federspiel and Popel 1986; Groebe and Thews 1989; Tsai and Intaglietta 1989; Wang and Popel 1993), should be experimentally measured to help answer questions concerning inter-RBC PO_2 resistance, oxygen flux at the capillary wall, and tissue PO_2 oscillations associated with PO_2 gradients between red blood cells and plasma gaps. A proposed technique to allow the determination of the values mentioned above would be the simultaneous measurement of EATs and video images of red blood cells and plasma gaps. With the dual acquisition of PO_2 and spatial data, the relationship between PO_2 and other variables such as RBC and capillary geometry and RBC flow dynamics could be compared with predictions of mathematical models.

Measurement of EATs in contracting skeletal muscle, where there is increased blood flow (with shorter residence time for RBCs in capillaries) and higher oxygen consumption, would be of interest to study the effects of the discrete nature of blood flow on oxygen transport to tissue via capillaries. A previous mathematical model (Groebe and Thews 1989), considered the impact of increased inter-erythrocyte plasma gap spacing and concluded that plasma gap spacing does not play an important role for tissue oxygen supply as long as overall capillary oxygen flux stays constant. Perhaps,

examining EATs under exercise conditions with increased red blood cell flux in capillaries and capillary recruitment can shed some light on this and similar issues. The effects of Oxyglobin and other hemoglobin-based oxygen carriers on EATs and capillary PO_2 under these conditions might also be considered.

Another proposal related to the present study would be the determination of functional capillary density following administration of Oxyglobin. Various studies have reported conflicting conclusions regarding the ability of hemoglobin-based oxygen carriers to maintain or decrease functional capillary density (Tsai, Friesenecker et al. 1995; Tsai 2001) with one study involving the use of Oxyglobin. Furthermore, FCD determination after the administration of an HBOC in conjunction with a vasodilator might also be of interest.

Finally, future study involving the comparison of Oxyglobin to the human HBOC product from Biopure would be worthwhile. The human product Hemopure®, or HBOC-201, is a bovine hemoglobin glutamer - 250. As a result of its increased size (32 to 500 kD compared to Oxyglobin molecular weight between 65 and 130 kD with no more than 10% having a molecular weight >500 kD) and improved purification, different results may be observed.

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