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Biotherapeutic Approaches, Apohemoglobin and Haptoglobin in Hemoglobin Toxicity Management

A Dissertation submitted in partial satisfaction of the requirements.
for the degree Doctor of Philosophy

in

Bioengineering

by

Carlos Jose Munoz

Committee in charge:

Professor Pedro Cabrales, Chair
Professor Marcos Intaglietta
Professor Kevin King
Professor Erik Kisler
Professor Christian Metallo
Professor Akif Tezcan

2023

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The Dissertation of Carlos Jose Munoz is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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University of California San Diego

2023

DEDICATION

I would like to dedicate this thesis to my parents Rachel and Jose Munoz, who gave me everything I needed to accomplish my dreams. I would also like to dedicate this thesis to my siblings George and Alejandra Munoz for their continuous support as a child and in adulthood. Finally, I would like to dedicate this thesis to my nieces Gabriala and Elise Paz, I hope to be the best role model for them as they grow up.

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ACKNOWLEDGEMENTS

I would like to acknowledge and thank Dr. Cabrales for his constant support, encouragement, and mentorship throughout this journey. I am very thankful that he gave me the opportunity to work in the laboratory for all this time. I would also like to thank Dr. Gopal, Dr. Kistler, Dr. Intaglietta and Dr. Tsai, whose guidance, support, and knowledge helped me throughout this program. I would especially like to thank Cynthia Walser for her knowledge and the countless hours preparing the animals for surgery, and out of lab being a great friend. I also would like to thank Ms. Daniela Lucas, Dr. Cynthia Muller, Dr. Fernando Dos Santos, Ms. Joyce Li, Mr. Alfredo Lucas, Mr. Vivek Jani, and Mr. Krianthan Govender, for their support and friendship. I would also like to thank the rest of my lab mates, past and present, for their friendship throughout the program. Finally, truly none of this would have been possible without the support of the rest of my friends and family for their support outside of the lab and giving me a space to express the highs and lows that this journey brought me.

Chapter 2, in full, is accepted material as it appears in "Apohemoglobin-haptoglobin complex attenuates the pathobiology of circulating acellular hemoglobin and heme" by Carlos J. Munoz, Ivan S. Pires, Jin Hyen Baek, Paul W. Buehler, Andre F. Palmer and Pedro Cabrales, in American Journal of Physiology- Heart and Circulatory Physiology Publish Ahead of Print (May 2020). The dissertation author was the primary investigator and author of this paper.

Chapter 3, in full, is accepted material as it appears in "Apohemoglobin-haptoglobin complex alleviates iron toxicity in mice with β-thalassemia via scavenging of cell-free hemoglobin and heme." by Carlos J. Munoz, Ivan S. Pires, Vinay Jani, Srila Gopal, Andre F. Palmer, and Pedro Cabrales, in Biomedicine & Pharmacotherapy Publish Ahead of Print (December 2022). The dissertation author was the primary investigator and author of this paper.

Chapter 4, in full, is being prepared for publication as it appears in “Apohemoglobin-Haptoglobin Complex Prevents Microvascular Dysfunction in Sickle Cell Mice: Potential Therapeutic Approach for Vaso-Occlusive Crisis.” by Carlos J. Munoz, Daniela Lucas, Ivan S. Pires, Cynthia Muller, Srila Gopal, Andre F. Palmer, and Pedro Cabrales. The dissertation author was the primary investigator and author of this paper.

Chapter 5, in full, is being prepared for publication as it appears in “Alpha-Alpha Hemoglobin/ Diaspirin-Crosslinked Hemoglobin (DCLHb) Benefits from Treatment using Apohemoglobin Haptoglobin Complex” by Carlos J. Munoz, Daniela Lucas, Jacinda Martinez, Mia Ricario, and Pedro Cabrales. The dissertation author was the primary investigator and author of this paper.

Chapter 6, in full, is being prepared for publication as it appears in “PEGylated Apo-Hemoglobin Haptoglobin can Effectively Limit Vascular Dysfunction in the Microcirculation and Prevent Acute Cardiac and Kidney Inflammation” by Carlos J. Munoz, Daniela Lucas, and Pedro Cabrales. The dissertation author was the primary investigator and author of this paper.

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PUBLICATIONS

Munoz CJ, Pires IS, Jani V, Gopal S, Palmer AF, Cabrales P. Apohemoglobin-haptoglobin complex alleviates iron toxicity in mice with β-thalassemia via scavenging of cell-free hemoglobin and heme. Biomed Pharmacother. 2022 Dec;156:113911. doi: 10.1016/j.biopha.2022.113911. Epub 2022 Oct 26. PubMed PMID: 36308920.

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ABSTRACT OF THE DISSERTATION

Biotherapeutic Approaches, Apohemoglobin and Haptoglobin in Hemoglobin Toxicity Management

by

Carlos Jose Munoz

Doctor of Philosophy in Bioengineering

University of California San Diego, 2023

Professor Pedro Cabrales, Chair

Pathophysiological conditions characterized by chronic hemolysis (e.g. genetic and acquired hemolytic diseases) increase circulatory cell free hemoglobin (fHb), free heme and iron.

These toxic species elicit a variety of adverse effects, including the scavenging of the potent natural vasodilator nitric oxide (NO) which decreases microcirculatory NO-bioavailability, decreased organ perfusion and renal function, end-organ damage, and increased mortality. Natural clearance mechanisms for fHb, free heme, and iron rely on the scavenger proteins hemopexin (Hpx) and haptoglobin (Hp), respectively. Unfortunately these proteins are depleted during hemolytic states. Thus, treatment of hemolytic conditions would benefit from administration of scavengers of fHb, free heme and iron. A possible functional alternative to Hpx is apohemoglobin (apoHb). ApoHb is produced by removing the heme from Hb and takes advantage of the natural high affinity for heme of the vacant heme-binding pockets in Hb. Hence, apoHb could serve as a novel, practical and scalable alternative to Hx *in vivo* scavenger for heme.

However, apoHb major limitations for in vivo use are its pour stability at physiological temperatures, and short circulatory half-life. Fortunately, Hp can stabilize and extend circulation time of apoHb by forming an anpohemoglobin-haptoglobin (apoHb-Hp) complex. This apoHb-Hp complex binds heme and is more stable at physiological temperature compared to free apoHb. In addition to being able to bind free heme, we have discovered that the apoHb-Hp complex can scavenge free Hb *in vivo* and *in vitro* by exchanging Hp bound apoHb αβ dimers for Hb αβ dimers in the circulation. Lastly, to ensure an ease of use for manufacturing conjugation of polyethylene glycol (PEG) to the apoHb (PEG-apoHb) overcomes issues of pour stability and does not diminish the safety and efficacy of the product in physiological conditions.

1. GENERAL INTRODUCTION

1.1 THE ISSUES WITH FREE HEMOGLOBIN IN THE CIRCULATION

Hemolytic anemias are a global health problem, impacting millions of individuals with costly treatment options and limited solutions[1]. According to the World Health Organization, 5% of the world's population are susceptible to sickle cell anemia and over 300,000 infants are born with other genetic hemoglobinopathies [1]. Greater than 200 million cases of malaria were reported worldwide in 2017 and pathogenesis of malaria is increasing in the America's [2], 30 million cases of severe sepsis happen worldwide each year [3], and approximately 70,000 suffer from thalassemia with more than half of these individuals experiencing severe forms of the disease (β-thalassemia major and HbE β-thalassemia)[4].

Erythropoiesis and hemolysis occur at similar rates, thus maintaining the total circulating number of red blood cells (RBCs) in a state of equilibrium. RBC homeostasis results in the cycle of erythropoiesis and hemolysis with effective transport and clearance of hemoglobin (Hb) and its degradation products[5]. However, genetic hemolytic diseases (e.g. sickle cell anemia and thalassemia), acquired hemolytic infections (e.g. Gram positive and malarial hemolysins) as well as hemolytic xenobiotic toxins (e.g. heavy metals, dapsone and phenyl hydrazine) damage the RBC, disrupting this delicate equilibrium by increasing the rates of both extra- and intra-vascular hemolysis [6–8]. As a result of this imbalance from accelerated hemolysis erythroid hyperplasia in the bone marrow is likely to occur, as well as extramedullary hematopoiesis and hepatosplenomegaly.[9] Notably, the constant drive for hematopoiesis and blood transfusion can lead to secondary hemochromatosis (i.e., iron overload) which is the primary cause for morbidity and mortality in thalassemic patients.[10] Since humans have no mechanism to eliminate excess iron, the quantity of iron in the body is carefully regulated primarily via control of gastrointestinal iron

absorption. In cases of chronic hemolytic anemia, increased iron absorption leads to iron accumulation in the spleen, liver, endocrine glands, and heart.[11] When left untreated, iron build-up in the heart can progress towards cardiomyopathy. Furthermore, iron build-up in the liver causes portal fibrosis, cirrhosis, and portal hypertension, which can exacerbate splenomegaly. Unfortunately, the frequent blood transfusion regimens in patients suffering from hemolytic anemia further increase iron levels, since one unit of packed RBCs contains approximately 200 mg of iron, while the normal intestinal iron absorption rate is 1-2 mg per day.[10,12] Thus, these patients receiving blood transfusions frequently require iron chelation therapies to neutralize and eliminate excess free iron.[13,14]

Hemolytic states are characterized by increased levels of circulating cell-free Hb, free heme and iron which are toxic oxidative species.[15] Furthermore, these toxicities are exacerbated by the frequent RBC transfusions patients receive, which can be up to 25% hemolyzed after 24 hours in circulation, and secondary hyperkalemia and hypocalcemia from the citrate contained in the transfused blood units.[16–19]

As a result of the excessive hemolysis, there is a release of arginase and cell-free Hb.[20,21] Arginase consumes l-arginine reducing the substrate availability for NO synthase (NOS), thus diminishing the concentration of available NO, a potent vasodilator. Furthermore, cell-free Hb reacts with NO, thus scavenging the already limited gaseous monoxide. As a result of the scavenging of nitric oxide (NO) there is an increase in oxidative stress, causing systolic and diastolic hypertension [20] mediated by NO depleted vasoconstriction[13, 21, 22], kidney injury [23], and cardiovascular lesions [24, 25]. The presence of Hb outside of the erythrocyte's protective membrane exposes Hb to a structurally unstable environment due to an increased dilutional dynamic equilibrium between tetrameric and dimeric components, which are prone to autoxidation [22] and

extravasation [23,24]. Acellular Hb ($\alpha_2\beta_2$) dissociation into αβ dimers resulting in autoxidation (Hb-Heme-($Fe^{2+}O_2$) → Hb-Heme-(Fe^{3+}) + $O_2^{\bullet-}$), structural destabilization of heme within its binding pocket [25,26] and subsequently transfer of heme prosthetic groups to reactive lipids [27], proteins [28] and bacterial receptor complexes[29] resulting in heme-induced oxidative injury.[30] Heme has also been associated with the activation of tool like receptor 4 and results in the expression of adhesive proteins such as iCAM, vCAM, p-selectin and e-selectin, inflammatory cytokines such as IL-1β, IL-6, TNF-α, heme also induces platelet activation as well as the release of other chemokines such as endothelin-1 (ET-1).[31–35]

Addressing the issue of acellular hemoglobin and the associated toxicities. In the systemic circulation, acellular Hb and heme, are bound and cleared by the plasma proteins haptoglobin (Hp)[36] and Hpx [37], respectively. Three major Hp phenotypes (Hp 1-1, Hp 2-1 and Hp 2-2) occur in human physiology[38,39]. Hp is produced in the liver and encode by one of three genes (*Hp1*, *Hp2* or *Hp3*), that have arisen as a result of intragenic polymorphisms on human chromosome 16[40]. Hp phenotypes are made up of the same Hb binding β subunit (Hpβ, 36 kDa) and intramolecular crosslinking α globin subunits (denoted Hpα1, 9 kDa or Hpα2, 18 kDa) that do not interact with Hb dimers [36,40,41]. The number of cysteines available for disulfide bond formation determines Hp molecular structure and size as follows: dimeric (Hp 1-1, 90 kDa) or multimeric (Hp 2-1, ~90-300 kDa; Hp 2-2, ~ 150-900 kDa)[40,41]. Individual Hp genotypes/phenotypes result in a range of plasma concentrations (0.3–1.9 mg/mL) that occur from highest to lowest: [Hp1-1] > [Hp2-1] > [Hp2-2][42]. The ability of Hp proteins to combat Hb exposure is dependent on the primary binding site for Hb dimers. Irreversible binding between the haptoglobin β chain and Hb αβ dimers occurs at a 1:1 stoichiometric ratio[36]. This interaction provides a critical antioxidant function by protecting key amino acids in Hb that are vulnerable to oxidation[36,43,44] thus preventing heme release [45,46] and

compartmentalizes Hb within a stable protein complex that does not extravasate into vascular or parenchymal sites[24,47]. To continue, Hpx is encoded by an 11 kilobase gene located on human chromosome 11 and functions as the putative heme binding beta-1B-glycoprotein with a molecular size of 57 ± 3 kDa[48]. The normal plasma concentration range for Hpx is 0.5 to 1.15 mg/ml[49], Hpx-Heme complex has been shown to have a N- and C-domain structure with a single high affinity (K_d < 1 pM) hydrophobic binding site comprised of aromatic amino acid residues from both domains[49]. Hpx binds heme in a 1:1 molar ratio, therefore the normal plasma concentration of Hpx is decreased, but not entirely depleted during many hemolytic disorders[50,51]. In such cases where the Hpx has been depleted, albumin can act as a secondary heme scavenger[52]. However, albumin's affinity for heme ($K_d = 1 \times 10^{-5}$ M) is much lower than that of Hpx ($K_d = 1 \times 10^{-12}$ M), and the heme-albumin complex cannot fully protect from heme-mediated toxicity[53].

Ultimately, during hemolytic diseases Hp acts as the primary defense, stabilizing the primary product of hemolysis, acellular Hb. Upon Hp saturation the dimerized excess Hb (αβ dimer) releases heme, which is captured by Hpx[37,54,55]. Both Hpx and Hp have been investigated and studied as viable therapeutic options to treat heme and hemoglobin (Hb) mediated toxicity in a variety of pre-clinical studies over the past decade. This work has evolved into investigations on the reversible structural modifications of Hb upon heme removal and kinetics of heme-binding. The absence of the prosthetic heme groups in Hb yields apohemoglobin (apoHb). This apoprotein has a high affinity region in the unoccupied hydrophobic heme-binding pocket for a number of ligands[56,57]. Yet, the natural affinity of apoHb for heme remains the highest [58,59]. Previous work has illustrated the binding of heme to apoHb and the complexation of apoHb to Hp *in vitro,* forming an apoHb-Hp complex[60,61]. In pathophysiological states of heme excess, use of apoHb or apoHb-Hp may offer an advantage by scavenging and clearing excess heme through the

monocyte/macrophage CD163 surface receptor, rather than heme-albumin plasma pooling with transfer to oxidizable lipoproteins (LDL, HDL, VLDL). However, no study has explored the potential *in vivo* heme scavenging properties of apoHb or apoHb-Hp. Moreover, we have observed that the apoHb-Hp complex, in addition to heme-binding, can exchange Hp bound apoHb dimers for holoHb dimers, serving a dual role of Hb and heme scavenger *in vivo* **(**Figure 1.1**Figure 1.1**).

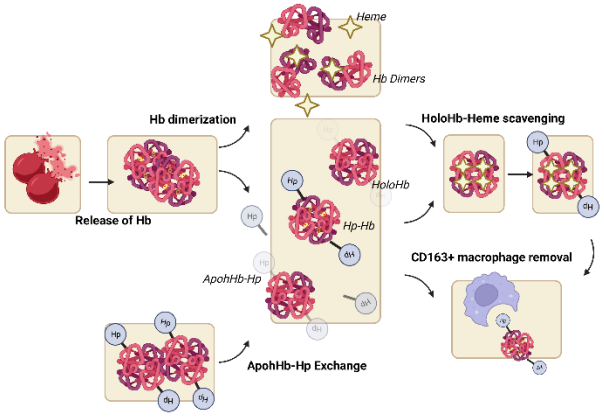

Figure 1.1 Apohemoglobin Haptoglobin Complex Mechanism of Action - Apohemoglobin haptoglobin complex scavenges free hemoglobin and heme and utilizes monocyte/ macrophage detoxification. The acellular hemoglobin released in the circulation becomes unstable causing dimerization of the protein and the release of heme as a result. However, prior to the dimerization Hp can transfer directly to the free hemoglobin protecting it from oxidative damages that force the dimerization. Furthermore, if the hemoglobin was to dimerize the HolohHb at such point will scavenge the free hemoglobin and be set to be removed through the tagging of Hp and the use of CD163 macrophages

These unique, biochemical characteristics of the apoHb-Hp complex target the efficient removal of circulatory heme and Hb through monocyte/macrophage detoxification. Although the apohb-hp complex would promote detoxification of hemoglobin and heme proteins, turning into holoHb to scavenge heme may as easily just reconstitute acellular hemoglobin. In such a scenario there maybe issues that arise of dimerization of the apohemoglobin complex via oxidation of the exposed hemoglobin. To address this issue the doubling the concentration of has been considered, but that would result in a dependence of Hp. An alternative to stabilizing the apoHb protein is the

use of poly(ethylene glycol) (PEG) a hydrophilic protein that modifies the surface of apoHb[62]. The newly formed PEG-apoHb complex is thought to increase circulatory half-life and aid in its stability when scavenging heme. The proposed protein has a newly estimated MW of 400 kDa, but only 30-40% of the original active binding heme concentration is accessible as a result.

Lastly, the use case of the apohb-hp complex expands past hemolytic animeas. With the evolving demand of whole blood or blood like products in austere conditions, hemoglobin based oxygen carriers have become a topic of interest for quite some time and only continue to be a point of research[41,63–65]. The need for these type of work are continually evolving particularly in today's society were the skew between transport times in EMS services is drastically different in urban areas and rural communities. HBOCs are finding a home in pre-hospital settings as a means to limit the use of whole blood, a commodity that has shown to be of utmost importance in the clinical setting where care is immediate and necessary. Apohb-hp can act as a scavenger protein to assure the use of the HBOC as a therepuetic only and not be left to dimerize and illicit the issues described above.

The following chapters of this work will focus on the initial purification and biosafety testing of the apohb-hp complex, the use of the apohb-hp complex for two different hemolytic anemia, the use of apoHb-Hp as a complementary product in the use HBOCs, and the use of PEG-ApoHb as an alternative to unPEGlyated apohb-hp complex.

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2. A NOVEL APO-HEMOGLOBIN-HAPTOGLOBIN COMPLEX ATTENUATES THE PATHOBIOLOGY OF ACELLULAR HEMOGLOBIN AND HEME

2.1 ABSTRACT

Haptoglobin (Hp) is the plasma protein that binds and clears cell-free hemoglobin (Hb), while apohemoglobin (apoHb, i.e. Hb devoid of heme) can bind heme. Therefore, the apoHb-Hp protein complex should facilitate holoHb-apoHb αβ dimer exchange and apoHb-heme intercalation. Thus, we hypothesized that apoHb-Hp could facilitate both Hb and heme clearance, which if not alleviated could have severe microcirculatory consequences. In this study, we characterized apoHb-Hp and Hb/heme ligand interactions, and assessed their *in vivo* consequences. Hb exchange and heme binding with the apoHb-Hp complex was studied with transfer assays using size exclusion-high performance liquid chromatography coupled with UV-visible spectrophotometry. Exchange/transfer experiments were conducted in guinea pigs dosed with Hb or heme-albumin followed by a challenge with equimolar amounts of apoHb-Hp. Finally, systemic and microcirculatory parameters were studied in hamsters instrumented with a dorsal window chamber via intravital microscopy. *In vitro* and *in vivo* Hb exchange and heme transfer experiments demonstrated proof-of-concept Hb/heme ligand transfer to apoHb-Hp. Dosing with the apoHb-Hp complex reversed Hb- and heme-induced systemic hypertension and microvascular vasoconstriction, reduced microvascular blood flow and diminished functional capillary density. Therefore, this study highlights the apoHb-Hp complex as a novel therapeutic strategy to attenuate the adverse systemic and microvascular responses to intravascular Hb and heme exposure.

2.2 INTRODUCTION

Erythropoiesis and hemolysis occur at similar rates in healthy organisms, thus maintaining the total population of red blood cells (RBCs) in the circulation. RBC homeostasis results in the balanced cycle of erythropoiesis and hemolysis, along with the effective transport and clearance of hemoglobin (Hb) and its degradation products (heme and iron) [1]. Genetic hemolytic diseases (e.g. sickle cell anemia and thalassemia), acquired hemolytic infections (e.g. gram positive and malarial hemolysins) as well as hemolytic xenobiotic toxins (e.g. heavy metals, dapsone and phenyl hydrazine) damage the RBC, disrupting this delicate equilibrium by increasing the rate of hemolysis [2]. Increased intravascular hemolysis and the Hb degradation product heme progress hemolytic diseases, such as sickle cell anemia, malaria and sepsis, [3-5] which affect millions of patients every year [6].

The presence of Hb outside of the erythrocyte's protective physiologic system exposes Hb to a structurally unstable environment due to the increased dilutional dynamic equilibrium between tetrameric ($\alpha_2\beta_2$) and dimeric components ($\alpha\beta$), which are more prone to autoxidation and extravasation relative to tetrameric Hb [8-10]. Acellular Hb ($\alpha_2\beta_2$) dissociation into $\alpha\beta$ dimers results in autoxidation, structural destabilization of heme within its binding pocket [11, 12], and subsequent transfer of heme prosthetic groups to lipids [13], proteins [14] and bacterial receptor complexes.[15]. In addition, acellular Hb and its' byproducts can scavenge nitric oxide (NO) and increase oxidative stress, cause hypertension [17], vasoconstriction[10, 18, 19], kidney injury [20], and cardiovascular lesions [21, 22].

In the circulatory intravascular comparment, acellular Hb and one of its' byproducts, heme, are bound and cleared by the plasma proteins haptoglobin (Hp)[23] and hemopexin (Hpx) [24], respectively.The ability of Hp to control acellular Hb exposure is dependent on the primary binding

site for Hb αβ dimers. Irreversible binding between the Hp β chain and Hb αβ dimers occurs at a 1:1 stoichiometric ratio [23]. This interaction provides a critical antioxidant function by protecting key amino acids in Hb that are vulnerable to oxidation [23, 30, 31], preventing heme release [32, 33] and compartmentalizing Hb within a stable protein complex that does not extravasate out of the circulatory intravascular compartment into the parenchymal tissues [10, 34]. During hemolytic diseases, Hp acts as the primary defense, stabilizing acellular Hb. Upon Hp saturation the dimerized excess Hb releases heme, which is captured by Hpx [24, 35, 36]. In addition to Hpx, serum albumin can act as a secondary heme scavenger [37]. However, albumin's affinity for heme is lower than that of Hpx and the heme-albumin complex does not fully protect from heme-mediated toxicity[38].

Apohemoglobin (apoHb) is produced by removing heme from Hb. This apoprotein has a high affinity region in the unoccupied hydrophobic heme-binding pocket for a number of ligands, but the highest affinity of apoHb is for heme [39, 40]. Previous work has illustrated the binding of heme to apoHb and the complexation of apoHb to Hp *in vitro,* forming an apoHb-Hp complex [41, 42]. In pathophysiological states of heme excess, the use of apoHb or apoHb-Hp may offer an advantage by scavenging and clearing excess heme through the monocyte/macrophage CD163 surface receptor. However, no study has explored the potential *in vivo* heme scavenging properties of apoHb or apoHb-Hp. Moreover, we observed that the apoHb-Hp complex, in addition to heme-binding, can exchange Hp bound apoHb αβ dimers for holoHb αβ dimers, serving a dual role of Hb and heme scavenger *in vivo* **(**Figure 2.1).

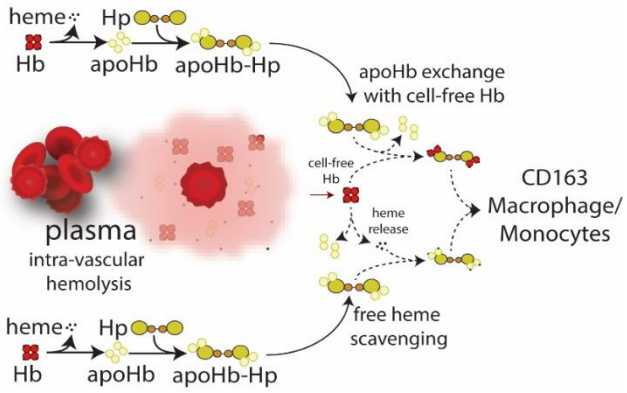

Figure 2.1 Proposed mechanisms for scavenging free heme and cell-free Hb via the apoHb-Hp complex.

We hypothesized that apoHb-Hp could facilitate heme transfer during states of high heme stress or holoHb exchange for apoHb αβ dimers during acellular Hb exposure. Further, we hypothesized that these ligand interactions could attenuate the adverse microcirculatory consequences of heme and acellular Hb. In the present study, we test a sequential approach characterizing in vitro and in vivo apo-Hb-Hp and heme/Hb ligand interactions using a guinea pig model to assess time dependent heme transfer and Hb exchange. To test the effect of apoHb for holoHb exchange on vascular response we evaluated (1) apoHb, Hp and apoHb-Hp complex pre-treatment followed by Hb administration as a 10% top-load. Next, we tested the effect of heme transfer to apoHb-Hp on the vascular response to heme-albumin after (2) apoHb-Hp as a 20% blood volume exchange. Both experimental designs were conducted in conscious Golden Syrian hamsters instrumented with the dorsal window chamber model to quantify systemic and microvascular hemodynamic responses to heme and acellular Hb.

2.3 METHODS

Hb Preparation

Hb (P68871, P69905) for this study was prepared via tangential flow filtration as previously described[36]. The concentration of Hb was determined spectrophotometrically.

Apohemoglobin Preparation

The apoHb used in this study was prepared via tangential flow filtration based on the acidic-ethanol heme-extraction procedure as previously described [37]. The absence of residual heme was verified by measuring the ratio of absorbance between the Soret peak (A_{max} = 412-413 nm) and 280 nm to ensure less than 1% residual heme in the product. The heme-binding capacity of apoHb preparations was 80% [38].

Haptoglobin Preparation

Hp was purified from human Cohn fraction IV (FIV) yielding a mixture of Hp2-1 and Hp2-2 Hp polymers with an average MW of 400-500 kDa.

Heme-Albumin Preparation

A 4 mM heme-albumin stock solution was prepared by dissolving 65 mg Hemin (Sigma) in 100 mM NaOH at 37°C and incubating for 1 h at 37°C with 20% human serum albumin (HSA). The pH was carefully adjusted to 7.45 with 14 mM orthophosphoric acid/317 mM NaCl followed by sterile filtration.

Total Protein Assays

To estimate the total protein concentration of the apoHb solution, a Bradford assay was performed (Coomassie Plus Protein assay kit, Pierce Biotechnology, Rockford, IL). Additionally, spectrophotometric analysis of the 280 nm peak for each sample was used to estimate the total protein concentration using the millimolar extinction coefficients of apoHb found in the literature[39].

In Vitro Hb Exchange and Heme Binding by the ApoHb-Hp Complex

The apoHb-Hp complex was formed by reacting apoHb with Hp, and complex formation was confirmed by analysis of the mixture via size exclusion HPLC (HPLC-SEC). Heme binding to the apoHb-Hp complex was assessed by mixing the apoHb-Hp complex with heme-albumin prior to HPLC-SEC. Furthermore, apoHb exchange for Hb within the apoHb-Hp complex was determined by reacting the apoHb-Hp complex with Hb. HPLC-SEC was performed using an Acclaim SEC-1000 (4.6 × 300 mm) column (Thermo Fisher Scientific, Waltham, MA) attached to a Dionex UltiMate 3000 system (Thermo Fisher Scientific, Waltham, MA). The mobile phase consisted of 50 mM potassium phosphate, pH 7.4 at a flow rate of 0.35 mL/min controlled by Chromeleon 7 software. Wavelength absorbance detection was set to $\lambda = 280$ nm to detect protein, $\lambda = 405$ nm to detect heme, and $\lambda = 413$ nm to detect Hb.

In Vivo *Hb Exchange and Heme Binding by the ApoHb-Hp Complex*

In vivo heme transfer (heme-albumin) and Hb (acellular Hb) exchange with apoHb-Hp was studied in male Hartley guinea pigs (400-600 grams). Guinea pigs were infused with acellular Hb (0.75 µmol protein) (n = 5) or heme-albumin (0.75 µmol protein) (n = 5) followed by apoHb (0.75 µmol protein) bound to Hp. Plasma concentrations of all components were evaluated over a 6-hour period by UV-visible spectrophotometry and analytical HPLC-SEC. Plasma concentrations versus time are expressed as heme (µg/ml). On days of surgery, guinea pigs were dosed subcutaneously with ketoprofen (5 mg/kg) for pain management and then anesthetized via intraperitoneal injection with a cocktail of ketamine HCl (100 mg/kg) and xylazine HCl (5 mg/kg) (Phoenix Scientific Inc., St. Joseph, MO USA). Sterilized PE50 tubing catheters were placed into the left external jugular vein, and left carotid artery and exteriorized at the back of the neck, all surgical sites were treated topically with bupivacaine HCl (2.5 mg/ml) (AuroMedics, Windsor, NJ, USA), and closed with 4-0 surgical silk internal sutures and external surgical staples. After 24-hours of post- surgical recovery, conscious guinea pigs were randomly allocated to dosing groups. Blood samples (150 µL) were collected through the carotid artery at baseline, after acellular Hb or heme-albumin albumin infusion, immediately after apoHb-Hp infusion (time 0) then at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50,

3.00 and 6.00 hours. All animal studies were approved by the FDA Institutional Animal Care and Use Committee, with all experimental procedures performed in adherence to the NIH guidelines on the use of experimental animals.

Plasma Hemoglobin Analysis

Plasma was diluted in 1× PBS, and Hb concentration was measured in 1-ml semi-micro cuvettes using a Cary 60 spectrophotometer (Agilent Technologies, Cary 60 UV-Vis, Model G6860A). Absorbance was measured over time every 2 nm between 350 and 650 nm with a 0.0125 s integration time per wavelength. Spectra were deconvoluted against standard extinction curves of the pure substances using a nonnegative least squares algorithm.

HPLC-SEC analysis of Hb-Hp Complexes in Plasma

Plasma samples (50 µL) were injected into a BioSep-SEC-S3000 (600 7.5 mm) SEC column (Phenomenex, Torrance, CA). The SEC column was attached to a Waters 2535 Quaternary Gradient Module pump and Waters 2998 Photodiode Array Detector controlled by a Waters 600 controller using Empower 2 software (Waters, Milford, MA), wavelength monitoring was the same as used for in vitro analysis (280, 405 and 413 nm).

Window Chamber Preparation in Golden Syrian Hamsters

Animal handling and care followed the NIH Guide for the Care and Use of Laboratory Animals and the study was approved by the University of California San Diego animal care committee. Studies were performed in 55 - 65 g male Golden Syrian Hamsters (Charles River Laboratories, Boston, MA) fitted with a dorsal window chamber. The hamster window chamber model is widely used for microvascular studies without anesthesia. The complete surgical technique has been described previously [40]. Two days after window implantation, arterial and venous catheters filled with heparinized saline solution (30 IU/mL) were implanted into the carotid

and jugular vessels. Catheters were tunneled under the skin, exteriorized at the dorsal side of the neck, and securely attached to the window frame.

Inclusion Criteria

Animals were considered suitable for experiments if systemic parameters were as follows: heart rate (HR) > 340 beats/min, mean arterial blood pressure (MAP) > 80 mm Hg, systemic Hct > 45%, and arterial O_2 partial pressure (pAO_2) > 50 mm Hg. Additionally, animals with signs of low perfusion, inflammation, edema, or bleeding in their microvasculature were excluded from the study.

Experimental Setup

The unanesthetized animal was placed in a restraining tube with a longitudinal slit from which the window chamber protruded, then fixed to the microscopic stage for transillumination with the intravital microscope (BX51WI, Olympus, Japan). Animals were given 20 minutes to adjust to the tube environment and images were obtained using a CCD camera (4815, COHU, San Diego, CA). Measurements were carried out using a 40× (LUMPFL-WIR, numerical aperture 0.8, Olympus) water immersion objective.

Systemic Parameters

The MAP and HR were monitored continuously (MP150, Biopac System Inc., Santa Barbara, CA). Hct was measured from centrifuged arterial blood samples taken in heparinized capillary tubes. Hb content was determined spectrophotometrically (B-Hemoglobin, Hemocue, Stockholm, Sweden). Arterial blood was collected in heparinized glass capillaries (50 μL) and immediately analyzed for pO_2, pCO_2, and pH (ABL90; Radiometer America, Brea, CA). Arterial Hb saturation was measured using an IL482 CO-Oximeter (Instrumentation Laboratory, Lexington, MA).

Microhemodynamics

Arteriolar and venular blood flow velocities were measured using the photodiode cross-correlation method (Photo-Diode/Velocity, Vista Electronics, San Diego, CA) [41]. The measured centerline velocity (V) was corrected according to blood vessel size to obtain the mean RBC velocity [42] . A video image-shearing method was used to measure blood vessel diameter (D) [43] . Blood flow (Q) was calculated from the measured values as $Q = \pi/4\ D^2\ V$.

Functional Capillary Density (FCD)

Functional capillaries, defined as capillary segments that have transit of at least one RBC in a 60 second period in 10 successive microscopic fields, were assessed in a region of 0.46 mm^2. The FCD (cm^{-1}) was calculated as the total length of RBC perfused capillaries divided by the viewing area (0.46 mm^2).

Vascular Response to Acellular Hb

Hamsters were pre-treated with apoHb (30 mg/mL, 100 µL, n = 6), Hp (15 mg/mL, 50 µL, n = 6), apoHb-Hp complex (45 mg/mL, apoHb 15 mg/mL + Hp 30 mg/mL, 150 µL, n = 6), or vehicle (saline equal volume as study groups, n = 6). Hamsters were then subjected to a 10% hypervolemic infusion of purified acellular human Hb (50 mg/mL, 500 µL) and systemic and microvascular responses were measured after 30 minutes.

Vascular Response to Heme-Albumin

Hamsters were either pre-treated with apoHb-Hp complex (45 mg/mL, apoHb 15 mg/mL + Hp 30 mg/mL - 0.15 mL and 6.75 mg total dose, n = 6), or vehicle (saline equal volume as study groups, n = 6). Then hamsters were dosed with heme-albumin (2.0 mg/mL, 2.0-2.8 mg total dose) via a 20% blood volume exchange transfusion. Systemic hemodynamics and microcirculatory function were measured after 30 minutes.

Statistical Analysis

For *in vivo* Hb exchange and heme transfer studies performed in guinea pigs (n = 5/group), all data are represented as mean values ± SD. AUC values were derived using the linear trapezoidal rule and data were compared using a One-way ANOVA with Tukey's multiple comparisons test for parametric data in GraphPad Prism 8.3, GraphPad Software Inc., San Diego, CA. For microcirculatory studies, data within each group were analyzed using a two-way ANOVA for repeated measurements. When appropriate, post hoc analyses were performed with the Dunnett's multiple comparisons test. Microhemodynamic data are presented as ratios relative to baseline values. A ratio of 1.0 signifies no change from baseline, while lower and higher ratios are indicative of changes proportionally lower and higher than baseline (i.e., 1.5 represents a 50% increase from the baseline level). The same blood vessels and capillary fields were monitored throughout the study, such that direct comparisons to their baseline levels could be performed, allowing for more reliable statistics on small sample populations. All statistics were calculated using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA). Changes were considered significant if p values were less than 0.05.

2.4 **RESULTS**

In Vitro HoloHb Exchange and Heme Binding by the ApoHb-Hp Complex

Formation of the apoHb-Hp complex *in vitro* can be achieved by reacting the two proteins (apoHb + Hp). The difference in molecular weight (MW) of Hp (~400 kDa in this study) compared to apoHb (~32 kDa) allowed for assessment of apoHb-Hp complex formation via HPLC-SEC. This is shown in the SEC chromatograms of **Figure 2.2 A-B.**

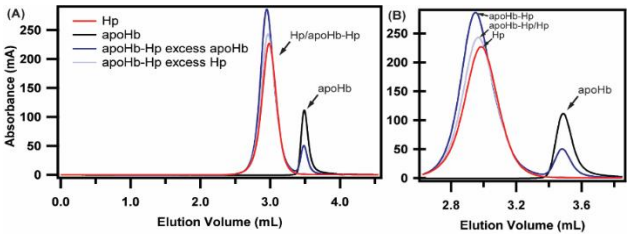

Figure 2.2 HPLC-SEC of apoHb binding to Hp - (A) HPLC-SEC of apoHb, Hp and mixtures of apoHb and Hp. **(B)** Close-up of elution curves of apoHb, apoHb-Hp and Hp.

Based on results from *in vitro* mixing, and analysis by HPLC-SEC, apoHb bound to Hp to form the apoHb-Hp complex. Based on the change in the AUC of the apoHb peak, the total mass of apoHb αβ dimers bound to Hp was comparable to the total mass of holoHb αβ dimers bound to Hp, demonstrating stoichiometric binding of apoHb to Hp. Further, *in vitro* heme-binding and Hb exchange with the apoHb-Hp complex was assessed via HPLC-SEC. The apoHb-Hp complex was mixed with either free Hb or heme-albumin. When holoHb was mixed with apoHb-Hp (**Figure 2.3 A**), Hb-apoHb exchange occurred, as evidenced by an increase in absorbance at 413 nm. Furthermore, free apoHb eluted in the same chromatogram, further indicating detachment of apoHb from the apoHb-Hp complex. This observation agrees with previous reports regarding the lower association equilibrium of apoHb to Hp compared to that of holoHb to Hp[44]. Moreover, when apoHb-Hp was mixed with heme-albumin (**Figure 2.3 B**), heme was transferred from albumin to the apoHb in the apoHb-Hp complex given that the absorbance of free albumin (405 nm) decreased while the absorbance of apoHb-Hp (405 nm) complex increased.

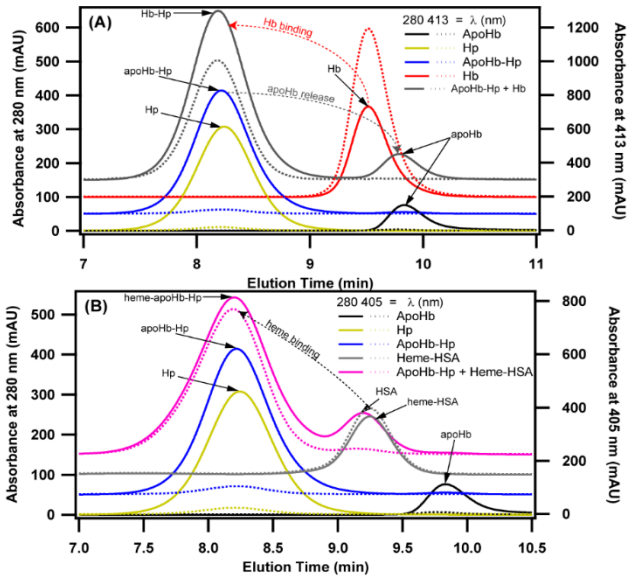

Figure 2.3 HPLC-SEC of Hb exchange for apoHb bound to Hp and heme exchange from heme-albumin to apoHb bound to Hp - (A) HPLC-SEC of apoHb, Hp, apoHb-Hp and mixtures of apoHb-Hp with Hb and **(B)** mixtures of apoHb-Hp with heme-albumin (heme-HSA).

In Vivo HoloHb Exchange for ApoHb within the Hb-Hp Complex

Guinea pigs were administered holoHb (denoted as Hb) followed by apoHb-Hp and time dependent blood sampling for analysis of Hp-bound and -unbound Hb plasma concentrations versus time are shown Figure 2.4 **A.** Maximum Hp-bound holoHb concentrations (C_{max}) occurred rapidly, with a time of maximum concentration (T_{max}) occurring within the 5 minute timeframe of holoHb administration (Hp-bound holoHb = 75.1 ± 16.8 µg/ml, as heme). Unbound holoHb concentrations in plasma remained low-to-undetectable (3.80 ± 2.17 µg/ml, as heme) after the initial exchange of apoHb for holoHb αβ dimers in Hp was observed in the plasma concentration versus time curves **(Figure 2.4 A**). The derived areas under the plasma concentration versus time curves (AUC $_{(0\text{-}360\ min)}$) were 9818 ± 1026 µg x minute/ml (total = Hp-bound + unbound), 9001 ± 986.1 µg x minute/ml (Hp-bound) and 805.4 ± 133.6 µg x minute/ml (unbound) for holoHb. These data demonstrate that Hp-bound apoHb αβ dimers were completely exchanged for holoHb αβ dimers, indicated by significantly higher Hp-bound holoHb versus unbound AUC $_{(0\text{-}360\ min)}$ values

(p<0.0001) shown in **Figure 2.4 B**. The rapid accumulation and stability holoHb bound to Hp is observed by the representative HPLC-SEC chromatographs with detection at 413 nm shown in **Figure 2.4 C**.

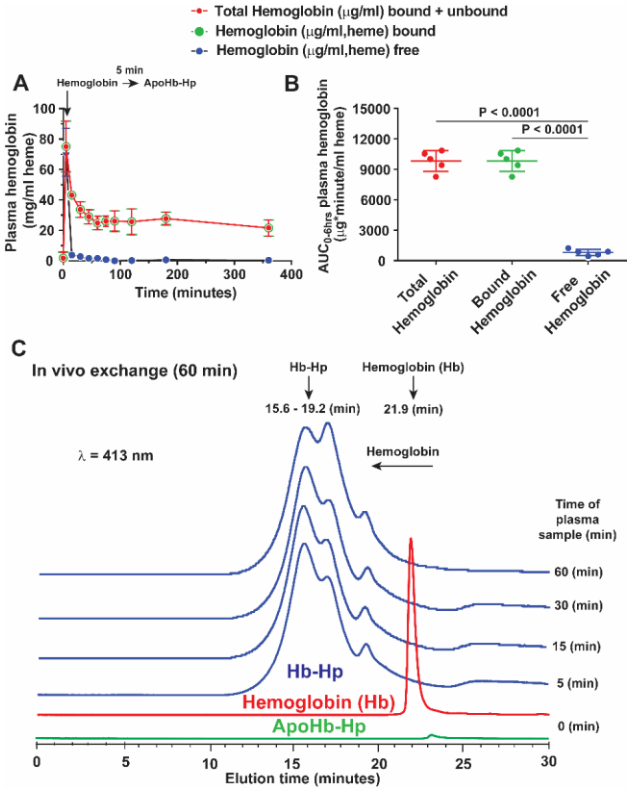

Figure 2.4 HoloHb exchange for apoHb in the apoHb-Hp complex in the plasma compartment of guinea pigs – (A) Plasma concentration versus time (0-360 minutes) of total holoHb (red line), Hp bound holoHb (green line, superimposed by red line) and unbound holoHb (blue line). Indicates nearly complete holoHb exchange for apoHb within the initial minutes following apoHb-Hp administration. **(B)** $AUC_{0\text{-}360min}$ values for total holoHb (Hp bound + unbound, red), Hp bound holoHb (green) and unbound holoHb (blue). Significant differences were observed between $AUC_{0\text{-}360min}$ for total versus free holoHb ($p<0.0001$) and Hp bound versus free holoHb ($p<0.0001$), based on a One-way ANOVA with a multiple comparisons test for parametric data (n=5/comparison). **(C)** HPLC-SEC traces (0-60 minutes) showing holoHb completely replacing apoHb in the Hb-Hp complex after the administration of holoHb and apoHb-Hp to guinea pigs. Representative plasma samples with holoHb (denoted (Hb), red trace), apoHb-Hp complex (green trace, no absorbance at 413 nm) and the rapid appearance of Hp bound holoHb (denoted Hb-Hp, blue trace) within the initial post dosing plasma samples, indicating rapid binding and detection (at l = 413 nm) of holoHb in exchange for apoHb. Note- representative chromatograms are shown until 60 minutes, due to rapid and complete holoHb exchange for apoHb.

In Vivo Heme Transfer from Heme-Albumin to ApoHb within the ApoHb-Hp complex

Guinea pigs were administered heme-albumin followed by apoHb-Hp. Time dependent blood sampling was conducted for analysis of heme transfer from heme-albumin to apoHb-Hp resulting in heme intercalated into apoHb αβ dimers, generating bound holoHb αβ dimers (denoted Hb-Hp) and their plasma concentrations over time as shown in **Figure 2.5 A.** The transfer of heme from heme-albumin to apoHb-Hp occurred more slowly in vivo. C_{max} values for heme intercalation into apoHb-Hp were 55.5 ± 2.60 µg/ml (as heme concentration) occurring at a T_{max} = 30 minutes. The areas under the plasma concentration versus time derived curves ($AUC_{(0\text{-}360\ min)}$) were 12,597 ± 4015.0 µg x minute/ml (total heme = heme-albumin + heme-apoHb-Hp), 10,163 ± 3922.0 µg x minute/ml (transferred heme = heme-apoHb-Hp, denoted Hb-Hp) and 2125 ± 511.6 µg x minute/ml (non-transferred heme = heme-albumin). Total and heme-albumin transferred heme were significantly greater ($p<0.0001$ and $p=0.0003$) compared to non-transferred heme, respectively as shown in **Figure 2.5 B**. The time course of heme transfer from heme-albumin to apoHb-Hp to generate Hb-Hp within the guinea pig plasma compartment is shown in the representative HPLC-SEC chromatographs with detection at 413 nm shown in **Figure 2.5 C**.

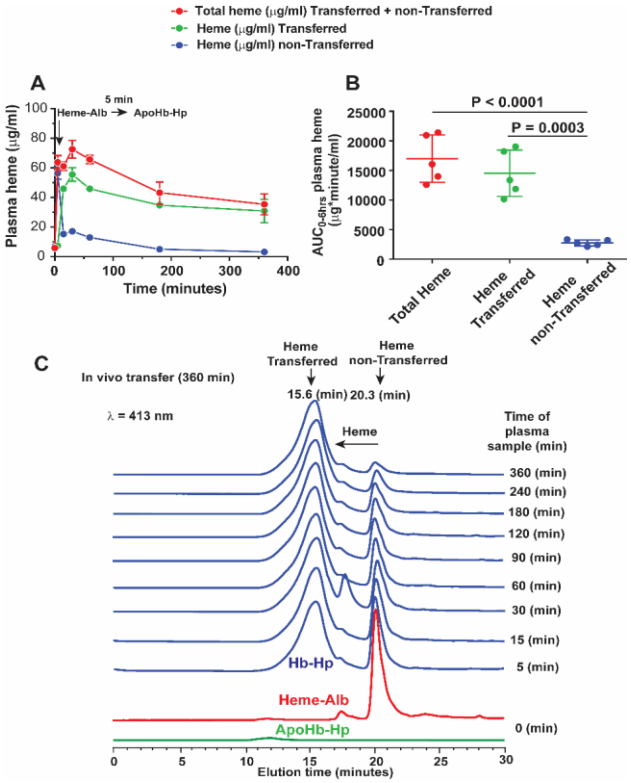

Figure 2.5 Heme transfer from heme-albumin to apoHb in the apoHb-Hp complex in the plasma compartment of guinea pigs – (A) Plasma concentration versus time (0-360 minutes) of total heme (red line), transferred heme (green line) and non-transferred heme (heme-albumin, blue line). Indicates transfer of heme over the initial 6 hours post heme-albumin and apoHb-Hp administration. (B) AUC values for total heme (transferred + non-transferred, red), heme (transferred, green) and heme (non-transferred, blue). Significant differences were observed between $AUC_{0\text{-}360min}$ values for total versus non-transferred heme ($p<0.0001$) and between $AUC_{0\text{-}360min}$ values for transferred versus non-transferred heme ($p=0.0003$), based on a One-way ANOVA with a multiple comparisons test for parametric data (n=5/comparison). (C) HPLC-SEC traces (0-360 minutes) showing heme-albumin (red), apoHb-Hp (green, no absorbance at 413 nm) in the Hb-Hp complex after the administration of heme-albumin followed by apoHb-Hp to guinea pigs. Representative plasma samples show the transfer of heme (at l = 413 nm) from heme-albumin to apoHb bound to Hp to ultimately generate holoHb-Hp (denoted Hb-Hp).

Microvascular Impact of Pretreatment with ApoHb, Hp and ApoHb-Hp Complex Followed by Acellular Hb Infusion

Systemic Parameters

The MAP and HR after pretreatment with vehicle, apoHb, Hp or apoHb-Hp, and subsequent challenge with acellular Hb dosing are presented in **Figure 2.6 A-B.** The MAP and

HR were not statistically different between groups at baseline or after pre-treatment with the test materials. After administration of acellular Hb, vehicle-dosed animals, and animals treated with apoHb or Hp alone demonstrated elevated MAP compared to baseline and pretreatment ($p < 0.05$). After Hb administration, both Hp and apoHb-Hp showed lower MAP than vehicle alone. However, only treatment with the apoHb-Hp complex maintained the animal's MAP at baseline and pretreatment levels. The HR after Hb infusion was inverse, but similar to the change in MAP. Acellular Hb infusion caused a statistically significant decrease in HR from baseline and pre-treatment for the vehicle and apoHb groups. There were no significant changes in HR in response to acellular Hb challenge for animals pretreated with Hp and apoHb-Hp. Furthermore, vehicle-treated, and apoHb-treated animals showed lower HR compared to the apoHb-Hp treated group post acellular Hb transfusion.

Functional Capillary Density

The FCD after pretreatment with vehicle, apoHb, Hp or apoHb-Hp, and challenge with acellular Hb dosing are presented in **Figure 2.6 C**. Pretreatment with test materials did not result in any adverse changes in FCD. FCD decreased for vehicle-treated and apoHb-treated animals following acellular Hb transfusion compared to baseline and pre-treatment. ApoHb-Hp attenuated the Hb-driven loss of FCD, as there were no statistical differences between time points, and this group's FCD was significantly higher than that of vehicle and apoHb alone following Hb transfusion. Hp alone also attenuated the loss of FCD due to Hb challenge, but to a lesser extent than the apoHb-Hp complex. These data suggest that Hp and apoHb-Hp pre-treatment result in a comparable response to Hb exposure and support the concept of holoHb-apoHb exchange within the apoHb-Hp complex.

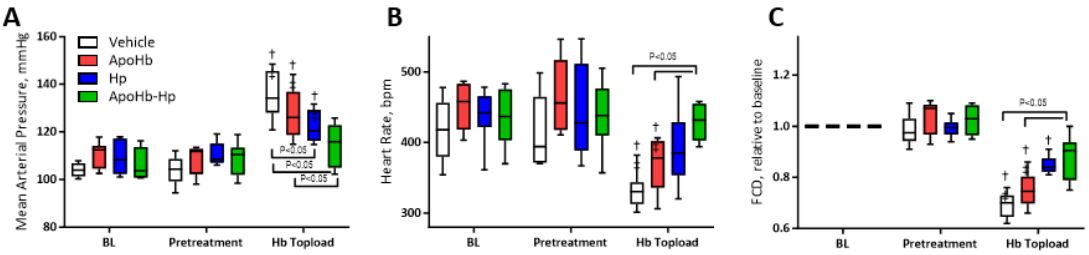

Figure 2.6 Systemic parameters (MAP and HR) and functional capillary density – Measurements made during baseline, following pretreatment and following Hb challenge for all experimental groups: the control, pretreatment with only apoHb, pretreatment with only Hp, and pretreatment with the apoHb-Hp complex. **(A)** Mean arterial pressure (MAP), **(B)** Heart rate (HR) and **(C)** functional capillary density. Significant differences are indicated with symbols: † compared to BL and ‡ compared to treatment (P<0.05).

Microhemodynamics

The changes in arteriolar and venular hemodynamics relative to baseline are shown for all four experimental groups. The arterioles were segmented into various vessel diameters: small arterioles from 20 to 40 µm, mid-size arterioles from 40 to 60 µm and large arterioles from 60 to100 µm. All venules were very consistent and grouped into a single venule group from 30 to 80 µm in vessel diameter.

***Small Arterioles (20-40 µm)*:** The diameter, velocity and flow, relative to baseline, for small arterioles, ranging from 20-40 µm in diameter are shown in **Figure 2.7 A**. There were no differences observed between baseline and pre-treatment groups. Following Hb administration, the vehicle group (untreated animals) (n=18 vessels) demonstrated decreased microvascular responses (i.e. decreased diameter, velocity and flow) compared to baseline and pre-treatment groups. Following Hb administration, animals infused with apoHb alone (n=14 vessels) also demonstrated decreased microvascular responses compared to baseline and pre-treatment groups in small vessel diameter, velocity, and flow. The small arteriolar diameter of animals infused with Hp alone (n=16 vessels) did not changed from baseline, however, their respective velocity and

flow decreased compared to the other pre-treatment groups. The small arteriolar diameter of animals dosed with Hp did not changed after Hb administration, thus, the Hp prevented vasoconstriction. The administration of apoHb-Hp improved diameter, velocity and flow compared to basal levels and in response to Hb dosing.

Mid-Sized Arterioles (40-60 µm): The diameter, velocity and flow, relative to baseline, for medium sized arterioles, ranging from 40-60 µm in diameter are shown in **Figure 2.7 B**. There were no differences observed between baseline and pre-treatment groups. Following Hb administration, the vehicle group (untreated animals) (n=18 vessels) demonstrated a decrease in vessel diameter, velocity and flow compared to baseline and pre-treatment. ApoHb alone (n=16 vessels) demonstrated a decrease in diameter, velocity, and flow compared to baseline and pre-treatment. Following Hb administration, animals infused with Hp alone (n=19 vessels) demonstrated no differences in diameter compared to baseline or pre-treatment, however, velocity and flow were decreased. Following Hb administration, the apoHb-Hp complex was not different from baseline or pre-treatment. However, after Hb dosing the apoHb-Hp group did demonstrate an increase in vessel diameter, velocity and flow compared to the control and apoHb groups but did increase in arteriole velocity compared to Hp dosing alone.

Large Arterioles (60-100 µm): The diameter, velocity and flow, relative to baseline, for large arterioles, ranging from 60-100 µm in diameter are shown in **Figure 2.7 C**. There were no differences observed between baseline and pre-treatment groups. Following Hb dosing, the vehicle group (n=22 vessels) demonstrated decreases compared to baseline and treatment for diameter, velocity and flow. Similarly, apoHb (n=16 vessels) and Hp (n=19 vessels) pre-treatment followed by Hb dosing, demonstrated decreases in diameter, velocity, and flow compared to baseline and pre-treatment. Following Hb dosing, the apoHb-Hp complex showed no change compared to

baseline or pre-treatment. The group comparisons demonstrated a difference in large arteriole diameter between the control and apoHb groups compared to apoHb-Hp group. Large arteriole velocity and flow were significantly decreased in control, apoHb and Hp groups following Hb dosing compared to the apoHb-Hp group.

Venules (30-80 µm): The diameter, velocity and flow, relative to baseline, for venules, ranging from 30-80 µm in diameter are shown in **Figure 2.7 D**. There were no differences observed between baseline and pre-treatment groups. After Hb dosing, the vehicle, apoHb, Hp and apoHb-Hp groups demonstrated similar diameter, velocity and flow-based responses.

Taken together, these data suggest that apoHb-Hp may offer an improvement over Hp alone in microvascular function of small, mid-size and large arteriole. To our knowledge these data are the first to demonstrate such an effect in the microcirculation and indicate the potential for a concomitant heme and Hb mediated pathobiology on the vasculature. To better understand this, we studied the systemic and microcirculatory hemodynamic response to pre-treatment with our novel apoHb-Hp construct followed by heme-albumin exposure.

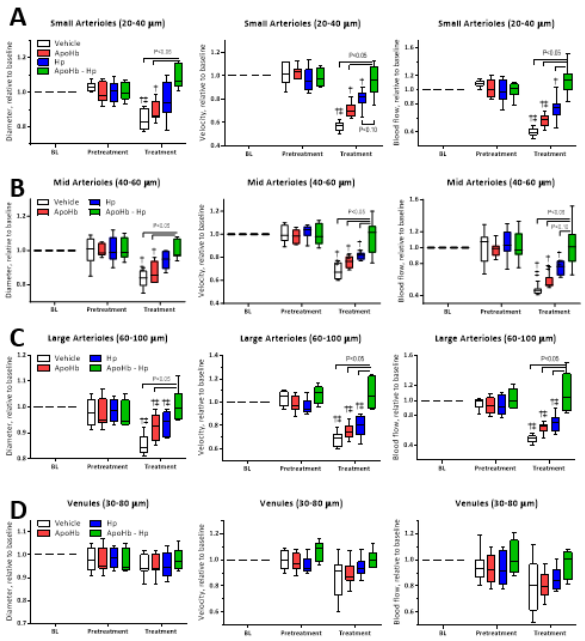

Figure 2.7 Diameter, velocity and flow of small, medium, large-sized arteriole vessels and venules – (A) small-sized arteriole vessels (20-40 µm), (B) mid-sized arteriole vessels (40-60 µm), (C) large arterioles (60-100 µm), (D) venules (30-80 µm) - relative to baseline during baseline, following pretreatment and following Hb challenge for all experimental groups: the control, pretreatment with only apoHb, pretreatment with only Hp, and pretreatment with the apoHb-Hp complex. †, compared to BL and ‡, compared to dosing groups ($P<0.05$).

In Vivo Pretreatment with ApoHb-Hp Followed by Heme-Albumin Exposure

This study was completed in twenty-four (N = 24) instrumented animals. Eight animals were randomly assigned to each experimental group. The first experimental group was a control (untreated animals), and the second group was infused with apoHb-Hp. All groups were then administered a single dose of heme-albumin. The impact of apoHb-Hp on mean arterial blood pressure (MAP) and heart rate (HR) following heme-albumin challenge are presented in **Figure 2.8 A** and **B**. Heme-albumin infusion decreased both MAP and HR decreased by 10% in control animals. Pretreatment with apoHb-Hp prevented this decrease in MAP and HR, suggesting modest cardiovascular depression in response to heme. Within the time frame of dosing and measurement (20 minutes), it is possible that heme metabolism by hemoxygenase-1 generated enough carbon

monoxide to explain this effect on both the macrovascular circulation and the myocardium. The changes in arteriolar hemodynamics (diameter and flow) are shown in **Figure 2.8 C** and **D**. Similar to MAP, control animals experienced a 20% and 10% decrease in flow and diameter, respectively, relative to baseline in response to heme-albumin. Pretreatment with apoHb-Hp prevented this decline in diameter and flow. The FCD is shown in **Figure 2.8 E** and demonstrates a 40% decrease in RBC transit through capillaries in response to heme-albumin exposure for control animals relative to baseline. Pretreatment with apoHb-Hp also prevented this decrease in FCD in response to heme-albumin exposure. This further demonstrates a pathophysiologic response to exposure to heme-albumin at the microcirculatory level and suggests that apoHb-Hp has the ability to attenuate this response.

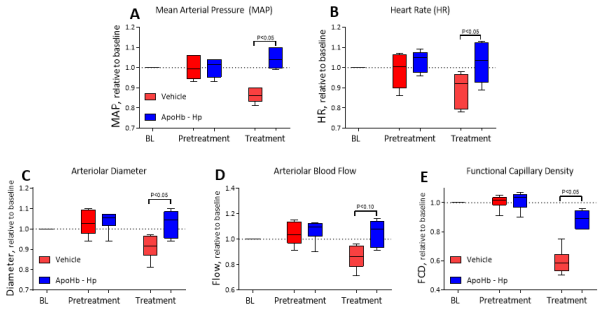

Figure 2.8 Systemic hemodynamic and microcirculatory response to heme-albumin and attenuation by apoHb-Hp – Measurements made relative to baseline during baseline, following pretreatment and following heme-albumin challenge for experimental groups - control (untreated), pretreatment with the apoHb-Hp complex. (A) Mean arterial pressure (MAP) and (B) Heart rate (HR). Microcirculatory changes: (C) Arteriolar diameter, (D) Arteriolar blood flow and (E) Functional capillary density (FCD). Significant differences are indicated within group comparisons ($P<0.05$).

2.5 DISCUSSION

Acellular Hb has multiple pathophysiologic effects when released into the intravascular space during hemolysis. Hb tetramers ($\alpha_2\beta_2$) dissociate into $\alpha\beta$ dimers quickly in the circulation which can easily oxidize into methemoglobin (metHb) and release free heme. Multiple

pathophysiological consequences are associated with heme and Hb [45]. To prevent the adverse effects from Hb and its degradation products, Hp binds dimeric Hb (i.e. αβ dimers), thus preventing Hb from mediating oxidative reactions and from releasing free heme [25, 26]. When Hp is depleted (during extensive acute or low-level chronic hemolysis), heme released by Hb is transferred and bound by Hpx. In addition, albumin serves as a secondary heme binding protein, but does not fully prevent heme-mediated toxicity due to its lower affinity for heme than highly lipophilic LDL, HDL and VLDL [12]. Hb binding to Hp is a biologically irreversible process, creating a stable and high-molecular-weight molecule that is cleared by macrophages and monocytes upon binding to the scavenger receptor, CD163 [16, 46]. ApoHb acts as a heme scavenging protein, binding heme with a higher affinity than albumin [33]. Moreover, the apoHb-heme protein can bind to Hp to be cleared through CD163+ macrophages and monocytes. To ensure apoHb-heme can be delivered to monocytes/macrophages we bound apoHb to Hp. This heme capture mechanism provides a specific pathway for heme clearance, in lieu of the described route of heme-Hpx uptake, which occurs through LRP-1 (low density lipoprotein receptor, CD91), a ubiquitous receptor that exists on the surface of numerous cell types and exhibits a multitude of functions [47].

This study demonstrates *in vitro* and *in vivo* holoHb exchange for apoHb when bound to Hp and highlights the unique property of heme intercalation into apoHb-Hp, following holoHb and heme-albumin exposure in guinea pigs. This combination of effects offers a potentially advantageous combination when concomitantly or independently addressing intravascular hemolysis and heme stress. Furthermore, microcirculatory studies in hamsters with apoHb, Hp, and the apoHb-Hp complex reduced adverse macrovascular and microvascular responses to acellular Hb exposure. More specifically, administration of Hp and apoHb-Hp complex prevented hypertension and vasoconstriction, as well as preserved microvascular diameter, blood flow, and

functional capillary density across a range of arteriolar sizes. The apoHb-Hp complex appears to demonstrate a complementary mechanism to address Hb vasoactive response by attenuating heme and acellular Hb vascular interactions in the circulation. Unless bound to Hp, Hb dissociates into αβ dimers and extravasates through the blood vessel wall and reacts with or scavenges NO. Furthermore, both heme within Hb [48] and heme bound to albumin [49] participate in oxygen radical reactions that covalently modify proteins, lipids, carbohydrates, and nucleotides, leading to tissue damage. Administration of the apoHb-Hp complex may provide a unique therapeutic strategy to simultaneously mitigate heme stress and plasma Hb exposure across a range of disease states that involve heme-protein circulatory exposures. This approach provides a distinctive physiologic method to target the well-established mechanism of Hb-Hp clearance and intra-cellular Hb/heme detoxification by targeting the monocyte/macrophage CD163 surface receptor.

In our microcirculatory function studies, infusion of acellular Hb induced arteriolar vasoconstriction and increased vascular resistance in small, mid and large arterioles. As Hb dissociates into αβ dimers it extravasates through the fenestrated capillaries and scavenges NO from the tissue. NO stimulates the production cGMP, decreasing the intracellular Ca^{2+} concentration, resulting in muscle relaxation; without the accumulation of cGMP the smooth muscles around the arterioles constrict. Hb mediated NO consumption leads to vasoconstriction, prevents perfusion of capillary beds, and reduces the number of functional capillaries, preventing metabolite washout. The vasoconstriction in the microcirculation results in an upstream response observed as an increase in MAP. The increase in MAP leads to a decrease in heart rate through the baroreceptor reflex. Finally, arteriole constriction hinders flow and velocity in all sized arterioles suggesting a reduction in tissue oxygen perfusion [50]. The severity of these physiological responses can be observed after a simple exogenously administered acellular Hb intravascular dose. During

active intravascular hemolysis, the body's natural levels of scavenger proteins (Hp and Hpx) cannot keep up with the demand to stabilize the Hb dimer or scavenge free heme and its associated iron.

In our studies, a single injection of Hp, at a concentration of 1.5 g/dL, prior to the 0.1 mL Hb dose resulted a greater reduction in vasoconstriction compared to the control and the apoHb infused animals. This was an expected result, since Hp stabilizes the Hb αβ dimer and prevents the release of heme [25, 26]. Arterioles experienced less vasoconstriction, and flow as well as velocity were preserved to a greater degree. The downstream effect is that MAP and HR are maintained closer to baseline. Hp dosing also prevented the loss of FCD in response to heme and Hb, supporting the concept of intravascular compartmentalization which prevents extravasation of Hb through fenestrated capillaries, less NO scavenging, and preservation of microvascular pressures. For animals pretreated with 3 g/dL of apoHb, hemodynamic side-effects were observed in animals after Hb exposure. Most of the adverse hemodynamic responses were still present, including constriction of the arterioles, a decrease in velocity and flow, elevated systemic blood pressure, decreased heart rate and a reduction in functional capillaries. However, apoHb-Hp pre-dosing followed by Hb exposure lead to the most optimal response in terms of preserving systemic and microhemodynamic parameters close to the values found at baseline. ApoHb-Hp prevented the rise in MAP and decrease in HR. Further apoHb-Hp optimized blood vessel diameter, velocity and flow at baseline levels and the number of functional capillaries were maintained at basal levels. These results demonstrate a proof-of-concept that the apoHb-Hp complex prevents Hb-mediated circulatory dysfunction. In addition, apoHb-Hp was effective at maintaining basal circulatory function when dosed prior to heme-albumin exposure.

2.6 CONCLUSION

In conclusion, the present data suggests that the apoHb-Hp complex can effectively exchange apoHb for holoHb *in vitro* and *in vivo*. Further, apoHb situated in the apoHb-Hp complex is an effective heme binding protein complex based on *in vitro* and *in vivo* heme-albumin transfer studies and *in vivo* microcirculatory experiments. These unique properties of the apoHb-Hp complex prevent adverse systemic and microvascular responses to Hb and heme-albumin exposure and introduces a novel therapeutic approach to facilitate simultaneous removal of extracellular Hb and heme.

2.7 ACKNOWLEDGMENTS

We thank Cynthia Walser (University of California San Diego) for surgicalpreparation of animals. We acknowledge Mani Grevenow (Transfusion Ser-vices, Wexner Medical Center, The Ohio State University) for generously donating expired RBC units.

Chapter 2, in full, is accepted material as it appears in "Apohemoglobin-haptoglobin complex attenuates the pathobiology of circulating acellular hemoglobin and heme" by Carlos J. Munoz, Ivan S. Pires, Jin Hyen Baek, Paul W. Buehler, Andre F. Palmer and Pedro Cabrales, in American Journal of Physiology- Heart and Circulatory Physiology Publish Ahead of Print (May 2020). The dissertation author was the primary investigator and author of this paper.

2.8 GRANTS

This work was supported by National Institutes of Health grants R01-HL-126945, R01-EB-021926, and R01-HL-138116, the Ohio State UniversityOffice of Undergraduate Research & Creative Inquiry Summer ResearchFellowship, and the Pelotonia Fellowship Program.

2.9 DISCLAIMERS

Any opinions, findings, and conclusions expressed in this material are thoseof the authors and do not necessarily reflect those of the Pelotonia FellowshipProgram.

2.10 DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

2.11 AUTHOR CONTRIBUTION

SA.F.P. and P.C. conceived and designed research; C.J.M. and I.S.P. per-formed experiments; C.J.M., I.S.P., J.H.B., A.F.P., and P.C. analyzed data;C.J.M., I.S.P., J.H.B., and P.C. interpreted results of experiments; P.W.B. andP.C. prepared figures; C.J.M. and P.W.B. drafted manuscript; C.J.M., I.S.P.,J.H.B., P.W.B., A.F.P., and P.C. edited and revised manuscript; C.J.M., I.S.P.,P.W.B., A.F.P., and P.C. approved final version of manuscript.

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3. APOHEMOGLOBIN-HAPTOGLOBIN COMPLEX ALLEVIATES IRON TOXICITY IN MICE WITH β-THALASSEMIA VIA SCAVENGING OF CELL-FREE HEMOGLOBIN AND HEME

3.1 ABSTRACT

β-thalassemia is a genetic hemoglobin (Hb) disorder that affects millions of people world-wide. It is characterized by ineffective erythropoiesis and anemia. The resultant chronic anemia can require life-long blood transfusion regimens, leading to secondary hemochromatosis. Moreover, the abnormal red blood cells (RBCs) from β-thalassemia patients are prone to hemolytic events that release cell-free Hb and heme causing a series of events that result in oxidative organ and tissue damage. In this study, β-thalassemic mice were treated with a protein scavenger for six weeks, apohemoglobin-haptoglobin (apoHb-Hp), this protein scavenges cell free Hb and heme. We hypothesize that scavenging cell-free Hb and heme will lead to a positive therapeutic event. After the apoHb-hp treatment it was observed to reduce the weight of the liver and spleen and show an improvement in liver function by a drop in ALT, AST, and ALP markers. ApoHb-hp treatment also hints at an improved RBC half-life as the number of reticulocytes decreased, the mean corpuscular volume (MCV) increased, mean corpuscular hemoglobin increase and the RBC distribution width decreased. Furthermore, apoHb-Hp treatment reduced circulating serum iron concentration and transferrin saturation concentration. Based on these outcomes, introducing a scavenger protein can benefit β-thalassemic mice. This study demonstrated that apoHb-Hp treatment may be a viable strategy to mitigate toxicities associated with cell free Hb and heme, a driver of β-thalassemic issues.

Keywords: Apohemoglobin, haptoglobin, iron toxicity, thalassemia, anemia, hemolysis

Key points:

- Apohemoglobin-haptoglobin complex alleviates iron toxicity and regulates RBC turnover in β-thalassemic mice
- Apohemoglobin-haptoglobin complex reduces hepatosplenomegaly in β-thalassemic mice

3.2 INTRODUCTION

Every year, more than 300,000 children are born with a genetic disorder involving hemoglobin (Hb)[1]. Of these births, approximately 70,000 suffer from thalassemia with more than half of these individuals experiencing severe forms of the disease (β-thalassemia major and HbE β-thalassemia)[1]. In thalassemia, deletions or mutation in genes coding for the α- or β-globin chain of Hb result in a decrease or absent production of one of the globin chains [2,3]. Unfortunately, excess α- or β-chains are unstable and cannot properly transport oxygen, reducing oxygen carrying capacity. Furthermore, the abnormal globin chains can lead to intracorpuscular hemolysis, and, in β-thalassemia, excess α-chains quickly precipitate within the RBC precursors (reticulocytes) in the bone marrow, damaging the erythropoietic system.

Considering the complications originating from defective globin chain production in β-thalassemia, chronic anemia is the most common clinical condition due to the defective production and accelerated destruction of RBCs. Hence, β-thalassemic patients often require frequent blood transfusions[4,5]. Moreover, ineffective erythropoiesis, anemia and accelerated hemolysis induce marked erythroid hyperplasia in the bone marrow, as well as extramedullary hematopoiesis and hepatosplenomegaly.[3] Notably, the constant drive for hematopoiesis and blood transfusion can lead to secondary hemochromatosis (i.e., iron overload) which is the primary cause for morbidity and mortality in thalassemic patients.[6] Since humans have no mechanism to eliminate excess iron, the quantity of iron in the body is carefully regulated primarily via control of gastrointestinal iron absorption. In thalassemic patients, chronic hemolytic anemia increases iron absorption leading to iron accumulation in the spleen, liver, endocrine glands, and heart.[7] When left untreated, iron build-up in the heart can progress towards cardiomyopathy. Furthermore, iron build-up in the liver causes portal fibrosis, cirrhosis, and portal hypertension, which can exacerbate splenomegaly.

Unfortunately, the frequent blood transfusion regimens in thalassemic patients further increase iron levels, since one unit of packed RBCs contains approximately 200 mg of iron, while the normal intestinal iron absorption rate is 1-2 mg per day.[6,8] Thus, thalassemic patients receiving blood transfusions frequently require iron chelation therapies to neutralize and eliminate excess free iron.[4,5]

In addition, β-thalassemic patients suffer from the toxicities associated with hemolysis. Hemolytic states are characterized by increased levels of circulating cell-free Hb, free heme and iron which are toxic oxidative species.[9] In thalassemia, these toxicities are exacerbated by the frequent RBC transfusions patients receive, which can be up to 25% hemolyzed after 24 hours in circulation, and secondary hyperkalemia and hypocalcemia from the citrate contained in the transfused blood units.[10–13] Under normal conditions, the damaging effects of these toxic molecules are mitigated by naturally occurring plasma proteins such as haptoglobin (Hp) and hemopexin (Hpx) which scavenge cell-free Hb and heme, respectively.[9] However, these scavenging proteins easily become saturated or depleted during chronic or acute hemolytic states, enabling the cell-free Hb and heme to elicit their toxic effects.

Currently there are no FDA approved therapeutics for hemolysis treatment. In addition to severe anemia, clinically severe thalassemia ($\beta^0/\beta^0, \beta^+/\beta^0$) can result in jaundice and hepatomegaly secondary to indirect bilirubinemia from excessive erythrocyte destruction; with few therapies having demonstrated improvement in indirect bilirubinemia in thalassemia. A logical approach for hemolysis treatment would be to supplement the patient with the depleted Hb and heme scavenger proteins Hp and Hpx.[6,14,15] However, none of these therapies have been clinically translated, likely due to the complex and costly purification techniques required for their isolation. Moreover, no studies have shown the effect of a dual treatment modality (i.e. dually treat the

effects of cell-free Hb and heme) in treating chronic hemolytic conditions such as β-thalassemia, and how such a treatment strategy relates to observed clinical conditions such as hepatosplenomegaly and hemochromatosis. We have recently demonstrated that the apohemoglobin-Hp (apoHb-Hp) complex can serve as a scavenger of both cell-free Hb and heme.[16] Apohemoglobin (apoHb) is the apoprotein obtained via the removal of heme groups from Hb.[17] ApoHb has unoccupied hydrophobic heme-binding pockets with high affinity for heme, allowing it to serve as a heme scavenger.[18,19] Moreover, when bound to Hp in the apoHb-Hp complex, apoHb is more stable and maintains its heme-binding activity. In addition to heme-scavenging, the apoHb-Hp complex can also scavenge cell-free Hb as it can exchange apoHb αβ dimers for holo-Hb αβ dimers. Thus, this single molecular complex is engineered to function as both a Hpx and Hp mimetic, scavenging cell-free Hb and heme during hemolytic states such as β-thalassemia. Notably, we have developed methods for large-scale manufacturing of these proteins enabling potential clinical translation of this novel therapeutic. [17,20]

In this study, β-thalassemic mice were treated with 50 µL of apohemoglobin-haptoglobin (apoHb-Hp, n=8) complex at a dose of 80 mg/kg or PBS (vehicle, n=8) over a six-week period to investigate its efficacy to alleviate cell free Hb and heme toxicities.

3.3 METHODS

Apohemoglobin Preparation. The apoHb used in this study was prepared via tangential flow filtration based on the acidic-ethanol heme-extraction procedure as previously described in the literature.[17] The heme-binding capacity of apoHb preparations was approximately 80%, with less than 1% residual heme present.

Haptoglobin Preparation. Human Hp was purified from human Cohn fraction IV (FIV) purchased from Seraplex (Pasadena, CA) via tangential flow filtration as previously described in the literature.[20] The final protein solution was composed of a mixture of Hp2-1 and Hp2-2 Hp polymers, with an average molecular weight of 400-500 kDa and > 95% purity.

Animal Model and Treatment. Thalassemic mice consisted of C57BL/6 heterozygous for the *Hbb* β-globin gene deletion ($Hbb^{td3th/}$BrjK) (beta-thalassemia, Jackson Laboratory). Animals were treated every other day for six weeks with the apoHb-Hp complex (Hp 22.5 mg/mL, apoHb 7.5 mg/mL, 50 µL, n = 8), or vehicle (PBS, equal volume as study group, n = 8) via tail vein injection at a dose of 80 mg/kg. Animal body weight was monitored at alternate treatment days. Animals were sacrificed after the final dose for analysis.

Hematological Parameters. Blood samples were obtained at baseline and every two weeks by retro-orbital puncture under isoflurane (2% for maintenance, Drägerwerk AG, Lübeck, Germany). Complete blood counts (CBC) were measured on an Hemavet blood analyzer (Drew Scientific, Oxford, CT) and confirmed via flow cytometry on selected samples.

Serum Iron Content and Tf Saturation. Serum iron and unsaturated iron-binding capacity (UIBC) were measured in non-hemolyzed mouse serum using an iron and total iron-binding capacity (TIBC) assay according to the manufacturer's instructions (LabCorp, Burlington, NC). TIBC and transferrin saturation were calculated from the measured serum iron and UIBC.

Determination of Tissue Iron Content. After the experimental end point was reached, half of the mice (n = 4/group) were euthanized and transcardially perfused via the aorta with PBS, and tissue non-heme iron content was determined with a colorimetric method using BPS (4,7-diphenyl-1,10-phenantroline disulfonic acid) as the chromogen. Briefly, 0.2 g of tissues were incubated overnight in a mixture of trichloroacetic (10%) and hydrochloric (4 N) acids, and 100 µl of supernatant reduced with thioglycolic acid (Sigma-Aldrich) and acetic acid-acetate buffer (pH 4.5). The ferrous iron content was determined spectrophotometrically (535 nm) with the addition of BPS and after 1 hr incubation at 37°C. The results were expressed as µg iron/g dry tissue weight.

Histology and Iron Staining. At the end of the study, half of the mice (n = 4/group) were transcardially perfused with PBS followed by perfusion of a fixative solution (4% paraformaldehyde in PBS). The liver and spleen were harvested and continued to be fixed in the same solution (4 hrs at 4°C). Tissues were washed in PBS, and cryoprotected by immersion in sucrose overnight. Tissues were cut and the free-floating sections were stored in cryoprotective solution at −20°C until processed. Iron was detected using Perls' staining for non-heme ferric iron (Fe^{3+}) followed by 3,3 diamino-benzidine (DAB, Sigma-Aldrich) in methanol. Iron staining was developed by incubation of tissues with DAB and hydrogen peroxide, and then transferred onto gelatin-coated slides, rinsed in PBS, counterstained with hematoxylin, dehydrated and mounted. Quantification of iron positive cells was performed with a high-resolution microscope using a digital CCD ORCA-285 camera (Hamamatsu, Hamamatsu City, Japan). Images for Perls'-stained areas and Hoechst stained areas were prepared using Wasabi Imaging Software (Hamamatsu). The ratio of pixels stained for Perls' Prussian Blue in each region compared to the total cellular area of the image was calculated. Ten images were analyzed, by sections, and the results were pooled to

determine the mean and standard deviation (SD). To indicate colocalization of Perls' Prussian Blue and Hoechst in cells, positive cells were counted.

Statistical Analysis. Results are presented as Tukey box plots. Data analysis between groups and time points were analyzed via two-way analysis of variance (ANOVA), with Tukey post-hoc test when appropriate. Before experiments were initiated, sample sizes were calculated based on $\alpha = 0.05$, and power = 0.9 to detect differences between primary end points (serum iron and transferrin saturation). All statistics were performed in GraphPad Prism 7 (GraphPad, San Diego, CA). Results were considered statistically significant if $p < 0.05$.

3.4 RESULTS

This study was completed in sixteen *Hbb*$^{th3/+}$ mice. Eight animals were randomly assigned to each experimental group. The first experimental group was untreated, receiving only PBS (vehicle group). The second group was treated with the apoHb-Hp complex (apoHb-Hp group). All animals were confirmed positive for β-thalassemia via genotyping performed by the vendor and tolerated the experiments without signs of pain or discomfort.

Six-weeks of apoHb-Hp treatment does not show signs of toxicity. The toxicity associated with continuous apoHb-Hp treatment was assessed by measuring the body weight of animals during the experiment. **Figure 3.1 A** shows the body weight of mice at the beginning and end of the study, while **Figure 3.1 B** shows the changes in weight of mice every four days during the study. As shown in **Figure 3.1**, the treated and vehicle control mice showed similar weight fluctuations over the length of the study with no significant differences. Moreover, there was no significant change in body weight over the study period for both groups.

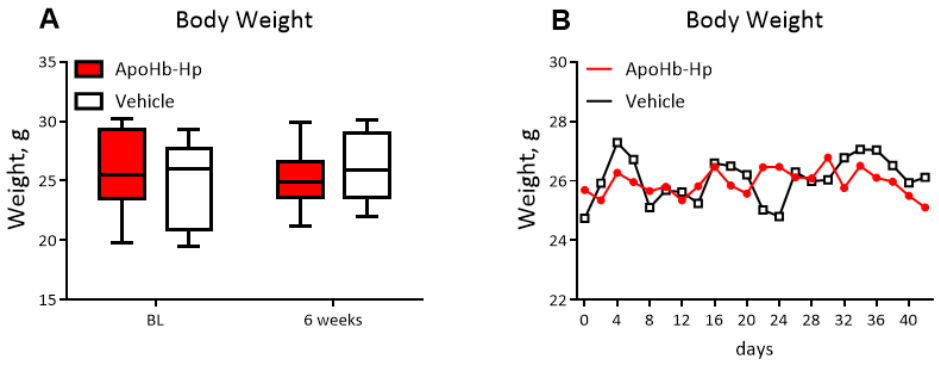

Figure 3.1 Animal body weight - (A) Animal body weight in grams at baseline (BL) and after 6 weeks for ApoHb-Hp treated animals and the vehicle. (B) Animal body weight tracked continuously over 6 weeks fot ApoHb-Hp treated animals and the vehicle.

ApoHb-Hp treatment reduces splenomegaly, hepatomegaly and markers of liver damage induced by β-thalassemia. The enlargement of the spleen (splenomegaly) and liver (hepatomegaly) are common pathological traits of β-thalassemia. **Figure 3.2A** compares the weight of the spleen and the liver at the end of the experiment for the apoHb-Hp complex treated group and vehicle control group. The spleen and liver weight for the apoHb-Hp complex treated group was significantly lower compared to the vehicle control group. **Figure 3.2 B** shows key markers of liver function [alanine amino transferase (ALT), aspartate amino transferase (AST) and alkaline phosphatase (ALP)]. AST and ALP levels were significantly decreased in the apoHb-Hp complex treated group compared to the vehicle control group. ApoHb-Hp treatment also decreased ALT values compared to the control group, but the difference was not significant.

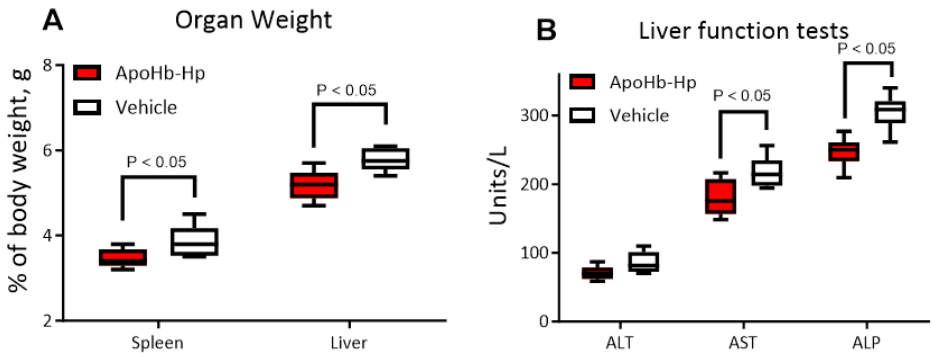

Figure 3.2 Organ weights and liver function - (A) Liver and spleen weight in grams after 6 weeks for ApoHb-Hp treated animals and the vehicle. (B) Liver function test looking at Alanine Amino Transferase (ALT), Aspartate Amino Transferase (AST), and Alkaline Phosphatase (ALP) after 6 weeks for ApoHb-Hp treated animals and the vehicle.

ApoHb-Hp treatment recovers RBC levels in β-thalassemic mice. Figure 3.3 A, **B**, and **C** show the RBC count, total Hb concentration (tHb), and hematocrit (Hct), respectively. **Figure 3.3 D**, **E**, and **F** show these parameters (RBC count, tHb, and Hct) normalized to baseline levels. The absolute RBC count and tHb were increased compared to baseline after six weeks of treatment with apoHb-Hp. Moreover, at the end of treatment, the apoHb-Hp group had higher absolute RBC count compared to the control group, and the control group had decreased tHb levels compared to baseline. Finally, there were no significant differences in the absolute Hct of treated and untreated groups.

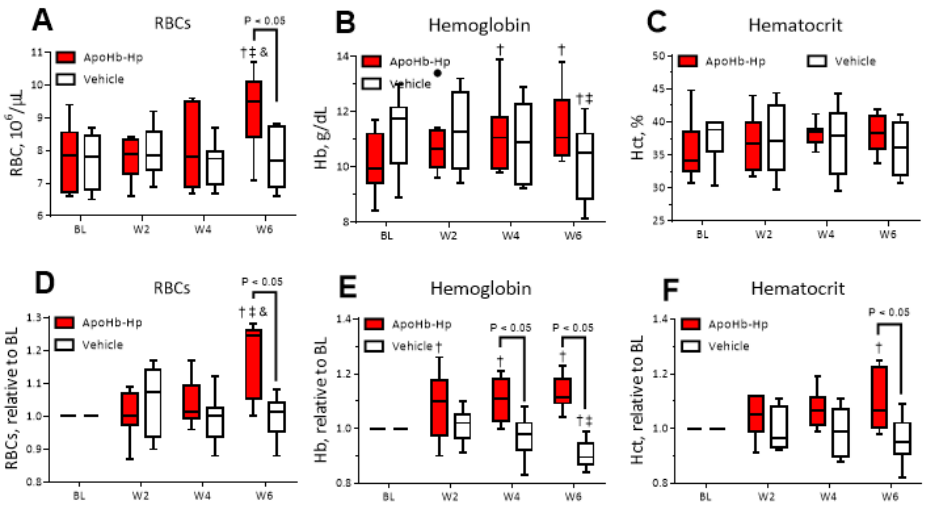

Figure 3.3 Hematological parameters - (A) Red blood cell (RBC) count at baseline (BL), week 2, week 4, and week 6 for ApoHb-Hp treated animals and the vehicle. **(B)** Hemoglobin concentration at baseline (BL), week 2, week 4, and week 6 for ApoHb-Hp treated animals and the vehicle. **(C)** Hematocrit at baseline (BL), week 2, week 4 and week 6 for ApoHb-Hp treated animals and the vehicle. **(D)** RBC count relative to baseline. **(E)** Hemoglobin concentration relative to baseline. **(F)** Hematocrit relative to baseline. †, compared to BL ‡, compared to week 2 ad &, compared to week 4 ($P<0.05$).

When normalized to baseline, the effects of apoHb-Hp treatment were more pronounced as the normalization accounts for the inherent differences between animals. After six weeks of treatment, the relative RBC count, tHb level, and Hct of the apoHb-Hp treated group increased compared to baseline and were significantly higher than the control group. Moreover, the relative tHb level of the apoHb-Hp treated group was significantly higher than baseline and vehicle controls at 4 and 6 weeks of treatment. Finally, the relative tHb levels of the control group decreased over the experimental study, becoming significantly lower than baseline at six weeks. These results indicate that apoHb-Hp treatment improved RBC levels, reducing the severity of anemia in β-thalassemic mice. Although the increase in tHb levels also indicated an improvement over the control, the increase could be an artifact of higher cell-free Hb retention when bound to the apoHb-Hp complex. Moreover, the relative decrease in tHb of the control group is indicative of worsening of anemia in β-thalassemic animals, which was not observed in the apoHb-Hp treated animals.

ApoHb-Hp treatment reduce reticulocytes levels in β-thalassemic mice. The percentage of reticulocytes (Retic) and percentage RBC distribution width (RDW) is shown in **Figure 3.4 A**, and **B**, while **Figure 3.4 C** and **D** shows the Retic and RDW normalized to baseline. Both the absolute and relative change in Retic showed a gradual increase for the control and a gradual decrease for the apoHb-Hp treated group over time. At six weeks, the absolute and relative Retic of the apoHb-Hp treated group was significantly lower than baseline. In hemolytic anemias, the high rate of hemolysis results in higher Retic levels due to the increase in erythropoiesis. Accordingly, apoHb-Hp treatment lowers the drive for erythropoiesis as seen by the increase in tHb and drop in Retic levels. The absolute RDW did not show any significant changes or differences during the six-week treatment. Yet, the relative RDW demonstrated that the untreated group had a significantly higher RDW compared to the apoHb-Hp treated animals at the second and sixth week of treatment. The higher RDW is indicative of the presence of various shaped and deformed RBCs which is consistent with thalassemia.[20]. Thus, apoHb-Hp treatment allowed for a normalization of RBC shape in the circulation.

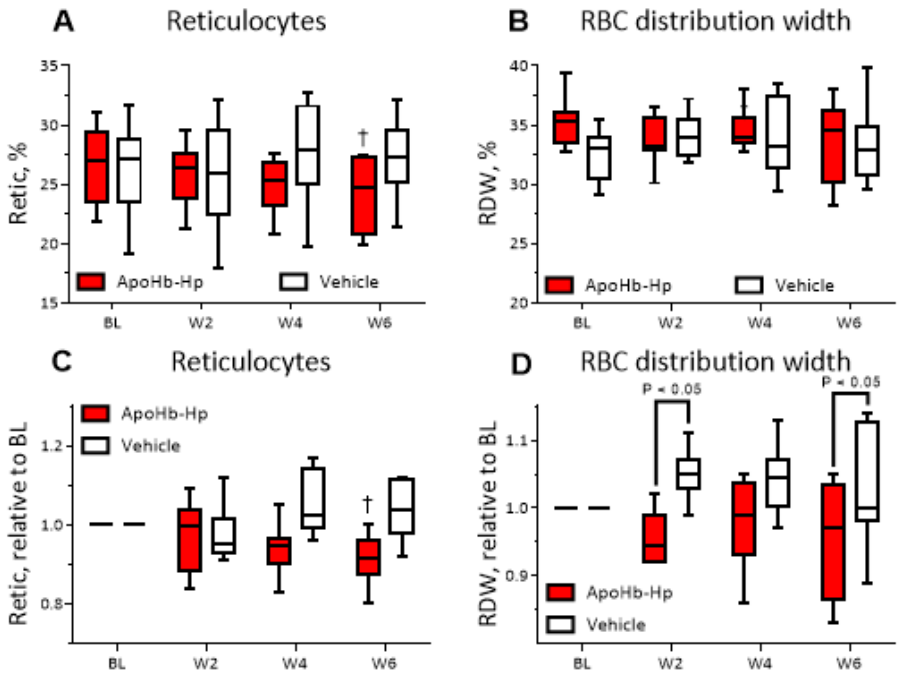

Figure 3.4 Hematological parameters – (A) Reticulocyte percentage at baseline (BL), week 2, week 4, and week 6 for ApoHb-Hp treated animals and the vehicle. **(B)** Red blood cell (RBC) distribution width at baseline (BL), week 2, week 4, and week 6 for ApoHb-Hp treated animals and the vehicle. **(C)** Reticulocyte percentage relative to baseline. **(D)** Red blood cell (RBC) distribution width relative to baseline. †, compared to BL ($P<0.05$).

ApoHb-Hp treatment lowers total iron levels and increases serum iron binding capacity of transferrin. Figure 3.5 A shows the serum iron concentration, while **Figure 3.5 B** shows the transferrin (Tf) saturation, **Figure 3.5 C** shows the serum Tf concentration, and **Figure 3.5 D** shows the total unsaturated Tf concentration. The serum iron concentration and Tf saturation in the apoHb-Hp treated group was significantly lower than the control after three weeks and six weeks of treatment. Moreover, Tf levels were significantly higher in the apoHb-Hp treated group. The results also showed that serum iron significantly increased comparing the third and sixth week of the control group, but not for the apoHb-Hp group. Furthermore, Tf levels significantly decreased when comparing the third and sixth week of treatment for the control group. Tf saturation increased for both the apoHb-Hp and control treated groups when comparing the third

and sixth week. Lastly, unsaturated Tf significantly increased in the apoHb-Hp treated group. These results showed that apoHb-Hp treatment lowered the free iron load in circulation and increased the total iron binding capacity of the serum, indicating a reduction of hemochromatosis associated with thalassemia.

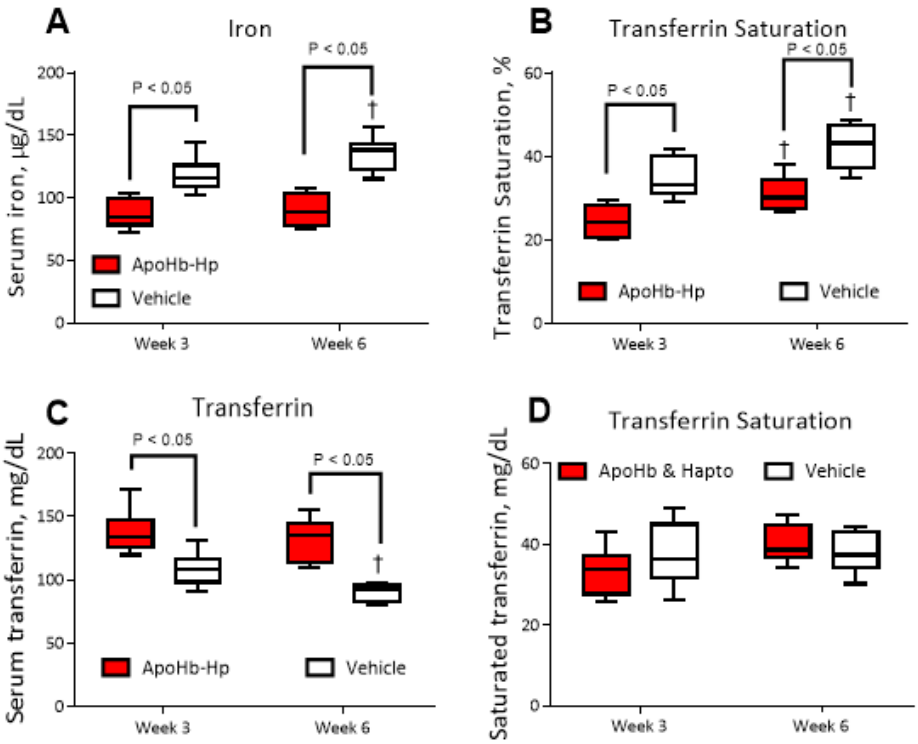

Figure 3.5 Serum Iron and Transferrin – (A) Serum iron concentration at week 3 and week 6 for ApoHb-Hp treated animals and the vehicle. **(B)** Transferrin saturation percentage at week 3 and week 6 for ApoHb-Hp treated animals and the vehicle. **(C)** Serum transferrin concentration at week 3 and week 6 for ApoHb-Hp treated animals and the vehicle. **(D)** Saturated transferrin concentration at week 3 and week 6 for ApoHb-Hp treated animals and the vehicle. †, compared to week 3 ($P<0.05$).

ApoHb-Hp treatment reduces iron accumulation in the liver and spleen. Figure 6 shows iron staining in the liver and spleen after six weeks of treatment. There was a decrease in iron staining in images of both the liver (**Figure 3.6 A and B**) and spleen (**Figure 3.6 D and E**) for apoHb-Hp treated animals. When total iron was quantified (**Figure 3.6 C and F**), this decrease became more apparent, but the difference was not statistically significant. Therefore, this data corroborates the

results shown previously that apoHb-Hp treatment reduces iron accumulation in β-thalassemic mice.

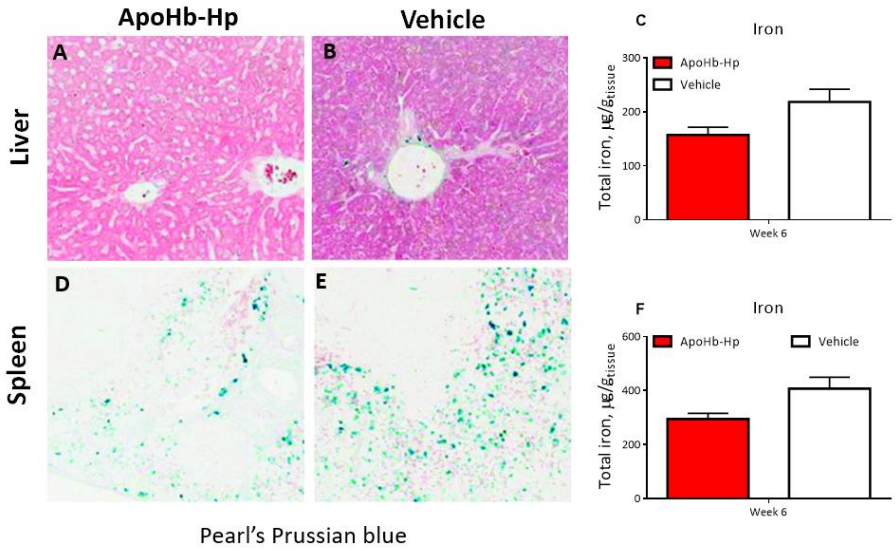

Figure 3.6 Iron Stains In the Liver and Spleen – (A) Liver iron stain following week 6 of ApoHb-Hp treatment. **(B)** Liver iron stain following week 6 of the vehicle animals. **(C)** Total iron per gram of tissue in the liver after week 6 of ApoHb-Hp treated animals and vehicle animals. **(D)** Spleen iron stain following week 6 of ApoHb-Hp treatment. **(E)** Spleen iron stain following week 6 of vehicle animals. **(F)** Total iron per gram of tissue in the spleen after week 6 of ApoHb-Hp treated animals and vehicle animals.

3.5 DISCUSSION

Following preclinical evaluation over a six week regimen of apoHb-hp, it was observed that reducing the amount of cell free hemoglobin and heme in β-thalassemic mice correlates to a drop in spleen and liver weight, drop in AST, ALT and ALP concentrations, a drop in RBC distribution width, a drop reticulocyte count, an increase in both mean corpuscular volume and hemoglobin, a drop in serum iron concentration and a drop in transferrin saturation percentage. The drop in spleen and liver weight, and drop in AST, ALT and ALP concentrations are associated with better organ function. A drop in RBC distribution width, a drop in reticulocyte count and increase in both mean corpuscular volume and hemoglobin are associated with increase in RBC

half-life. A drop in serum iron concentration and a drop in transferrin saturation percentage are associated with a better regulation of iron. An interpretation of these results are that increasing the concentration of scavenger proteins decreases iron accumulation in the spleen and liver from excess RBC turnover, leading to better organ functionality in mice with β-thalassemia. One hypothesis to explain the promising results are that apoHb-hp lowers work hypertrophy and extramedullary hematopoiesis known to induce enlargement of these organs. Briefly, work hypertrophy and extramedullary hematopoiesis results from either an increased activity of splenic macrophages in response to thalassemic erythrocytes[21, 22] or to chronic anemia and tissue hypoxia, where erythroblast production is increased at sites of fetal erythropoiesis, namely the liver and spleen. The latter of these is believed to be the primary mechanism for hepatomegaly in thalassemia[23,24, 25, 26,27]., We hypothesize 57poHb that scavenging cell-free Hb and heme reduces vascular dysfunction and increasing RBC half-life alleviating chronic anemia and tissue hypoxia. . Cell free hb and heme are known to increase intravascular oxidative stress because they trigger a cascade of events: nitric oxide scavenging, a release of inflammatory cytokines, a release of adhesion proteins, and an increase in reactive oxygen species (ROS) [28–31]. Thus, controlling the concentrations of cell free hemoglobin and heme can prevent the negative downstream affects known to cause vascular injury and cell death thus improving anemic states. Considering the former, a decreased activity of splenic macrophages by apohb-hp despite thalassemic erythrocytes, is due in part to the reduction of RBC sequestration in the spleen[33]. Previous studies have demonstrated that vaso-occlusion induced by hemolysis can increase capture of RBCs within the spleen.[34] Thus, by reducing the hemolysis induced side-effects of β-thalassemia, apoHb-Hp treatment may reduce RBC capture, leading to the observed decrease in the size of the organ.

Both hypothesis associated with splenomegaly point to an increase RBC half-life. As previously mentioned by lowering the concentration of free hemoglobin and free heme you may lower the concentration of other proteins, cytokines and enzymes that may cause oxidative damage to the vasculature, but also to the RBCs themselves. Oxidative damage on the RBC membrane induces hemolysis by activating phospholipid scramblase and increasing the exposure of phosphatidylserine (PS) on the membrane of the RBC initiating cell death[37]. Apohb-hp may decrease the concentration of proteins causing oxidative stress. More importantly, regardless of the mechanism the reduction of splenomegaly is vital for clinical management of thalassemic patients. Both extramedullary hematopoiesis and RBC sequestration strain spleen function which can lead to a hyperactive spleen (hypersplenism). In hypersplenism, the spleen destroys RBCs at an accelerated rate, exacerbating anemia and increasing transfusion requirements. Treatment at severe stages of hypersplenism primarily consists of splenectomy, which can make patients more susceptible to infection and sepsis.[35,36]

In addition to splenomegaly, β-thalassemic patients commonly suffer from hepatomegaly (enlarged liver). Similar to splenomegaly, extramedullary hematopoiesis can also occur in the liver, leading to its enlargment.[25] Furthermore, the increased liver iron pool in β-thalassemic patients is associated with cellular toxicity and its enlargement.[23,24] Notably, the hemolytic environment associated with β-thalassemia can lead to excess heme uptake within the liver, resulting in liver damage and congestion due to inflammation and oxidative stress.[26,27] This causes chronic liver injury and fibrosis in β-thalassemic patients.[38] One of the consequences from extensive liver injury and fibrosis is portal hypertension, which can contribute to splenomegaly and increases the risk of hypersplenism.[39,40] Moreover, liver disease can also lead to abnormal RBCs as the liver controls lipid metabolism.[41] Thus, the damaged liver and excess hemolytic

species (i.e. Hb and heme) can lead to abnormal RBCs, further increasing congestive splenomegaly and anemia. With apoHb-Hp treatment, both extramedullary hematopoiesis and hemolytic stress can be reduced, leading to normal liver function. As mentioned previously, one of the causes of splenomegaly in β-thalassemic patients is work hypertrophy mediated by thalassemic erythrocytes. In β-thalassemia, these abnormalities in RBCs are not only derived from the genetic deficiency in β-globin production, but hemolytic species (i.e. Hb, heme, and iron) can also lower the lifespan of RBCs. In β-thalassemia, there is an excess of free alpha chains and a release of intra-erythrocytic heme which triggers a hemoglobin oxidation pathway, thus causing damage to the RBCs[42]. With beta-thalassemia, auto-oxidation reflects the formation of superoxide radicals (0_2) and hydrogen peroxide (H_2O_2) resulting in oxidative stress. This occurs due to exposure to high levels of ROS, resulting in oxidative damage to the RBC membrane, leading to premature macrophage capture.[43–46] Therefore, apoHb-Hp treatment may also contribute in reducing the oxidative stress experienced by the liver and RBCs in thalassemic animals, leading to the observed clinical benefits from apoHb-Hp treatment.

Furthermore, serum iron studies confirmed that treatment with apoHb-Hp resulted in decreased erythrocyte turnover, as evidenced by decreased serum iron, decreased transferrin saturation, and increased transferrin. These data support the hypothesis that treatment with apoHb-Hp independently reduces splenic extravascular hemolysis thereby reducing anemia. In cases of chronic anemia, there is a physiologic increase in iron absorption from decreased hepcidin inhibition of ferroportin activity. In the context of thalassemia, this is clinically problematic in the case of blood transfusion, as repeated transfusions of packed RBCs provide an external source of iron which result in iron overload from decreased hepcidin activity[43,47]. Our results consequently suggest that apoHb-Hp may have increased hepcidin activity relative to vehicle treatment, though

we did not measure serum hepcidin. Future directions would be to assess hepcidin activity with treatment with apoHb-Hp treatment. Based on the results shown in this study and the described mechanisms of β-thalassemia induced hepatosplenomegaly, a mechanism of action for apoHb-Hp treatment was proposed and is illustrated in **Figure 3.7**.

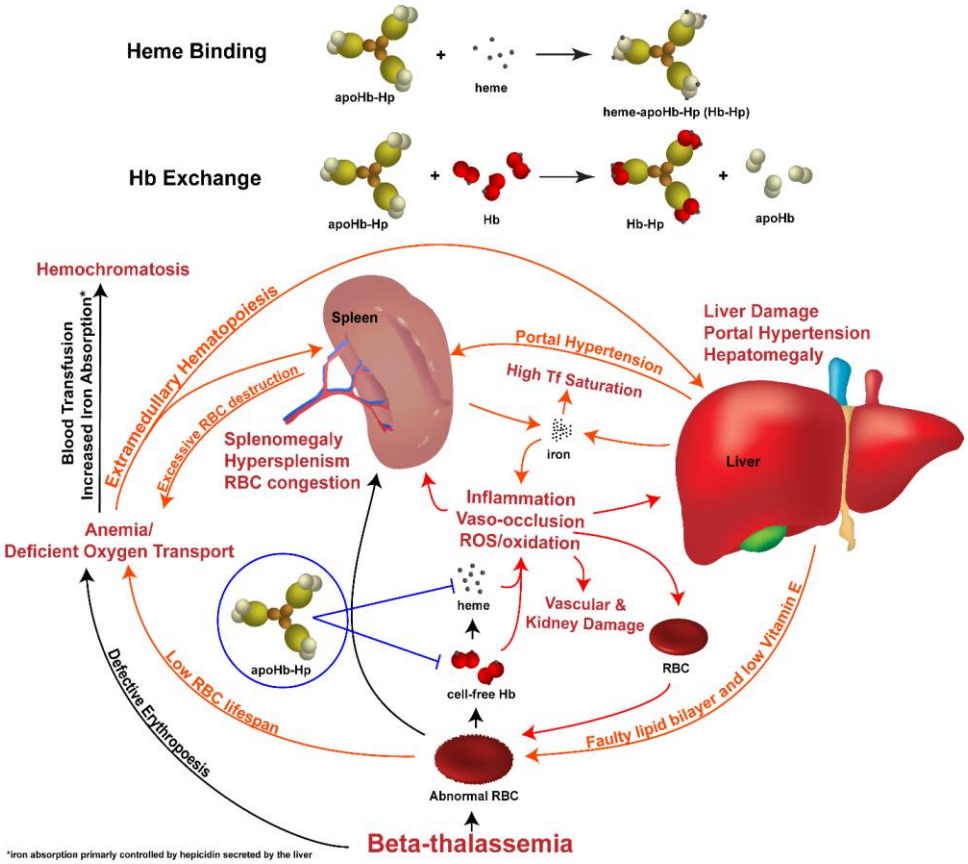

Figure 3.7 Mechanism of action in apoHb-Hp treatment of β-thalassemia - ApoHb-Hp scavenges cell-free Hb and heme, which prevents the cascade of effects that lead to more severe spleen and liver damage and associated pathophysiology. Red lines indicate the effects of cell-free Hb and heme directly reduced upon apoHb-Hp treatment. Orange lines indicate the secondary effects of apoHb-Hp treatment.

Consistent with the data presented here and the proposed mechanism of action for apoHb-Hp, previous studies have demonstrated that Hp and Hpx double knock-out mice suffer from splenomegaly and severe liver inflammation and fibrosis when exposed to hemolytic stress.[48] In these prior studies, splenomegaly was primarily caused by the accumulation of RBCs, which was

attributed to free heme, leading to vascular alterations that caused adhesion of RBCs to the endothelium.[48] Additionally, Hpx treatment has been shown to reduce liver damage and endothelial function in β-thalassemia and sickle-cell disease.[49] However, Hpx treatment alone did not improve RBC levels in β-thalassemic mice, unlike the apoHb-hp treatment implemented in our study. Moreover, unlike the results shown in our study, one month of Hpx treatment alone increased iron levels in the liver in β-thalassemic mice. While this increase in liver weight would be favorable since it suggests that the excess-heme was directed to cells specialized in the detoxification of heme, the study demonstrated that Hpx treatment alone could not prevent the morbidities of β-thalassemia.

Of all our observations, the observation that treatment with apoHb-Hp improved total RBC count is the most promising for its potential as a therapeutic. These results suggest that apoHb-Hp may offer an alternative to splenectomy, which is known to result in an increased incidence of bacterial sepsis from decreased IgM production[35,36]. Our study showed that only after prolonged treatment, apoHb-Hp increased Hb, reduced reticulocyte count, and RDW. Briefly, anemia results in an artificially increased RDW and reticulocyte count due to the relative decrease in RBC concentration. More importantly, the observed reduction in reticulocyte count indicates a suppression of erythropoiesis suggesting that treatment with apoHb-Hp improved systemic oxygenation and reduced extravascular hemolysis due to increased circulation time[50]. The murine CD163 does not recognize human Hp, so it is unlikely that the apoHb-Hp complex helps with erythropoiesis by recycling heme for Hb synthesis, thus the increase Hb and Hct has to be due to increase circulation time. In summary, our results demonstrate that treatment of thalassemia with apoHb-Hp improves anemia and reduces RBC turnover without the need for external intervention (e.g. transfusion).

3.6 CONCLUSION

In summary, we have presented preclinical evaluation of apoHb-Hp for treatment of beta-thalassemia major in a mouse animal model. Our study demonstrates that treatment with apoHb-Hp over a 6-week period improved anemia and normalized erythrocyte turnover in thalassemia. Furthermore, we observed that absolute RBC count increased despite the lack of change in Hb and Hct, suggesting that apoHb-Hp treatment reversed extravascular microcytic anemia in thalassemia. These results demonstrate that apoHb-Hp has the potential to treat thalassemia without the need of serial transfusions or splenectomy, the current standard of care. Future directions would be to mechanistically evaluate the physiology of apoHb-Hp treatment, particularly with regard to suppression of splenic macrophage mediated extravascular hemolysis and the role of hepcidin. Additionally, future studies should investigate whether apoHb-Hp has the potential to mediate transfusion induced iron overload in this same population.

3.7 ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health (NIH) grants T32HL105373, R56HL123015, R01HL126945, R01EB021926, and R01HL138116, the Ohio State University Office of Undergraduate Research & Creative Inquiry Advanced Summer Research Fellowship, and the Pelotonia Fellowship Program. Any opinions, findings, and conclusions expressed in this material are those of the author(s) and do not necessarily reflect those of the Pelotonia Fellowship Program.

Chapter 3, in full, is accepted material as it appears in “Apohemoglobin-haptoglobin complex alleviates iron toxicity in mice with β-thalassemia via scavenging of cell-free hemoglobin and

heme.” by Carlos J. Munoz, Ivan S. Pires, Vinay Jani, Srila Gopal, Andre F. Palmer, and Pedro Cabrales, in Biomedicine & Pharmacotherapy Publish Ahead of Print (December 2022). The dissertation author was the primary investigator and author of this paper.

3.8 AUTHOR CONTRIBUTIONS

C.J.M. and I.S.P. wrote the main manuscript text and prepared the figures; C.J.M. and I.S.P. performed the experiments and analyzed the data; I.S.P., C.J.M., A.F.P., and P.C. reviewed and revised the manuscript; and all authors contributed to the design and implementation of the experiment.

3.9 CONFLICT OF INTEREST DISCLOSURE

ISP and AFP are inventors on a pending patent application outlining methods related to the production of proteins such as apohemoglobin and haptoglobin (PCT/US2020/016267). Furthermore, ISP, AFP and PC are inventors on a pending patent application outlining biomedical applications of the apohemoglobin-haptoglobin complex (PCT/US2020/033836).

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4. Apohemoglobin-Haptoglobin Complex Prevents Microvascular Dysfunction in Sickle Cell Mice: Potential Therapeutic Approach for Vaso-Occlusive Crisis

4.1 ABSTRACT

Sickle cell disease (SCD) is associated with episodes of vaso-occlusive crisis (VOC) caused by the effects of hemoglobin S (HbSS) polymerization in red blood cells (RBCs). In the acute setting, treatment for VOC is limited to supportive care such as hydration and aggressive pain management as no specific therapies can alter the course of acute VOC. The apohemoglobin-haptoglobin (apoHb-Hp) complex is a two-protein formulation that is capable of scavenging and neutralizing the toxic effects of cell-free hemoglobin (Hb) and heme released from the hemolysis of HbSS RBCs. The primary objective of this study was to evaluate the microcirculatory effects of apoHb-Hp during hypoxia-induced VOC in sickle cell transgenic mice (HbSS-Towne's mice), compared to mice expressing normal human Hb (HbAA-Towne's mice). Intravital microscopy on the dorsal window chamber instrumented to the animal was used to observe microcirculatory effects of apoHb-Hp, haptoglobin (Hp), and vehicle control (saline). ApoHb-hp (59 mg/mL) and Hp (86 mg/mL) were injected intravenously to yield equimolar total heme and Hb binding capacity between treatments prior to hypoxia exposure. ApoHb-Hp treatment significantly improved microcirculatory hemodynamics by preventing hypoxia-induced reduction of blood flow in arterioles and venules and their vasoconstriction compared to vehicle control and Hp treatment. Furthermore, the functional capillary density (FCD) was kept at homeostatic conditions in apoHb-Hp treated mice compared to control and Hp treated groups. Also, the use of apohb-hp improved cardiac function (cardiac output and stroke volume). Lastly, the number of lung macrophages, neutrophils and lymphocytes decreased favorably (not significantly) in mice treated with apoHb-Hp compared to HbSS vehicle mice and HbSS mice treated with Hp. These results confirm the potential therapeutic activity of the apoHb-Hp complex to reduce the microvascular burden from

VOC in SCD and highlight the benefit of a dual heme and Hb scavenger therapy relative to single treatments.

4.2 INTRODUCTION

Sickle cell disease (SCD) is a group of inherited blood disorders where genetic mutations of hemoglobin (Hb) can lead to abnormally shaped ('sickle') red blood cells (RBCs) due to Hb polymerization upon deoxygenation. This disease manifests as acute episodes of severe pain also referred to as vaso-occlusive events (VOC). Each year, about 60% of patients with SCD will experience an episode of severe pain in which they are left with less than satisfactory treatment.[1] Treatment of a VOC involves supportive care, including fluids, identification and management of possible triggers, pain control and blood transfusions.[2] RBC transfusions are meant to decrease the HbS concentration in hopes of potentially modifying the course of the disease. However, RBC transfusions during VOC can be complicated by alloimmunization, decreasing the pool of viable blood donors for transfusion and increasing risks of complications.[3,45,6] Furthermore, over the patient's lifespan, frequent transfusions can result in iron overload and organ dysfunction, hence current recommendations suggest not to transfuse during an acute VOC unless there is an organ or life threatening indication.[2] Another common treatment for patients with severe pain is to prescribe them opiates parenterally until pain is under control and then tapered to oral analgesic therapy.[7] However, despite advances in pain management, and in the management of SCD, physicians are often reluctant to give adequate dosages of narcotic analgesics because of concerns of addiction, tolerance, and possible side effects.

While there are several therapies aimed to decrease the rate of VOCs and modify the course of the disease in SCD, treatment options for acute VOC are limited. Currently, the FDA has approved long term prophylactic treatments for SCD (hydroxyurea, L-glutamine, Voxelotor, Crizanlizumab)[7–10] which work towards reducing the number of VOCs a patient may experience, however, there is no current drug in the market that focuses on alleviating a VOC episode while it

occurs. VOCs are commonly connected with microcirculatory dysfunction.[11–13] A hallmark sign of VOC is vasculopathy; this pathophysiology involves a variety of events such as NO scavenging, oxidative damage, inflammation, platelet adhesion and several others.[14] Some of the hypotheses for why these occur revolve around the chronic hemolysis associated with SCD.[15,16] As a result of hemolysis, there is a release of arginase and cell-free Hb.[17,18] Arginase consumes l-arginine reducing the substrate availability for NO synthase (NOS), thus diminishing the concentration of available NO, a potent vasodilator, and cell-free Hb reacts with NO, thus scavenging the gaseous monoxide. Furthermore, cell-free Hb is prone to oxidation into methemoglobin which readily releases heme. Heme is associated the expression of adhesive proteins such as iCAM, vCAM, p-selectin and e-selectin, inflammatory cytokines such as IL-1β, IL-6, TNF-α, heme also induces platelet activation as well as the release of other chemokines such as endothelin-1 (ET-1).[12,15,19–21] The cascade of events initiated by the large amounts of hemolysis all lead to microcirculatory dysfunction.

Based on the known contributions of cell-free Hb and heme to VOC, prevention or treatment of Hb and heme toxicity should be a point of consideration to control and end a VOC episode. Hence, there are ongoing clinical trials aiming to use of hemopexin (Hpx) to scavenge free heme during VOC.[22] The use of hp to scavenge hemoglobin is vital to prevent and control microcirculatory dysfunction and oxidative damage. However, since Hpx only scavenges heme putting this approach in question. Purely scavenging heme is troublesome since the hemoglobin is free to dimerize in circulation resulting in the concentration of heme to increase 4x. Ultimately, the use of both, a heme and hemoglobin scavenger, is necessary to prevent the VOC in SCD mice as past literature has deduced.[23,24] An alternative to Hpx is apoHb (an apoprotein used to scavenge free heme[25–27]) and combining it with hp (a protein used to scavenge cell-free Hb) as an intriguing

therapeutic since it can rapidly scavenge both cell free Hb and free heme. Previously, this therapeutic has been shown to prevent the microvasculature effects of these hemolytic byproducts in acute settings.[27]

Our work introduces apHb-Hp as a novel therapeutic strategy for SCD patients, that leverages natural scavenger proteins to alleviate the hemolytic toxicities and resultant VOCs, by proactively scavenging cell-free Hb and heme from the circulation.[27] Apohb is a more favorable heme scavenger then the native hemopexin as it can collect 4x the concentration of heme and can be easily purified and manufactured at scale with favorable price points compared to the purification of hemopexin.[28] To determine its feasibility we studied this complex in a mouse model of SCD (HbSS Townes) mice , specifically in the context of ischemia reperfusion.

We evaluated the role of this complex by studying four groups of mice: 1) HbSS Townes /SCD mice treated with the scavenger protein complex (apoHb-Hp); 2) HbSS Townes/ SCD mice treated with Hemopexin Hp 3) HbSS Townes/ SCD vehicle control; and 4) HbAA Townes/ Control mice. Each group contained 11 mice and were challenged by inducing hypoxia via environmental manipulation over 60

4.3 **METHODS**

This study was completed in forty-four mice. Thirty-three sickle cell mice were randomly assigned to each experimental group, while the remaining eleven mice did not experience the sickle cell trait and were used as a control group. The first experimental group was the control group that did not have the sickle cell trait, these mice followed the experimental protocol through its entirety. The second experimental group was the vehicle, these mice did have the sickle cell trait, and were subjected to the entire experimental protocol. The third experimental group was mice that were treated with Hp, a Hb scavenger protein. These mice did have the sickle cell trait and were

subjected to the entire experimental protocol. The fourth and final experimental group was mice that were treated with apoHb-Hp complex, a Hb and heme scavenger protein complex, these mice did have the sickle cell trait and were subjected to the entire experimental protocol. All animals were confirmed positive for HbS or HbA via genotyping performed by the vendor and tolerated the experiments without signs of pain or discomfort.

4.4 RESULTS

The following results are written to either express the quantified measurement as an average of all mice with the SS-gene (HbSS), the individual groups with a given treatment type (HbSS no treatment, HbSS treated with Hp, or HbSS treated with Apohb-Hp), or the mice without the sickle cell gene (HbAA). The fractional oxygen amount at baseline is 21%, in hypoxia it is 7% and in normoxia it is 21%.

ApoHb-Hp or Hp treatment does not alter mean arterial pressure (MAP), heart rate (HR), or hematological parameters during hypoxia challenge in Hb SS-mice.

At baseline, the MAP (**Figure 4.1 A**) and HR (**Figure 4.1 B**) of HbSS mice (P=0.002) were both significantly lower than HbAA mice, consistent with observations reported in the literature.[29,30] HbSS mice recorded 76.60 ± 1.22 mmHg and 431.42 ± 8.68 bpm and HbAA mice recoded 91.24 ± 5.79 mmHg and 505 ± 31.55 at baseline.

Under hypoxic conditions the MAP of HbSS mice and HbAA mice dropped significantly (P<0.0001) compared to baseline. When the mice's fractional oxygen returned to normoxia, MAP in all the HbSS mice was significantly lower than HbAA mice. Compared to HbAA mice (83.87 ± 7.97 mmHg), HbSS mice with no treatment (77.54 ± 11.74 mmHg, P=0.0206), HbSS mice with Hp treatment (74.91 ± 9.85 mmHg, P=0.0024), and HbSS with Apohb-Hp treatment (77.79 ± 10.30

mmHg, P=0.0248) were all significantly lower. These results indicate that MAP is regulated by other factors different from hemoglobin and heme scavenging.

Furthermore, the HR under hypoxic conditions showed significantly lower values when comparing to HbAA mice (487.51 ± 27.37 bpm) to HbSS mice without treatment (430.96 ± 34.73 bpm, P=0.0026) and HbSS mice treated with Apohb-Hp (429.67± 36.40 bpm, P= 0.0019), but HbSS mice treated with Hp had no significant drop. Furthermore, when comparing HbAA mice in normoxia no group showed any significant difference across groups on the values for HRs. Apohb-hp treatment did not have any impact on MAP or HR in HbSS mice.

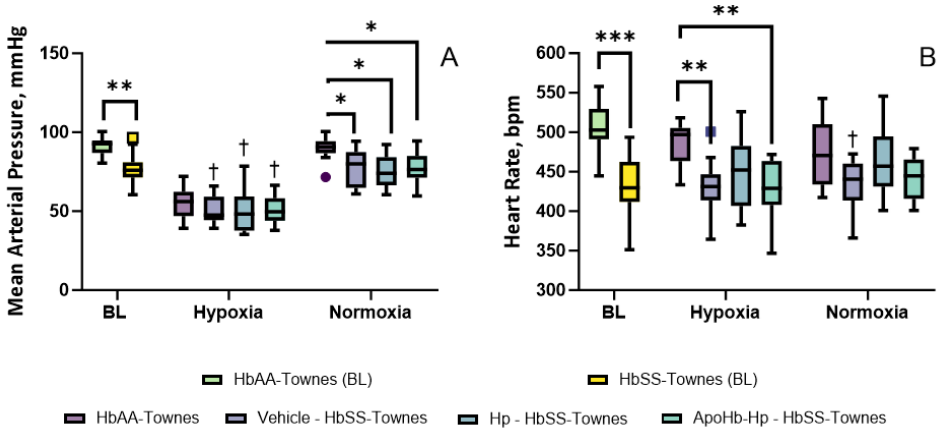

Figure 4.1 Mean Arterial Pressure and Heart Rate in Sickle Cell Mice. A) Mean arterial pressure and B) Heart rate, are shown for HbAA-Townes mice, HbSS-Townes mice (vehicle), HbSS-Townes mice (treated with Hp) and HbSS-Townes mice (treated with apoHb-Hp). All four experimental groups started at a fractional inspired oxygen (FiO_2) concentration of 21%, they were then treated, following an hour of rest, the mice were challenged with hypoxia-reoxygenation (7% O_2/ 93% N_2 for 1 hour followed by room air for 1 hour). Each group contained 11 mice. † vs HbSS-Townes

Table 4.1 portrays hematological properties associated with each experimental group. At baseline, HbSS mice had lower Hb (p<0.0001), hematocrit (Hct) (p<0.0001), and elevated cLac (P<0.0001) compared to HbAA mice, indicating anemia and decreased tissue perfusion, consistent with previously reported data [31,32] During hypoxia, HbSS experienced a decrease in Hb (p<0.0001) by 7% and in Hct (p<0.0032) by 5%, and to a lesser extent in HbAA (Hb 4%, P=0.004 Hct by

2%, P=0.618) mice. An increase in cLac by 150% was seen in HbSS mice and by 212% in HbAA mice. Apohb-hp treatment did not have any impact on Hb, Hct or cLac in the HbSS mice.

Table 4.1 Total hemoglobin concentration, hematocrit, and lactate concentration are shown for HbAA-Townes mice, HbSS-Townes mice (vehicle), HbSS-Townes mice (treated with Hp) and HbSS-Townes mice (treated with apoHb-Hp). All four experimental groups started at a fractional inspired oxygen (FiO_2) concentration of 21%, they were then treated, following an hour of rest, the mice were challenged with hypoxia-reoxygenation (7% O_2/ 93% N_2 for 1 hour followed by room air for 1 hour). Each group contained 11 mice. † vs HbAAA-Townes, ‡ vs HbSS-Townes (Hp), ¶ vs HbSSS-Townes (Apohb-Hp)

Hematological Properties					
		HbAA-Townes (Control)	HbSS-Townes (Vehicle)	HbSS-Townes (Hp)	HbSS-Townes (ApoHb-Hp)
tHb (g/dL)	*Baseline*	11.59 ± 1.20	8.55 ± 1.10†	8.03 ± 1.44†	8.09 ± 1.16†
	Hypoxia	11.08 ± 1.25	8.01 ± 1.18†	7.61 ± 1.28†	7.36 ± 1.07†
	Normoxia	10.51 ± 1.10	7.51 ± 1.17†	7.21 ± 1.26†	7.08 ± 1.01†
Hct (%)	*Baseline*	34 ± 3	25 ± 3†	24 ± 5†	23 ± 4†
	Hypoxia	33 ± 4	24 ± 3†	23 ± 4†	21 ± 3†
	Normoxia	32 ± 4	22 ± 4†	21 ± 4†	21 ± 3†
cLac (mmol/L)	*Baseline*	1.41 ± 0.11	2.48 ± 0.32†	2.49 ± 0.38†	2.28 ± 0.23†
	Hypoxia	4.39 ± 0.62	6.54 ± 0.65†‡¶	5.73 ± 0.62†	5.67 ± 0.52†
	Normoxia	2.33 ± 0.19	3.46 ± 0.36†¶	3.37 ± 0.44†‡	2.81 ± 0.32†

Partial Pressure and Saturation of Oxygen Showed a Neutral Response to ApoHb-Hp Treatment

The partial pressure of oxygen and the saturation of oxygen in both arterial and venous ends were recorded, the values are referenced on **Table 4.2** and **Table 4.3**, respectively. At baseline the pO_2 and the SO_2 in the arterial and venous end all have similar values across groups and no group showed any significance between each other at baseline. However, in hypoxic conditions the pO_2 and sO_2 in the arterial and venous end all dropped significantly compared to their respective baseline values. Returning to normoxia the mice arterial and venous pO_2 and sO_2 regardless of the sickle cell trait and treatment returned to baseline conditions.

Table 4.2 Arterial partial pressure of oxygen and venous partial pressure of oxygen is shown for HbAA-Townes mice, HbSS-Townes mice (vehicle), HbSS-Townes mice (treated with Hp) and HbSS-Townes mice (treated with apoHb-Hp). All four experimental groups started at a fractional inspired oxygen (FiO_2) concentration of 21%, they were then treated, following an hour of rest, the mice were challenged with hypoxia-reoxygenation (7% O_2/ 93% N_2 for 1 hour followed by room air for 1 hour). Each group contained 11 mice. † vs HbAAA-Townes, ‡ vs HbSS-Townes (Hp), ¶ vs HbSS-Townes (Apohb-Hp), * vs BL.

Partial Pressure of Oxygen					
		HbAA-Townes (Control)	HbSS-Townes (Vehicle)	HbSS-Townes (Hp)	HbSS-Townes (ApoHb-Hp)
$pO_{2,A}$ (mmHg)	*Baseline*	101.4 ± 16.6	89.9 ± 10.4	91.0 ± 7.9	100.4 ± 16.2
	Hypoxia	41.5 ± 7.0*	46.0 ± 7.1*	46.3 ± 8.5*	41.2 ± 5.4*
	Normoxia	97.2 ± 15.7	94.3 ± 10.5	93.6 ± 8.4	96.9 ± 18.7
$pO_{2,V}$ (mmHg)	*Baseline*	35.4 ± 10.9	31.9 ± 7.1	31.1 ± 3.7	34.9 ± 10.8
	Hypoxia	15.3 ± 3.6*	19.5 ± 4.0*	19.3 ± 3.3*	15.3 ± 3.8*
	Normoxia	35.7 ± 10.3	33.4 ± 5.2	32.4 ± 1.7	35.8 ± 9.3

Table 4.3 Arterial hemoglobin saturation percentage and venous hemoglobin saturation percentage is shown for HbAA-Townes mice, HbSS-Townes mice (vehicle), HbSS-Townes mice (treated with Hp) and HbSS-Townes mice (treated with apoHb-Hp). All four experimental groups started at a fractional inspired oxygen (FiO_2) concentration of 21%, they were then treated, following an hour of rest, the mice were challenged with hypoxia-reoxygenation (7% O_2/ 93% N_2 for 1 hour followed by room air for 1 hour). Each group contained 11 mice. † vs HbAA-Townes, ‡ vs HbSS-Townes (Hp), ¶ vs HbSS-Townes (Apohb-Hp)

Hemoglobin O_2 Saturation					
		HbAA-Townes (Control)	HbSS-Townes (Vehicle)	HbSS-Townes (Hp)	HbSS-Townes (ApoHb-Hp)
$sO_{2,A}$	*Baseline*	0.98 ± 0.01	0.96 ± 0.02†	0.95 ± 0.02†	0.97 ± 0.01
	Hypoxia	0.77 ± 0.08*	0.75 ± 0.08*	0.75 ± 0.08*	0.67 ± 0.11*
	Normoxia	0.98 ± 0.01	0.96 ± 0.02	0.96 ± 0.01	0.96 ± 0.01†
$sO_{2,V}$	*Baseline*	0.63 ± 0.15	0.51 ± 0.13	0.52 ± 0.15	0.56 ± 0.18
	Hypoxia	0.16 ± 0.08*	0.21 ± 0.09*	0.21 ± 0.09*	0.10 ± 0.05*
	Normoxia	0.66 ± 0.13	0.54 ± 0.11	0.57 ± 0.12	0.56 ± 0.15

ApoHb-Hp treatment prevents hypoxia-induced vasoconstriction and reduction in blood flow in HbSS mice to a greater extent than Hp alone.

We studied the microvascular affects associated with hemoglobin toxicities in sickle cell mice, by measuring diameter and flow in arterioles below 40um (**Figure 4.2**), above 40 um (**Figure 4.3**) and venules between 20-60 µm (**Figure 4.4**). In arterioles less than 40 µm when comparing

the diameter and the flow between HbSS mice (32.70 ± 5.25 µm, 1.30 ± 0.63 nL/s) and HbAA mice (37.13 ± 2.58 µm, 1.80 ± 1.44 nL/s) at baseline, the diameter and flow of HbSS mice was significantly lower than HbAA mice (P=0.020, P=0.003). In the case of arterioles > 40 µm the diameter did not vary among groups. However, the flow was significantly lower (p<0.014) in HbSS mice (4.10 ± 1.43 nL/s) than in HbAA mice (7.24 ± 1.84 nL/s) at baseline. Lastly, the venules showed some degree of vasoconstriction and a reduction in flow for HbSS mice (37.67 ± 9.35 µm, 1.87 ± 1.40 nL/s) compared to HbAA mice (44.58 ± 5.07 µm, 2.38 ± 0.87 nL/s) at baseline (P=0.019, P=0.575).

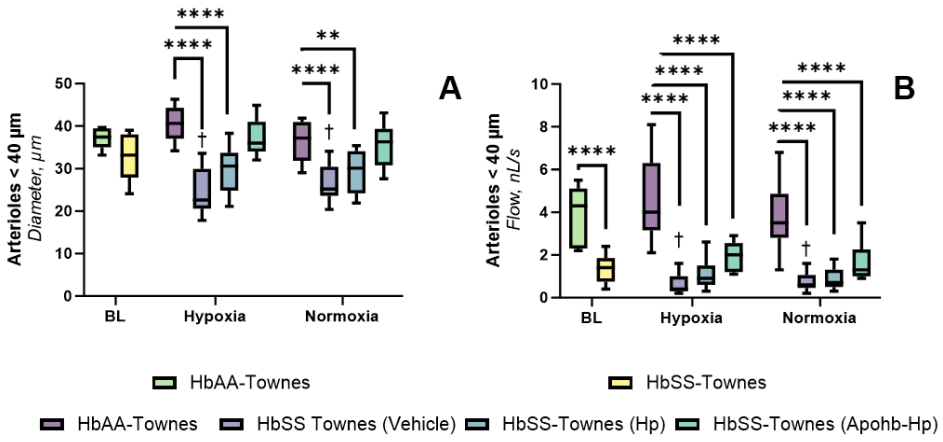

Figure 4.2 Microcirculation of Arterioles < 40 µm: A) Arteriole diameter relative to baseline and B) Arteriole flow relative to baseline is shown for HbAA-Townes mice, HbSS-Townes mice (vehicle), HbSS-Townes mice (treated with Hp) and HbSS-Townes mice (treated with apoHb-Hp). All four experimental groups started at a fractional inspired oxygen (FiO_2) concentration of 21%, they were then treated, following an hour of rest, the mice were challenged with hypoxia-reoxygenation (7% O_2/ 93% N_2 for 1 hour followed by room air for 1 hour). Each group contained 13 vessels. † vs HbAA-Townes

In hypoxic conditions, the arterioles < 40 µm of HbAA mice (40.57 ± 4.26 µm, 4.57 ± 2.08 nL/s) were compared to each treatment group of HbSS mice with HbSS mice treated with apohb-hp having the best response to the hypoxic challenge. HbSS mice with no treatment experienced severe vasoconstriction (25.13 ± 5.47 µm, P<0.0001) and reduced flow (0.65 ± 0.49 nL/s,

P=0.0204), while), HbSS mice treated with hp observed a level vasoconstriction and reduced flow (29.46 ± 5.95 µm, P=0.0029) (1.08 ± 0.92 nL/s, P=0.0307), and HbSS mice treated with apohb-hp had no significant vasoconstriction or flow reduction. The same diameter and flow comparisons were done with arterioles > 40 µm in hypoxia. Similarly, to what was seen with the arterioles < 40 µm, HbSS mice treated with apohb-hp had the best response to the hypoxic challenge with an increase in diameter (52.93 ± 5.18 µm, P=0.2222) and a better flow profile (5.380 ± 2.183 nL/s, P=0.0.2772) when compared to that of HbAA mice (48.76 ± 4.01 µm, 7.21 ± 2.21 nL/s). Finally, observing how the diameter and flow of the venules in HbSS mice compared to HbAA mice (47.69 ± 8.35 µm, 1.59 ± 0.87 nL/s) during hypoxic conditions proved that apohb-hp treatment shows the most promising results showing an improvement in diameter (42.21 ± 6.39 µm, P=0.1484) and flow (1.07 ± 0.50 nL/s, P=0.1452).

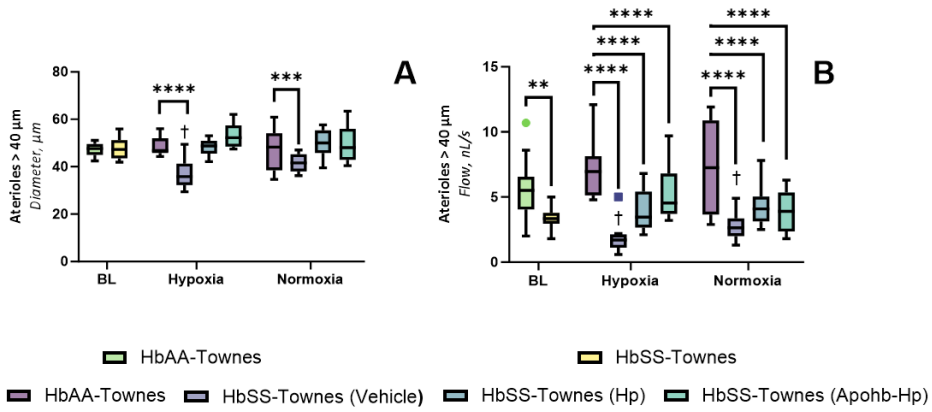

Figure 4.3 Microcirculation of Arterioles > 40 µm: A) Arteriole diameter relative to baseline and B) Arteriole flow relative to baseline is shown for HbAA-Townes mice, HbSS-Townes mice (vehicle), HbSS-Townes mice (treated with Hp) and HbSS-Townes mice (treated with apoHb-Hp). All four experimental groups started at a fractional inspired oxygen (FiO_2) concentration of 21%, they were then treated, following an hour of rest, the mice were challenged with hypoxia-reoxygenation (7% O_2/ 93% N_2 for 1 hour followed by room air for 1 hour). Each group contained 9 vessels. † vs HbSS-Townes

Although the amount of oxygen has been reverted to baseline levels in normoxia, during hypoxia there is still an accumulation of reactive oxygen species that are sensitive to oxygen. The literature has shown that this leads to hemolysis and microvascular injury.[33,34] The comparisons done in hypoxia have also been done in normoxia. The diameters and flow of arterioles < 40 µm were compared to HbAA mice (36.45 ± 5.07 µm, 3.77 ± 1.78 nL/s) versus all groups of HbSS mice. HbSS mice with no treatment had the most significant vasoconstriction (26.80 ± 4.18 µm, P=0.0142) and impaired flow (0.74 ± 0.40 nL/s, P=0.0305), HbSS mice treated with hp showed improvement in diameter changes (29.39 ± 4.96 µm, P=0.0786) and flow (0.92 ± 0.54 nL/s, P=0.0377), and HbSS mice treated with apohb-hp had the largest improvement diameter and flow with no significant difference to HbAA. Furthermore, arterioles >40 µm were considered and HbSS mice were compared against HbAA mice (47.59 ± 8.75 µm, 7.48 ± 3.49 nL/s). HbSS mice without treatment had hindered flow (2.76 ± 1.09 nL/s, P=0.0085), HbSS mice treated with Hp and HbSS treated with apoHb-Hp improved in diameter and flow with now significant difference

to the vehicle. Lastly, the venules between 20 and 60 µm of HbSS mice were assesed against HbAA mice (46.80 ± 11.19 µm, 2.04 ± 1.15 nL/s). HbSS mice with no treatment had the greatest vasoconstriction (32.95 ± 8.53 µm, P<0.001) and reduction in flow (0.91 ± 0.72 nL/s, P=0.0044), HbSS mice treated with Hp and HbSS treated with ApoHb-Hp showed no significant difference in diameter or flow.

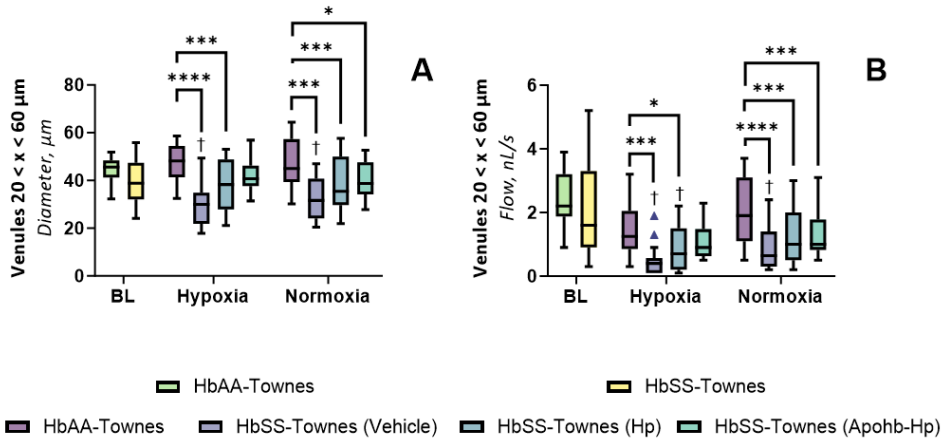

Figure 4.4 Microcirculation of venules 20 < x < 60 µm: A) Venule diameter relative to baseline and B) Venule flow relative to baseline is shown for HbAA-Townes mice, HbSS-Townes mice (vehicle), HbSS-Townes mice (treated with Hp) and HbSS-Townes mice (treated with apoHb-Hp). All four experimental groups started at a fractional inspired oxygen (FiO_2) concentration of 21%, they were then treated, following an hour of rest, the mice were challenged with hypoxia-reoxygenation (7% O_2/ 93% N_2 for 1 hour followed by room air for 1 hour). Each group contained 22 vessels. † vs HbSS-Townes

Lastly, we measured functional capillary density (FCD) which measures the number of capillaries that possess RBC transit as an indicator of capillary health. **Figure 4.5** shows the dynamic characteristics of the FCD relative to baseline in all groups over a period of 72 hours following hypoxia challenge. In Hb AA mice, there was no change in FCD following hypoxia. In Hb SS mice, FCD decreased steadily over the course of the 72-hour period following hypoxia challenge Treatment with hp partially improved the reduction in FCD (40% at 0 hours, 59% at 24 hours, 48% at 48 hours, and 44% at 72. Finally, HbSS treated with ApoHb-Hp improved this

reduction in FCD to a greater extent (36% at 0 hours, 50% at 24 hours, 35% at 48 hours, and 27% at 72 hours).

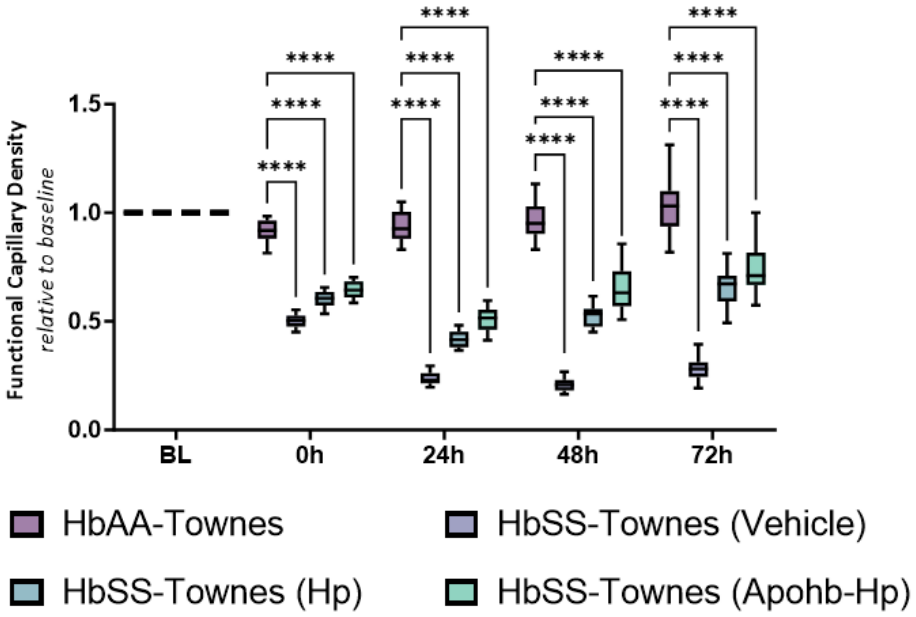

Figure 4.5 Functional Capillary Density - Functional capillary density relative to baseline is shown for HbAA-Townes mice, HbSS-Townes mice (vehicle), HbSS-Townes mice (treated with Hp) and HbSS-Townes mice (treated with apoHb-Hp). All four experimental groups were tracked over 72 hours following the conclusion of the experimental hypoxia-reoxygenation protocol.

ApoHb-Hp treatment may improve already compromised cardiovascular function.

Cardiac function results are displayed in **Figure 4.6**. At baseline, HbSS mice had a higher cardiac output (15.24 ± 1.34 mL/min, P=0.0004), stroke volume (33.12 ± 5.61 µL, 0.0017) and end diastolic volume (3.55 ± 0.80 µL, P=0.0101) when compared to HbAA mice (11.68 ± 1.06 mL/min, 24.18 ± 3.49 µL, 2.37 ± 0.28 µL), consistent with previously reported literature.[30]

During hypoxia, no significant difference was noticeable when comparing HbAA mice groups's cardiac output (12.60 ± 1.33 mL/min) and stroke volume (26.65 ± 4.91 µL) to all HbSS mice groups. Comparing end diastolic volume to HbAA mice in hypoxia (2.65 ± 0.44 µL), all HbSS mice groups were significantly larger with values as follows; HbSS mice without treatment

(5.04 ± 1.09 µL, P<0.0001), HbSS mice treated with Hp (5.03 ± 1.16 µL, P<0.0001), and HbSS treated with apohb-hp (4.97 ± 1.06 µL, P<0.0001).

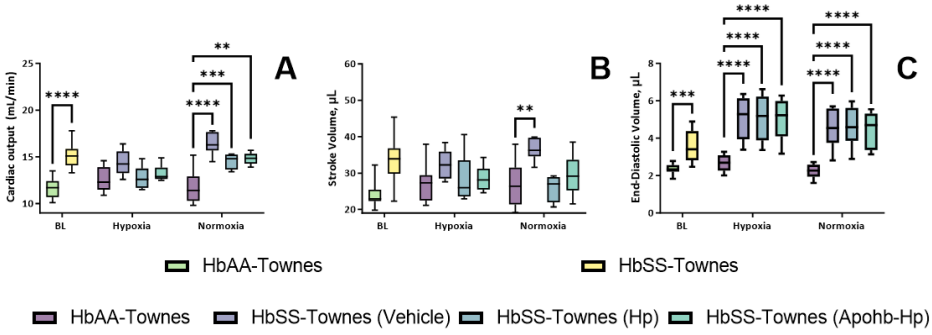

Figure 4.6 Heart Function. A) Cardiac output, and B) Stroke volume and C) End-Diastolic Volume are shown for HbAAA-Townes mice, HbSS-Townes mice (vehicle), HbSS-Townes mice (treated with Hp) and HbSS-Townes mice (treated with apohb-Hp). All four experimental groups started at a fractional inspired oxygen (FiO_2) concentration of 21%, they were then treated, following an hour of rest, the mice were challenged with hypoxia-reoxygenation (7% O_2/ 93% N_2 for 1 hour followed by room air for 1 hour). Each group contained 11 mice.

During normoxia, comparing the cardiac output of HbAA mice (11.81 ± 1.71 mL/min) HbSS mice without treatment saw the largest significant difference (16.43 ± 1.19 mL/min, P<0.0001). Comparing stroke volume to HbAA mice in nomoxia (26.86 ± 6.04 µL) only HbSS mice without treatment experienced a significant change (36.53 ± 3.05 µL, P=0.0.0004) and HbSS mice treated with and HbSS mice treated with apohb-hp saw no significant change. Comparing end diastolic volume to HbAA mice in normoxia (2.22 ± 0.39 µL) all HbSS mice groups were significantly larger; HbSS mice without treatment (4.58 ± 0.96 µL, P<0.0001), HbSS mice treated with Hp (4.64 ± 1.02 µL, P<0.0001), and HbSS treated with apohb-hp (4.48 ± 0.96 µL, P<0.0001). Treatment with Apo hb-hp in Hb SS mice decreased cardiac output and stroke volume at baseline but had no effect on response to hypoxia.

ApoHb-Hp treatment and Hp treatment reduce inflammation in the lungs and markers of pulmonary hypertension.

We characterized pulmonary pressures after the conclusion of the experiment (**Figure 4.7**). Compared to HbAA mice (12.01 ± 1.98 mmHg), the HbSS mice had elevated pulmonary arterial pressure, with HbSS mice without treatment having the largest significant difference (18.76 ± 4.37 mmHg, P<0.0001), HbSS mice treated with Hp was not as large (16.50 ± 3.24 mmHg, P=0.0053) and HbSS mice treated with apohb-hp having the least change (15.44 ± 1.536 mmHg, P=0.0368). Furthermore, the right atrium (RA) pressure was measured to further understand the changes in pulmonary pressure. The RA pressure for HbAA mice (4.26 ± 0.77 mmHg) was compared against the groups, showing a significant difference only in HbSS mice without treatment (5.56 ± 0.81 mmHg, P=0.0234). HbSS mice treated with Hp (4.79 ± 0.66 mmHg, P=0.5742), and HbSS mice treated with apohb-hp (4.67 ± 0.76 mmHg, P=0.7404). Finally, the Fulton ratio was evaluated for HbAA mice (0.068 ±0.010) and was only significantly different when compared against HbSS mice without treatment (0.097 ± 0.024, P=0.0400). The Fulton index (a measurement of right ventricular hypertrophy) was higher in HbSS mice when compared to HbAA mice, indicating right ventricular hypertrophy. The Fulton index did not change with apohb-hp treatment, possibly because these changes are more chronic in nature and difficult to change with an acute intervention.

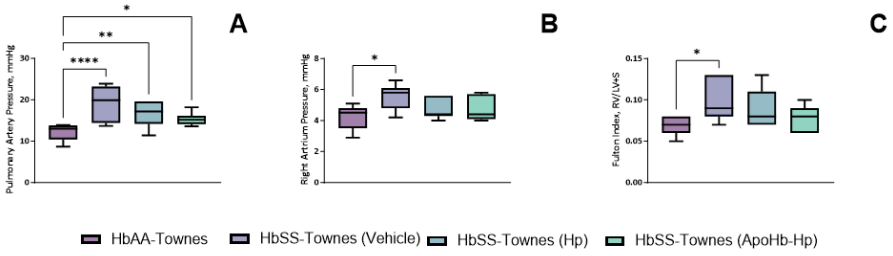

Figure 4.7 Pulmonary Hypertension After Hypoxia and Reoxygenation. A) Pulmonary artery pressure B) Right atrium pressure and C) Fulton index are shown for HbAA-Townes mice, HbSS-Townes mice (vehicle), HbSS-Townes mice (treated with Hp) and HbSS-Townes mice (treated with apoHb-Hp). All four experimental groups started at a fractional inspired oxygen (FiO_2) concentration of 21%, they were then treated, following an hour of rest, the mice were challenged with hypoxia-reoxygenation (7% O_2/ 93% N_2 for 1 hour followed by room air for 1 hour). Each group contained 11 mice.

To study lung immune response, macrophage, neutrophil and lymphocytes were quantified in the pulmonary tissue collected after the experimental protocol was completed. The macrophage count was compared to HbAA mice (11.17 ± 1.95) showing that HbSS mice without treatment had a significant increase in macrophage count (18.17 ± 3.16, P<0.0001) and HbSS mice treated with apohb-hp had the best result (14.58 ± 2.35, P=0.0096). The HbSS mice's neutrophil count was also compared to HbAA mice (3.33 ± 0.49), HbSS mice without treatment saw the largest number of neutrophils (9.17 ± 1.90, P<0.0001) and HbSS mice treated with apohb-hp had the best result (5.25 ± 0.87, P=0.0025). Finally, lymphocytes were quantified for all groups with the HbSS mice being compared to the HbAA mice (14.00 ± 2.22), HbSS mice without treatment experienced the largest rise (22.25 ± 3.91, P<0.0001) and HbSS mice treated with apohb-hp saw the best result (18.25 ± 3.62, P=0.0075).

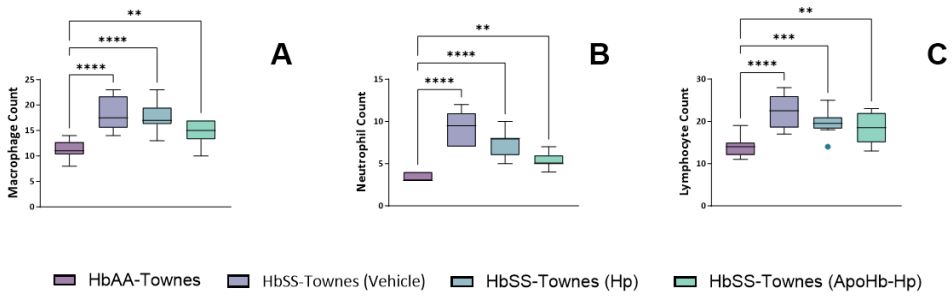

Figure 4.8 Lung Inflammatory Cells After Hypoxia and Reoxygenation - A) Number of macrophages B) Number of neutrophils and C) Number of lymphocytes are shown for HbAA-Townes mice, HbSS-Townes mice (vehicle), HbSS-Townes mice (treated with Hp) and HbSS-Townes mice (treated with apoHb-Hp). All four experimental groups started at a fractional inspired oxygen (FiO_2) concentration of 21%, they were then treated, following an hour of rest, the mice were challenged with hypoxia-reoxygenation (7% O_2/ 93% N_2 for 1 hour followed by room air for 1 hour). Each group contained 11 mice.

4.5 DISCUSSION

The principal finding of this data is that ApoHb-Hp can help with the preservation of microcirculatory health, cardiac function, and pulmonary health during an ischemia reperfusion injury in sickle cell mice.

A key component in microcirculatory health begins with the arterioles. Arterioles are pressurized vessels with greater flow rates then its counterpart the venule. Arterioles are covered in smooth muscles to aid on the dilation and constriction of these vessels. The ability to vasoconstrict and vasodilate is tied to the myogenic response. Being able to regulate the size of the vessel helps control the flow rate, allowing control over the amount of oxygen offloaded to the tissue. It is important to understand that oxygen delivery is reliant on convection not diffusion and the greater the flow the more oxygen that is available to the tissue.[18] By inducing hypoxic conditions, the amount of available oxygen is diminished and the shunting of vessels to organs of less importance is noticed.[35,36] By introducing this novel approach of studying microhemodynamics on the dorsal skin fold, it allows us to observe the immediate response of the microcirculation in non-vital organs under hypoxia. This dorsal skin fold works as a tool to measure events tgar occur during chronic diseases, such as sickle cell disease. Under the disease state, it is assumed that these mice (HbSS) are suffering from inflammation, oxidative damage, high HbS levels, activation of adhesion markers and a plethora of other chronic issues. Therefore, the introduction of hypoxia to the model should only exacerbate the issues.

Interestingly enough, HbSS mice that received no treatment showed the largest amount of vasoconstriction, while HbSS mice that received a Hp or ApoHb-Hp had less vasoconstriction. The hypothesis behind this positive result tales back to Hp alleviating the dimerization of cell free hemoglobin (preventing NO scavenging) that might be present during hypoxia. However, ApoHb-

Hp shows the best outcome during hypoxia as its ability to scavenge hemoglobin and heme, preventing a further activation of reactive oxygen species, adhesion molecules and DAMPs, and immune response associated with heme's interaction with Toll like receptor 4 (TLR-4).[24] Previous findings have shown the concentration of TLR-4 will increase under hypoxia. Therefore, preventing heme from interacting with TLR-4 you can stop further downstream adverse effects.[24]

To continue, the second portion of this model involves the reperfusion of oxygen. However, reperfusing the tissue with oxygen can result in leukocyte recruitment creating tissue dysfunction, causing a substantial increase in highly reactive superoxide, hydroxyl radicals, and uric acid.[36–39] This will further compromise the model as the level of hemolysis increase, an overacting immune system exists, and the potential of vaso-occlusion is high.[14,36] In such events, the arterioles and venules both had a more positive outcome when treated with Hp or ApoHb-Hp. The reasoning is the same during hypoxic challenges. The scavenging of Hb, heme, or both prevents further vascular dysfunction in the HbSS mice.

Another key aspect of microcirculatory health that benefits from the scavenging of Hb, heme, or both is seen in the capillary bed. Capillaries are single cell vessels responsible for metabolite washout. It is commonly understood that the density of functional capillaries is correlated to tissue health.[35,40,41]. The ability of the capillary to remain functional is dependent on enough hydrostatic pressure brought on by the arterioles. As presented in our data, the HbSS mice with no treatment 0, 24, 48 and 72 hours after reperfusion continuously remain at a dangerously low amount relative to baseline. This suggests some level of vaso-occlusion increased or remained the same as time continued. This is based on data of previous studies, where they show how vascular dysfunction persisted in the model. On the other hand, the HbSS mice treated with Hp and ApoHb-Hp showed an improvement trend in the number of functional capillaries through time,

with ApoHb-Hp treatment promoting the best results. The only difference between ApoHb-Hp and Hp treatment is that ApoHb-Hp can scavenge heme in addition to cell free Hb. This added benefit suggest that heme triggers a greater amount of vascular dysfunction than hemoglobin alone.

Cardiac function (cardiac output, stroke volume, and end-diastolic volume) is known to be affected in sickle cell patients resulting in increased cardiac output and cardiomegaly (enlarged heart).[42] Our data aligns with these notions and showed that an Hp or ApoHb-Hp improves the cardiac output regardless if the HbSS mice were under ischemic conditions or if they were reperfused with oxygen. This continues to tease out the substantial difference that heme has on the pathophysiology of SCD. The EDV remained high for all HbSS mice, which could be indicative of subacute/ chronic cardiac remodeling.

As previously mentioned, the oxygen reperfusion creates a plethora of issues, including an exaggerated immune response.[43] The data presented illustrates this point since the pulmonary immune cells count showed a significant increase of macrophages, neutrophils, and lymphocyte in the HbSS mice without treatment. Interestingly the HbSS mice with Hp or ApoHb-Hp treatment diminished the concentration of these immune cells present in the lungs, with ApoHb-Hp having the most important protective effect. To evaluate the benefits of the treatment to avoid pulmonary dysfunction, the pulmonary artery pressure, right atrium pressure and the Fulton index were assessed. Although not significant, the use of ApoHb-Hp does help to some extent the pulmonary hypertension seen in the HbSS mice. Lastly, it can be highlighted that ApoHb-Hp complex's the ability to scavenge both hemoglobin and heme does allude to a positive result, given that the ischemia reperfusion model in SCD mice can promote a high probability of hemolysis leading to several toxicities.

4.6 CONCLUSION

Taken together, this work demonstrates that ApoHb-Hp complex and Hp prevent microvasculature alterations in a SCD mice model challenged with a hypoxia-reoxygenation protocol. However, the ApoHb-Hp complex fared better and more consistently than Hp treatment alone indicating the vital role of scavenging both heme and Hb.

4.7 ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health (NIH) grants T32HL105373, R56HL123015, R01HL126945, R01EB021926, and R01HL138116, the Ohio State University Office of Undergraduate Research & Creative Inquiry Advanced Summer Research Fellowship, and the Pelotonia Fellowship Program. Any opinions, findings, and conclusions expressed in this material are those of the author(s) and do not necessarily reflect those of the Pelotonia Fellowship Program.

Chapter 4, in full, is being prepared for publication as it appears in "Apohemoglobin-Haptoglobin Complex Prevents Microvascular Dysfunction in Sickle Cell Mice: Potential Therapeutic Approach for Vaso-Occlusive Crisis." by Carlos J. Munoz, Daniela Lucas, Ivan S. Pires, Cynthia Muller, Srila Gopal, Andre F. Palmer, and Pedro Cabrales. The dissertation author was the primary investigator and author of this paper.

4.8 AUTHOR CONTRIBUTIONS

C.J.M., D.L., and I.S.P. wrote the main manuscript text and prepared the figures; C.J.M. and I.S.P. performed the experiments and analyzed the data; C.J.M., D.L., I.S.P., S.G., A.F.P., and P.C. reviewed and revised the manuscript; and all authors contributed to the design and implementation of the experiment.

4.9 CONFLICT OF INTEREST DISCLOSURE

ISP and AFP are inventors on a pending patent application outlining methods related to the production of proteins such as apohemoglobin and haptoglobin (PCT/US2020/016267). Furthermore, ISP, AFP and PC are inventors on a pending patent application outlining biomedical applications of the apohemoglobin-haptoglobin complex (PCT/US2020/033836).

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5. Alpha-Alpha Hemoglobin/ Diaspirin-Crosslinked Hemoglobin (DCLHb) Benefits from Treatment using Apohemoglobin Haptoglobin Complex

5.1 ABSTRACT

Alpha-Alpha crosslinked human hemoglobin (ααHb) or diaspirin-crosslinked hemoglobin (DCLHb), was a promising hemoglobin-based oxygen carrier (HBOC). This HBOC resulted from a collaborative effort between the Army Medical Research and Development Command and Baxter Healthcare. The core idea behind its development was hemoglobin's chemical stabilization ('crosslinking') to prolong its intravascular retention and reduce its renal toxicity. DCLHb aimed to provide soldiers in the field with a chance of survival in the event of life-threatening blood loss. However, extensive research revealed several issues associated with the use of DCLHb that contributed to high mortality rates in clinical trials. This study explores whether increasing hemoglobin and heme scavenging can reduce the toxicities associated with DCLHb. The Apohemoglobin-haptoglobin (ApoHb-Hp) complex, engineered as a dual-use protein complex that scavenges hemoglobin and heme, serves as the focus. The experiment involved Golden Syrian hamsters equipped with a window chamber on the dorsal skin fold to visualize the microcirculation and observe deviations. Each group was first infused with lactated ringers or ApoHb-Hp followed by a hypovolemic infusion of 10% of the animal's blood volume of DCLHb. The results indicate that animals treated with ApoHb-Hb exhibited improved microhemodynamics when treated with ApoHb-Hp. Furthermore, the number of functional capillaries in those treated with lactated ringers was significantly less than those treated with ApoHb-Hp. However, regardless of treatment, all groups displayed acute inflammation in multiple organs, hypertension, and decreased heart rate. Although underlying issues persist in DCLHb, utilizing scavenger proteins can limit the effects, although complete prevention remains elusive.

Key Points

1. Understand the usefulness of combining an HBOC solution with a hemoglobin scavenger to prevent microcirculatory effects.
2. Describe the negative effects of DCLHb (aaHb) as a HBOC solution.
3. Create a better understanding of the use of ApoHb-Hp as a scavenger protein.

5.2 INTRODUCTION

Blood transfusions are vital in clinical medicine, serving as the primary treatment for numerous diseases like sickle cell anemia, thalassemia, and trauma. However, challenges such as the quality of stored blood and donor scarcity have fueled research into the creation of blood alternatives[1–3]. One promising avenue has been the development of hemoglobin-based oxygen carriers (HBOCs) to serve as oxygen carriers for tissue reperfusion and delivery[3]. Unfortunately, no research group has successfully produced HBOCs without adverse side effects[2]. One HBOC, alpha-alpha hemoglobin (aaHb), created through collaboration between the US Army and Baxter Pharmaceuticals, showed great promise[4,5]. This product involved crosslinking the alpha subunits of the hemoglobin tetramer to prevent its dimerization into smaller subunits, thereby avoiding extravasation into tissues and potential harm[6–8].

Regrettably, aaHb failed during phase III clinical trials, demonstrating a higher mortality rate than the control group during hemorrhagic shock[9]. The increase in blood pressure (BP) observed with aaHb was attributed to its tight binding to nitric oxide (NO), a known vasorelaxation signal in smooth muscle[5]. aaHb was not the only HBOC to face such challenges; others produced by Biopure, Northfield Laboratories, Sangart, and HemoBioTech encountered similar issues and met a similar fate. These solutions were engineered to have therapeutic effects using hemoglobin that proved highly unstable and unpredictable.

To address the instability and unpredictability of hemoglobin-based solutions, we propose harnessing naturally occurring Hb detoxification mechanisms to mitigate systemic hypertension. The naturally occurring Hb-scavenging protein, haptoglobin (Hp), plays a pivotal role in detoxifying stroma-free Hb in the blood[10]. Previous studies have shown that Hp administration can normalize vascular NO signaling following hemolysis[11,12]. Hp preferentially binds to low

molecular weight (LMW) polymerized human hemoglobin (polyhHb), increasing its molecular size. This increase may reduce tissue extravasation and subsequent NO scavenging[13].

The second component of stroma-free Hb detoxification is hemopexin, which scavenges heme released from stroma-free Hb after Hb auto-oxidation[14,15]. By isolating heme within the hemopexin complex, heme is prevented from catalyzing oxidative reactions with blood and tissue components, thus averting lipid, protein, and nucleic acid oxidation[16,17]. A potential cost-effective alternative to hemopexin is heme-free apohemoglobin (apoHb). This molecule can scavenge free heme due to its specificity for heme and the highly hydrophobic nature of its vacant heme-binding pocket[18,19]. Combining the apoHb with Hp results in a protein complex (apoHb-Hp), that is capable of potentially scavenging both Hb and heme in the plasma[11].

It is hypothesized that coupling aaHb with scavenger proteins associated with capturing free hemoglobin dimmers and free heme would positively affect the performance of the engineered protein. This protein scavenger has been used previously to scavenge free circulating hemoglobin and heme in the circulation and has even shown therapeutic effects in hemolytic anemia, and thalassemia. It is hypothesized that coupling apoHb-Hp with the aaHb will subdue some of the adverse effects associated with the HBOC and promote improved microhemodynamic health and inflammation in vital organs.

In this study, the use of aaHb coupled with apohb-Hp to test its efficacy in the microvasculature was done using golden Syrian hamsters instrumented with a dorsal window chamber, and a catheter in the left common carotid artery by administering a 10% top load of test solution based on their blood volume (estimated as 7% of body weight). The hamsters were given an infusion of apohb-Hp prior to a top load of aaHb solution and observed 20 minutes after injection of the HBOC solution. Microvascular perfusion was characterized by blood vessel

diameter, flow, and functional capillary density. Systemic variables, such as mean arterial pressure (MAP) and heart rate (HR) were also monitored. A histology report was generated to assess toxicity in the spleen, kidney, liver, and heart.

5.3 METHODS

Apohemoglobin Preparation

The apoHb used in this study was prepared via tangential flow filtration based on the acidic-ethanol heme-extraction procedure as previously described.[19] The absence of residual heme was verified by measuring the ratio of absorbance between the Soret peak (λ_{max} = 412-413 nm) and 280 nm to ensure less than 1% residual heme in the product. The heme-binding capacity of apoHb preparations was 80%.[18]

Haptoglobin Preparation

Human Hp was purified from human Cohn fraction IV (FIV) purchased from Seraplex (Pasadena, CA). The final protein solution was composed of a mixture of Hp2-1 and Hp2-2 Hp polymers with an average MW of 400-500 kDa.

Total Protein Assays

To estimate the total protein concentration of the apoHb solution, a Bradford assay was performed (Coomassie Plus Protein assay kit, Pierce Biotechnology, Rockford, IL). Additionally, spectrophotometric analysis of the 280 nm peak for each sample was used to estimate the total protein concentration using the millimolar extinction coefficients of apoHb found in the literature.[20]

In Vitro Hb Exchange and Heme Binding by the ApoHb-Hp Complex

The apoHb-Hp complex was formed by reacting apoHb with Hp, and complex formation was confirmed by analysis of the mixture via size exclusion HPLC (HPLC-SEC). Heme binding

to the apoHb-Hp complex was assessed by mixing the apoHb-Hp complex with heme-albumin prior to HPLC-SEC. Furthermore, apoHb exchange for Hb within the apoHb-Hp complex was determined by reacting the apoHb-Hp complex with Hb. HPLC-SEC was performed using an Acclaim SEC-1000 (4.6 × 300 mm) column (Thermo Fisher Scientific, Waltham, MA) attached to a Dionex UltiMate 3000 system (Thermo Fisher Scientific, Waltham, MA). The mobile phase consisted of 50 mM potassium phosphate, pH 7.4 at a flow rate of 0.35 mL/min controlled by Chromeleon 7 software. Wavelength absorbance detection was set to $\lambda = 280$ nm to detect protein, $\lambda = 405$ nm to detect heme (bound to apoHb or albumin), and $\lambda = 413$ nm to detect Hb (intermediate wavelength between residual heme in apoHb and prevalent holoHb form used in the study, oxyHb).

In Vivo Hb Exchange and Heme Binding by the ApoHb-Hp Complex

In vivo heme transfer (heme-albumin) and Hb (acellular Hb) exchange with apoHb-Hp was documented previously in another study. [11]

Animal Preparation

Investigations were performed in 55 – 65 g male Golden Syrian Hamsters (Charles River Laboratories, Boston, MA) fitted with a dorsal skinfold window chamber. Animal handling and care followed the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the local animal care committee. The hamster window chamber model is widely used for microvascular studies in the unanesthetized state, and the complete surgical technique is described in detail elsewhere. [21,22] The experimental animal was allowed at least 2 days for recovery before the preparation was assessed under the microscope for any signs of edema, bleeding, or unusual neovascularization. Animals were anesthetized again, and arterial and venous catheters filled with a heparinized saline solution (30 IU/ml) were implanted. Catheters

were tunneled under the skin, exteriorized at the dorsal side of the neck, and securely attached to the window frame. The microvasculature was examined 3 to 4 days after the initial surgery and only animals with window chambers whose tissue did not present regions of low perfusion, inflammation, and edema were entered into the study.[23] The animal preparation is illustrated in **Figure 5.1**.

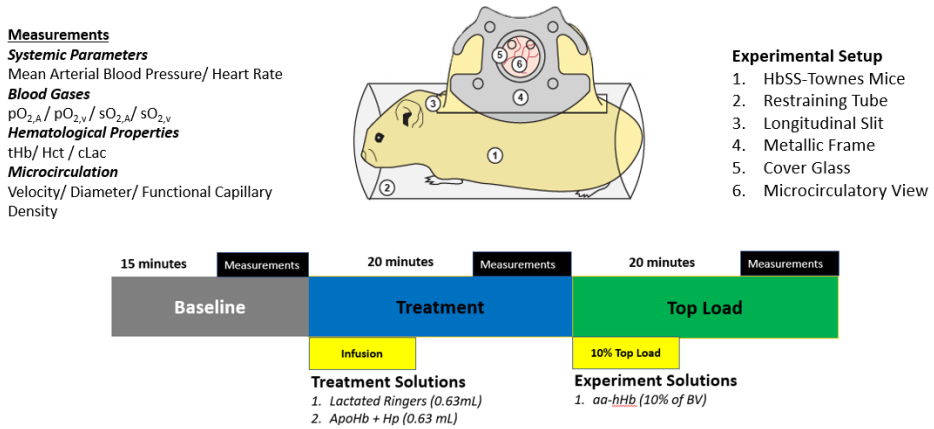

Figure 5.1 Experimental Timeline. The animals were subjected to a three-phase experimental process, baseline, treatment and experiment. The baseline values of microcirculation and laboratory parameters were collected, the treatment phase saw an IA injection of either lactated ringers or ApoHb-Hp complex after 20 minutes the same information as baseline was collected in treatment, and finally the experimental group has an IA injection of ααHb following 20 minutes after the injection the same values from baseline were collected.

Inclusion Criteria

Animals were suitable for the experiments if 1) systemic variables were within normal range, namely, heart rate (HR) > 340 beat/min, mean arterial blood pressure (MAP) > 80 mmHg, systemic hematocrit (Hct) > 45%, and arterial oxygen partial pressure (PaO2) > 50 mmHg; and 2) microscopic examination of the tissue in the chamber observed under a 650 X magnification did not reveal signs of edema or bleeding. Hamsters are a fossorial species with a lower arterial PO_2 than other rodents due to their adaptation to the subterranean environment. However,

microvascular PO_2 distribution in the chamber window model is the same as in other rodents such as mice.[24]

Hypervolemic Infusion (Top-Load) Protocol

Experimental groups were labeled based on the infusion of the treatment solution, this included the control (lactated ringers) or ApoHb-Hp an amount of 0.6mL was infused. The concentration of the apoHb-Hp solution was 62.01 mg/mL this was an average dose of 550 mg/kg per animal of apohb-hp. Followed by aaHb at a concentration of 50 mg/mL at an average dose of 300 mg/kg per animal this was given as a bolus through the carotid artery. After each infusion, animals were allowed 15 to 20 minutes to stabilize before systemic and microvascular characterization. The experimental timeline is described in figure 1 and the solution characteristics are described in **Table 5.1**.

Table 5.1 Apohemoglobin-Haptoglobin Characteristics: The total protein concentration, hemoglobin binding capacity, and heme binding capacity of lactated ringers, apoHb-Hp, and ααHb are shown.

Treatment	Total Protein Conc. (mg/mL)	Hb Binding Capacity (mg/mL)	Heme Binding Capacity (mg/mL)
Lactated Ringers	*0*	*0*	*0*
ApoHb + Hp	*62.01*	*25.23*	*0.74*
Experiment	**Total Protein Concentration (mg/mL)**		
ααhHb	*50*		

Systemic Variables and Blood Chemistry

The arterial cannula was connected to a pressure transducer and recording system (MP150, Biopac, Santa Barbara, CA), and blood pressure signals was recorded, along with mean arterial pressure (MAP), and heart rate (HR). The Hct was measured from centrifuged arterial blood samples taken in heparinized capillary tubes. Hb content was determined spectrophotometrically from a single drop of blood (B-Hemoglobin, Hemocue, Stockholm, Sweden). Arterial blood was

collected in heparinized glass capillaries (50 μL) and immediately analyzed for pO_2, pCO_2, pH, electrolytes, lactate, and total Hb content (ABL90; Radiometer America, Brea, CA).

Microvascular Experimental Setup

The unanesthetized animal was placed in a restraining tube with a longitudinal slit from which the window chamber protruded, then fixed to the microscopic stage of a transillumination intravital microscope (BX51WI, Olympus, New Hyde Park, NY). The animals were given 20 min to adjust to the change in the tube environment before measurements were made. The tissue image was projected onto a charge-coupled device camera (COHU 4815) connected to a videocassette recorder and viewed on a monitor. Measurements were carried out using a 40X (LUMPFL-WIR, numerical aperture 0.8, Olympus) water immersion objective. The same sites of study were followed throughout the experiment so comparisons could be made directly to baseline levels.

Microhemodynamics

Arteriolar and venular blood flow velocities were measured online by using the photodiode cross-correlation method[25] (Photo Diode/Velocity Tracker Model 102B, Vista Electronics, San Diego, CA). The measured centerline velocity (V) was corrected according to vessel size to obtain the mean RBC velocity.[26] A video image-shearing method was used to measure vessel diameter (D).[27] Blood flow (Q) was calculated from the values measured as $Q = \pi \times V (D/2)2$. The data was separated into three groups arteriole diameter and flow < 60μm, arteriole diameter and flow > 60μm, and venules diameter and flow between 20 and 80 μm.

Functional Capillary Density (FCD)

Functional capillaries, defined as those capillary segments that have RBC transit of at least one RBC in a 45s period in 10 successive microscopic fields, were assessed, totaling a region of

0.46 mm^2. Each field had between two and five capillary segments with RBC flow. FCD (cm^{-1}), i.e., the total length of RBC perfused capillaries divided by the area of the microscopic field of view, was evaluated by measuring and adding the length of capillaries that had RBC transit in the field of view. The relative change in FCD from baseline levels after each intervention is indicative of the extent of capillary perfusion.[28,29]

Pharmacokinetics— Exchange Transfusion Protocol

Pharmacokinetics was studied for 48 hours after exchange transfusion with 5 g/dL ααHb or 5 g/dL ααHb treated with apoHb-Hp solution. Briefly, C57BL/6 mice fitted with the dorsal window chamber were exchanged with 40% of the estimated BV (7% of body weight). Test solutions were infused into the jugular vein catheter at a rate of 100 µl/min with simultaneous blood withdrawal at the same rate from the carotid artery catheter via a dual syringe pump (Harvard Apparatus, Holliston, MA). Blood samples (50 µL) were taken after exchange transfusion at 0.5, 1, 2, 4, 8, 12, 24, and 48 hours.

Organ Harvesting and Analysis

Following experimental protocol, blood was collected and centrifuged to separate the plasma. Animals were sacrificed with Fatal Plus (sodium pentobarbital, 300 mg/kg), urine was collected, and kidneys, liver, spleen, and lungs were harvested. Markers of inflammation, function, and organ injury were evaluated. These analyses were performed by the UC San Diego Histology Core via ELISA and flow cytometric analysis of tissue homogenates and plasma. Markers of inflammation, function, and organ injury were evaluated using the ELISA Kits/ Assays described in table S1.

Statistical Analysis

Results are presented as the mean ± standard deviation. Data within each group were analyzed using analysis of variance for repeated measurements (ANOVA, Kruskal-Wallis test). When appropriate, post hoc analyses were performed with the Dunn's multiple comparison test. Comparison between samples was performed using two-way ANOVA (Hb plasma concentration); post hoc analyses were performed with Bonferroni posttests. Microhemodynamic data are presented as absolute values and ratios relative to baseline values. A ratio of 1.0 signifies no change from baseline, while lower and higher ratios are indicative of changes proportionally lower and higher than baseline (i.e., 1.5 represents a 50% increase from the baseline level). The same blood vessels and capillary fields were monitored throughout the study, such that direct comparisons to their baseline levels could be performed, allowing for more reliable statistics on small sample populations. All statistics were calculated using computer software (GraphPad Prism 9, GraphPad Software, Inc., San Diego, CA). Changes were considered significant if the p values were less than 0.05. The sample size per experimental group is based on the ANOVA test using an alpha of 0.05 and a power of 0.9 resulting in a minimum acceptable sample size of 15 per group.

5.4 **RESULTS**

Mean Arterial Pressure, Heart Rate, and Hematological Parameters

Mean arterial pressure (MAP) and heart rate (HR) is presented in **Figure 5.2**. Both MAP and HR values reverted to baseline after treatment with ApoHb-Hp showing no significance. However, with ringers, the control group, the HR did not return to baseline showing a significant decrease.

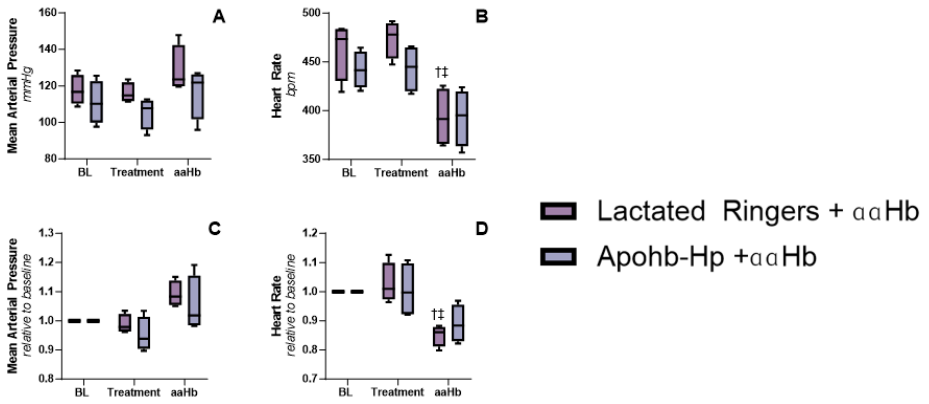

Figure 5.2 Mean Arterial Pressure and Heart Rate of Golden Syrian Hamsters - The Golden Syrian Hamsters's mean arterial pressure and heart rate were evaluated for the duration of the experiment. The values were normalized to baseline to observe relative changes. Each group has 4 hamsters. † vs baseline, ‡ vs Treatment

Total hemoglobin concentration (tHb), hematocrit (Hct), pH, pO2, and pCO2 are presented in **Table 5.2**. It can be observed from the data that the group treated with ApoHb-Hp reverted the values of tHb, Hct, pH, pO2, and pCO2 back to baseline. However, the control group showed a significant decrease in Hct compared to baseline.

Table 5.2 Hematological Properties. Total hemoglobin concentration, hematocrit, pH, pCO2, and pO2 were measured at each phase of the experiment. The values are shown as the mean +/- standard deviation with a single dagger to distinguish a significant change to baseline.

Hematological Properties				
tHb (g/dl)	Baseline Lactated Ringers ααHb	15.5 ± 1.0 15.2 ± 1.1 15.3±1.0	Baseline ApoHb-Hp ααHb	16.1 ± 0.4 14.7 ± 0.3† 14.9 ± 0.5
Hct (%)	Baseline Lactated Ringers ααHb	49 ± 2 48 ± 3 46 ± 3†	Baseline ApoHb-Hp ααHb	49 ± 1 46 ± 1† 45 ± 3
pH	Baseline Lactated Ringers ααHb	7.362 ± 0.032 7.368 ± 0.037 7.343 ± 0.015	Baseline ApoHb-Hp ααHb	7.351 ± 0.026 7.348 ± 0.038 7.343 ± 0.010
pCO_2 (mmHg)	Baseline Lactated Ringers ααHb	54.8 ± 4.6 51.7 ± 6.0 49.8 ± 4.7	Baseline ApoHb-Hp ααHb	59.1 ± 6.2 55.2 ± 2.6 53.4 ± 3.0
pO_2 (mmHg)	Baseline Lactated Ringers ααHb	59.1 ± 9.7 69.1 ± 14.2 69.4 ± 19.4	Baseline ApoHb-Hp ααHb	68.0 ± 15.1 68.2 ± 4.5 66.4 ± 12.1
				† vs Baseline

Pharmacokinetics

The pharmacokinetics and oxidation rate are presented in **Figure 5.3** and **Figure 5.4**. The data reported a half-life of 9.544 hours when lactated ringers was the treatment and 6.087 hours when ApoHb-Hp was the treatment. Furthermore, the data also reported that the tau or the rate of oxidation is 14.50 hours when treated with lactated ringers and 15.39 hours when treated with ApoHb-Hp.

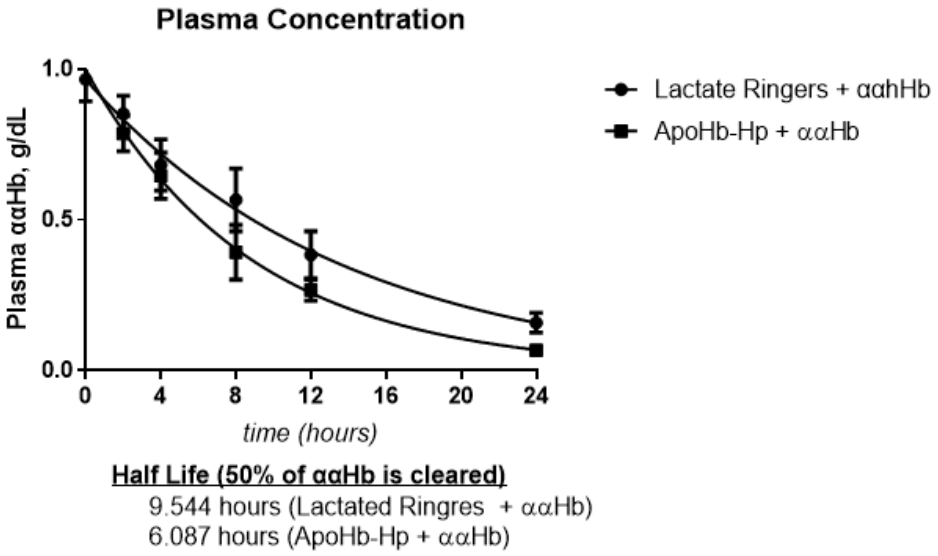

Figure 5.3 Pharmacokinetics. A C57BL/6 mouse was used to perform pharmacokinetic of the ααHb solution at 5g/dL with and without the apohb-Hp complex. Lactated ringers or apohb-Hp was injected via IA followed by the ααHb solution at 5g/dL.

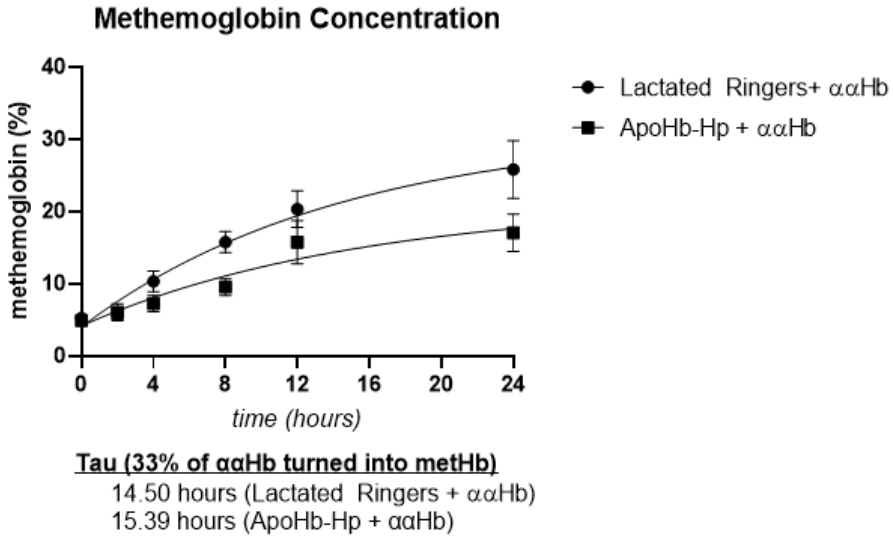

Figure 5.4 Oxidation Rate. A C57BL/6 mouse was used to evaluate the rate of oxidation of the ααHb solution at 5g/dL with and without the apohb-Hp complex. Lactated ringers or apohb-Hp was injected via IA followed by the ααHb solution at 5g/dL.

Microvascular Hemodynamics

Microvascular measurements are presented in **Figure 5.5, Figure 5.6, and Figure 5.7**. The data for this section was separated by flow and diameter for arterioles greater than 60um, arterioles

less than 60um, and venules in between 20 to 80um. Considering the flow and diameter of the arterioles >60um, we can observe that both groups showed no significant difference in flow or diameter compared to baseline. Considering the flow and diameter of the arterioles <60um, we can observe that both groups showed a significant difference in diameter compared to baseline. Interestingly, it can also be highlighted how the ApoHb-Hp treatment has a significantly higher flow compared to baseline. However, both the flow and diameter in the ApoHb-Hp treated group was significantly higher than in the control group. Considering the flow and diameter of venules in between 20um and 80um. The diameter and flow for both groups were not significantly different to baseline, showing how the values revert3ed to baseline within both the control group and the treatment of ApoHb-Hp group. However, it can be observed that the flow in the treatment group was significantly higher than the control group.

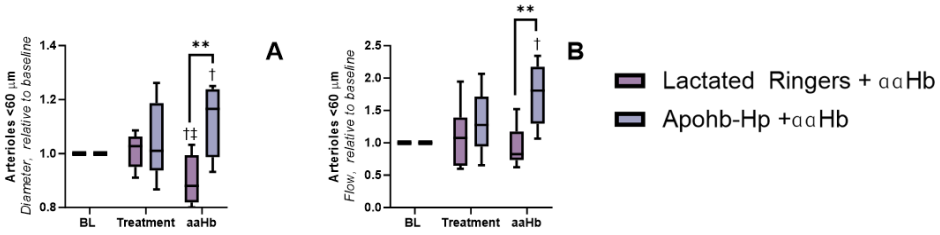

Figure 5.5 Arterioles < 60µm - Golden Syrian Hamsters underwent microvascular observation using intra-vital microscopy. Hamsters infused with ringer solution (control) saw a drop in relative diameter and relative flow compared to baseline. However, these effects were reduced when treated with apohb-hp. † vs baseline, ‡ vs Treatment

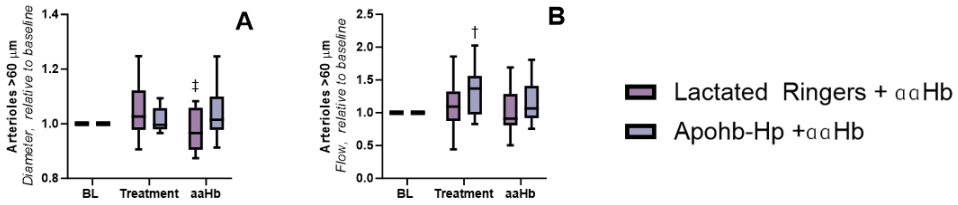

Figure 5.6 Arterioles > 60µm. Golden Syrian Hamsters underwent microvascular observation using intra-vital microscopy. Hamsters infused with ringer solution (control) saw a drop in relative diameter and relative flow compared to baseline. However, these effects were reduced when treated with apohb-hp. † vs baseline, ‡ vs Treatment

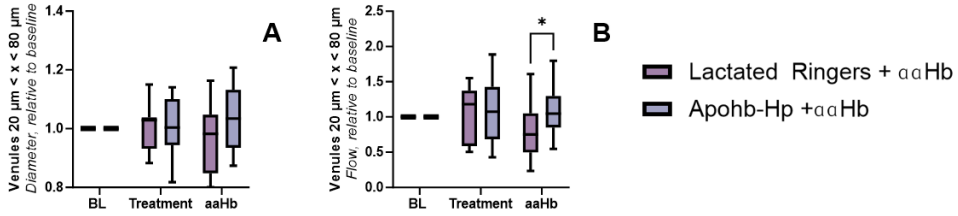

Figure 5.7 Venules 20 < x < 60µm. Golden Syrian Hamsters under went microvascular observation using intra-vital microscopy. Hamsters infused with ringer solution (control) saw a drop in relative diameter and relative flow compared to baseline. However, these effects were reduced when treated with apohb-hp. † vs baseline, ‡ vs Treatment

Functional Capillary Density (FCD)

Functional capillary density normalized to the baseline value is showed in **Figure 5.8**. When comparing the groups to baseline, both the control group and the treated ApoHb-Hp group are significantly different. However, compared to baseline the decrease in FCD is significantly lower for the control group than the treatment group, showing a better FCD recovery with the ApoHb-Hp treatment.

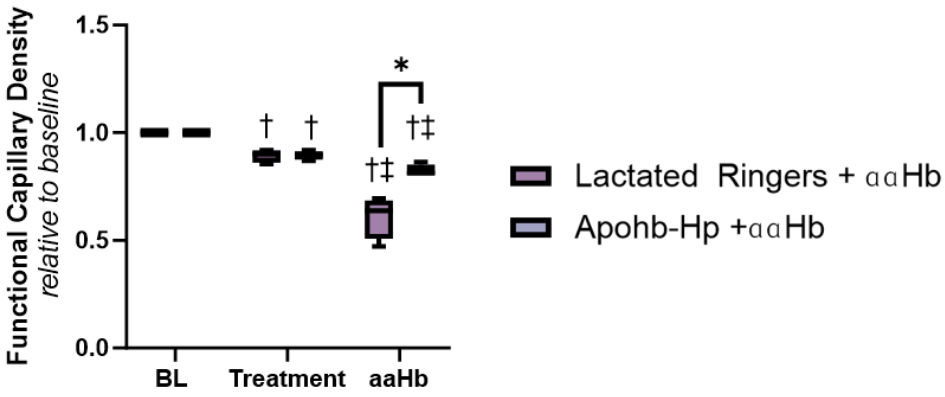

Figure 5.8 Functional Capillary Density - Golden Syrian Hamsters underwent microvascular observation using intra-vital microscopy. Hamsters infused with lactated ringer solution as its treatment saw a drop in the number of functional capillaries when ααHb was infused. When apoHb-Hp was infused as the treatment amount of functional capillaries. † vs baseline, ‡ vs Treatment

Inflammatory Markers

Heart, liver, kidney, urine, and spleen were harvested for all subjects at the end of the protocol. To evaluate the inflammation in the heart, cardiac TNF-α, IL-6, IL-1, Troponin, CRP, MCP-1 were measured and are presented in **Figure 5.9.** Cardiac TNF-α, IL-6, and CPR showed a significant increase in the control group compared to the sham and ApoHb-Hp groups. However, for cardiac TNF-α, the ApoHb-Hp group showed a significant increase compared to sham, but the control group had the highest significant difference. Cardiac Troponin, CRP and MCP-1 showed no significant difference between the groups, but it can be observed how the inflammation is still higher in the control group compared to ApoHb-Hp or sham group.

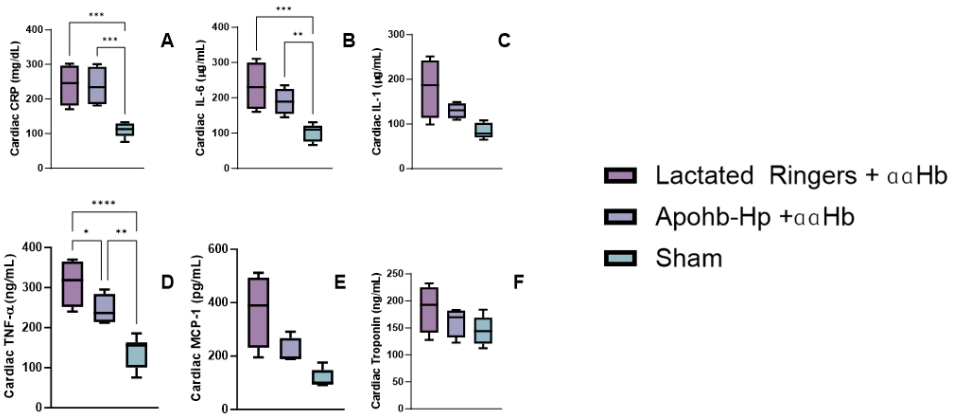

Figure 5.9 Cardiac Inflammation - Following the conclusion of the experiment the Golden Syrian Hamsters were sacrificed and the heart was harvested for analysis. The biomarkers recorded were cardiac CRP, cardiac IL-6, cardiac IL-1, cardiac TNF-α, cardiac MCP-1, cardiac Troponin

5.5 DISCUSSION

The principal findings of this study are nothing short of groundbreaking, offering a glimmer of hope in the quest to make blood transfusions safer and more effective. At the heart of these findings lies the discovery that the use of apoHb-Hp can enhance microvascular health and reduce inflammation in vital organs resulting from acellular Hb (ααHb) infusion. This revelation could revolutionize the HBOCs field, potentially paving the way for their safer and more widespread clinical application. The injection of aaHb, while not demonstrating significant effects on systemic parameters when combined with apoHb-Hp, should not be dismissed as harmless. It's essential to acknowledge that the dosage used in the current model might have been too conservative to induce aaHb's notorious hypertensive effects. This is a critical revelation, underscoring the need for precise dosing and further exploration into aaHb's behavior.

Microhemodynamics emerged as a central theme in this study, highlighting the pivotal role of aaHb and apoHb-Hp in shaping the microcirculation. The arterioles' diameter in the microcirculation proved a crucial indicator, shedding light on aaHb's vasoconstrictive tendencies,

a well-documented concern[5]. However, when coupled with apoHb-Hp, aaHb's vasoconstriction effect was miraculously nullified. This is a revelation with profound implications. The differential impact on blood flow further solidified the importance of molecular size, suggesting that aaHb's vasoconstriction might indeed be tied to its molecular dimensions. Previous research has hinted at the possibility that apoHb-Hp facilitates holoHb-apoHb αβ-dimer exchange and apoHb-heme intercalation, possibly leading to the formation of Hp-ααHb. The increase in molecular size resulting from these interactions appears to play a pivotal role in preventing extravasation through the vasculature, increasing plasma viscosity, and elevating oncotic pressure in circulation. Working in harmony, these factors contribute to the remarkable improvement in microcirculatory health observed in this study.

Moreover, the implications of the observed shorter half-life of HBOCs when combined with apoHb-Hp hold profound significance. This could be the pivotal factor in augmenting their therapeutic potential. HBOCs, while undeniably valuable, inherently exhibit instability in circulation, and this accelerated elimination could hold the solution to preventing the accumulation of oxidative damage over time.

Beyond these revelations, the incorporation of apoHb-Hp introduces an exhilarating dimension to the study's significance. By augmenting the heme-binding capacity within the bloodstream, apoHb-Hp presents a unique avenue for mitigating the vasoconstrictive effects typically associated with free heme. The implications of this discovery extend into the innate immune system, where hemes often trigger responses that can exacerbate existing medical conditions.

Lastly, the preservation of arteriolar microcirculation functionality and the subsequent increase in hydrostatic pressure at the arteriole end of the capillaries signify a momentous

achievement within this study. This fosters a greater number of functional and denser capillary beds, a physiological state inherently advantageous for tissue oxygenation and overall health. The marked disparity in functional capillary density when compared to aaHb's effects, underscores the immense potential of the apoHb-Hp complex in mitigating the adverse outcomes associated with HBOCs. Perhaps most strikingly, the findings strongly suggest that apoHb-Hp could be the missing piece in the HBOC puzzle. The notable reduction in inflammation observed in vital organs, including the heart, spleen, liver, kidney, and plasma, cannot be overstated. This discovery alone harbors tremendous promise for the future of blood transfusion therapies, as inflammation remains a common adverse effect capable of significantly impacting patient outcomes.

In conclusion, the results of this study mark a significant leap forward in pursuing safer and more effective blood substitutes. The apoHb-Hp complex represents a beacon of hope, potentially rendering HBOCs a viable and dependable option for clinical applications. These findings not only broaden the horizons of research and treatment but also promise to mitigate the risks associated with blood transfusions, ultimately leading to improved patient care and the preservation of lives.

5.6 **ACKNOWLEDGEMENT**

The authors thanks Cynthia Walser for the surgical preparation of the animals. The authors will also like to thank the National Institutes of Health (NIH) for funding through grants R01HL162120 and R01HL159862.

Chapter 5, in full, is being prepared for publication as it appears in “Alpha-Alpha Hemoglobin/ Diaspirin-Crosslinked Hemoglobin (DCLHb) Benefits from Treatment using Apohemoglobin Haptoglobin Complex” by Carlos J. Munoz, Daniela Lucas, Jacinda Martinez, Mia Ricario, and Pedro Cabrales. The dissertation author was the primary investigator and author of this paper.

5.7 **AUTHORSHIP**

Contribution: C.J.M. performed experiments; C.J.M., D.L., J.M., M.R., and P.C., analyzed results and made the figures; C.J.M. and P.C. designed the research, and C.J.M. and D.L. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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6. PEGylated Apo-Hemoglobin Haptoglobin can Effectively Limit Vascular Dysfunction in the Microcirculation and Prevent Acute Cardiac and Kidney Inflammation

6.1 ABSTRACT

Introduction: Hemoglobin (Hb) toxicities can occur in a variety of situations including hemolytic anemia, surgical procedures, or bacterial infections. Hemoglobin is commonly found inside the membrane of red blood cells, if chronic hemolysis arises hemoglobin will be found circulating freely in the vasculature resulting in a series of lethal events including renal failure, cardiac dysfunction, and vascular reactivity. In most cases native scavenger proteins such as haptoglobin (Hp) and hemopexin can prevent these issues. Unfortunately, during pathological conditions such as sickle cell anemia, thalassemia, malaria, or even during some surgical procedures like extracorporeal membrane oxygenation (ECMO) chronic hemolysis occurs and a rush of hemoglobin enters the circulation. This results in several complications such as nitric oxide scavenging, tool like receptor 4 over stimulation, and cardiac contractility dysfunction. To address this concern bioengineers, look to create a supplemental scavenger protein to aid patients in cases of chronic hemolysis. This paper looks at the next evolution of a newly engineered scavenger protein, apoHb, and the en-vivo result associated with PEGylating ApoHb and its ability to have a dual use by conjugating it with Haptoglobin creating PEG-ApoHb-Hp.

Methods Utilizing intravital microscopy on male Golden Syrian hamsters instrumented with dorsal window chamber, we evaluate the in vivo effects of PEG-apoHb-Hp complex by challenging it against a bolus injection (10% of the animal's blood volume) of human Hb (hHb, 5 g/dL). This study evaluated four experimental treatment groups saline, PEG-apoHb only, Hp only, and PEG-apoHb-Hp under the hHb challenge. In this study the microvascular hemodynamics (flow) in arterioles < 50 μm, arterioles > 50 μm and venules 20<x<100 μm in diameter were

recorded at baseline, 20 minutes after treatment, and 20 minutes after hHb challenge. Systemic parameters (blood pressure and heart rate), blood gases (pH, pCO2, and pO¬2), blood parameters (Hb concentration and hematocrit) and multiorgan functionality/ inflammation was recorded.

Results The results suggest that microvascular hemodynamics improve as more hemoglobin and its associated products are scavenged. In relation to the hypovolemic infusion of hHb, PEG-ApoHb treatment alone scavenged 0% of hemoglobin resulting in its dimerization and at such point only 12% of heme was scavenged. This illustrated marginal improvements compared to the control (saline) treatment in the microcirculation, however, it did substantially alleviate cardiac and kidney inflammation. Hp treatment alone scavenged 31% of Hb and prevents 31% of heme release. This significantly improved microcirculatory hemodynamics and reduced cardiac and kidney inflammation. Finally, the use of PEG-ApoHb-Hp scavenged 31% of Hb and 42% of total heme, such a substantial amount of hemoglobin scavenging had the best improvement in the microcirculation and prevent cardiac and kidney inflammation.

Conclusion: PEGlyation of apoHb does not compromise its effectiveness to scavenge hemoglobin in the circulation.

6.2 INTRODUCTION

Through the years scientist have spent countless hours understanding and engineering methods to overcome toxicities due to hemoglobin (Hb).[1–3] Hb is the key protein in providing oxygen to the tissues in an effective manner that allows biological tissue to meet its metabolic needs. Hb is carried inside red blood cells and separated from the vasculature via a plasma membrane. This separation and isolation inside the red blood cells plays a key role in preventing Hb from oxidization, dimerized, and extravasated through the surrounding tissue. Hb in the circulation leads to oxidize damage resulting in a new species of Hb to form, methemoglobin (metHb). Furthermore, as Hb becomes oxidized and moves into new redox states it generates reactive oxygen species, ferrous (Fe^{2+}) to ferric (Fe^{3+}) to ferryl (Fe^{4+}) and accelerates the release of heme, a dangerous cytotoxin[4,5]. At the same time of the release of heme, Hb dimerization will occur and the Hb subunits will result in scavenging of nitric oxide, a potent vasodilator, resulting in hypertension, drop-in heart rate, and vasoconstriction.[6–8]

In mammalian species cellular free hemoglobin is stabilized and scavenged through a protein found in the plasma, haptoglobin (Hp). Hp binds itself to key amino acids on the exposed free Hb and prevents oxidative damage and dimerization of the Hb protein[9,10]. Hp acts as a tag for CD163 macrophage phagocytosis to remove it from circulation and degradation of the protein will commence returning key pieces such as iron to the bone marrow for continued erythropoiesis.[11] However, in situations of high rates of hemolysis, haptoglobin cannot function as an efficient method of preventing the pathophysiological issues of free circulating plasma Hb. As mentioned previously the dimerization of the hemoglobin occurs. Upon its dimerization the prosthetic heme moiety. This leads to a host of other pathophysiological consequences. Heme is known to be a protein that triggers kidney disfunction, cytokine storms, an overactive immune system, and other

challenges.[5,12,13] Similar to Hp, there are native proteins who are responsible for the scavenging of heme and iron. Hemopexin (Hpx) is a protein that resides in the free circulating plasma. Hpx function is to scavenge free circulating heme. The protein is taking the liver for further uptake in the hepatocytes and catabolized by heme-oxygenase proteins (HO-1 and HO-2).[14,15]

Ultimately, during hemolytic anemias the native scavengers are unable to prevent inevitable toxicities. Thus, supplemental dosages of these proteins have been explored as means to aid patients. The difficulty of such comes from the price and efforts necessary to purify concentrations large enough to have therapeutic events.[16] To address this issue researchers have explored the use of apohemogloibn (apoHb) as a heme scavenger. ApoHb has four times the molar capacity to scavenge heme compared to Hpx[17,18]. Unfortunately, apoHb is unstable in room temperature environments unless bounded to Hp forming an apohemoglobin haptoglobin complex (apoHb-Hp). Addressing this concern, polyethylene glycol (PEG) is used to stabilize the apoHb. PEGlyation of apohemoglobin formulates (PEG-ApohHb). PEG-ApohHb is hypothesized to be a new stable molecule in the circulation that can scavenge heme keep its tetrameric shape and can interact with Hp similar to the results seen previously in ApoHb-Hp complexes[18]. In this study, we utilize golden Syrian hamsters instrumented with a window chamber to observe microcirculatory events and determine that PEG-ApoHb is effective. We will be injecting free hemoglobin and testing against PEG-ApohHb, Hp, and PEG-apoHb-Hp treatments.

6.3 METHODS

Apohemoglobin (ApoHb) Preparation

The apoHb used in this study was prepared via tangential flow filtration based on the acidic-ethanol heme-extraction procedure as previously described.[17] The absence of residual heme was verified by measuring the ratio of absorbance between the Soret peak (λ_{max} = 412-413 nm)

and 280 nm to ensure less than 1% residual heme in the product. The heme-binding capacity of apoHb preparations was 80%.[19] Further testing associated to its stability and analysis of the PEG-ApoHb was done previously in another study. [18]

Haptoglobin Preparation

Human Hp was purified from human Cohn fraction IV (FIV) purchased from Seraplex (Pasadena, CA). The final protein solution was composed of a mixture of Hp2-1 and Hp2-2 Hp polymers with an average MW of 400-500 kDa. Further testing associated to its stability and analysis of the PEG-ApoHb was done previously in other studies. [16,20]

PEGylation of ApoHb

ApoHb was thawed from −80 ° C at 4 °C over the course of 5 h. ApoHb was then diluted to ∼10 mg/mL in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) and filtered through a 0.2 μm syringe filter prior to the PEGylation reaction. The sample was incubated with 10× molar excess (on a globin basis of apoHb) of 2-iminothiolane hydrochloride (IT) and a 20× molar excess of mPEG-maleimide-5000 for 16 h or overnight at 4 °C. The thiol-maleimide chemistry for PEGylation of apoHb was used as previously described for PEGylation of Hb.[21–23] The excess mPEG-maleimide-5000 and IT were removed via TFF facilitated diafiltration using a 30 kDa mPES hollow fiber membrane. This procedure was tested on batches using 0.1 and 1 g of apoHb. Further testing associated to its stability and analysis of the PEG-ApoHb was done previously in another study. [20]

Haptoglobin Binding to PEG-ApoHb and Heme-Bound PEG-ApoHb

Haptoglobin (Hp) was purified from human Cohn fraction IV yielding a mixture of Hp2-1 and Hp2-2 phenotypes with an average MW of ∼400 kDa. The binding capacity of Hp to Hb and apoHb was determined using high-performance liquid chromatography (HPLC) size exclusion

chromatography (SEC) (HPLC-SEC). The change in the AUC of free Hb/apoHb when Hp was added to the sample was used to quantify the amount of Hb/apoHb bound to Hp. To analyze the binding of Hp to PEG-apoHb, 2× excess PEG-apoHb was mixed with Hp (based on the Hb/apoHb binding capacity of Hp). The mixture was then separated on an HPLC-SEC column. The change in the chromatogram when monitoring the 280 nm absorbance was then used to determine the formation of the Hp-PEG-apoHb complex. The same process was repeated using a PEG-apoHb sample incubated with 5× excess heme−albumin to saturate the heme-binding hydrophobic pockets of PEG-apoHb. In this case, instead of analyzing Hp binding via the 280 nm absorbance, fluorescence quenching of Hp (monitored via 285 nm excitation and 330 nm emission) was used to determine the amount of bound heme-PEG-apoHb to Hp.

Animal Preparation

Investigations were performed in 55 – 65 g male Golden Syrian Hamsters (Charles River Laboratories, Boston, MA) fitted with a dorsal skinfold window chamber. Animal handling and care followed the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the local animal care committee. The hamster window chamber model is widely used for microvascular studies in the unanesthetized state, and the complete surgical technique is described in detail elsewhere. [24,25] The experimental animal was allowed at least 2 days for recovery before the preparation was assessed under the microscope for any signs of edema, bleeding, or unusual neovascularization. Animals were anesthetized again, and arterial and venous catheters filled with a heparinized saline solution (30 IU/ml) were implanted. Catheters were tunneled under the skin, exteriorized at the dorsal side of the neck, and securely attached to the window frame. The microvasculature was examined 3 to 4 days after the initial surgery and only animals with window chambers whose tissue did not present regions of low perfusion,

inflammation, and edema were entered into the study.[26] The animal preparation is illustrated in **Figure 6.1**.

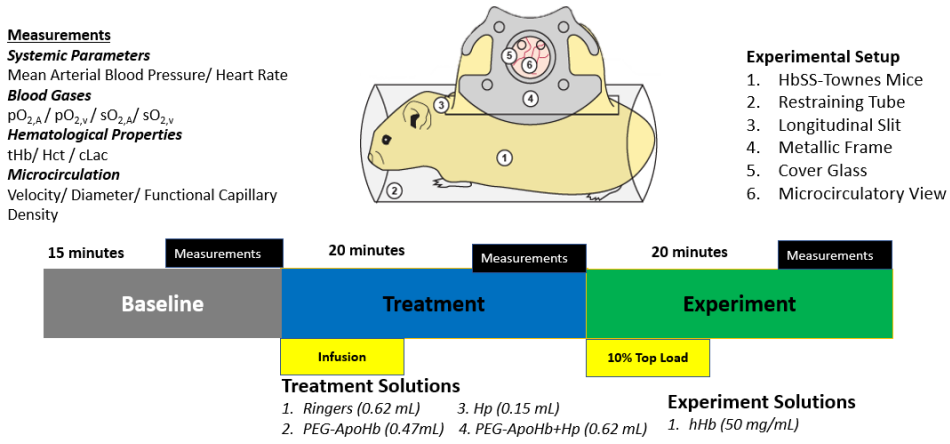

Figure 6.1 Experimental Timeline, Testing Treatment of PEG-ApoHb, Hp, and PEG-ApoHb+Hp. The animals were subjected to a three-phase experimental process, baseline, treatment and experiment. The baseline values of microcirculation and laboratory parameters were collected, the treatment phase saw an IA injection of either lactated ringers or ApoHb-Hp complex after 20 minutes the same information as baseline was collected in treatment, and finally the experimental group has an IA injection of ααHb following 20 minutes after the injection the same values from baseline were collected.

Inclusion Criteria

Animals were suitable for the experiments if 1) systemic variables were within normal range, namely, heart rate (HR) > 340 beat/min, mean arterial blood pressure (MAP) > 80 mmHg, systemic hematocrit (Hct) > 45%, and arterial oxygen partial pressure (PaO2) > 50 mmHg; and 2) microscopic examination of the tissue in the chamber observed under a 650 X magnification did not reveal signs of edema or bleeding. Hamsters are a fossorial species with a lower arterial PO_2 than other rodents due to their adaptation to the subterranean environment. However, microvascular PO_2 distribution in the chamber window model is the same as in other rodents such as mice.[6]

Hypovolemic Infusion (Top-Load) Protocol

Experimental groups were labeled based on the infusion of the treatment solution, this included the control (lactated ringers), PEG-ApoHb, Hp, or PEG-ApoHb-Hp, each solution received an infusion of 0.62mL, 0.47 mL, 0.15 mL, 0.62 mL, respectively. Followed by a hypovolemic infusion given as a bolus through the carotid artery (10% of the animals blood volume) of stroma free human hemoglobin (hHb) at a concentration of 50 mg/mL. After each infusion, animals were allowed 15 to 20 minutes to stabilize before systemic and microvascular characterization. The experimental timeline is described in figure 1 and the solution characteristics are described in **Table 6.1**.

Table 6.1 Apohemoglobin-Haptoglobin Characteristics: The total protein concentration, hemoglobin binding capacity, and heme binding capacity of lactated ringers, PEG-apoHb, Hp, and ααHb are shown.

Treatment	Total Protein Conc. (mg/mL)	Hb Binding Capacity (mg/mL)	Heme Binding Capacity (mg/mL)
Lactated Ringers	0	0	0
PEG-ApoHb	15	0	0.24
Hp	101.3	50.6	0
PEG-ApoHb-Hp	35.56	11.98	0.18
Experiment	**Total Protein Concentration (mg/mL)**		
hHb	*50*		

Systemic Variables and Blood Chemistry

The arterial cannula was connected to a pressure transducer and recording system (MP150, Biopac, Santa Barbara, CA), and blood pressure signals was recorded, along with mean arterial pressure (MAP), and heart rate (HR). The Hct was measured from centrifuged arterial blood samples taken in heparinized capillary tubes. Hb content was determined spectrophotometrically from a single drop of blood (B-Hemoglobin, Hemocue, Stockholm, Sweden). Arterial blood was collected in heparinized glass capillaries (50 μL) and immediately analyzed for pO_2, pCO_2, pH, electrolytes, lactate, and total Hb content (ABL90; Radiometer America, Brea, CA).

Microvascular Experimental Setup

The unanesthetized animal was placed in a restraining tube with a longitudinal slit from which the window chamber protruded, then fixed to the microscopic stage of a transillumination intravital microscope (BX51WI, Olympus, New Hyde Park, NY). The animals were given 20 min to adjust to the change in the tube environment before measurements were made. The tissue image was projected onto a charge-coupled device camera (COHU 4815) connected to a videocassette recorder and viewed on a monitor. Measurements were carried out using a 40X (LUMPFL-WIR, numerical aperture 0.8, Olympus) water immersion objective. The same sites of study were followed throughout the experiment so comparisons could be made directly to baseline levels.

Microhemodynamics

Arteriolar and venular blood flow velocities were measured online by using the photodiode cross-correlation method[27] (Photo Diode/Velocity Tracker Model 102B, Vista Electronics, San Diego, CA). The measured centerline velocity (V) was corrected according to vessel size to obtain the mean RBC velocity.[28] A video image-shearing method was used to measure vessel diameter (D).[29] Blood flow (Q) was calculated from the values measured as $Q = \pi \times V (D/2)2$. The data was separated into three groups arteriole diameter and flow < 60μm, arteriole diameter and flow > 60μm, and venules diameter and flow between 20 and 80 μm.

Functional Capillary Density (FCD)

Functional capillaries, defined as those capillary segments that have RBC transit of at least one RBC in a 45s period in 10 successive microscopic fields, were assessed, totaling a region of 0.46 mm^2. Each field had between two and five capillary segments with RBC flow. FCD (cm^{-1}), i.e., the total length of RBC perfused capillaries divided by the area of the microscopic field of

view, was evaluated by measuring and adding the length of capillaries that had RBC transit in the field of view. The relative change in FCD from baseline levels after each intervention is indicative of the extent of capillary perfusion.[30,31]

Organ Harvesting and Analysis

Following experimental protocol, blood was collected and centrifuged to separate the plasma. Animals were sacrificed with Fatal Plus (sodium pentobarbital, 300 mg/kg), urine was collected, and kidneys, liver, spleen, and lungs were harvested. Markers of inflammation, function, and organ injury were evaluated. These analyses were performed by the UC San Diego Histology Core via ELISA and flow cytometric analysis of tissue homogenates and plasma. Markers of inflammation, function, and organ injury were evaluated using the ELISA Kits/ Assays described in table S1.

Statistical Analysis

Results are presented as the mean ± standard deviation. Data within each group were analyzed using analysis of variance for repeated measurements (ANOVA, Kruskal-Wallis test). When appropriate, post hoc analyses were performed with the Dunn's multiple comparison test. Comparison between samples was performed using two-way ANOVA (Hb plasma concentration); post hoc analyses were performed with Bonferroni posttests. Microhemodynamic data are presented as absolute values and ratios relative to baseline values. A ratio of 1.0 signifies no change from baseline, while lower and higher ratios are indicative of changes proportionally lower and higher than baseline (i.e., 1.5 represents a 50% increase from the baseline level). The same blood vessels and capillary fields were monitored throughout the study, such that direct comparisons to their baseline levels could be performed, allowing for more reliable statistics on small sample populations. All statistics were calculated using computer software (GraphPad Prism 9, GraphPad

Software, Inc., San Diego, CA). Changes were considered significant if the p values were less than 0.05.

6.4 RESULTS

Microvascular Hemodynamics

Microvascular measurements are presented in **Figure 6.2**, for arterioles, and in **Figure 6.3**, for venules. The data was separated between three groups, arterioles <50 um, arterioles >50 um, and venules between 20 and 80 um. The data is represented via a normalization to baseline. Considering the diameter and flow of the arterioles < 50 µm, all values were similar to baseline during the infusion of the treatment. However, during the experimental protocol, there was a significant increase in diameter and flow for the group treated with PEG-ApoHb-Hp compared to the control and the PEG-apoHb groups. Considering the diameter and flow of the arterioles > 50 µm, there was no significance between any of the groups, however all groups except for PEG-ApoHb-Hp showed significant decrease to baseline for both diameter and flow in the experimental timepoint.

The venules' diameter shows no significant difference between the groups. Moreover, the flow of the venules showed a significant increase in the experiment timepoint in the PEG-ApoHb-Hp group compared to the control and PEG-apoHb groups.

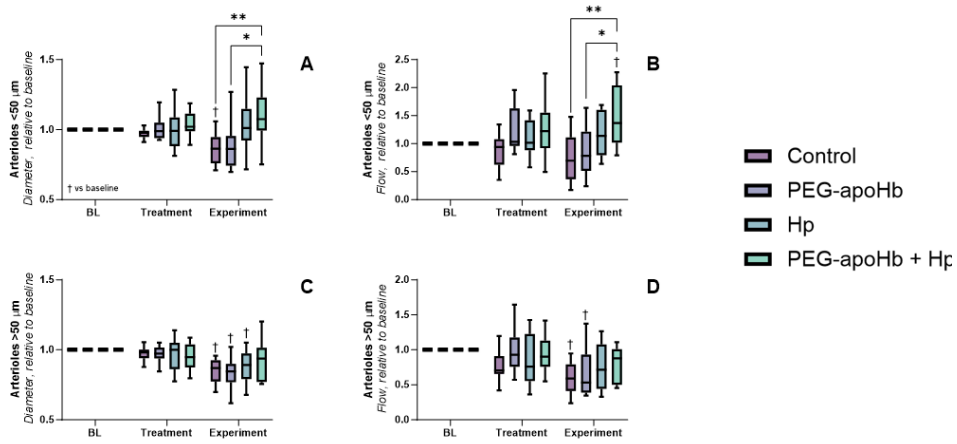

Figure 6.2 Microvascular Arterioles. The microvascular arterial diameter and flow were recorded in vessels <50 µm and vessels >50 µm. Animal subjects first received treatment of lactated ringers (control), PEGlyated apohemoglobin (PEG-apoHb), haptoglobin (Hp), or the combination of PEG-apoHb + Hp followed by a hypovolemic infusion (top load) of 10% of their blood volume using human Hb (hHb). Animal treated with the combination of PEG-Apohb + Hp showed an increase in diameter and flow compared to the others.

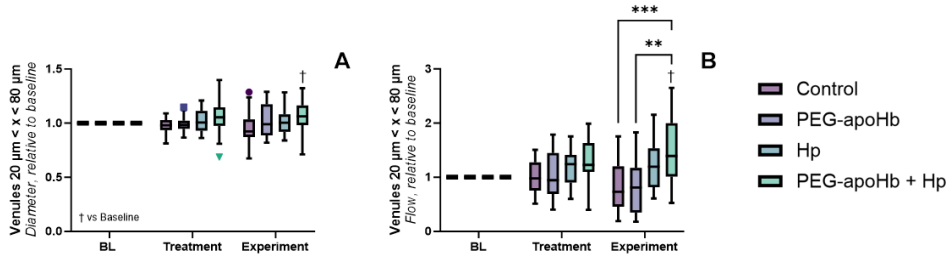

Figure 6.3 Microvascular Venules. The microvascular venule diameter and flow were recorded in vessels 20 < x <80 µm. Animal subjects first received treatment of lactated ringers (control), PEGlyated apohemoglobin (PEG-apoHb), haptoglobin (Hp), or the combination of PEG-apoHb + Hp followed by a hypovolemic infusion (top load) of 10% of their blood volume using human Hb (hHb). Animal treated with the combination of PEG-Apohb + Hp showed anincrease in diameter and flow compared to the others.

Functional Capillary Density

The normalized values for functional capillary density (FCD) are shown in **Figure 6.4**. At the infusion of the treatment solutions all groups had a decrease in FCD values. However, this decrease was not significant between the groups or the values at baseline. At the experimental timepoint,

all groups were significantly lower than baseline values. Moreover, the PEG-ApoHb-Hp and Hp groups showed a significant higher FCD value when compared to the control group.

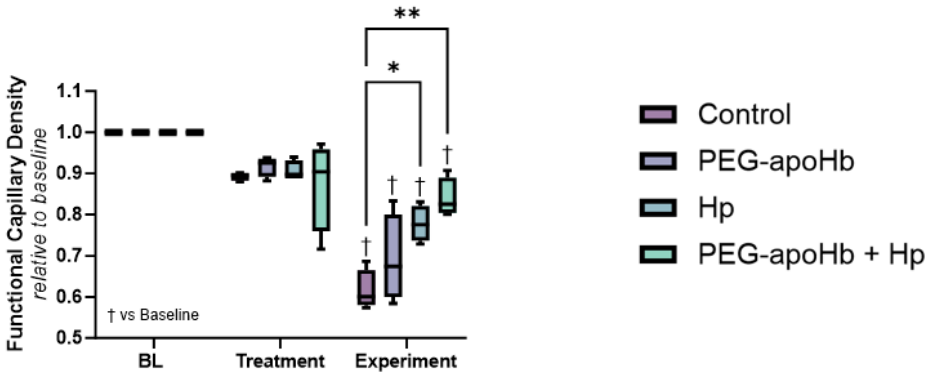

Figure 6.4 Functional Capillary Density. The functional capillary density in the microvasculature was quantified to give a understanding of microvascular health.Animal subjects first received treatment of lactated ringers (control), PEGlyated apohemoglobin (PEG-apoHb), haptoglobin (Hp), or the combination of PEG-apoHb + Hp followed by a hypovolemic infusion (top load) of 10% of their blood volume using human Hb (hHb). Animal treated with the combination of PEG-Apohb + Hp showed an increase in functional capillary density.

Markers for Cardiac Inflammation

The inflammatory markers for the heart, including CRP, IL-6, IL-1, TNF-alpha, MCP-1, and Troponin are presented in **Figure 6.5**. Cardiac CRP, IL-6, IL-1, TNF-alpha, MCP-1 were significantly higher in the hHb group compared to all other groups. hHb, PEG-Apo-Hb, and Hb showed a significant increase in cardiac CRP, IL-6, TNF-alpha, and MCP-1 levels when compared to sham. PEG-ApoHb-Hp showed no significance in CRP, IL-6, TNF-alpha, and MCP-1 levels against the sham group, however it showed significantly lower levels when compared to hHb, PEG-Apo-Hb, and Hb. IL-6 levels for PEG-ApoHb-Hp showed no significance when compared to sham and PEG-ApoHb, however it showed a significant decrease compared to hHb and Hp. Levels of cardiac troponin in the hHb grouo showed a significant when compared to all groups. However, all other groups including PEG-ApoHb, Hp, and PEG-ApoHb-Hp showed no significant difference in troponin against the sham group.

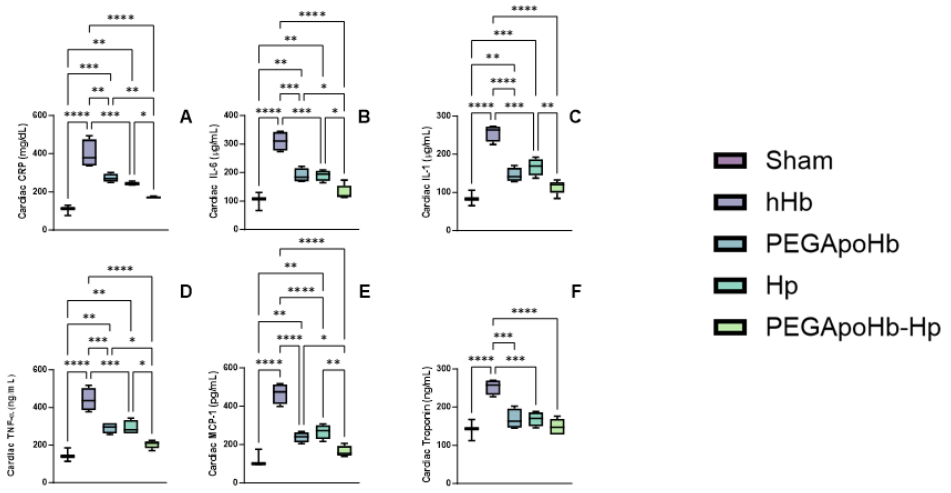

Figure 6.5 Cardiac Inflammation - Following the conclusion of the experiment the Golden Syrian Hamsters were sacrificed and the heart was harvested for analysis. The biomarkers recorded were A) cardiac CRP, B) cardiac IL-6, C) cardiac IL-1, D) cardiac TNF-α, E) cardiac MCP-1, and F) cardiac Troponin. The combination treatment of both PEG-ApoHb + Hp had a significant effect on cardiac biomarkers compared to the control.

Markers for Kidney Inflammation

The inflammatory markers to show renal injury are presented in **Figure 6.6**. Plasma BUN levels showed to be significantly higher in the hHb group when compared to all groups. Moreover, there was no significant difference between PEG-ApoHb, Hp, and PEG-ApoHb-Hp when compared to sham. U-NGAL levels showed significance between all groups, however it was clear that hHb group had the highest u-NGAL values compared to all groups and sham had the lowest values. Moreover, PEG-ApoHb-Hp showed significant lower values when compared to PEG-ApoHb and hHb but higher values compared to sham. PEG-ApoHb-Hp and Hp groups showed no significance between each other. Urine creatinine levels showed to be significantly higher in hHb when compared to all groups except for Hp. Moreover, PEG-ApoHb-Hp showed no significant difference to sham, however Hp and PEG-ApoHb where significantly higher when compared to sham.

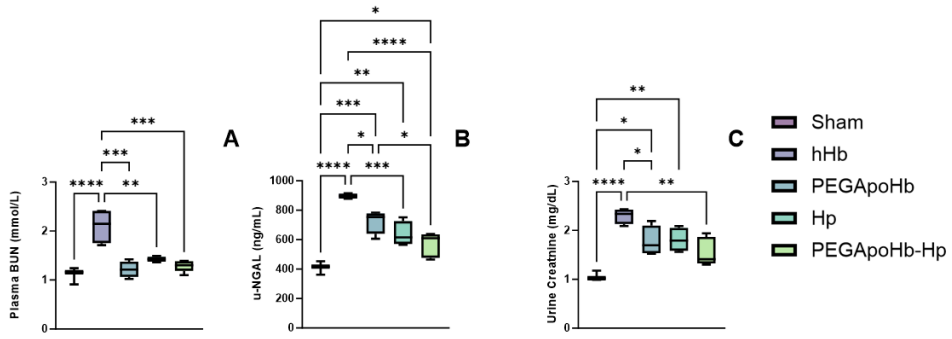

Figure 6.6 Kidney Function – Following the conclusion of the experiment the Golden Syrian Hamsters were sacrificed where the kidney, urine and plasma were collected to measure biomarkers of overall kidney functionality. The biomarkers measured where A) plasma blood urea nitrogen BUN, B) urine neutrophil gelatinase-associated lipocalin NAGL, and C) urine creatinine to evaluate kidney function. The combination treatment of both PEG-ApoHb + Hp had a significant effect on cardiac biomarkers compared to the control.

6.5 DISCUSSION

This study is builds upon proving that PEGylating apohemoglobin does not negatively affect its efficacy as a scavenger protein when bio conjugated to haptoglobin, however, there is a drop in active scavenging ability resulting in the need for a more concentrated product. Previous work established that PEGlyation of apohemoglobin increased its thermal stability, a major issue of apohemoglobin in the past.[20] The PEG-apoHb-Hp solution improved microcirculatory hemodynamics, functional capillary density, showed less inflammation and less damage to the heart, liver, spleen, and kidneys.

One of the most controversial effects of hemoglobin (Hb) is the increase in systemic vascular resistance. The balance between vasodilation and vasoconstriction closely follows the activity of nitric oxide (NO), a ubiquitous highly diffusible gas that acts as a signaling molecule.[32] When NO is produced in the vascular endothelium, it interacts with the enzyme guanylate cyclase to produce vasodilation. NO that diffuses into blood reacts with oxyhemoglobin (its principal scavenger) and deoxyhemoglobin to form S-nitroso hemoglobin.[33] The intravascular half-life of

NO is approximately 2 milliseconds in whole blood but is more than 500 times shorter when exposed to Hb.[34] Hb can also generate the release of reactive oxygen species that can react with NO, inhibit NO synthase, and possibly increase the release of endothelin, another potent vasoconstrictor.[35] Ultimately, the administration of Hb can dramatically reduce NO concentrations, thereby reducing the vasodilation effects of NO and promoting vasoconstriction. Furthermore, the reduced NO may also affect contractility in the heart, the cardiomyocytes calcium dynamics will slow because of NO concentrations wavering, thus, the heart rate will drop in theory.[32]

NO concentration was not directly measured; however, the mean arterial pressure (MAP), heart rate (HR), and microcirculatory hemodynamics do provide insight on these issues regarding Hb and the therapeutic event associated with scavenging the Hb from the circulation. The control group showed an increase in MAP, a decrease in heart rate, and vasoconstriction was attenuated as more Hb and Hb related proteins were removed from circulation.

Hb is a protein made up of two alpha beta dimers and four heme moieties each carrying iron. As previously mentioned, the oxidative damage of Hb from entering the circulation without the protection of the RBC membrane leads to the dimerization of this protein. Dimerization leads to the extravasation and scavenging of larger amounts of NO and the release of heme, a cytotoxin to the vasculature.[5,12,17,35–37] Pretreatment with PEG-ApoHb scavenges 12% of all the released heme from the hypovolemic infusion of human Hb, but this means that all Hb dimers are assumed to extravasate and consume NO. This is reflected in the diameters of the arterioles and venules in the microcirculation as they show minimal effect compared to the control group, especially in the arterioles. Arterioles are known to be more vaso-active due to the amount of smooth muscles surrounding these vessels in comparison to the venules[38]. Furthermore, the MAP and heart rate

associated with the control and PEG-ApoHb pretreatment both show hypertension characteristics and a reduced heart rate. However, the 12% of heme that was scavenged does make a difference in organ inflammation and organ damage biomarkers as they have comparable results to the other two experimental groups. Lastly, the microcirculatory pressure, which is associated with functional capillary density[30], does show marginal improvement compared to the control. Treatment with Hp scavenges 31% of Hb dimers which associates to 31% of heme not being released as a result in regards of the Hb infused. Hp stabilizes the Hb tetrameric structure preventing dimerization and thus the release of heme[9,14,39]. Preventing the release of Hb dimers into the circulation does provide a greater therapeutic effect in terms of the microcirculation as arterioles and venules both have improved microcirculatory flow and subsides a greater amount of the vasoconstriction seen in the arterioles then just scavenging heme alone (PEG-ApoHb). This also corresponds to improved MAP and HR tendencies as there is less hypertension and less of a drop-in heart rate compared to baseline. Interestingly enough, the organ inflammatory and damage biomarkers show minimal improvement compared to PEG-ApoHb. This demonstrates that acute organ damage associated with Hb may be independent of the concentration of circulating Hb dimers or heme protein. Lastly, microcirculatory pressure associated with the functional capillary density was significantly improved compared to the control. This result can be correlated to the reduced vasoconstriction seen in the arterioles as the pressure in the branching capillaries remain high enough to keep them open. Ultimately, introducing PEG-ApoHb-Hp prior to the infusion of Hb proves to have superior results compared to all other experimental groups. PEG-ApoHb-Hp scavenges 30% of all Hb dimers and 42% of all heme. PEG-ApoHb-Hp subsides much of the vasoconstriction and improves microvascular flow, it prevents hypertension and a reduced heart rate, and the microcirculatory pressure is preserved even more compared to baseline. However,

similar results seen from in the Hp treatment, the use of PEG-ApoHb-Hp does not significantly better organ inflammation or damage when compared to PEG-ApoHb.

In conclusion, PEGlyation of ApoHb and conjugating it with Hp does hold its effectiveness in preventing Hb toxicities that are common in the vasculature particularly in the microcirculation. PEG-ApoHb-Hp scavenged 30% of Hb dimers and prevented/ scavenged the 42% of heme from circulating freely. This result proves an effective method to address the thermal instability of ApoHb once had without compromising its therapeutic ability.

6.6 ACKNOWLEDGEMENT

The authors thanks Cynthia Walser for the surgical preparation of the animals. The authors will also like to thank the National Institutes of Health (NIH) for funding through grants R01HL162120 and R01HL159862.

Chapter 6, in full, is being prepared for publication as it appears in "PEGylated Apo-Hemoglobin Haptoglobin can Effectively Limit Vascular Dysfunction in the Microcirculation and Prevent Acute Cardiac and Kidney Inflammation" by Carlos J. Munoz, Daniela Lucas, and Pedro Cabrales. The dissertation author was the primary investigator and author of this paper.

6.7 AUTHORSHIP

Contribution: C.J.M. performed experiments; C.J.M., D.L., and P.C., analyzed results and made the figures; C.J.M. and P.C. designed the research, and C.J.M. and D.L. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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7. Conclusion

The work presented in this dissertation was performed to achieve 4 specific aims, all with the overarching goal of establishing a safe and efficacious hemoglobin and heme scavenger using apohemoglobin and haptoglobin complex. The first specific aim assessed was achieving four times the molar scavenging of heme compared to hemopexin in vivo and establish the biocompatibility in a health animal model for the apoHb-Hp complex. The second specific aim was to establish efficacious pre-clinical data of the treatment of apoHb-Hp versus Hp in two specific hemoglobinopathies, β-Thalassemia and sickle cell disease. The third specific aim was establishing an efficacious use of apoHb-Hp to treat a hemoglobin-based oxygen carrier that is commonly known for its toxicity. Lastly, the last specific aim was to determine the safety and efficacy of the use of PEGlyation of apoHb when comparing it to apoHb without PEGlyation. This chapter briefly summarizes conclusions from each aim, suggests the strengths and limitations of the work, and briefly explores future work that can be derived from this dissertation.

7.1 SPECIFIC AIM 1 CONCLUSION

The present data suggests that the apoHb-Hp complex can effectively exchange apoHb for holoHb *in vitro* and *in vivo* similar to Hemopexin. Further, apoHb situated in the apoHb-Hp complex is an effective heme binding protein complex based on *in vitro* and *in vivo* heme-albumin transfer studies and *in vivo* microcirculatory experiments. These unique properties of the apoHb-Hp complex prevent adverse systemic and microvascular responses to Hb and heme-albumin exposure and introduces a novel therapeutic approach to facilitate simultaneous removal of extracellular Hb and heme.

7.2 SPECIFIC AIM 2 CONCLUSION

We have presented preclinical evaluation of apoHb-Hp for treatment of beta-thalassemia major in a mouse animal model. Our study demonstrates that treatment with apoHb-Hp over a 6-week period improved anemia and normalized erythrocyte turnover in thalassemia. Furthermore, we observed that absolute RBC count increased despite the lack of change in Hb and Hct, suggesting that apoHb-Hp treatment reversed extravascular microcytic anemia in thalassemia. These results demonstrate that apoHb-Hp has the potential to treat thalassemia without the need of serial transfusions or splenectomy, the current standard of care. Future directions would be to mechanistically evaluate the physiology of apoHb-Hp treatment, particularly with regard to suppression of splenic macrophage mediated extravascular hemolysis and the role of hepcidin. Additionally, future studies should investigate whether apoHb-Hp has the potential to mediate transfusion induced iron overload in this same population.

7.3 SPECIFIC AIM 3 CONCLUSION

This work demonstrates that ApoHb-Hp complex and Hp prevent microvasculature alterations in a SCD mice model challenged with a hypoxia-reoxygenation protocol. However, the ApoHb-Hp complex fared better and more consistently than Hp treatment alone indicating the vital role of scavenging both heme and Hb.

7.4 SPECIFIC AIM 4 CONCLUSION

In conclusion, the results of this study mark a significant leap forward in pursuing safer and more effective blood substitutes. The apoHb-Hp complex represents a beacon of hope, potentially rendering HBOCs a viable and dependable option for clinical applications. These findings not only broaden the horizons of research and treatment but also promise to mitigate the

risks associated with blood transfusions, ultimately leading to improved patient care and the preservation of lives.

7.5 SPECIFIC AIM 4 CONCLUSION

PEGlyation of ApoHb and conjugating it with Hp does hold its effectiveness in preventing Hb toxicities that are common in the vasculature particularly in the microcirculation. PEG-ApoHb-Hp scavenged 30% of Hb dimers and prevented/ scavenged the 42% of heme from circulating freely. This result proves an effective method to address the thermal instability of ApoHb once had without compromising its therapeutic ability.