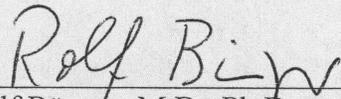


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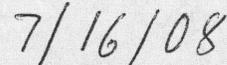
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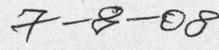
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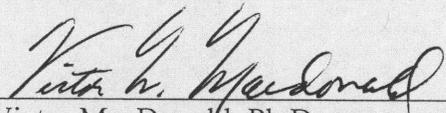
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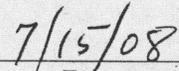
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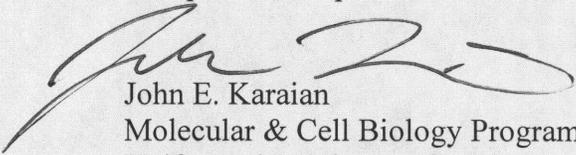


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ABSTRACT

Title of Dissertation: “Use of Isolated Mitochondria and Pulmonary Artery Endothelial Cell Systems In Studies of Oxygen Utilization and the Effects of Hemoglobin-Based Oxygen Carriers”

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Introduction: Hemoglobin-based oxygen carriers (HBOCs) with various oxygen affinities are currently being evaluated for clinical use. We tested whether HBOCs with high vs. low oxygen affinities can affect molecular oxygen on- and offloading, using isolated mitochondria and Bovine Pulmonary Artery Endothelial Cells (BPAECs) as model systems.

Methods: Isolated mitochondria in the absence/presence of low and high oxygen affinity HBOCs were assessed for changes in State 3 (active)/State 4 (resting) respiration rates and changes in the Respiratory Coupling Ratio (RCR). Furthermore, HBOC treated mitochondria were evaluated by immunoblotting for various mitochondrial electron transport proteins and the VDAC/Porin protein complex. A cell culture model of confluent BPAECs, passages 3-6, were maintained in RPMI media, supplemented with 10% FBS, L-glutamine, and antibiotics. BPAECs (5×10^7 cells) were placed in a respirometer containing room air saturated buffer in the absence or presence of bovine hemoglobin (HBOC-201®) ($p50=26$ torr, 21.5°C) or human hemoglobin (HbA_0) ($p50=6.9$, 21.5°C). Uncoupler $0.3 \mu\text{M}$ carbonyl cyanide 4(tri-fluoromethoxy) phenylhydrazine (FCCP) was used to trigger

maximal mitochondrial VO_2 . Oxygen uptake was measured at 95% oxygen depletion (VO_{295}) and at physiological microvascular oxygen tension (PO_2) ranges (35-0 torr). An MTS (a modified MTT) assay was performed at Baseline ($t=0$), 4 hours post treatment, and 24 hours post treatment.

Results: Isolated mitochondria proved to be an inadequate model system to study oxygen uptake in the absence/presence of low vs. high oxygen affinity HBOCs. This was due to the following reasons: Treatment of mitochondria with various doses of either low vs. high oxygen affinity HBOCs led to a decrease in State 3 (active) respiration, along with a reduction of the RCR. Also treatment of mitochondria with various doses of these two HBOCs caused the loss of immunoreactivity of antibodies to mitochondrial electron transport proteins and the VDAC/Porin protein complex as well. In the BPAEC system we observed differences among the effects of low vs. high oxygen affinity HBOCs when assessing cellular oxygen uptake up to 95% oxygen depletion (VO_{295}): There was an expected enhancement of oxygen uptake by BPAECs treated with low oxygen affinity HBOC-201 relative to HbAo. However, there were **no differences** in BPAEC oxygen utilization when the VO_2 was measured over the physiological range of microcirculatory PO_2 's (35-0 torr). In addition, the MTS (modified MTT) assay revealed no differences between HBOC treatments in mitochondrial metabolic activity at 4 hours and 24 hours post treatment with low vs. high oxygen affinity HBOCs.

Conclusion: 1) Isolated rat liver mitochondria are not a useful model to assess molecular oxygen on- and offloading in the presence low vs. high oxygen affinity HBOCs; 2) As physiologically expected in the BPAEC system, oxygen uptake was increased with the low oxygen affinity HBOC-201 up to 95% oxygen depletion (VO_{295}) compared to high oxygen affinity HbAo; 3) However, no differences in BPAEC oxygen uptake were observed over a range of physiological microcirculatory PO_2 values from 35-0 torr; this suggests that oxygen affinity of HBOCs per se **may not** be the key determinant for cellular oxygen availability under microcirculatory conditions. 4) No differences were observed in an MTS assay at 4 hours and 24 hours post treatment with low vs. high oxygen affinity HBOCs indicating that the BPAEC-HBOC protocol did not result in any measurable changes to mitochondrial redox metabolism under the conditions of this study.

DEDICATION

I dedicate this dissertation to all the men and women in the U.S. military who put their lives at risk to protect us from harm's way. Also I dedicate this dissertation to my parents whose love and guidance has helped me throughout my life.

**Use of Isolated Mitochondria and Pulmonary Artery Endothelial Cell Systems
In Studies of Oxygen Utilization and the Effects of Hemoglobin-Based Oxygen
Carriers**

By:

John Edward Karaian

Dissertation submitted to the Faculty of the
Molecular & Cell Biology Program
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LIST OF ABBREVIATIONS

BME	Bis N-Makeimidomethyl Ether
BPAEC	Bovine Pulmonary Artery Endothelial Cells
CS	Citrate Synthase
DNTB	Dithio-Bis(-2-Nitrobenzoic Acid)
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol-bis(2-aminoethylether)-N-tetraacetic Acid
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FCCP	Carbonyl Cyanide 4(tri-fluoromethoxy) Phenylhydrazone
FCD	Functional Capillary Density
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic Acid
HbAo	High oxygen affinity-Human Hemoglobin
HBOC	Hemoglobin-Based Oxygen Carrier
HBOC-201	Low oxygen affinity-Bovine Hemoglobin
LDH	Lactate Dehydrogenase
MAP	Mean Arterial Blood Pressure
MetHb	Methemoglobin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (modified MTT)
NO	Nitric Oxide
O ₂	Oxygen
PEG	Polyethylene Glycol

PFC	Perfluorocarbon
PO ₂	Partial Pressure of oxygen (torr)
RBC	Red Blood Cell
RCR	Respiratory Coupling Ratio/Respiratory Control Ratio
TRALI	Transfusion-Related Acute Lung Injury
VDAC	Voltage Dependent Anion Channel
VO ₂	Oxygen Uptake

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Chapter 1- Historic Background of Hemoglobin-Based Oxygen Carrier (HBOC) Development

The transfusion of red blood cells (RBCs) in clinical medicine is one of the oldest, most trusted procedures, but it is the least scientific of medical practices; least scientific because the practice is not supported by clinical data showing efficacy and safety which would be required by any new therapeutic regimen based on Food and Drug Administration (FDA) standards (1). The clinical decision to transfuse a patient is based largely on the principle that infusion of RBCs, containing the physiological oxygen carrier hemoglobin, will increase the oxygen available to hypoxic or ischemic tissues (1).

Currently, the transfusion of blood to patients remains a common practice in the modern clinical setting, but may lead to severe reactions attributed to immunological mechanisms, non-immunological mechanisms, or infection (2, 3). Most cases of clinical transfusion reactions are attributable to cross-matching errors between the donor and patient blood types (2, 3). However, even when the correct blood type is given a mild transfusion reaction can occur. The most common immunologically mediated transfusion reaction is non-hemolytic fever, causing fever and chills without destruction (hemolysis) of the red blood cells (2, 3). It can occur even when the blood has been correctly matched and administered. The more transfusions a patient receives, the greater the risk for this type of reaction. Also, immunological hemolytic transfusion reactions can occur and destroy the transfused red blood cells. This most commonly occurs if there is a mismatch with the ABO or Rh blood types, but some of the minor blood subtypes can also cause this severe reaction, which can be life-threatening (2, 3).

Allergic reactions may be mild or severe (2, 3). Mild allergic reactions may involve itching, hives, and wheezing. Severe allergic reactions can involve anaphylactic shock, a life-threatening

reaction. An immune reaction to platelets in transfused blood results in the destruction of the transfused platelets. People who develop this type of reaction may have difficulty finding blood that can be transfused without causing a reaction. On rare occasions, an immune reaction may take place that attacks the person's lungs, leading to transfusion-related acute lung injury (TRALI) (2-3). This complication often results in difficulties in breathing and can ultimately lead to lung failure. At the microcirculatory level there is cytokine activation and vascular leakage resulting in edema (4).

Non-immune reactions involve physical alterations to the blood because of a transfusion (2,3). Intravascular volume overload can develop when a patient receives too much fluid due to transfusion, especially if there is no blood loss before the transfusion. The clinical signs associated with volume overload are often iron overload and could also lead to heart failure in patients with cardiovascular disease (2,3) Also, viral/bacterial contamination of donor blood is a risk of receiving blood transfusions; of major concern in this respect include human immunodeficiency virus (HIV), hepatitis B/C, as well as more recent pathogens of concern, including west Nile virus and mad cow disease.

Recent evidence from human trauma studies have demonstrated improved outcomes in patient populations receiving a modified transfusion consisting of a high ratio of plasma/platelets to RBCs (5). The use of traditional 'transfusion triggers' in trauma care have been widely discussed (6) and two recent studies in the journal "The Proceedings of the National Academy of Sciences" (PNAS) have examined whether stored blood is more dangerous compared to freshly drawn blood or RBCs (7, 8). According to these recent studies, RBCs stored for more than 3 hours begin to lose s-nitrosylation (S-NO) on cysteine moieties of the hemoglobin tetramer (7, 8). Reduced s-nitrosylation of these key amino acids appears to impair hemoglobin's ability to restore coronary blood flow to ischemic hearts in an in-vivo canine model (7). This interpretation was supported by the finding that

restoring S-NO hemoglobin, by incubating cell-free hemoglobin with a chemical nitric oxide donor, also restored coronary blood flow to an extent comparable to fresh S-NO containing hemoglobin (7). Thus, such studies could suggest that the conventional practice of 'blood banking' is not optimal; if not problematic. In the post 9/11 era, there is also a greater concern for bioterrorism, eg. contamination of the blood banking supply by biological or chemical toxins such as bioengineered viruses/bacteria and chemical toxins such as cyanide.

A promising solution to the existing risks of transfusion reactions with RBCs appears to be the development of artificial Hemoglobin-Based Oxygen Carriers (HBOCs), which can theoretically be engineered to serve as universally compatible oxygen delivery vehicles that ideally can be readily administered to any patient at reduced transfusion risks. Various different HBOCs have been developed with varying degrees of oxygen affinity, i.e. p_{50} 's, the partial pressure of oxygen at 50% hemoglobin oxygen saturation (9). Two major classes of HBOCs are being developed and tested: 'low oxygen affinity' HBOCs (high p_{50}) or 'high oxygen affinity' HBOCs (low p_{50}). However, it still is debated whether these different oxygen affinities of these HBOCs have a physiological and significant impact on the delivery of oxygen at the microcirculatory level.

The class of blood substitutes, referred to as the 'HBOCs' are the focus of this study. HBOCs are derived from human or bovine hemoglobin. Non-hemoglobin oxygen carriers such as perfluorocarbons (PFCs) have the ability to bind dissolved oxygen with a very high oxygen affinity. Therefore, supplemental oxygen had to be administered with PFCs so that bound oxygen could be released at microcirculatory oxygen partial pressures (9). Another effort focused on encapsulated HBOC formulations, for example by liposomal formulations, but this type of HBOC is not the focus of this study.

Already in the 17th century there were attempts to infuse various "HBOC type" solutions into patients as blood substitutes, such as wine; but efficacy could not be proved (9, 10). A major paradigm shift in the use of blood substitutes occurred late in the 19th century, when it was discovered that antigens were located on the surface of red blood cells (9, 10). This observation led to the idea to use cell-free isolated hemoglobin, free of red cell membrane antigens (stroma free hemoglobin). Early reports from Germany in the 1890's by Von Stark et al. (9, 10) described the first attempts to treat anemic patients subcutaneously with a stroma free hemoglobin application (9, 10). Then in 1916, Sellards and Minot at Massachusetts General Hospital, tested a stroma free hemoglobin solution, in patients with anemia, which however led to subsequent hemoglobinuria in 33 patients (9, 10).

In a later study by Amberson et al. in 1949 it was demonstrated that use of stroma free hemoglobin in a woman undergoing postpartum hemorrhage, while resulting in hemodynamic stability, led to oliguria and renal failure, and ultimately death (9, 10). Hence, due to a variety of severe clinical complications, including hypertension, bradycardia, and renal failure, the development of stroma free hemoglobin as a blood substitute was abandoned.

The next major paradigm shift in the efforts to produce a pathogen and pyrogen free blood substitute occurred only in the mid-1950's, after the chemical structure of hemoglobin had been elucidated. It was established that hemoglobin was a protein that was a tetramer, having a molecular weight of 64.5kD (9, 10). The tetrameric protein was composed of two alpha and two beta subunits. It was considered that the renal problems associated with the administration of cell free hemoglobin's were due to the subunit dissociation from the hemoglobin tetramer releasing free alpha/beta dimers (32kD); these dimers could be ultrafiltered into Bowman's space of the glomerular capsule, entering the proximal renal tubule, thereby causing obstruction and tubular

necrosis (8, 9). It seemed conceivable that chemical modifications of hemoglobin to increase its molecular size and radius could prevent subunit dissociation and the deleterious glomerular filtration. It was hoped that the renal toxicity of such stroma-free hemoglobins should be alleviated by such new large hemoglobins. Work performed by Bunn et al. in the 1950-60's demonstrated that the chemical cross-linker bis N-maleimidomethyl ether (BME) could be employed as an effective cross-linker, cross-linking the beta subunits of the hemoglobin tetramer and preventing subunit dissociation under in vivo conditions. This type of cross-link resulted in an artificial hemoglobin with a higher than physiological oxygen affinity (a lower p50), in comparison to native human hemoglobin found in RBCs (p50 of 26-28torr at 37°C, pH=7.4) (9, 10).

In the mid 1980's Diasprin-(α - α)-crosslinked human hemoglobin was developed and tested as an HBOC. In this preparation crosslinking of the alpha-alpha lysine residues of the hemoglobin tetramer were demonstrated to prevent hemoglobin subunit dissociation. This HBOC was developed in cooperation between the U.S. Army and Baxter Healthcare Inc.; it had an oxygen affinity similar to that of whole RBC hemoglobin at 37°C with a p50 of 26-28torr (11).

Around the same time that Diasprin-(α - α)-crosslinked human hemoglobin was being developed, other HBOC models were studied (9). For example, new chemical modifications were employed to increase the hemoglobin molecular weight, thereby increasing its plasma half life (9); this was accomplished using polymerization, which links multiple hemoglobin tetramers together. It became evident that bifunctional aldehydes such as glutaraldehyde were useful as polymerization agents (9). However, because it was difficult to control the glutaraldehyde polymerization process itself the resulting hemoglobin polymers had greatly varying molecular

weights (from 258kD-64kD) (12). It was also realized that glutaraldehyde polymerization of bovine hemoglobin decreased its oxygen affinity to a p_{50} of 37.8torr at 37°C, pH=7.4 from a p_{50} of 27torr at 37°C, pH=7.4 (13). Nevertheless, it was argued that such a ‘high p_{50} ’ (low oxygen affinity) hemoglobin would facilitate the offloading of oxygen at low partial pressures of oxygen (PO_2) in the microcirculation, i.e. where oxygen transport to parenchymal cells occurs physiologically. The proponents of low oxygen affinity hemoglobin (high p_{50}) believed that a lowered oxygen affinity would be beneficial in facilitating oxygen transport in the microvasculature or capillary beds (9, 14).

Support for this concept came from studies that suggested that a lower oxygen affinity could indeed benefit highly metabolically active tissues such as the myocardium and the brain (14). Further indirect support came from studies by Woodson et al. who used 2,3-DPG depleted rodent RBCs to increase oxygen affinity, reporting that these 2,3-DPG depleted RBCs were inferior to using untreated RBCs during resuscitation of hemorrhagic shock (15).

This information would eventually lead to the development of HBOC-201 by Biopure Corporation (Cambridge, MA). This HBOC is a **bovine** ‘low oxygen affinity’ glutaraldehyde polymerized hemoglobin with a p_{50} of 36.8torr at 37°C, pH=7.4 and a molecular weight ranging between 87.2-502.3kD (13, 16). Another company, Northfield Laboratories (Chicago, IL) also developed a polymerized HBOC, PolyHeme, which was a glutaraldehyde polymerized **human** hemoglobin, and had an oxygen affinity similar to that of whole human RBC hemoglobin at 37°C with a p_{50} of 26-28torr (17).

As various HBOC preparations were being developed by private industry in the late 1980’s, and were being tested in clinical phase trials, many clinically adverse side-effects/toxicities of the various HBOCs still became apparent. Many of the clinical side-

effects/toxicities were similar for the various HBOCs including elevations in systemic and pulmonary arterial pressures (MAP), decreased cardiac output, gastrointestinal symptoms including, but not limited to, abdominal pain, elevated plasma liver enzyme activities, and even acute pancreatitis (18). Clinical testing of Diasprin-(α - α)-crosslinked human hemoglobin in a phase III study to assess the efficacy and safety in human trauma patients compared to standard treatment demonstrated increased morbidity/mortality in patients transfused with Diasprin-(α - α)-crosslinked human hemoglobin (45% vs. 32%, $p < 0.05$) after 28 days (19). The failure of these clinical trials with Diasprin-(α - α)-crosslinked human hemoglobin eventually lead to the abandonment by Baxter Healthcare of HBOCs based on α - α cross-linking technologies. Furthermore, the multitude of adverse reactions encountered during clinical testing of all the various HBOCs led the Food and Drug Administration (FDA) to put a hold on many of the ongoing Phase III clinical trials with HBOCs of all types.

Many of the known adverse clinical reactions caused by intravenous HBOCs are hypothesized to be related to or caused by the scavenging of nitric oxide (NO). According to this hypothesis when cell-free hemoglobin solutions are administered they can scavenge NO, thereby removing a physiological vasodilator resulting in vasoconstriction and pulmonary and systemic hypertension (20).

In an effort to reduce NO scavenging, Hemospan, an HBOC by Sangart Inc. (San Diego, CA) has been designed, characterized by polyethylene glycol (PEG) coating of purified human hemoglobin (21). A non-antigenic layer of PEG is not known to trigger immune reactions and was used to encapsulate the hemoglobin. This technique produces an HBOC with a large molecular radius and a highly negative charge, which could shield the positive amino acid residues of the hemoglobin tetramer thereby making it less likely that this pegylated HBOC

would be bound by the negatively charged endothelial cell membranes. Hemospan has a very 'high oxygen affinity' with a p50 of 4-5 torr at 37°C, pH=7.4. Animal studies using this HBOC have demonstrated improved survival in a swine in vivo model of uncontrolled hemorrhagic shock (22). Furthermore microvascular functional capillary density (FCD) measurements in an in vivo hamster pouch model have demonstrated improved FCD upon serial transfusions of this very 'high oxygen affinity' Hemospan compared to the 'low oxygen affinity' HBOC-201, which is not pegylated (24). More recently, Sangart Inc. has reported that they have completed Phase I and Phase II clinical trials of Hemospan in Sweden at the Karolinska Institute with favorable outcomes in initial dose-response studies (21, 23). The reported side effects were mildly elevated hepatic enzymes and lipase and was associated with less hypotension and more bradycardic events in comparison to Ringer's acetate. The company is in the process of enrolling patients for a large multicenter European Phase III clinical trial for orthopedic surgical cases. The company has not submitted an application to the FDA for approval in the United States of America.

Based on these complex data and results, and considering the existing demand for pyrogen and pathogen free HBOCs with suitable oxygen binding and offloading characteristics we attempted to develop a new in vitro model system that allowed measurement and quantification of the effect of differing oxygen affinity HBOCs on oxygen delivery to and consumption by mitochondria as well as intact endothelial cells. We tested two model systems: 1) isolated rat liver mitochondria and 2) Confluent cultured whole cell bovine pulmonary artery endothelial cells (BPAECs). The following thesis describes and analyzes the efforts and results developing and working with such never-before-tested model systems. Another goal of these studies was to examine the utility of these two systems for assessing offloading and cellular

uptake of oxygen and utilization at microvascular PO_2 's, i.e. in an environment where gas exchange and diffusion occur physiologically in vivo.

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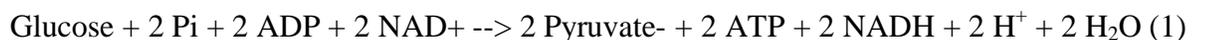
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Chapter 2- Tests of Isolated Rat Liver Mitochondrial Preparations in The Presence of HBOCs

Introduction:

Considering the continued interests in providing pyrogen and pathogen-free hemoglobin based oxygen carriers (HBOCs) we attempted to develop and test an in-vitro system that would allow us to quantitatively to assess the oxygen delivery and offloading features of these HBOCs. Since mitochondria are the loci of oxidative phosphorylation it seemed promising to examine whether mitochondrial oxygen uptake could be influenced by low vs. high oxygen affinity HBOCs.

In eukaryotic cells, the mitochondria serve as energy producing organelles (1). Most of cellular oxygen is utilized by the mitochondria to reduce molecular oxygen to water during the process of oxidative phosphorylation. For example, aerobic respiration with glucose as substrate first involves the cytosolic cleavage of glucose ($C_6H_{12}O_6$) to yield 2 molecules of pyruvate:



When oxygen is present pyruvate can be transported into the mitochondria through the monocarboxylate transporter and feed in to the Krebs's cycle to yield 34 ATPs via oxidative phosphorylation as follows (assuming a classical P/O ratio of 3) (16):



We hypothesized that isolated mitochondrial preparations could potentially be used as a model system to quantitate the delivery of oxygen and use of oxygen by the mitochondrial respiratory chain tested in the presence of low vs. high oxygen affinity HBOCs.

Materials & Methods:

A.) Preparation of the Hemoglobin & Myoglobin Solutions

HBOC-201, a glutaraldehyde polymerized bovine hemoglobin solution, with low oxygen affinity ($p_{50} = 36.8$ torr @ 37°C) (7) at a concentration of 13.5g/dl bag was provided for testing from the Biopure corporation (Cambridge, Massachusetts, USA). Since previous studies have demonstrated that low oxygen affinity HBOC-201 can rapidly be converted to methemoglobin, and loss its ability to bind to oxygen (2), samples of HBOC-201 were drawn up using a gas tight syringe, placed into cryovials, immediately immersed into liquid nitrogen, and maintained at -80°C , until usage. Human HbAo, a high oxygen affinity HBOC ($p_{50} = 10$ torr @ 37°C) was purchased from Sigma/Aldrich Corp. (USA) as a lyophilized powder solution, and was reported to be free of excess methemoglobin. A stock solution of 13.5g/dl human HbAo was prepared by dilution of the lyophilized powder into mitochondrial respiration buffer containing no EDTA (please refer to the section titled, 'Isolation of Rat Liver Mitochondrial Preparations' for a more detailed description), and was placed into cryovials, which were immediately immersed into liquid nitrogen, and maintained at -80°C , until usage. For use, hemoglobin solutions were gently thawed in an ice-water bath to prevent methemoglobin formation (3). This method led to the formation of $\leq 3\%$ methemoglobin in the hemoglobin solutions (3). After thawing the

methemoglobin content in the hemoglobin solutions were verified by the Evelyn-Malloy Assay (4) as described in Section B.) below.

B.) Determination of the Methemoglobin (Fe^{3+}) Concentrations in the various Hemoglobin Solutions:

To control for the effects of spontaneous methemoglobin production, the method of Evelyn-Malloy (1938) was utilized (4). This method is spectrophotometrically based and is a superior technique to quantitate the % of methemoglobin in the various hemoglobin solutions. The % MetHb in a solution is determined as follows: hemoglobin solutions 13.5g/dl were diluted in a quartz cuvette with mitochondrial respiratory buffer, by adding 990 μl of mitochondrial respiratory buffer and 10 μl of cell-free hemoglobin. This dilution standardized the spectrophotometer to give maximal absorbances below values of 1.0. To determine the amount of oxyhemoglobin present a quartz cuvette containing 990 μl of mitochondrial respiratory buffer and 10 μl of cell-free hemoglobin was scanned (450-650nm) on a Shimadzu UV-2401 PC spectrophotometer. This scan represented the Oxyhemoglobin spectra. To obtain the total amount of Met-Hb (where the oxyhemoglobin is chemically oxidized to Met-Hb) 990 μl of mitochondrial respiratory buffer and 10 μl of cell-free hemoglobin along with a small amount of potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) was placed into a quartz cuvette and scanned (450-650nm). In another scan, which was needed to determine how much Met-Hb was originally present, 990 μl of mitochondrial respiratory buffer and 10 μl of cell-free hemoglobin along with a small amount of potassium cyanide (KCN) were placed into a quartz cuvette and scanned (450-650nm). Finally, to determine the amount of cyanmethemoglobin in our samples 990 μl of mitochondrial

respiratory buffer and 10 μ l of cell-free hemoglobin along with a small amount of potassium cyanide (KCN) + potassium ferricyanide (K₃Fe(CN)₆) into a quartz cuvette and scanned (450-650nm). All samples were run in triplicate and each individual cuvette was scanned in triplicate, with the reads averaged.

The %MetHb was determined as follows after performing all the different scans:

1. No added reagents, Oxy-Hb spectrum
2. With K₃Fe(CN)₆, Total Met-Hb spectrum
3. With KCN, Met-Hb initially present
4. With K₃Fe(CN)₆ + KCN, totally CN-Met Hb

The following formula was used to calculate the %MetHb:

$$\frac{A_{540}(3) - A_{540}(1)}{A_{540}(4) - A_{540}(2)} \times 100 = \% \text{ Met Hb}$$

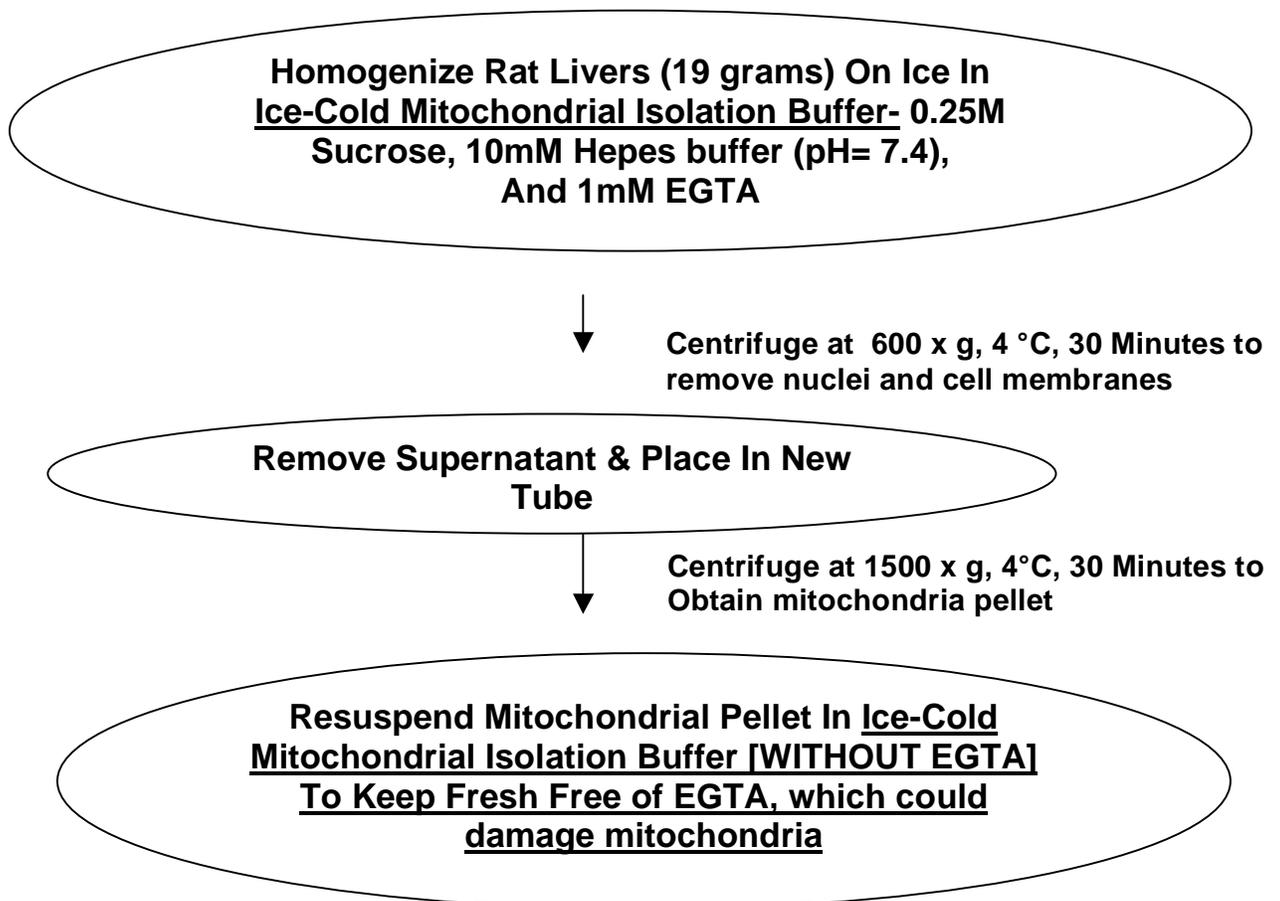
Whereby, A₅₄₀ is defined as the absorbance of the sample at 540nm (4).

C.) Isolation of Rat Liver Mitochondria

All animals were handled according to Institutional Animal Care & Use Committee (IACUC) guidelines. Using Sprague-Dawley rats (300-350grams), livers (19grams) were surgically exposed through a right sided mid-thoracic transverse incision, followed by immediate excision of the anatomical lobes of the liver; which were then placed into cold mitochondrial isolation buffer on ice. Mitochondrial isolation was accomplished through differential centrifugation at 4°C, as previously described (5, 6, 7). Initially, the liver tissue was mechanically homogenized in ice-cold mitochondrial isolation buffer containing 0.25M Sucrose, 10mM HEPES, and 1mM EGTA, 20mM KCl, 5mM potassium phosphate, and 2mM MgCl₂,

pH=7.4. Afterwards, the crude liver homogenates were centrifuged at a speed of 600 g for 30 minutes to remove phospholipid cell membranes and nuclei, and the supernatants were collected. The collected supernatants were then centrifuged at 1500 g for 30 minutes to obtain a mitochondrial pellet. The pellet was washed three times with ice-cold mitochondrial isolation buffer (without EGTA) to obtain the final isolated mitochondrial preparations (See **Flowchart #1**).

Flowchart #1: Protocol for isolation of rat liver mitochondrial preparations (5, 6, 7).

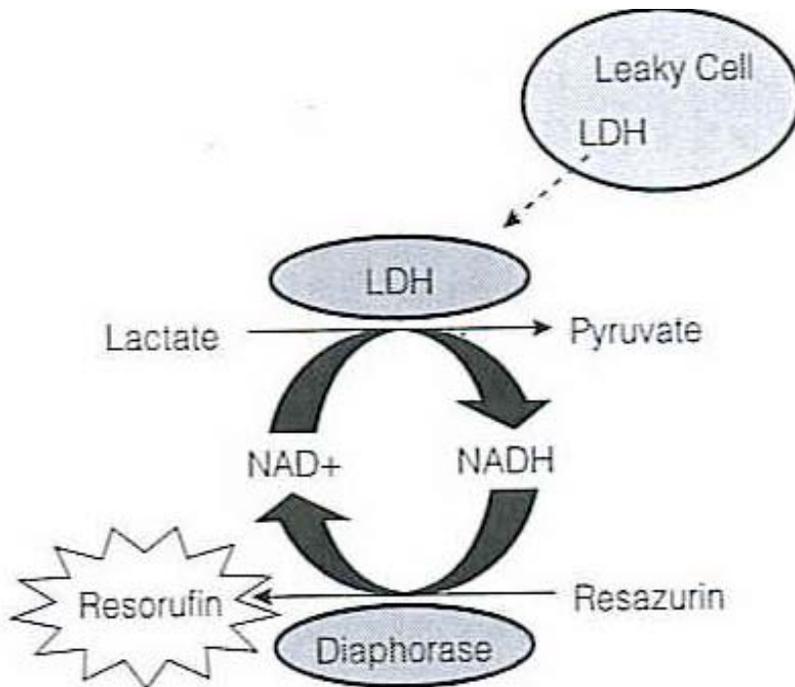


D.) Mitochondrial Purity & Protein Quantification-

1.) Lactate Dehydrogenase (LDH)

Lactate Dehydrogenase (LDH) is a cytosolic protein. A fluorimetric LDH assay was performed to assess cytosolic protein contamination in the final isolated mitochondrial pellet, as compared to initial crude liver homogenates. Fluorescence determination was performed using a commercially available CytoTox-ONE Homogenous Membrane Integrity Assay (Cat# G7890; Promega Corp., USA). The product was supplied as a lyophilized substrate mixture which was reconstituted with Assay buffer to form the active CytoTox One Reagent; containing resazurin, lactate, NAD⁺, and diaphorase. LDH was measured with a 10 minute coupled enzymatic assay that results in the conversion of resazurin into resorufin (See **Figure 1**), a fluorescent product with a maximal excitation wavelength of 560nm ,and a maximal emission wavelength of 590nm. 200µl of either crude liver homogenates or final mitochondrial preparations were added to 3ml clear coated disposable polystyrene cuvettes (Sigma Corp., USA). 2ml of the Cyto-Tox One Solution were also added to the cuvettes and incubated at room temperature for 10 minutes. Afterwards, fluorescence was determined at peak excitation & emission wavelengths, using a Perkin Elmer LS-50B Fluorimeter (Perkin Elmer, USA).

Figure 1: The CytoTox-One® Homogenous Membrane Integrity Assay and the coupled enzymatic reaction¹.



¹- This Illustration is from Promega Corporation's online Technical Bulletin for the CytoTox-ONE Homogenous Membrane Integrity Assay (Cat# G7890; Promega Corp., USA)

2.) Citrate Synthase

Citrate synthase (CS) is a metabolic Krebs (tricarboxylic acid) cycle enzyme, and is found mainly in the mitochondrial matrix (8). Therefore, its measured enzymatic activity serves as an index of mitochondrial quantity in mitochondrial preparations (8). CS activity was measured as previously described (5). The following reagents were prepared: A.) 1mM 5-5'-dithio-bis (-2-nitrobenzoic acid) (DNTB) (Sigma Corp, USA) dissolved in 0.5M Tris-HCl buffer, pH=8.1, B.) 10mM acetyl coenzyme a (Sigma Corp, USA), trilithium salt, dissolved in deionized water, and C.) 10mM oxalacetate (Sigma Corp, USA) dissolved in deionized water. In a 2ml disposable polystyrene cuvette (Sigma Corp, USA) the following components were mixed and

added: 100µl of the 1mM DNTB solution, 30µl of the 10mM acetyl coenzyme a solution, and 720µl of deionized water (Total volume = 850µl). Then 50µg of the mitochondrial protein, as determined by a BioRad protein assay, was diluted in 500µl of 0.5M Tris-HCl buffer, pH=8.1. 30µl of the diluted mitochondrial protein solution was added to each cuvette containing 850µl of DNTB, Acetyl Coenzyme A, and deionized water. Using a Shimadzu UV 2401 Spectrophotometer (Shimadzu Corp, Japan), the cuvettes were scanned for 5 minutes at room temperature using a set wavelength of 412nm. This allowed for background/endogenous CS activity corrections. To determine the total CS activity, 50µl of the oxalacetate solution was added to the cuvettes, and the cuvettes were rescanned for 5 minutes at room temperature using a set wavelength of 412nm. The net CS activity was calculated by subtracting the change in absorbance at 412nm/minute of the background/endogenous CS activity from the change in absorbance at 412nm/minute of the total CS activity. The CS activity was calculated according to the equation:

$$\text{Units of CS Activity } (\mu\text{mole/ml/min}) = \frac{(\Delta\text{CS}_{\text{Total}}/\text{minute} - \Delta\text{CS}_{\text{Background}}/\text{minute}) \times V(\text{ml}) \times \text{dil}}{\epsilon^{\text{mM}} \times L(\text{cm}) \times V_{\text{enz}}(\text{ml})}$$

Later conversion of CS activity from µmole/(min * mg pt) to nmol/(min * mg pt):

$$\text{CS Activity } (\mu\text{mole}/(\text{ml} \cdot \text{min})) \times 10^3 = \text{CS Activity } (\text{nmol}/(\text{ml} \cdot \text{min}))$$

Whereby: dil – the dilution factor of the original sample

V (ml)- the reaction volume = 1ml

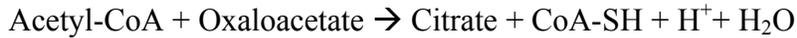
V_{enz} (ml)- the volume of the enzyme sample (ml)

ε^{mM} (mM⁻¹cm⁻¹)- the extinction coefficient of TNB at 412nm is 13.6.

L (cm)- pathlength defined as 1cm.

Pt (mg)- protein content

Reaction catalyzed by Citrate Synthase:



Colorimetric reaction:



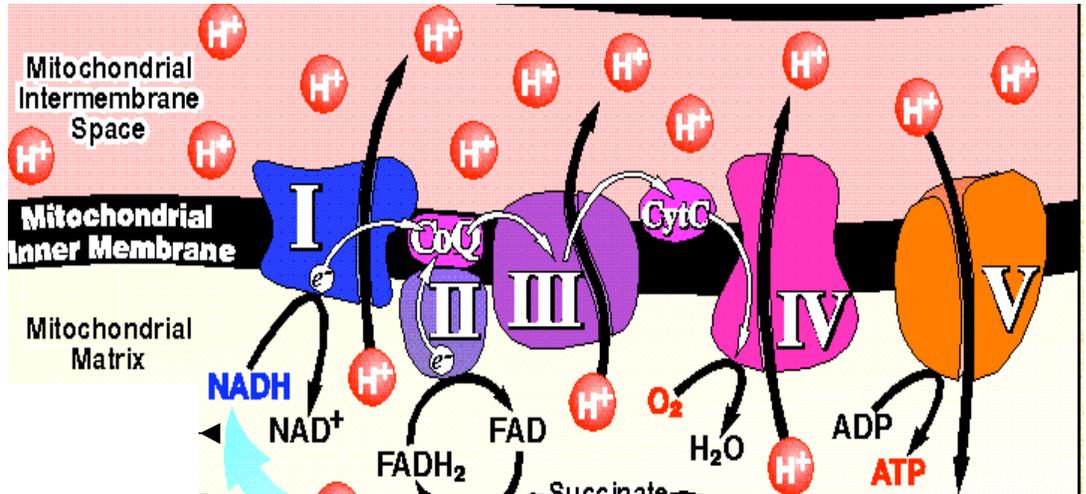
3.) Mitochondrial Protein Assay

The protein content of isolated liver mitochondria was determined by performing a BioRad protein assay. This is based upon the method of Bradford M et al. and uses a dye, Commasie Blue which reacts with proteins to give a colorimetric product with a maximal absorbance at a wavelength of 595nm (9). Protein Standard for Bovine Serum Albumin protein was purchased from Pierce and standards were serially diluted to a range of 2mg/ml-0mg/ml protein. Standard curves for protein concentration versus absorbances were generated from the serially diluted protein standards by mixing 10 μ l of a standard protein solution with 990 μ l of Biorad Protein assay reagent in a cuvette. After 5 minutes absorbances were measured at 595nm and a calibration curve relating protein concentrations versus absorbances was generated. To determine the protein concentration in the mitochondrial samples 10 μ l of the mitochondrial sample was mixed with 990 μ l of Biorad Protein assay reagent in a cuvette. After 5 minutes absorbances were measured at 595nm and the protein contents were obtained from the calibration curve from the standards. The average protein concentration of the mitochondria were

200-250mg protein per 10 grams of liver, which was in the range of protein yield reported by others using a similar mitochondrial isolation technique (6).

E.) State 3/ State 4 Respiration Rates & Respiratory Coupling Ratio (RCR)

The oxygen consumption rates in 10 μ l of the isolated rat liver mitochondrial preparations in the presence of 100 μ l of respiratory buffer containing 13.5g/dl solutions were determined using a Multichannel Strathkelvin 928 Oxygen Meter. This was equipped with multiple MT200 microrespirometers (housed with Clark type oxygen electrodes)/for measuring dissolved oxygen consumption rates (Strathkelvin Corp., UK) as previously described (5). Before measurements were performed the oxygen electrodes were calibrated with a 100 μ l solution of room-air-saturated respiration buffer containing a total of 23.85 nmoles of oxygen (10). The State 4 respiration rate (resting state respiration) is defined as the mitochondrial oxygen consumption rate measured in the absence of ADP but in the presence of an electron transport substrates (10mM Malate and 5mM Glutamate). These substrates provide electrons to Complex I via NADH reducing equivalents. Note the schematic provided below shows the mitochondrial respiratory chain complexes:



Complex I dependent respiration was assessed in the presence and absence of various hemoglobin/myoglobin solutions. The State 3 respiration rate is defined as the mitochondrial oxygen consumption rate measured in the presence of an electron-donating substrate (10mM Malate + 5mM Glutamate) plus 1mM of ADP and Pi to drive oxidative phosphorylation physiologically and we tested this in the presence of various hemoglobin/myoglobin solutions.

Respiratory Coupling Ratios (RCR) were computed as ratios of the State 3/State 4 oxygen consumption rates, and served as a measure of how tightly mitochondria could couple the process of electron transport and oxidative phosphorylation, whereby $\text{ADP} + \text{Pi} \rightarrow \text{ATP}$. Previously using this technique others have reported similar RCR values ranging from 4-5 for

control isolated rat liver mitochondria using our mitochondrial isolation techniques (5, 6, and 11).

F.) Immunoblotting of Mitochondrial Electron Transport Proteins & VDAC/Porin

An increase of mitochondrial membrane permeability is one of the key events in apoptotic or necrotic cell death. The mitochondrial membrane permeability transition (MPT) is a Ca^{2+} -dependent increase of mitochondrial membrane permeability that leads to loss of $\Delta\Psi$, mitochondrial swelling, and rupture of the outer mitochondrial membrane. The MPT is thought to occur after the opening of a channel that is known as the permeability transition pore (PTP), which putatively consists of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), cyclophilin D (Cyp D: a mitochondrial peptidyl prolyl-cis, trans-isomerase), and other molecule(s) (12). Therefore we wanted to evaluate mitochondria for possible damage or loss of the VDAC/Porin complex and various other proteins in the mitochondrial respiratory chain in the absence/presence of various cell-free hemoglobins.

100 μl of the isolated rat liver mitochondria (containing 150 μg of mitochondrial protein) were incubated at room temperature (21.5°C) for thirty minutes with 1ml of mitochondrial respiration buffer (maintaining a similar ratio of mitochondria protein: buffer as used in our respirometry experiments) containing either 13.5-0.135 g/dl HBOC-201, 1.35g/dl-0.135g/dl HbAo, 13.5g/dl Metmyoglobin, or 3-3mM H_2O_2 . Control experiments were performed with mitochondria treated only with 1ml of mitochondrial respiration buffer. After incubation samples were centrifuged at 1000g for 10 minutes and 20ul of the mitochondrial pellet protein was prepared for immunoblotting. As previously described (4), proteins were dissolved in sample buffer [62.5mM Tris HCl (pH=6.8), 2% Sodium Dodecyl Sulfate (SDS), 10% glycerol, 5% β -

mercaptoethanol, and 0.005% bromophenol blue] and were subjected to SDS-polyacrylamide gel electrophoresis using a NOVEX® mini- gel apparatus (Invitrogen Corp., USA). Proteins were electrophoretically separated out on pre-cast 10-14% polyacrylamide gels (Invitrogen Corp., USA), and the proteins were transferred onto a nitrocellulose membrane (BioRad Corp., USA) using a NOVEX® mini-gel apparatus (Invitrogen Corp., USA). Non-specific proteins were blocked in 5% non-fat dried milk dissolved in phosphate buffered saline, pH= 7.4 (Quality Biologicals, Gaithersburg, MD) containing 0.05% Tween 20 (TBS). Primary antibodies were diluted in blocking buffer (1:1000) and incubated with the blots at 4°C overnight. For the detection of mitochondrial electron transport chain components a rodent total OXPHOS complex detection kit (MitoSciences, USA) was employed. This kit contained appropriate positive controls and a mixture of 5 monoclonal antibodies for detecting the following mitochondrial electron transport components: 1) The 20 kDa subunit of Complex I (probably ND6), 2) The 30 kDa subunit of Complex II, 3) Core Protein 2 of Complex III, 4) Subunit 1 of Complex IV, and 5) The alpha subunit of Complex V (F1F0 ATPase). Additional blots were prepared using primary antibodies designed to recognize the 39 kDa mitochondrial protein Porin/the Voltage Dependent Anion Channel (VDAC) (14). The membranes were subsequently incubated with secondary antibodies directed towards goat anti-mouse IgG (Chemicon, USA) (1: 10,000). Chemiluminescence as signaled by horseradish peroxidase was detected by autoradiography, using a Super Signal detection kit (Pierce Biotechnology, USA). The optical density (OD) of the protein bands was measured and integrated using the NIH image/Scion image software program.

G.) Data Presentation and Statistics

Data are presented as means \pm Standard Error (SE). Differences between treatment groups were evaluated using the LSD (Least Significant Differences) correction in a generalized ANOVA (analysis of variance) to control for Type I error¹. The error bars in the figures represent SE.

¹Type I (α) error is defined as a false rejection of the null hypothesis. This error is quite often predetermined the value of the assigned P value (14).

Results:

Oxygen Consumption By Isolated Rat Liver Mitochondria In The Presence/Absence of Various Hemoglobin/Myoglobin Solutions- 1.) From **Table 1** treatment of isolated rat liver mitochondrial preparations with either 13.5g/dl HbAo, metmyoglobin, or 3mM hydrogen peroxide led to depressed State 4 (resting) respiration rates, which were respectively lowered to values of 22.2 ± 3.5 , 5.0 ± 0.9 , and 2.9 ± 0.7 nmoles O_2 /min* mg mitochondrial protein, when compared to vehicle control treated (respiration buffer) isolated rat liver mitochondrial preparations (32.0 ± 4.2 nmoles O_2 /min* mg mitochondrial protein). Interestingly, the State 4 (resting) respiration rates of mitochondrial preparations treated with 13.5g/dl HbAo (22.2 ± 3.5 nmoles O_2 /min* mg mitochondrial protein) were significantly different from those treated with either 3mM hydrogen peroxide (2.9 ± 0.7 nmoles O_2 /min* mg mitochondrial protein) or 13.5g/dl metmyoglobin (5.0 ± 0.9 nmoles O_2 /min* mg mitochondrial protein). Furthermore, no statistically significant differences in State 4 (resting) respiration were observed for mitochondrial preparations treated with 13.5g/dl HBOC-201 (30.0 ± 2.2 nmoles O_2 /min* mg mitochondrial protein) when compared to vehicle treated control preparations (32.0 ± 4.2 nmoles O_2 /min* mg mitochondrial protein).

2.) State 3 (active) respiration rates, measure mitochondrial oxygen consumption in the presence of an electron transport substrate (Glutamate + Malate) and ADP + Pi; allowing measure of mitochondrial oxygen consumption during the metabolic process of oxidative phosphorylation. From **Table 1** it was observed that treatment of the mitochondrial preparations with 13.5g/dl of HBOC-201, HbAo, metmyoglobin, or 3mM hydrogen peroxide resulted in statistically significant lower State 3 (active) respiration rates, which were respectively 46.0 ± 11.7 , 43.7 ± 14.3 , 5.5 ± 1.1 , and 8.0 ± 0.5 nmoles O_2 /min* mg mitochondrial protein, in

comparison to control vehicle treated preparations (121.2 ± 19.1 nmoles $O_2/\text{min}^* \text{mg}$ mitochondrial protein). No major differences in State 3 respiration rates were observed for mitochondrial preparations treated with either 13.5g/dl HBOC-201 (46.0 ± 11.7 nmoles $O_2/\text{min}^* \text{mg}$ mitochondrial protein) vs. 13.5g/dl HbAo (43.7 ± 14.3 nmoles $O_2/\text{min}^* \text{mg}$ mitochondrial protein). Furthermore, the treatment of mitochondrial preparations with either 13.5g/dl metmyoglobin or 3mM hydrogen peroxide resulted in significantly lower State 3 (Active) respiration rates, which were respectively 5.5 ± 1.1 and 8.0 ± 0.5 nmoles $O_2/\text{min}^* \text{mg}$ mitochondrial protein, when compared to either of the two hemoglobin treatments (HBOC-201 & HbAo).

3.) The Respiratory Coupling Ratio (RCR), is a ratio of the State 3 (Active) respiration rate to the State 4 (Resting) respiration rate, and provides a measure of mitochondrial function, by serving as an index of the mitochondrial ability to metabolically couple electron transport and the process of oxidative phosphorylation in the overall production of mitochondrial ATP. According to **Table 1** the RCR values of isolated mitochondrial preparations treated with either 13.5g/dl HBOC-201, HbAo, or metmyoglobin were statistically significantly lower, which were respectively 1.6 ± 0.5 , 2.0 ± 0.7 , and 1.2 ± 0.3 , in comparison to controls treated with vehicle (3.9 ± 0.3). Interestingly, the RCR values of the 3mM hydrogen peroxide treated mitochondrial preparations (3.6 ± 0.8) were not statistically significantly different in comparison to controls treated with vehicle (3.9 ± 0.3).

Immunoblot Analysis of various Mitochondrial Electron Transport Proteins and the VDAC/Porin Protein Complex In The Presence/Absence of Various Hemoglobin/Myoglobin Solutions- According to **Table 2** & **Figure 2A** the incubation of rat liver mitochondrial preparations with varying doses of HBOC-201 or human HbAo (13.5g/dl-0.135g/dl) led to a

statistically significant lowering of the integrated optical densities of the mitochondrial electron transport complex proteins III, IV, I, and V, when compared with controls treated with vehicle. In the 13.5g/dl HBOC-201 treatments the integrated optical densities of the mitochondrial electron transport complex proteins III, IV, I, and V, were reduced to $0.6 \pm .04$, $0.5 \pm .03$, $0.5 \pm .01$, and $0.7 \pm .02$ respectively. In the 1.35g/dl HBOC-201 treatments the integrated optical densities of the mitochondrial electron transport complex proteins III, IV, I, and V, were reduced to $0.4 \pm .03$, $0.5 \pm .03$, $0.2 \pm .04$, and $0.6 \pm .07$ respectively. In the 0.135 g/dl HBOC-201 treatments the integrated optical densities of the mitochondrial electron transport complex proteins III, IV, I, and V, were reduced to $1.3 \pm .03$, $0.9 \pm .03$, $0.6 \pm .02$, and $0.9 \pm .05$ respectively. In the 13.5g/dl human HbAo treatments the integrated optical densities of the mitochondrial electron transport complex proteins III, IV, I, and V, were reduced to $0.3 \pm .03$, $0.6 \pm .04$, $0.3 \pm .01$, and $0.4 \pm .02$ respectively. In the 1.35g/dl human HbAo treatments the integrated optical densities of the mitochondrial electron transport complex proteins III, IV, I, and V, were reduced to $0.8 \pm .04$, $1.1 \pm .02$, $0.8 \pm .03$, and $0.8 \pm .02$ respectively. Whereas, in controls treated with vehicle the integrated optical densities of the mitochondrial electron transport complex proteins III, IV, I, and V, were $1.9 \pm .04$, $1.3 \pm .09$, $1.4 \pm .04$, and $1.5 \pm .04$ respectively.

In most cases treatment of isolated mitochondrial preparations with varying doses of HBOC-201 and human HbAo (13.5g/dl-0.135g/dl) did not alter the integrated optical densities of the complex II proteins in the mitochondrial electron transport chain. In the 13.5g/dl HBOC-201 treatments the integrated optical density of the mitochondrial electron transport complex II protein was $2.3 \pm .14$. In the 1.35g/dl HBOC-201 treatments the integrated optical density of the mitochondrial electron transport complex II protein was $1.9 \pm .08$. In the 0.135g/dl HBOC-201

treatments the integrated optical density of the mitochondrial electron transport complex II protein was $1.9 \pm .05$. Treatment with 13.5g/dl human HbAo led to a statistically significant lowering of the integrated optical density of the mitochondrial electron transport complex II proteins to $0.6 \pm .13$. However, treatment with 1.35g/dl human HbAo did not alter the integrated optical density of the complex II proteins ($1.9 \pm .09$), when compared with controls treated with vehicle ($2.3 \pm .04$).

Also, treatment of isolated mitochondrial preparations with high or low doses of hydrogen peroxide (3mM & 0.3mM) did not result in any alterations of the integrated optical densities of the proteins in Complex III, IV, I, and V of the mitochondrial electron transport chain when compared to vehicle treated controls. In the 3mM hydrogen peroxide treatments the integrated optical densities of the mitochondrial electron transport complex proteins III, IV, I, and V, were $1.7 \pm .03$, $1.4 \pm .02$, $1.4 \pm .03$, and $1.7 \pm .02$ respectively. In the 0.3mM hydrogen peroxide treatments the integrated optical densities of the mitochondrial electron transport complex proteins III, IV, I, and V, were $2.3 \pm .03$, $1.3 \pm .04$, $1.9 \pm .01$, and $1.7 \pm .02$ respectively. In the vehicle treated controls the integrated optical densities of the mitochondrial electron transport complex proteins III, IV, I, and V, were $1.9 \pm .04$, $1.3 \pm .09$, $1.4 \pm .04$, and $1.5 \pm .04$ respectively.

On the other hand, the incubation of mitochondrial preparations with varying doses of HBOC-201 or human HbAo (13.5g/dl-0.135g/dl) led to a lowering of the integrated optical densities of the mitochondrial VDAC/Porin protein complex, when compared with vehicle treated controls (**Figure 2B** & **Table 2**). In the 13.5g/dl, 1.35 g/dl, and 0.135g/dl HBOC-201 treatment groups the integrated optical density of the VDAC/Porin protein complex was 0.9, 1.3, and 2.2 respectively. In the 13.5g/dl and 1.35g/dl human HbAo treatment groups the integrated

optical density of the VDAC/Porin protein complex was 0.5 and 0.5 respectively. Whereas, vehicle treated control had an integrated optical density of 2.5.

Treatment of isolated mitochondrial preparations with high or low doses of hydrogen peroxide (3mM & 0.3mM) did not result in any measurable alterations in the optical density to the VDAC/Porin protein complex when compared to vehicle treated controls. In the 3mM and 0.3mM treatment groups the integrated optical density of the VDAC/Porin protein complex was 2.6 and 2.7, respectively, which compared well with the vehicle treated control of an integrated optical density of 2.5.

Summary of Results:

- 1) We find that isolated rat liver mitochondria are not an appropriate system to quantitate the delivery of oxygen to and the use of oxygen by the respiratory chain in the presence of low vs. high oxygen affinity HBOCs.
- 2) Mitochondrial failure was indicated by the following toxic effects of HBOCs:
 - A) Substantial inhibition of the mitochondrial State 3 respiration
 - B) Loss of the normal coupling ratio (RCR)
 - C) Loss of immunoreactivity of antibodies against various electron transport proteins and the VDAC/Porin protein complex
- 3) Control experiments with H₂O₂-induced oxidative stress demonstrated a different mechanism of toxicity and mitochondrial failure when compared with HBOCs: H₂O₂ did greatly reduce State 3 respiration but RCR was maintained normal.

For Discussion of Results Refer to Chapter 4-General Discussion

Table 1: State 3 (active) & 4 (resting) respiration rates, and RCR values of rat liver mitochondria measured in the absence/presence of various 13.5 g/dl hemoglobin/myoglobin solutions. Treatment of mitochondria preparations with 3mM H₂O₂ served as a negative control.

Treatments (Mean ± SEM) (n=6)	State 3 (active) (nmoles O₂)/ (min* mg mito protein)	State 4 (resting) (nmoles O₂)/ (min* mg mito protein)	Respiratory Coupling Ratio (RCR)
Control	121.2 ± 19.1	32.0 ± 4.2	3.9 ± 0.3
Mito + HBOC	46.0 ± 11.7 *, **	30.0 ± 2.2	1.6 ± 0.5*
Mito + 3mM H₂O₂	8.0 ± 0.5 *	2.9 ± 0.7 *	3.6 ± 0.8 ***
Mito + HbAo	43.7 ± 14.3 *, **	22.2 ± 3.5*, **	2.0 ± 0.7*
Mito + Metmyoglobin	5.5 ± 1.1 *	5.0 ± 0.9 *	1.2 ± 0.3*

* p < 0.05 relative to CTRL, Metmyoglobin, or H₂O₂

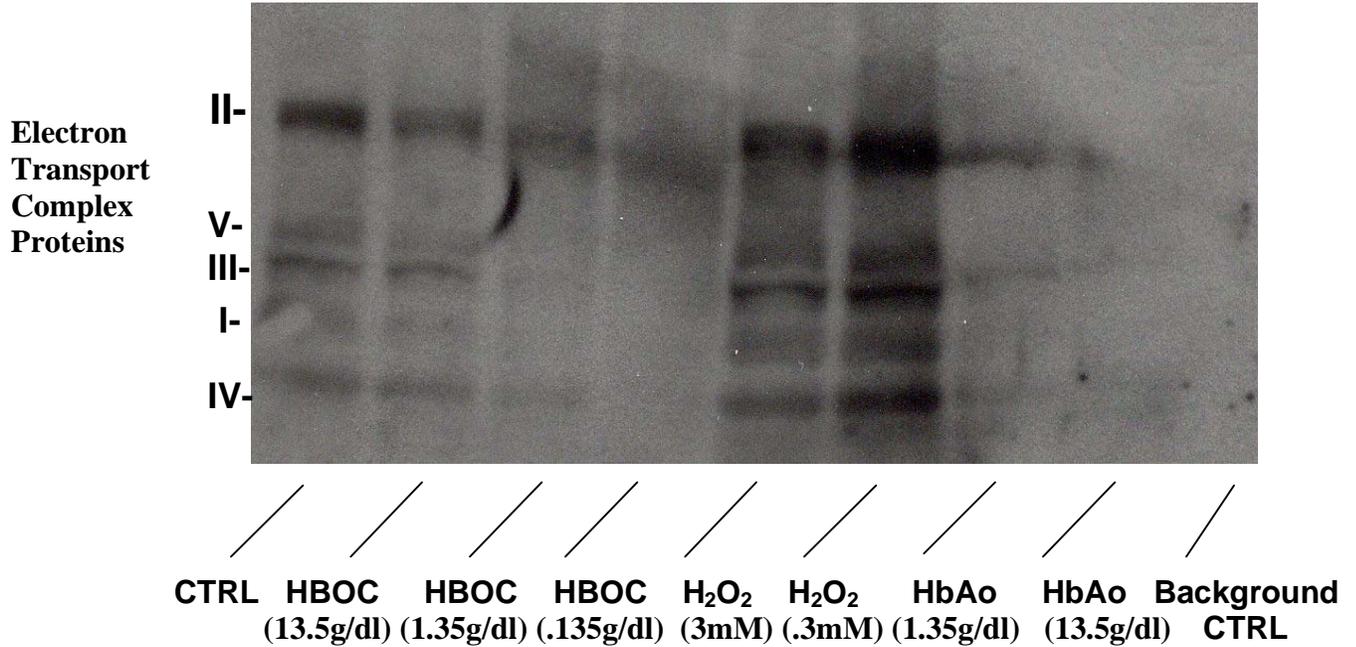
** p < 0.05 relative to Metmyoglobin or H₂O₂

*** p < 0.05 relative to HbAo, HBOC, and Metmyoglobin

Using a one-way ANOVA analysis with the LSD correction
Each chamber contained 15mg/ml mitochondrial protein

Figure 2: A.) Representative immunoblot showing loss of immunoreactivity in various mitochondrial electron transport proteins after treatment with various doses of Hemoglobin/Myoglobin solutions. The total number of preparation tested was N=3
B.) Immunoblot showing loss of immunoreactivity to VDAC/Porin protein complex after treatment with various doses of Hemoglobin/Myoglobin solutions. (N=1)

A.) Representative Immunoblot of Electron Transport Complex Proteins (N=3):



B.) Immunoblot of Porin/VDAC protein (N=1):

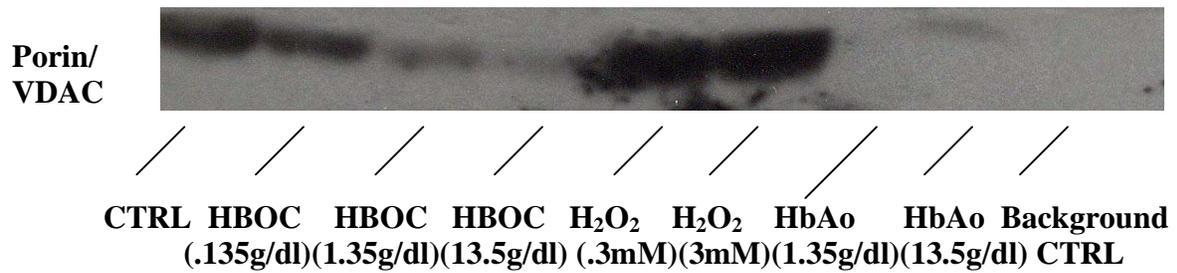


Table 2: Table of Integrated Optical Densities for Immunoblots of Mitochondrial Electron Transport ($x \pm \text{SEM}$; N=3) & Porin Proteins ($x \pm \text{SEM}$; N=1).

	N	II	V	III	I	IV	Porin/VDAC
CTRL	3	2.3 \pm .04	1.5 \pm .04	1.9 \pm .04	1.4 \pm .04	1.3 \pm .09	2.5 (n=1)
HBOC: (13.5g/dl)	3	2.3 \pm .14	0.7 \pm .02*	0.6 \pm .04*	0.5 \pm .01*	0.5 \pm .03*	0.9 (n=1)
(1.35g/dl)	3	1.9 \pm .08	0.6 \pm .07*	0.4 \pm .03*	0.2 \pm .04*	0.5 \pm .03*	1.3 (n=1)
(0.135g/dl)	3	1.9 \pm .05	0.9 \pm .05*	1.3 \pm .03*	0.6 \pm .02*	0.9 \pm .03*	2.2 (n=1)
H₂O₂: (3mM)	3	2.2 \pm .09	1.7 \pm .02	1.7 \pm .03	1.4 \pm .03	1.4 \pm .02	2.6 (n=1)
(0.3mM)	3	2.7 \pm .08	1.7 \pm .02	2.3 \pm .03	1.9 \pm .01	1.3 \pm .04	2.7 (n=1)
HbAo: (13.5g/dl)	3	0.6 \pm .13*	0.4 \pm .02*	0.3 \pm .03*	0.3 \pm .01*	0.6 \pm .04*	0.5 (n=1)
(1.35g/dl)	3	1.9 \pm .09	0.8 \pm .02*	0.8 \pm .04*	0.8 \pm .03*	1.1 \pm .02*	0.5 (n=1)

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Chapter 3- Use of Bovine Pulmonary Artery Endothelial Cells (BPAECs) To Quantitate Whole Cell Oxygen Uptake (VO₂) In The Presence of HBOCs

Introduction:

The continued interests in providing pyrogen and pathogen-free hemoglobin based oxygen carriers (HBOCs) prompted us to develop and test an in-vitro system that would allow quantitative assessments of the oxygen delivery and offloading features of various HBOCs. As isolated liver mitochondria failed in the presence of HBOCs (see **Chapter 2**), we tested an intact cultured endothelial cell culture model using Bovine Pulmonary Endothelial Cells (BPAECs) which proved to enable the measurements of oxygen delivery by these HBOCs under the conditions of actively respiring cells in-vitro.

Materials & Methods:

A.) Cell culture

Bovine Pulmonary Artery Cells (BPAECs) used in these experiments, were purchased from Lonza Corporation (Walkersville, MD, Cat#BW-6004, Lot #3F0352). Cells were maintained in RPMI media without L-Glutamine (Lonza Corp.), containing 10% Fetal Bovine Serum (FBS) (Lonza Corp.), and supplemented with L-glutamine and antibiotics (Gibco Life Sciences) in a 37°C 5% carbon dioxide (CO₂) water jacketed Forma Scientific cell culture incubator. All cells were used between passages 3-6. At the time of the experiments, culture flasks were first trypsinized for 30 seconds, and adherent endothelial cells were scraped using a cell scraper. Then RPMI media containing FBS was added to stop the trypsinization reaction.

The scraped cells were then transferred to a 15ml conical tube and were briefly centrifuged at 1000rpm. 20µl of pelleted cells containing $\sim 5 \times 10^7$ cells determined by hemocytometry) were placed into a closed chamber MT200 microrespirometer (Strathkelvin Instruments, UK) with a Clark type electrode for measuring dissolved oxygen.

B.) Preparation of Hemoglobin solutions

Please refer to **Chapter 2-Section A.)**

C.) Determination of the Methemoglobin (Fe^{3+}) Concentrations in the various Hemoglobin Solutions:

Please refer to **Chapter 2-Section B.)**

D.) Whole cell respiration experiments- Whole cell respiration experiments were performed using a Multichannel Strathkelvin respirometry system, equipped with multiple MT200 microrespirometry chambers, housing Clark type microelectrodes for the measurement of dissolved oxygen (Strathkelvin Instruments, UK). The respirometry system is also equipped with Strathkelvin data acquisition software, which records and stores whole cell respirometry data. Each dissolved oxygen microelectrode was calibrated to contain 23.85nmole of oxygen in room air saturated buffer solution containing 250mM Sucrose, 10mM Hepes, pH=7.2, 20mM KCl, 5mM potassium phosphate, and 2mM MgCl_2 at 21.5° C (1). Room air saturated buffer solution was prepared by vigorous stirring in an open air glass beaker for 1 hour at room temperature (21.5°C). The zero point calibration of the oxygen electrodes were performed by the addition of sodium sulfite to the buffer solution at room temperature. Hemoglobin solutions were

used at 1.35g/dl diluted in HEPES buffer. This concentration of acellular hemoglobin solutions has been shown to elicit equivalent vasoconstrictive responses in the in-vivo isolated perfused heart model (2). In some experiments the mitochondrial uncoupler, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), was used at a concentration of 0.3 μ M. The different treatment groups are shown below:

Table 1- The different experimental treatment conditions that we tested with BPAECs in the presence or the absence of either low or high oxygen affinity hemoglobins. Under certain experimental conditions FCCP (carbonyl cyanide 4(tri-fluoromethoxy) phenylhydrazone), an uncoupler of oxidative phosphorylation was used to drive maximal oxygen uptake (VO_2).

TABLE 1. Experimental Groups:

- 1) 20 μ l BPAEC Cells Only
- 2) 20 μ l BPAEC Cells + 0.3 μ M FCCP (uncoupler)
- 3) 20 μ l BPAEC Cells + 1.35g/dl Low Oxygen Affinity Hb (HBOC-201)
- 4) 20 μ l BPAEC Cells + 1.35g/dl Low Oxygen Affinity Hb (HBOC-201) + 0.3 μ M FCCP (uncoupler)
- 5) 20 μ l BPAEC Cells + 1.35g/dl High Oxygen Affinity Hb (HbAo)
- 6) 20 μ l BPAEC Cells + 1.35g/dl High Oxygen Affinity Hb (HbAo) + 0.3 μ M FCCP (uncoupler)

Whole cell respiration tracings were recorded and analyzed using the Strathkelvin respiratory data acquisition software.

E.) Determination of Oxygen uptake up to 95% Oxygen Depletion (VO_{295}) and at Physiological Microvascular PO_2 levels

BPAEC oxygen uptake (VO_2) at 95% oxygen depletion of the respirometer in the presence of high and low oxygen affinity hemoglobins were estimated using the following equation:

$$(VO_2)_{95} = \Delta \text{Total Oxygen (nmoles of oxygen)} / \Delta \text{Time Until 95\% Oxygen Depletion (minutes)}$$

Total Oxygen content of the test system is the sum of physically dissolved plus hemoglobin-bound oxygen. Dissolved oxygen is given by the Bunsen Coefficient α . Bound oxygen is a function of the PO_2 and the hemoglobin oxygen dissociation curve, which relates the amount of bound hemoglobin saturation to the PO_2 (3-5).

Therefore, the Total Oxygen content of the test system at any point in time was determined according to:

$$\text{Total Oxygen} = \alpha(PO_2) + 4(SO_2\%)([Oxyhemoglobin] - [Methemoglobin]) \quad (3)$$

In a physiologically more relevant analysis oxygen uptake ΔVO_2 was estimated over the known range of microvascular capillary bed PO_2 , from of 35 to 0 torr (4).

F.) Assessing cellular NADH/NAD⁺ levels in the absence/presence of Hemoglobin solutions

Cellular NADH/NAD⁺ levels were assessed through an MTS assay (a modified one step MTT assay), whereby cells containing intact mitochondrial respiratory enzymes, are able to convert a tetrazolium salt into a formazan product, with a maximal absorbance at 490nm. Experiments were performed on confluent BPAECs cultured in Corning 96 well plates (get serial number and company info). Each well of cells were grown up in 200ul of RPMI media, supplemented with 10% FBS and antibiotics, and maintained in a 37°C 5% CO₂ cell culture incubator as previously described. At the time of the experiments, media was removed, and a total of 70µl volume was added to each well. Positive controls for cell growth were performed by incubating cells with 70µl of cellular respiration buffer, and negative controls for cell viability were performed by treating cells with Promega® Lysis buffer (10µl + 60µl cellular respiration buffer). After incubation at room temperature for 1 hour, 20µl of MTS solution was placed into each well, and incubated in a 37°C 5% CO₂ cell culture incubator for 4 hours. Plates were analyzed using a Fluostar fluorimetric/absorbance plate reader, set to record at an absorbance wavelength of 490nm. Data was recorded and transferred to Microsoft Excel using the Fluostar software package. Each experiment was performed in triplicate and represents an average of those data.

G.) Data Presentation and Statistics

Data are presented as means \pm Standard Error (SE). Differences between treatment groups were evaluated using multiple group comparisons (general ANOVA) with the Tukey correction. All data are expressed as $x \pm$ SEM.

Results:

Oxygen Uptake of BPAECs in the absence/presence of low vs. high oxygen affinity hemoglobins up to 95% Oxygen Depletion (VO_2 95)- The oxygen uptake of BPAECs under the experimental conditions listed in **Table 1** were assessed at 95% oxygen depletion (VO_2)95. Untreated BPAECs had an oxygen uptake of 2.7 ± 0.4 nmoles O_2 /min, which increased to 8.7 ± 1.1 nmoles O_2 /min with the uncoupler FCCP. The VO_2 of BPAECs treated with HBOC-201 (low oxygen affinity) was 3.4 ± 0.3 nmoles O_2 /min, which increased to 12.3 ± 1.8 nmoles/ O_2 with uncoupler. BPAECs treated with HbAo had a VO_2 of 1.1 ± 0.1 nmoles O_2 /min, which increased to 2.6 ± 0.2 nmoles O_2 /min with the uncoupler FCCP.

Oxygen Uptake of BPAECs in the absence/presence of low vs. high oxygen HBOCs over Physiological Microvascular PO_2 ranges- After an extensive literature search for microvascular PO_2 ranges shown in **Table 3** a PO_2 range of 0-35 torr was selected. The change in the oxygen uptake of BPAECs (ΔVO_2) under the experimental conditions listed in **Table 1** were assessed over a range of partial pressures of oxygen (PO_2) from 35-0 torr, which represent physiologically relevant microvascular capillary bed PO_2 's. Untreated BPAECs had a VO_2 of 1.3 ± 0.4 nmoles O_2 /min, which increased to 3.9 ± 0.6 with the uncoupler FCCP. The VO_2 of BPAECs treated with HBOC-201 was 8.3 ± 1.2 nmoles O_2 /min, which increased to 21.6 ± 7.8 nmoles O_2 /min with uncoupler. BPAECs treated with HbAo had a VO_2 of 3.1 ± 0.4 nmoles O_2 /min, which increased to 18.8 ± 5.7 with FCCP.

MTS Assay of BPAECs in the absence/presence of low vs. high oxygen HBOCs- In **Figure 4A** BPAECs were assessed at 4 hours and 24 hours post treatment after subtraction of initial Baseline background absorbance values.

At Baseline untreated BPAECs had an absorbance of 0.166 ± 0.01 . BPAECs treated with the uncoupler FCCP had an absorbance of 0.219 ± 0.01 . BPAECs treated with HBOC-201 had an absorbance of 0.439 ± 0.12 . BPAECs treated with HBOC-201 + FCCP had an absorbance of 0.444 ± 0.02 with uncoupler. BPAECs treated with HbAo had an absorbance of 0.448 ± 0.02 . BPAECs treated with HbAo + FCCP had an absorbance of 0.453 ± 0.03 .

After 4 hours of treatment untreated BPAECs had an absorbance at 490nm of 0.531 ± 0.09 , which decreased to 0.000 ± 0.01 upon treatment with the uncoupler FCCP. BPAECs treated with HBOC-201 had an absorbance of 0.293 ± 0.09 , which decreased to 0.034 ± 0.02 with uncoupler. BPAECs treated with HbAo had an absorbance of 0.226 ± 0.11 , which decreased to 0.007 ± 0.05 in the presence of uncoupler.

After 24 hours of treatment untreated BPAECs had an absorbance at 490nm of 0.564 ± 0.07 , which decreased to -0.085 ± 0.02 upon treatment with the uncoupler FCCP. BPAECs treated with HBOC-201 had an absorbance of 0.968 ± 0.06 , which decreased to 0.254 ± 0.07 with uncoupler. BPAECs treated with HbAo had an absorbance of 0.906 ± 0.09 , which decreased to -0.018 ± 0.02 in the presence of uncoupler.

Summary of Results:

- 1) Confluent BPAECs may serve as a useful in-vitro model to study cellular oxygen availability in the absence/presence of low vs. high oxygen affinity HBOCs.

- 2) In the presence of low oxygen affinity HBOC-201 reached 95% oxygen depletion was reached faster than with high oxygen affinity HbAo, as expected.
- 3) However, no differences in oxygen uptake with low vs. high oxygen affinity HBOCs were observed when oxygen uptake rates were determined at physiologically relevant microvascular PO₂ levels (35-0 torr).
- 4) There were also no differences with low vs. high oxygen affinity HBOCs in the MTS assay at post 4 hour and post 24 hours treatment suggesting that the mitochondrial metabolic activity was not altered by HBOC treatment of the confluent BPAEC system.

For Discussion of Results Refer to Chapter 4-General Discussion

Figure 1: The determination of BPAEC oxygen uptake in the absence/presence of low vs. high oxygen affinity HBOCs up to 95% oxygen depletion (VO_{295}). The VO_{295} was determined by taking the sum of the [dissolved oxygen in our system + the amount of hemoglobin bound oxygen] divided by the time until 95% oxygen depletion in minutes.

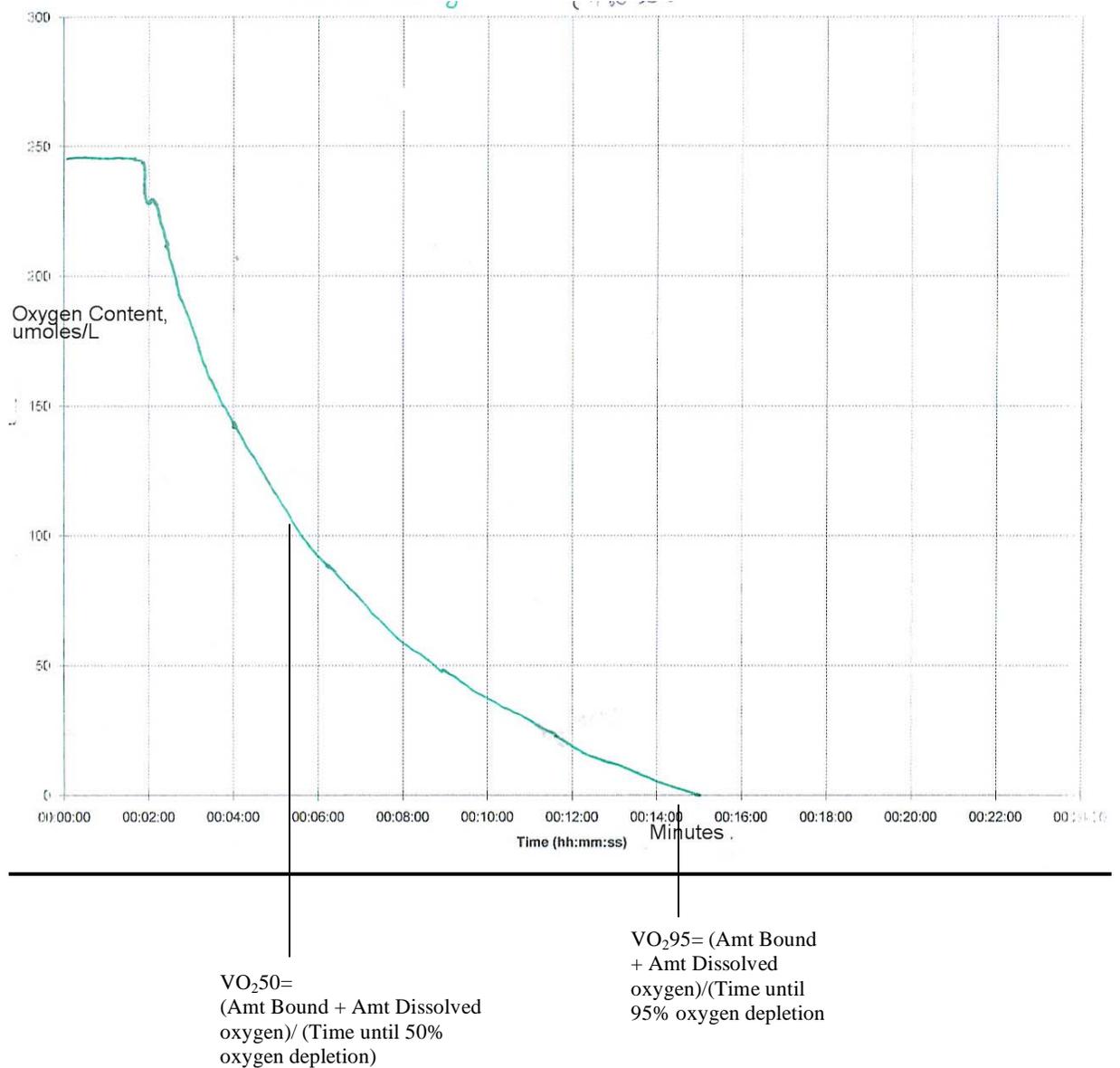


Table 2: The VO₂95 of BPAECs in the absence/presence of low vs. high oxygen affinity HBOCs. In some experiments FCCP was added to drive maximal oxygen consumption.

Treatment	Total Oxygen (nmoles O₂)	Time to 95% O₂ Depletion (Min) ± SE	VO₂95 (nmoles O₂/min)
Untreated (CTRL)(N=7)	28.6	11.3 ± 1.7	2.7 ± 0.4
FCCP(N=6)	28.6	3.4 ± 0.4	8.7 ± 1.1
HBOC-201 (N=7) Low oxygen affinity	101.6	27.3 ± 2.3	3.4 ± 0.3
HBOC-201 + FCCP (N=6)	101.6	8.0 ± 0.9	12.3 ± 1.8
HbAo (N=6) High oxygen affinity	110.6	65.2 ± 5.0	1.1 ± 0.1
HbAo + FCCP(N=7)	110.6	25.8 ± 1.6	2.6 ± 0.2

All data were represented as the mean ± SEM.

Figure 2: The oxygen uptake of BPAECs under the experimental conditions listed in **Table 1** assessed up to 95% oxygen depletion (VO_2)₉₅. At 95% respirometer depletion of oxygen, HBOC had released increased amounts of oxygen (available for cellular uptake) relative to HbAo.

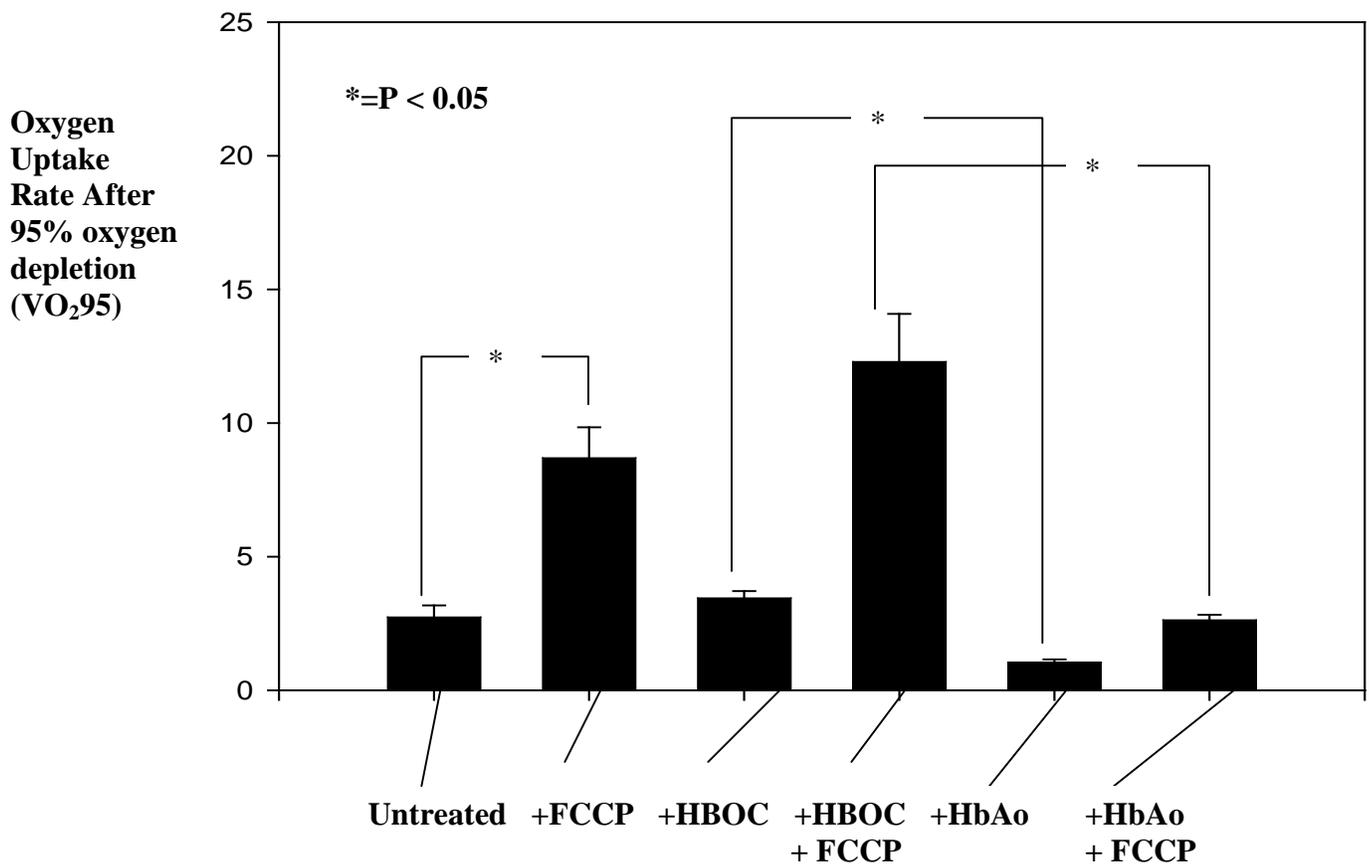


Table 3: List of various microcirculatory PO₂ levels for various tissues reported in the literature.

Tissue	PO ₂ (Torr)	Reference(s)
Skt Muscle	15-20 torr “Critical Capillary PO ₂ ”	Wittenberg BA and Wittenberg JB. Ann Rev Physiol 51:857-878, 1989.
Liver	PO ₂ reduced from 32.4 to 16.1 torr during shock	Makisalo HJ et al. Crit Care Med. 1998; 16:857-861. Kessler M. Anesthesiology 1976; 45:184-197.
Small Intestine	Arteriole= 45 torr Venule= 35 Capillary= 22 torr	Sinaasappel M et al. Am J Physiol 1999, 276: G1515-20.
Kidney	Cortex reduced from 47 to 35 torr after ARF (renal failure) and Outer Medulla from 32torr to 24	Liss P et al. Oxygen Transport to Tissues XXI, NY, 1999, 353-359.
Heart	Epicardium 40-20 torr Endocardium 20-10 torr	RA Olsson. The Heart and Cardiovascular System, NY, 1986, 987-1037.
Brain	<20 torr “Critical Capillary PO ₂ ”	Severinghaus J et al. Oxygen Transport to Tissues XXI, NY 1999, 595-604.

Figure 3: The change in the oxygen uptake of BPAECs (ΔVO_2) under the experimental conditions listed in **Table 1** were assessed over a range of partial pressures of oxygen (PO_2) from 35-0 torr, which represent physiologically relevant microvascular capillary bed PO_2 's. There were no differences in VO_2 between HBOC and HbAo treated BPAEC's, whether or not treated by uncoupler.

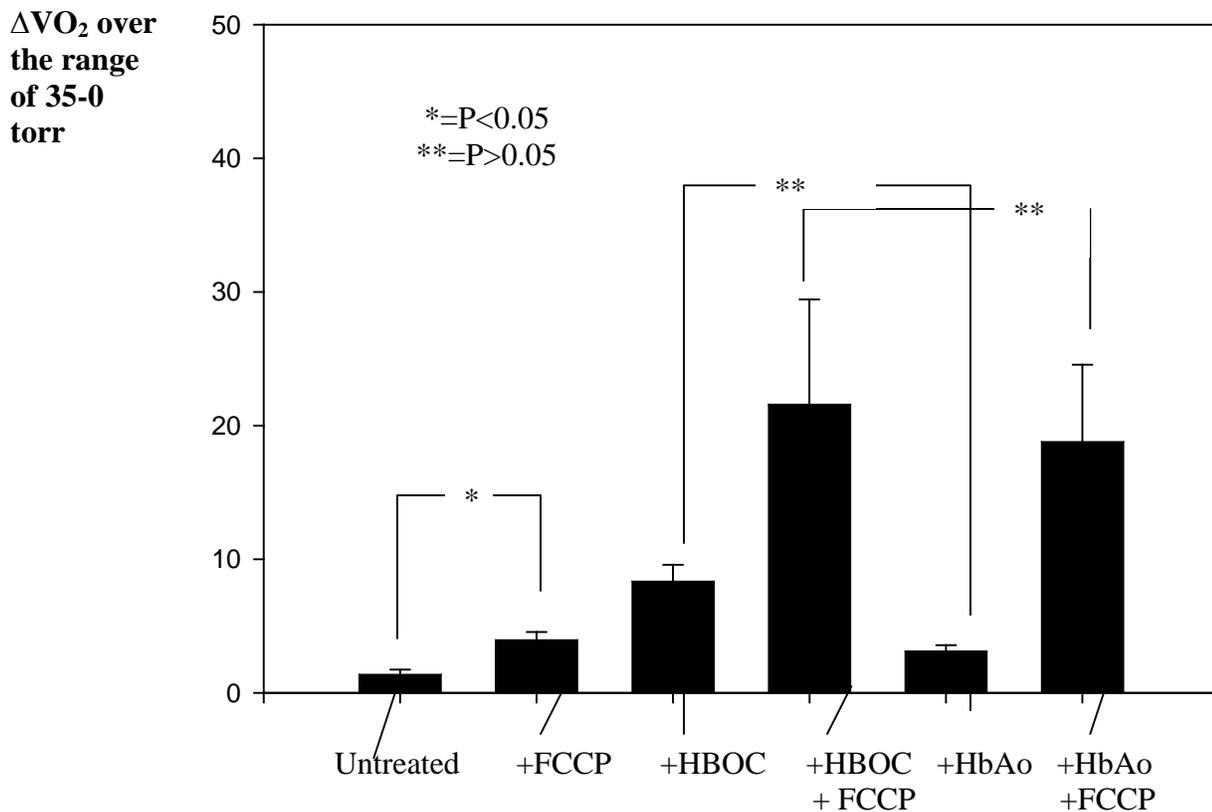


Figure 4: MTS assay of BPAECs treated in the absence/presence of low vs. high oxygen affinity hemoglobins at Baseline, T=0.

MTS Assay of BPAECs following various treatments at Baseline- (T=0)
(N=8 for different treatment grps, 2 different cell culture preparations)

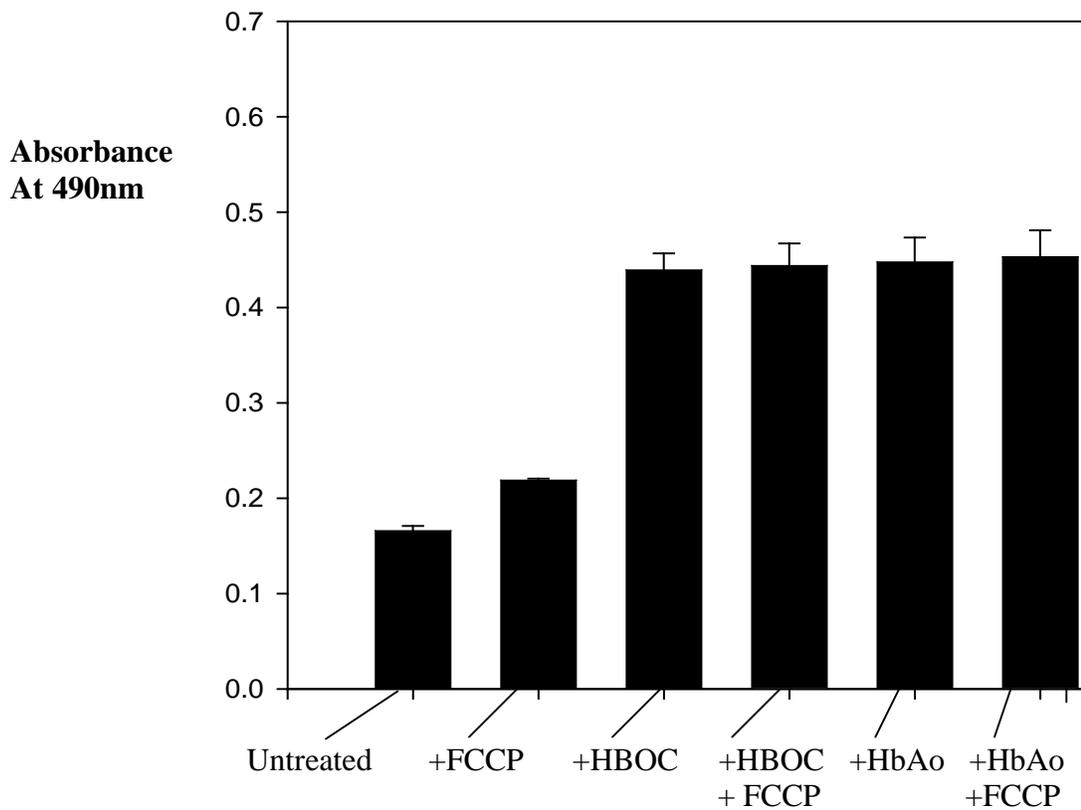
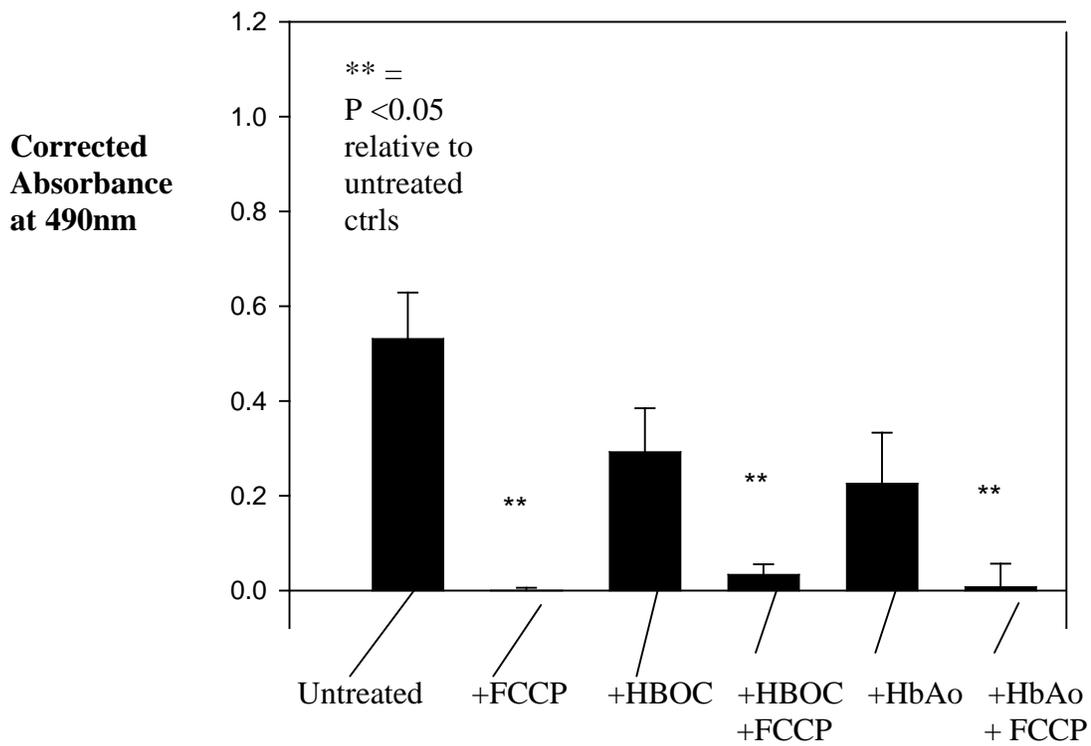


Figure 5: MTS assay after correction for baseline absorbances at 490nm. A) Post 4 hours Treatment, B) Post 24 hours Treatment

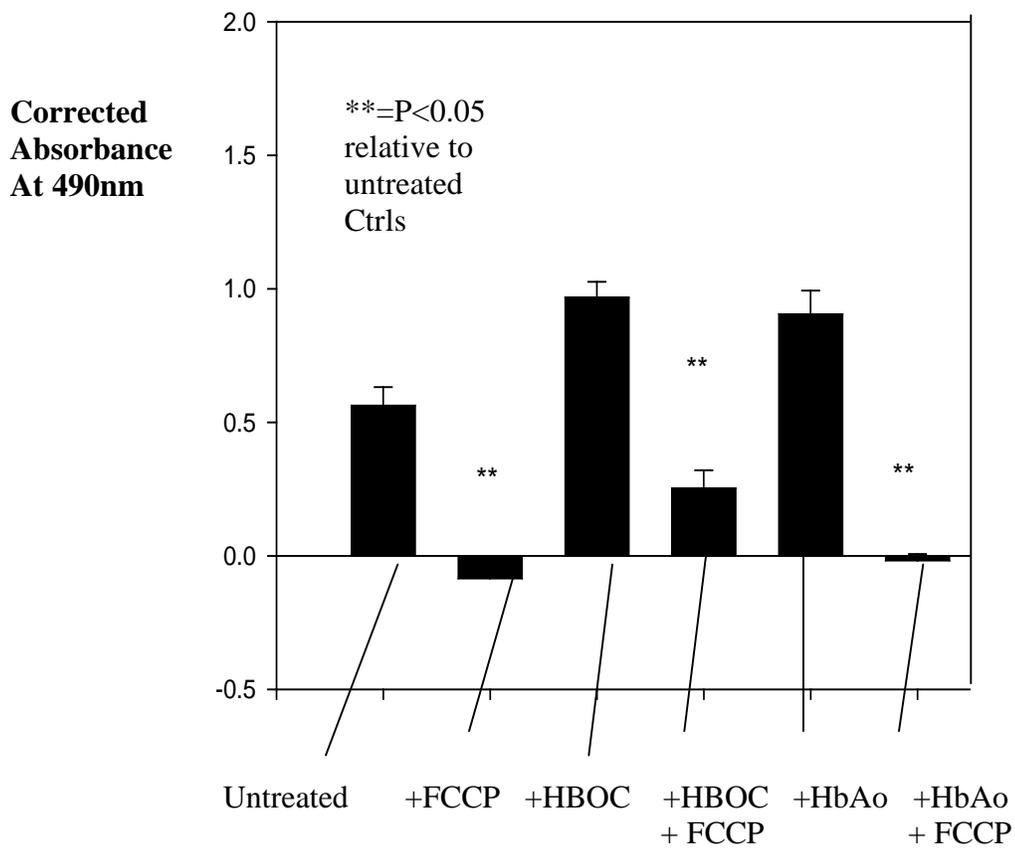
A.)

BPAEC MTS Assay After Various Treatments
Post 4hrs. Baseline Corrected Data



B.)

**BPAEC MTS Assay After Various Treatments
Post 24hrs. Baseline Corrected Data**



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Chapter 4: General Discussion

A.) Isolated Mitochondria Results:

The present study indicates that isolated rat liver mitochondrial preparations are not an appropriate model system to quantitate the delivery of oxygen to and use of oxygen by the respiratory proteins in the presence of low vs. high oxygen affinity HBOCs. This is indicated by the following effects of the HBOCs: 1) the lowering of the physiologically active mitochondrial respiration rate in the presence of substrate (1mM Glutamate + Malate) + 1mM ADP + Pi (State 3 respiration), 2) the lowering of the respiratory coupling ratio (RCR), and 3) the loss of immunoreactivity of antibodies against various mitochondrial electron transport complex proteins and the VDAC/Porin protein complex as well.

These findings are consistent with previous studies that demonstrated that cell-free hemoglobin's could potentially act as pro-oxidants causing severe free-radical mediated membrane damage (1). Incubation of modified and unmodified HBOCs with artificial liposome vesicles containing cytochrome c oxidase (mitochondrial electron chain complex IV) resulted in an increase in the level of free-radical mediated lipid peroxidation of the artificial liposome vesicles (1). Taken together these observations indicate that HBOCs can damage biological membranes, especially at the subcellular level.

In control experiments mitochondrial preparations were incubated with high vs. low doses of hydrogen peroxide, which acts as a free-radical donor, through the Fenton reaction as follows:



We observed the following effects of oxidative stress due to hydrogen peroxide: 1) the lowering of the State 4 resting respiration rate in the presence of substrate (1mM Glutamate + Malate) but in the absence of ADP, 2) the lowering of the physiologically active State 3 mitochondrial respiration rate in the presence of substrate (1mM Glutamate + Malate) + 1mM ADP + Pi, 3) no changes in the respiratory coupling ratio (RCR), and 4) there was no loss of immunoreactivity of the antibodies against various mitochondrial electron transport complex proteins and the VDAC/Porin protein complex.

These results are consistent with other studies that have demonstrated that hydrogen peroxide leads to a decrease in both the State 4 (resting) and State 3 (active) respiration rates in isolated rat liver mitochondria (1). Although we don't know the exact mechanism of the lowering of the State 3/State 4 respiration by hydrogen peroxide, this effect could result from some subpopulation of the mitochondria that are resistant to oxidative stress by hydrogen peroxide thereby maintaining a normal RCR. Indeed a recent study has demonstrated that the enzymatic activities of the electron transport chain complex proteins are not affected by hydrogen peroxide treatment (2), which is consistent with our results from the immunoblots. The results indicate that the mechanism of hydrogen peroxide-dependent mitochondrial toxicity is different mechanistically from the respiratory failure induced by low vs. high oxygen affinity HBOCs.

Because of the damaging effects of HBOCs on the respiration rate and coupling, the present study indicates that isolated rat liver mitochondrial preparations are not an appropriate

model system to quantitate the delivery of oxygen and the use of oxygen in the presence of various doses of low vs. high oxygen affinity HBOCs.

B.) Cultured BPAEC Results:

These studies demonstrate that BPAECs cultured to confluence may serve as a suitable in-vitro model system to study cellular oxygen availability in the absence/presence of low vs. high oxygen affinity HBOCs. The rate of oxygen uptake of BPAECs to the point of 95% respirometer oxygen depletion (VO_{295}) demonstrated that low oxygen affinity, HBOC-201, had released oxygen faster (available for cellular uptake) relative to high oxygen affinity HbAo; this was expected. However, when the rate of oxygen uptake was assessed in the range of physiological microvascular PO_2 levels between 0-35 torr no differences were observed in cellular VO_2 between HBOC-201 and HbAo, whether or not cells were treated by uncoupler. Furthermore, the MTS assay results demonstrated that there were no substantial difference between HBOC-201 and HbAo treated cells in terms of mitochondrial NADH availability. The HBOC treated BPAECs also compared well with untreated controls in the MTS assay suggesting that mitochondrial metabolic activity of confluent BPAECs was not seriously altered in incubations with low vs. high oxygen affinity HBOCs.

While we observed expected differences in the release of oxygen up to 95% oxygen depletion (VO_{295}) of the respirometer in the presence of low vs. high oxygen affinity HBOCs, a more physiologically relevant analysis of the data that focused on microvascular PO_2 ranges (35-0 torr), demonstrated no differences among low vs. high oxygen affinity HBOCs in terms of BPAEC respiration. These results compare with recent observations from Tsai et al. (3) who reported that microvascular functional capillary density (FCD) in an in-vivo hamster pouch

model was improved upon infusion of a high oxygen affinity HBOC relative to the low oxygen affinity HBOC-201 (3). These hamster pouch results taken together with our BPAEC data would indicate that perhaps increased FCD, not oxygen affinity of HBOCs per se, is the key factor influencing microvascular oxygen availability in-vivo during blood substitute regimens.

Our MTS data from the BPAECs compare well with a report by Ortegon et al. who examined cultured neurons and found that cultures exposed to either low or high oxygen affinity HBOCs (at concentrations similar to those in our studies) proved to sustain substantial mitochondrial metabolic redox activity (4).

In summary these results taken together demonstrate that BPAECs cultured to confluence may serve as a useful in-vitro model to study cellular oxygen uptake in the absence/presence of low vs. high oxygen affinity HBOCs. While we observed that BPAECs in the presence of 'low oxygen affinity' HBOC-201 reached 95% oxygen depletion (VO_{295}) faster than 'high oxygen affinity' HbAo, no differences in oxygen uptake were observed when oxygen uptake rates were measured at physiologically relevant microvascular PO_2 ranges (35-0 torr). Since there were no differences in the MTS assay in the absence/presence of low vs. high oxygen affinity HBOCs, the confluent BPAEC system seemed to remain metabolically intact under our experimental conditions. Our analysis of VO_2 over the range of physiological microvascular PO_2 's indicated that physical binding and release characteristics of HBOCs may not be the key determinant factor in oxygen availability in the microcirculation in-vivo.

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