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UNIVERSITY OF CALIFORNIA SAN DIEGO

Reducing the Dependence on Blood with Hemoglobin Based Oxygen Carriers

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

in

Bioengineering

by

Alexander Thomas Williams

Committee in charge:

Professor Pedro Cabrales, Chair
Professor Marcos Intaglietta
Professor Erik Kistler
Professor Frank Powell
Professor John Watson

2019

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University of California San Diego

2019

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ABSTRACT OF THE DISSERTATION

Reducing the Dependence on Blood with Hemoglobin Based Oxygen Carriers

by

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Doctor of Philosophy in Bioengineering

University of California San Diego, 2019

Professor Pedro Cabrales, Chair

Red blood cell (RBC) transfusions have been a life-saving procedure following trauma for decades. *Ex vivo* storage of RBCs is limited to 42 days, as the RBCs degrade during storage. Recent evidence suggests that transfusions of stored RBCs are associated with adverse events and increased mortality. Blood shortages are frequent and are expected to increase as the population ages. My research explored new methods to prolong *ex vivo* RBC storage, and alternatives to RBCs. A novel improvement to RBC storage is storage under anaerobic conditions to minimize oxidative stress. Anaerobically stored RBCs improved RBC recovery and resuscitation from

hemorrhagic shock compared to conventionally stored RBCs. However, anaerobic storage only slowed the degradation of RBCs, without further extending the RBC storage period. Therefore, we explored hemoglobin (Hb)-based O₂ carriers (HBOCs) as an alternative to RBCs. HBOCs are advantageous relative to blood, as they are shelf-stable, not limited by donors for supply, and do not require blood-type matching before infusion. Clinical trials of previous generations of HBOCs failed due to unforeseen side-effects. The next generation of HBOCs involves high molecular weight Polymerized Hb (PolyHb), which is the most promising and scalable HBOC formulation that alleviates the side effects of previous HBOCs. We compared fresh and stored PolyHb to RBCs in a hemorrhagic shock resuscitation model. The results confirm that PolyHb was as effective as fresh RBCs to recover from shock and significantly superior to stored RBCs, and that PolyHb's properties or efficacy did not change during long term storage. Finally, we assessed the safety of PolyHb in guinea pigs, as they are more human-relevant than other preclinical models. This study demonstrated that increasing the molecular size of PolyHb alleviates side-effects of previous HBOC formulations, and that PolyHb toxicity is transient. Future work will focus on the safety of PolyHb, particularly in vulnerable patient population.

CHAPTER 1: GENERAL INTRODUCTION

1.1: Trauma and Current Problems with Red Blood Cell Storage

Ten percent of deaths worldwide are due to trauma, which represents the third most common cause of death in the United States.¹ Apart from other causes of trauma death, hemorrhagic shock (HS) accounts for roughly half of trauma deaths,² and nearly 90% of potentially survivable battlefield deaths.³ HS rapidly decreases oxygen (O₂) carrying capacity and induces cardiovascular collapse, which prevents O₂ delivery to tissues and washout of metabolic waste, resulting in multiorgan failure if not corrected.⁴ In addition to achieving hemostasis, clinicians correct HS by infusing large volumes of red blood cells (RBCs). RBC transfusion restores O₂ carrying capacity and blood volume, which ensures hemodynamic stability.⁵ The accepted protocols of managing hemorrhagic shock are suggested by the Advanced Trauma Life Support® (ATLS®) guidelines.⁶ The ATLS® mandates evaluation of circulatory status, followed by control of hemorrhage and reversal of hypovolemia in patients presumed to be in HS.⁶ Independent of the fluid used to resuscitate from HS, the time to achieve hemodynamic stability and restore O₂ delivery determines the incidence of organ failure, and extent of HS sequelae.⁷ The ATLS advocates for a rapid fluid resuscitation strategy after surgical control of bleeding is achieved, and cross-matched RBCs transfusions are recommended.⁶

Despite widespread clinical use, transfusion of blood stored for extended periods of time has been shown to be correlated with higher levels of morbidity and mortality in transfusion recipients.⁸ As stored RBCs age, they undergo biochemical and biophysical changes, known collectively as the “storage lesion”.⁸ During extended *ex vivo* storage, a time-dependent series of physical and biochemical changes alter RBCs,⁸ and when transfused these RBCs with decreased deformability could theoretically be detrimental to the function of the microcirculation and prevent

O₂ delivery.⁹ Currently, the Food and Drug Administration (FDA) limits the RBC storage to 42 days in approved additive solutions, based on 24-hour recovery of RBCs and hemolysis measurements.¹⁰ However, the 24-hour recovery is a limited metric to measure the functionality of transfused RBCs, as it does not consider their contribution to hemodynamic stability, the ability of RBCs to deliver O₂, or any other response in the receiving subject.

In the United States, it is estimated that approximately 11 million units of whole blood/RBCs are transfused annually, while only 12 million units are collected.¹¹ As a result, shortages are frequent, and are expected to occur more often as the population ages.¹² These shortages, and the fear of transfusion-associated infections, transfusion related acute lung injury (TRALI), and other transfusion-related illnesses have led to increasingly restrictive transfusion approaches.¹³ There is no doubt that blood transfusions are a life-saving strategy; however, it has become evident that blood transfusion has immunomodulating effects that can increase the risk of nosocomial infections, cancer recurrence, and the possible development of autoimmune diseases later in life.¹⁴ Thus, transfusion-related adverse events, both short- and long-term, are among the costliest contributors to health care expenditures.¹⁵

Ten years ago, controversial retrospective studies raised concerns about the safety and efficacy of units stored longer than 2 weeks when transfused in certain categories of recipients.⁷ These observations have fostered recent randomized clinical trials addressing the potential issues associated with the age of blood, which provide overall reassuring evidence about the general safety and efficacy of current standards of practice.¹⁶ Recent clinical trials have also failed to show any difference between the fresh (less than a week old) and standard care blood (~ 3 weeks old).^{17,18} as they do not take into account the wide variability among stored RBCs units,¹⁹⁻²² which camouflages RBC age-related differences. Most of the evidence to date confirms that in-hospital

mortality rates do not differ for patients who receive the fresher blood during a transfusion compared to those who receive the older blood.⁸ On the other hand, compelling evidence has been collected by hundreds of studies showing that prolonged RBC storage negatively affects RBC physiology and functionality.²³ These observations have led to question whether the storage lesion could thus impair the efficacy of the transfusion therapy and likely mediate untoward transfusion-related events (e.g. transfusion-related acute lung injury [TRALI], transfusion-related immune modulation [TRIM]) or aggravate underlying conditions (e.g. sepsis).

1.2: New RBC Storage Methods

New additive solutions and storage methods are being explored to decrease storage lesions.²⁴ It has recently been shown that RBC units degrade at varying rates,¹⁹ possibly due to non-uniform Hb O₂ saturation at the beginning of storage.²⁵ Anaerobic storage RBCs has recently been proposed as a way to improve the uniformity and quality of RBC units by preserving RBC deformability, decreasing the extent of metHb formation and preserving metabolic homeostasis.²⁶ Additionally, anaerobic storage with a small amount of CO₂ has also been explored as a method of preserving ATP balance in anaerobically stored RBCs.²⁷ However, no *in vivo* studies have been performed to assess the functionality of anaerobically stored RBCs.

1.3: Alternatives to RBCs for Transfusion Medicine

Hemoglobin (Hb)-based O₂ carriers (HBOCs) have been in development as an alternative to blood for many years, with initial trials involving the transfusion of acellular Hb suspended in saline.²⁸ These trials were met with a number of tolerability concerns and a short intravascular half-life, which motivated research into modifying acellular Hb.²⁹ New methods of purifying and modifying acellular Hb improved intravascular retention and tolerability concerns in pre-clinical trials.³⁰ However, clinical trials of these HBOCs were terminated after Hb-

associated sequelae such as vasoconstriction, hypertension, and heart attacks were observed.^{31,32} Research has demonstrated that these sequelae stem from the presence of small molecular size Hb in the solution, which can extravasate, scavenge NO, and release heme.^{33,34} Further analysis revealed that the low molecular size Hb causes microvascular dysfunction, which is alleviated by increasing the molecular size of the HBOC transfused.^{35,36} The most scalable and cost-effective method of producing a high-molecular size HBOC is via the production of Polymerized Hb (PolyHb).

Given the heme-mediated production of reactive oxygen species, a model that mimics human anti-oxidant capacity and distribution is necessary for the evaluation of an HBOC's safety profile.²⁹ Pigs, dogs, mice, rats, and hamsters endogenously produce ascorbic acid (AA), the primary small-molecule reductant that regulates heme-mediated oxidative stress.³⁷ One potential candidate for preclinical testing of HBOCs is the guinea pig. Guinea pigs, like humans, lack functional hepatic L-gulonolactone oxidase (LGO), and are incapable of endogenous ascorbic acid (AA) production.³⁷ Humans, non-human primates, and guinea pigs interestingly have evolved in similar ways to distribute antioxidant capacity within the tissue and circulation.³⁷ As such, guinea pigs may be a more relevant small-animal model for assessing HBOC toxicity for human safety. The development of a human-safe HBOC is critical, as HBOCs can be stored under ambient environmental conditions without loss of O₂ carrying capacity, are not limited by blood type compatibility, and can be generated from products that are otherwise considered waste, such as expired blood units or bovine blood.³⁸ This results in a more flexible product that can be easily administered, and widely distributed for emergency preparedness.

1.4: Aims and Approaches

This dissertation proposed that the dependence on RBCs in fluid resuscitation from trauma can be alleviated with new RBC storage techniques, or alternatives to RBCs, such as PolyHb, that demonstrate efficacy and safety in human-relevant models. Three specific aims were proposed and investigated.

Aim 1: Develop a clinically relevant model of hemorrhagic shock resuscitation to assess the safety and efficacy of stored blood. Our objective is to study resuscitation from hemorrhagic shock with blood, which is considered the gold standard of care for hemorrhagic shock. Transfusions restore volume and O₂ carrying capacity, which favors the recovery of homeostasis during resuscitation. I developed a goal-based hemorrhagic shock resuscitation model in rats to evaluate the effectiveness of blood transfusions. My approach used rats instrumented with a miniaturized pressure-volume catheter placed in the left ventricle, which allowed us to assess systemic hemodynamics and cardiac function. The rats were subjected to a hemorrhage of 50% of animal's blood volume (BV), and resuscitated with fresh and stored blood until 90% of baseline mean arterial pressure was reached. This research aimed to expose the limits of resuscitation from hemorrhagic shock via blood transfusions, while simultaneously defining a model to evaluate the efficacy of PolyHb to be tested in aims 2 and 3.

Aim 2: Evaluate the efficacy of high molecular weight PolyHb to restore perfusion and oxygenation following hemorrhagic shock. My objective was to directly test the hypothesis that PolyHb with high molecular weight will allow for recovery of central hemodynamics and oxygenation without the negative side effects commonly associated with blood transfusions. Furthermore, my objective was to determine the biophysical properties of PolyHb that contribute to the recovery of these parameters following hemorrhagic shock. Blood transfusions restore

oxygen carrying capacity during hypovolemia, but fail to address the changes in central hemodynamics and cardiac perfusion that occur during resuscitation from hemorrhage. Like aim 1, animals were instrumented with a miniaturized pressure-volume catheter and subjected to 50% hemorrhage, and resuscitated to the achieve 90% of their baseline mean arterial pressure. This aim identified biophysical properties of PolyHb that minimize reperfusion injury and provide effective and stable restoration of systemic hemodynamics.

Aim 3: Analyze cardiac function in the presence of PolyHb in a model of limited anti-oxidant capacity. Incidences of myocardial injury due to oxidative stress in clinical trials of HBOCs slowed the commercialization and spread of HBOCs as an alternative to blood in transfusion medicine. In this aim, we tested the hypothesis that by controlling different parameters of PolyHb, such as size and O₂ affinity, we can attenuate cardiovascular dysfunction and organ damage. To complete this aim, we transfused healthy guinea pigs with PolyHb of varying size and molecular weight. To fully understand changes in cardiac function and evaluate any myocardial damage that occurred from the administration of the PolyHb solutions, animals were given up to 24 hours post-transfusion before changes in systemic blood pressure, left ventricular function, and markers of organ damage were assessed. In this aim, performed pharmacokinetic analysis of PolyHb and identified optimized PolyHb formulations that reduce toxicity and preserve cardiac function for further analysis.

1.5: Organization of the Text

This chapter provides a brief overview of trauma, the RBC storage lesion, and the impact of the RBC storage lesion on trauma resuscitation. We briefly discussed an alternative method of RBC storage that improves *in vitro* measures of RBC quality. Next, we established the need for alternatives to blood. A brief review of the clinical failure of previous generations of HBOCs was

provided to establish the rationale for testing high-molecular weight PolyHb for efficacy in an animal model of trauma, and for toxicity in a human-relevant animal model. We further discussed our study aims and briefly touched on the approaches to achieve these aims.

Chapter 2 describes the effect of anaerobic storage of RBCs on resuscitation from hemorrhagic shock to achieve Aim 1. This chapter first establishes that 3-week-old rat blood is similar to 35-day-old human blood, and that anaerobic storage improves parameters that are necessary for regulatory approval of anaerobic storage of human RBCs. Next, chapter 2 demonstrates that anaerobically stored RBCs improves resuscitation from hemorrhagic shock, requires a lower volume to maintain hemodynamic stability, and decreases markers of organ damage compared to conventional stored RBCs.

Chapters 3 and 4 describe the effects of PolyHb on cardiac function and organ damage after resuscitation from hemorrhagic shock to achieve Aim 2. Chapter 3 demonstrates that 2 year storage of PolyHb does not impair its efficacy in resuscitation from hemorrhagic shock. Furthermore, chapter 3 demonstrates that PolyHb is as efficacious as fresh blood in restoring systemic hemodynamic during resuscitation from hemorrhagic shock. Chapter 4 then demonstrates that the efficacy of PolyHb is highly dependent on the high solution viscosity, and that the O₂ carrying capacity is a secondary benefit of PolyHb transfusion.

Chapters 5 and 6 examine the toxicity of PolyHb in a healthy guinea pig model, as described in Aim 3. Chapter 5 establishes the importance of the purity of PolyHb, as related to the extent of hypertension and organ damage. Chapter 6 discusses the impact of PolyHb's quaternary state on changes to cardiac function. Chapter 6 also demonstrates the transience of organ damage following PolyHb transfusion.

Chapter 7 concludes the important findings of this dissertation. This chapter also discusses strengths, weaknesses, and future directions that stem from this dissertation.

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CHAPTER 2: TRANSFUSION OF ANAEROBICALLY OR CONVENTIONALLY STORED BLOOD AFTER HEMORRHAGIC SHOCK

2.1: Abstract

BACKGROUND: Resuscitation from hemorrhagic shock (HS) by blood transfusion restores oxygen (O₂) delivery and provides hemodynamic stability. Current regulations allow red blood cells (RBCs) to be stored and used for up to 42 days. During storage, RBCs undergo many structural and functional changes. These storage lesions have been associated with adverse events and increased mortality after transfusion, increasing the need for improved RBC storage protocols. This study evaluates the efficacy of anaerobically stored RBCs to resuscitate rats from severe HS compared to conventionally stored RBCs.

METHODS AND RESULTS: Rat RBCs were stored under anaerobic, anaerobic/hypercapnic, or conventional conditions for a period of 3 weeks. Hemorrhage was induced by controlled bleeding, shock was maintained for 30 minutes, and RBCs were transfused to restore and maintain blood pressure near the pre-hemorrhage level. All storage conditions met current regulatory 24-hour posttransfusion recovery requirements. Transfusion of anaerobically stored RBCs required significantly less RBC volume to restore and maintain hemodynamics. Anaerobic or anaerobic/hypercapnic RBCs restored hemodynamics better than conventionally stored RBCs. Resuscitation with conventionally stored RBCs impaired indices of left ventricular cardiac function, increased hypoxic tissue staining and inflammatory markers, and affected organ function compared to anaerobically stored RBCs.

CONCLUSIONS: Resuscitation from HS via transfusion of anaerobically stored RBCs recovered cardiac function, restored hemodynamic stability, and improved outcomes.

2.2: Introduction

Trauma accounts for 10% of deaths worldwide, and represents the third most common cause of death in the United States.^{1,2} Excluding other causes of trauma, like traumatic brain injury, sepsis, and multiple organ failure, hemorrhagic shock (HS) from exsanguination accounts for roughly half of trauma deaths.³ HS decreases oxygen (O₂) carrying capacity and induces cardiovascular collapse, limiting O₂ delivery to tissues and the washout of metabolic waste, and can result in multiorgan failure if not corrected in time.⁴⁻⁶ In addition to stopping the bleeding, severe HS is frequently corrected by infusing large volumes of red blood cells (RBCs) to restore volume, O₂ carrying capacity, and to recover blood flow and hemodynamic stability.⁷

Transfusion-associated infections, transfusion related acute lung injury (TRALI), and acellular hemoglobin (Hb) toxicity have been partly responsible for increasingly restrictive transfusion approaches.⁸⁻¹⁰ Although there is no debate about the need for blood transfusions, as blood products remain a vital resource for clinical care, transfusion of stored RBCs is associated with adverse events and increased mortality.¹¹ During storage RBCs undergo many physical, functional, and morphological changes, known as “storage lesions”.¹²⁻¹⁴ The Food and Drug Administration (FDA) limits the RBC storage to 42 days in approved additive solutions, based on 24-hour posttransfusion recovery of RBCs and hemolysis measurements.¹⁵ The 24-hour posttransfusion recovery is a limited metric of functionality of transfused RBCs, as it only considers the number of surviving RBCs after 24-hours in healthy volunteers, and it does not consider the hemodynamic stability, O₂ delivery, or any other response in the transfusion recipient. Transfusion-related adverse short- and long-term events, including infection, stroke, and multiorgan failure, among others, are among the costliest contributors to health care expenditures, accounting for up to \$64 billion per year in the US alone.^{16,17}

New additive solutions have been shown to improve in vitro quality of stored RBCs, and new methods are being explored to decrease storage lesions.¹⁸⁻²⁰ Recent studies show that the quality of RBCs degrade at varying rates,^{21,22} possibly due to non-uniform Hb O₂ saturation during storage.²³ Anaerobic storage of blood has been proposed to improve the quality and uniformity of RBC units by decreasing oxidative changes occurring during storage (RBC deformability, metHb formation, and metabolic changes, such as pentose phosphate pathway upregulation).^{24,25} Additionally, anaerobic storage with a small amount of CO₂ has also been explored as a method of preserving ATP balance in stored RBCs.²⁶

No *in vivo* study has been performed before to assess the functionality of anaerobically stored RBCs. This study aims to evaluate the implications of goal-directed resuscitation from severe HS with anaerobically stored RBCs. In efforts to translate these results from rats to humans, we chose to use the 24-hour posttransfusion recovery of stored RBCs as the parameter to determine the age of the rat RBCs transfused. A goal-directed resuscitation with aggressive administration of RBCs is a more clinically relevant model, as animals are transfused until a set central hemodynamic goal is met based on mean arterial pressure (MAP) rather than transfusing an arbitrary infusion volume, according to the Advanced Trauma Life Support® guidelines based on the recommendations of the American College of Surgeons²⁷.

2.3: Methods and Materials

Animal preparation. Studies were performed in male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 200-250 g. Animal handling and care followed the NIH Guide for Care and Use of Laboratory Animals. The experimental protocol was approved by the UCSD Institutional Animal Care and Use Committee. Animals were anesthetized with isoflurane in compressed room air (Drägerwerk AG, Lübeck, Germany) and placed on a heating pad to

maintain core body temperature at 37 °C for the duration of the experiment (**Figure 2.1A**. For more details see : Supplemental Methods).

Cardiac function. A 2F pressure-volume (PV) conductance catheter (SPR-858, Millar Instruments, TX) was inserted into the left ventricle (LV) using the closed chested method and the signal acquired continuously (MPVS300, Millar Instruments, Houston, TX, and PowerLab 8/30, AD Instruments, Colorado Springs, CO).²⁸ For the closed chest method, the right carotid artery is isolated with blunt dissection, and the PV catheter is advanced through the aorta until a PV loop is acquired.

Cardiac pressure-volume indices. Cardiac function parameters were calculated from 15 to 20 cardiac cycles at each time point. Stroke volume (SV), stroke work (SW), cardiac output (CO), ejection fraction (EF), cardiac contractility ($dP/dt/V_{ed}$), and arterial elastance (E_a) were directly calculated in the PowerLab software. Systemic vascular resistance (SVR) was calculated as: $SVR = \frac{MAP}{CO}$. The internal energy utilization (IEU) was used as a measure of internal metabolism of the LV²⁹ and was calculated as $IEU = ([V_{es} - V_0] * P_{es})/2$.

Hemorrhagic shock resuscitation protocol. Anesthetized rats were hemorrhaged by withdrawing 50% of the animal's blood volume (BV, estimated as 7% body weight) via the femoral artery catheter over 30 minutes. Hypovolemia was maintained for 30 minutes before resuscitation. Resuscitation was implemented by infusing the 3-week-old stored blood through the jugular vein catheter at a rate of 300 μ L/min, until the animal reached 90% baseline MAP. If the animal's MAP fell below 80% baseline MAP, additional blood was infused (**Figure 2.1B**). The total resuscitation time did not exceed 1 hour.

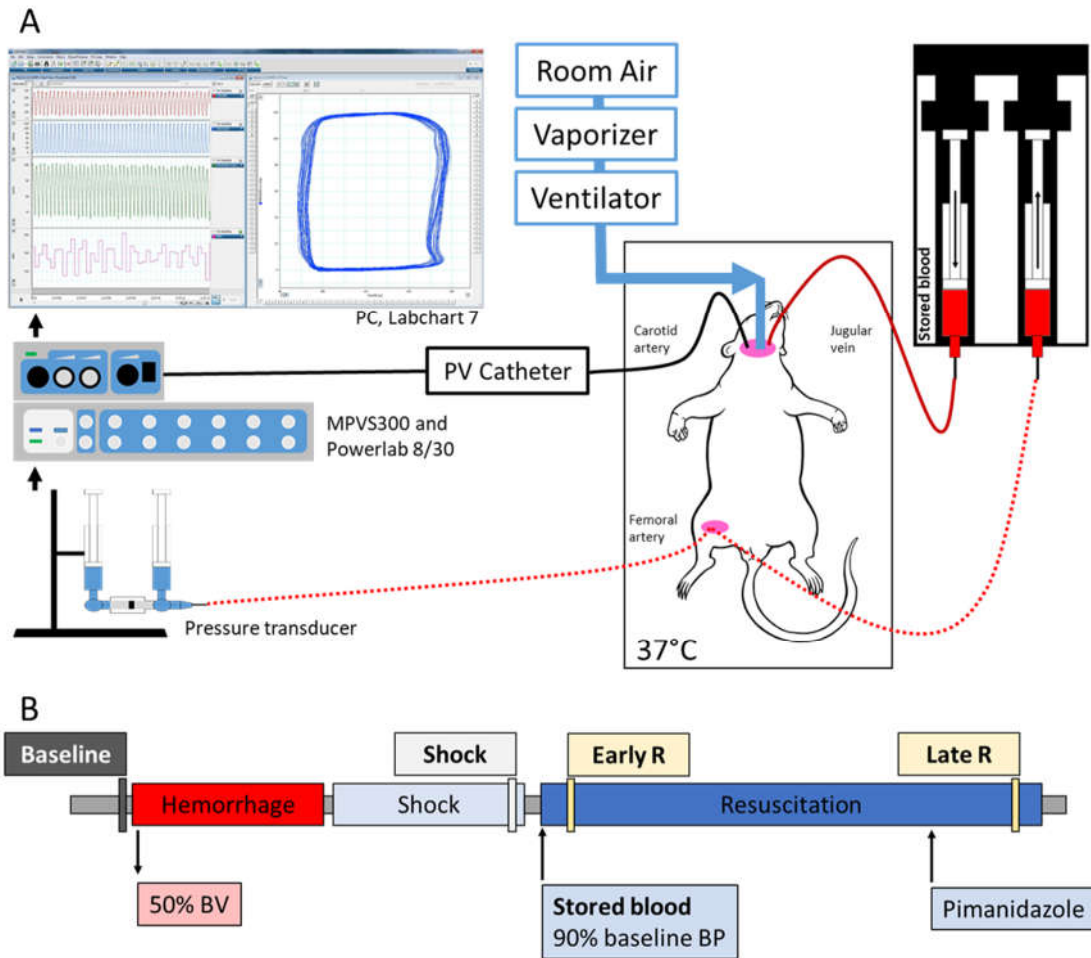


Figure 2.1: Experimental setup. (A) Rats were instrumented with catheters in the left femoral artery and left jugular vein, a tracheal cannula, and a miniaturized pressure-volume catheter introduced through the right carotid artery. (B) Animals were hemorrhaged for half their blood volume, held in shock for 30 minutes, and then transfused to a goal blood pressure. Resuscitation phase was 1 hour long, and pimonidazole (stain for hypoxic tissues) was injected 15 minutes before the end of the resuscitation period.

Blood collection and preparation. Briefly, male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 300-450g were anesthetized with a ketamine/xylazine cocktail, and a femoral artery catheter was implanted. Animals bled freely into CP2D (taken from AS-3 blood preparation kit, Haemonetics Corporation, Braintree, MA), and final CP2D concentration as adjusted to the manufacturer recommended concentration, and was pooled. Pooled blood was then centrifuged and the supernatant was removed. Finally, AS-3 was added to the manufacturer's recommended concentration, and units were passed through a neonatal leukocyte reduction filter (Haemonetics Corporation, Braintree, MA). RBC units intended for

conventional storage (**Conventional**) were stored at 4°C. Units for anaerobic storage (**Anaerobic**) and Anaerobic + CO₂ storage (**AN+CO₂**) were deoxygenated by filling the storage bag with nitrogen (or 5% CO₂, balance nitrogen), and mixing by rotation at approximately 30 RPM and then stored at 4°C. All blood units were gently mixed and brought to room temperature before infusion.

24-hour recovery. Donor rat blood from each group was radiolabeled with Technetium-99 (Tc⁹⁹) after 3 and 4 weeks of storage as described by Zink et. al.³⁰. 200 µL of Tc⁹⁹ radiolabeled blood (approximately 2% of blood volume) was delivered I.V. to male Sprague-Dawley rats (n=2 per pool, per storage condition) and samples were drawn at 5 minutes and 24 hours post-injection via tail clip. Samples were all run for radioactivity on a Cobra II gamma counter (Packard Instrument Co., Meriden, CT) at the same time so counts reported are independent of sample time.

UHPLC-MS metabolomics. Metabolomics analyses were performed on rat plasma at baseline and after transfusion of 3-week old conventionally or anaerobically stored blood. Detailed descriptions of the metabolomics extraction protocols and analyses have been recently published.^{31,32}

Statistical analysis. Results are presented as median with 95% confidence interval of the median. The Grubbs' method was used to assess closeness for all measured parameters at baseline and shock. Sample size was calculated using an α of 0.05 and a 1- β of 0.9, resulting in a minimum acceptable sample size of 5 per group. Additional animals were included due to the complexity of the experimental setup. Statistically significant changes between solutions and time points were analyzed using two-way analysis of variance (ANOVA), followed by *post hoc* analyses using Tukey's multiple comparisons test when appropriate. All statistics were calculated using GraphPad Prism 6 (GraphPad, San Diego, CA). Results were considered statistically significant if $P < 0.05$.

2.4: Results

Stored RBC characterization. In vitro analysis of stored RBCs was performed in 2 pools of blood, split 3 ways each. Hemolysis during storage occurred at similar rates. After the 4th week of storage the anaerobic RBCs (Anaerobic, AN+CO₂) had significantly higher hemolysis compared to conventional storage (**Fig. 2A**). ATP decreased for all methods, but after the 4th week of storage AN+CO₂ and anaerobic RBCs had 51% and 34% higher ATP than conventionally stored RBCs, respectively (**Figure 2.2B**). After one week of storage, anaerobic RBCs had 81% higher 2,3 DPG ($P<0.05$) than conventional or AN+CO₂, but 2,3 DPG was depleted by week 2 of storage (**Figure 2.2C**). The viscosity of all units increased during storage, and after 4 weeks storage, conventional RBCs had significantly higher viscosity than anaerobically stored RBCs (**Figure 2.3B**). At 3 weeks of storage, anaerobic RBC units had significantly higher lactate (31.0 [CI: 26.2-35.8 mmol/L]) compared to conventional (22.0 mmol/L [CI: 20.5-23.4 mmol/L]) or AN+CO₂ units (24.0 mmol/L [CI: 22.6-25.4 mmol/L], respectively).

After 3 weeks of storage, 24-hour posttransfusion recovery was >75% for all RBCs (n=4 per storage condition). AN+CO₂ RBCs showed statistically significantly higher 24-hour posttransfusion recovery than conventional RBCs. After 4 weeks of storage, 24-hour posttransfusion recovery was below 75% for conventionally stored RBCs, while recovery of anaerobically stored RBCs was above 75% (**Figure 2.3C**). No significant correlation was observed between the 24-hour posttransfusion recovery and the ATP, 2,3 DPG, hemolysis, or viscosity. Hemorrhagic shock and resuscitation studies were completed with RBCs stored for 3 weeks, as the 4 weeks 24-hour posttransfusion recovery was below FDA requirements (75% recovery).

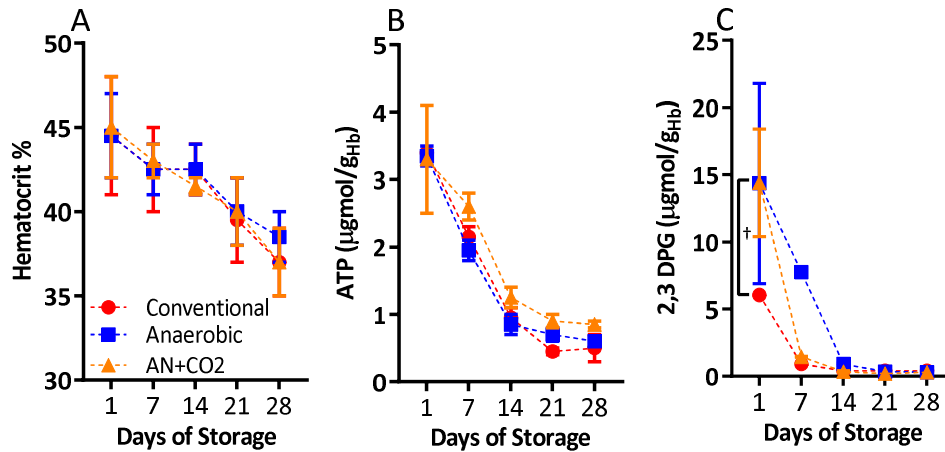


Figure 2.2: Additional parameters of the stored blood over time. (A) Hematocrit decreases with storage time due to hemolysis. (B) ATP decreases during storage. (C) 2,3 DPG decreases with storage but starts elevated for anaerobically stored units. †P<0.05.

In vivo studies. A total of 19 animals were entered into the hemorrhagic shock/resuscitation study. The animals were randomly assigned to the following groups: Conventional (n=6), Anaerobic (n=7), and AN+CO₂ (n=6). All animals survived the protocol. All groups were similar at baseline determined by Grubb's method.

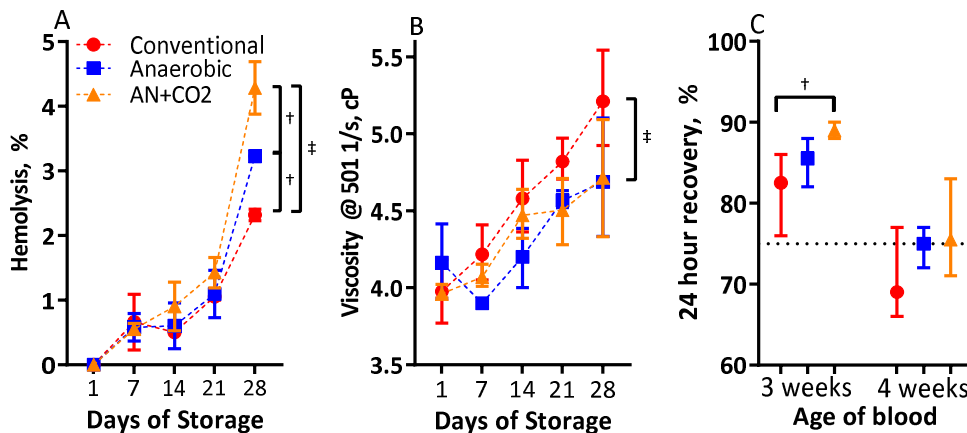


Figure 2.3: Properties of stored rat RBCs change during storage (2 pools of blood, split 3 ways each). (A) Hemolysis relative to day 1 – hemolysis increased during storage (n = 2 per group). (B) Viscosity (measured at 500 s⁻¹) increased for all units during storage (n = 4 per group). (C) Storage time decreases 24-hour recovery of Tc99 radiolabeled RBCs (n = 4 per group); At 3 weeks of storage, all units showed >75% 24-hour recovery. At 4 weeks of storage, 24-hour recovery decreased from 3 weeks of storage. Data are presented as median ± 95% confidence interval. †P<0.05, ‡P<0.01.

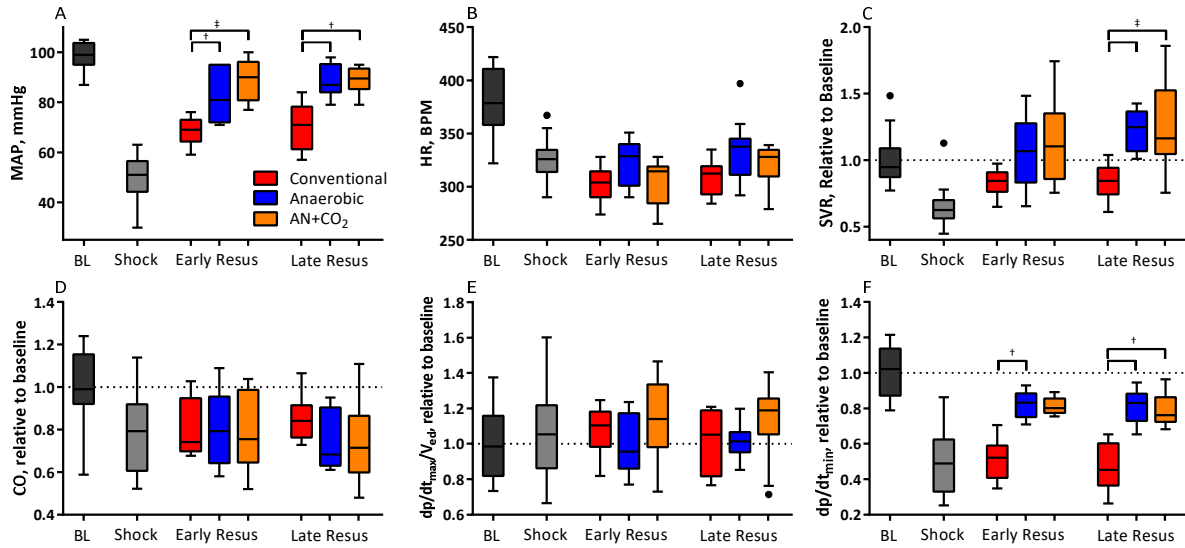


Figure 2.4: Hemodynamics and cardiac function during the hemorrhagic shock/resuscitation protocol. (A) Mean arterial pressure was not restored after transfusion of conventionally stored cells. (B) Heart rate decreased during shock and stayed depressed through the remainder of the protocol. (C) Systemic vascular resistance increased after transfusion of anaerobic and AN+CO₂ stored cells. (D) Cardiac output did not increase after resuscitation. (E) Isovolemic contraction normalized to end diastolic volume shows that preload independent contraction increases after hemorrhagic shock. (F) Isovolemic relaxation was slowed during hemorrhagic shock and transfusion of anaerobically stored blood restored isovolemic relaxation rate better than conventionally stored blood. †P<0.05, ‡P<0.01.

Systemic hemodynamics. All animals had similar MAP at baseline and at the end of the shock period (**Figure 2.4A**). Early in resuscitation, when similar volumes of stored blood had been given to all animals, MAP was significantly higher for animals that received anaerobically stored RBCs than conventionally stored RBCs. Despite continued infusion, infusion of conventionally stored RBCs did not restore MAP, while anaerobically stored RBCs restored MAP to near baseline. Hemorrhage induced bradycardia in all animals (**Figure 2.4B**) and transfusion did not revert heart rate changes in any of the groups. However, conventionally stored RBCs resulted in more severe bradycardia. SVR decreased during shock (**Figure 2.4C**). Conventionally stored RBCs was not able to restore SVR to baseline levels, while anaerobically stored RBCs resulted in increased SVR compared to conventionally stored RBCs.

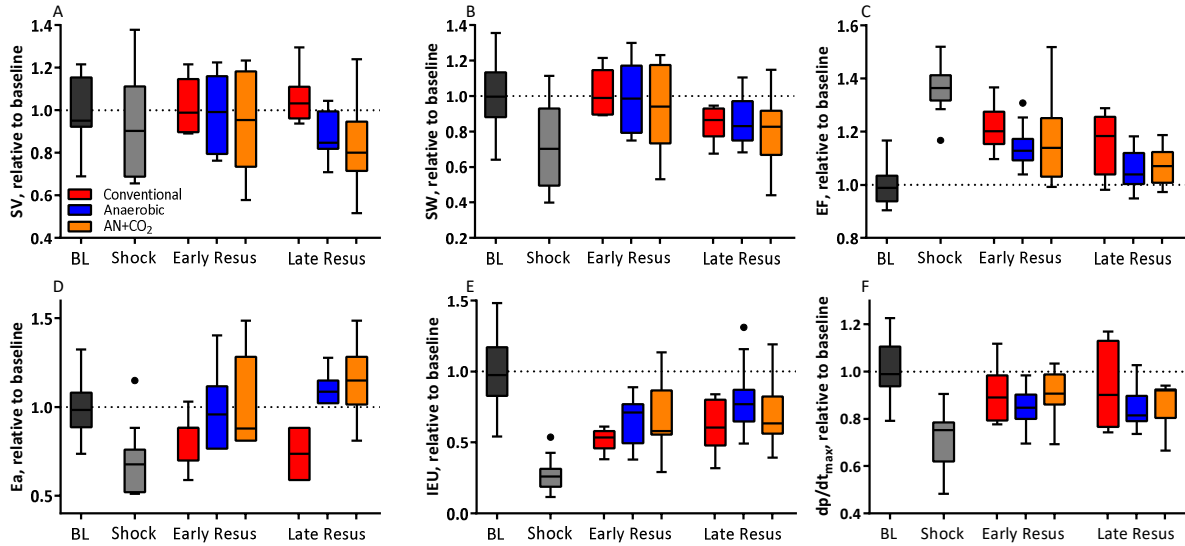


Figure 2.5: Additional cardiac parameters. (A) Transfusion increases stroke volume, and stroke volume stays elevated in animals transfused with conventionally stored blood. (B) Stroke work increases shortly after transfusion but does not remain elevated. (C) Ejection fraction increases during shock and stays elevated post-resuscitation. Ejection fraction remains slightly higher for animals transfused with conventionally stored blood. (D) Arterial elastance improves in animals transfused with anaerobically stored blood. (E) Conventionally stored blood slightly impairs internal energy utilization post-resuscitation. (F) Isovolumic contraction rate decreases during shock and is partially restored with transfusion of any condition.

Cardiac function. SV decreased after the hemorrhage and recovered with transfusion. As a result of the goal-based resuscitation, cardiac function during resuscitation was similar between groups (**Figure 2.5A**). Conventionally stored RBCs maintained SV after resuscitation compared to baseline, while anaerobically stored RBCs slightly decreased SV compared to conventional storage (**Figure 2.5A**). CO decreased during shock, and increased after transfusion, although baseline CO was not achieved during resuscitation (**Figure 2.4D**). Preload-independent cardiac contractility (dp/dt_{max} normalized to end diastolic volume, ie $dp/dt_{max}/V_{ed}$) increased during shock and remained elevated for the duration of the protocol (**Figure 2.4E**). Isovolumic relaxation rate (dp/dt_{min}) decreased with shock and did not recover with conventionally stored RBCs, while it recovered with anaerobically stored RBCs (**Figure 2.4F**).

Table 2.1: Hematology of rats during the hemorrhagic shock/resuscitation protocol. Data are presented as median with 95% confidence interval. †P<0.05 vs conventional. ‡P<0.01 vs conventional.

Time point	Group	Hct, %	Hb, g/dL	pHb, g/dL
BL		43 [42-43]	13.8 [13.4-14.0]	
Shock		28 [27-29]	8.9 [8.5 - 9.4]	
Early Resus	Conventional	40 [36-42]	13.1 [11.8-14]	0.1 [0.0-0.1]
	Anaerobic	†35 [33-37]	‡11.9 [10.6-11.9]	0.1 [0.0-0.2]
	AN+CO₂	37 [35-39]	12.6 [12.1-13.3]	0.1 [0.1-0.2]
Late Resus	Conventional	54 [42-57]	18.4 [15.1-18.5]	0.4 [0.2-0.4]
	Anaerobic	‡39 [35-46]	‡13.5 [11.1-16.4]	‡0.1 [0.0-0.1]
	AN+CO₂	‡40 [35-42]	‡13.7 [11.6-15.0]	‡0.1 [0.0-0.1]

Hematology. Hemorrhage reduced hematocrit to a similar level in all groups (**Table 2.1**). Following the goal-based protocol, animals were transfused until MAP reached 90% of the baseline MAP. A significantly higher volume of conventionally stored RBCs was transfused in attempts to reach this blood pressure goal (**Figure 2.6**). At the end of the observation period, animals transfused with anaerobically stored RBCs required half the volume of those transfused with conventionally stored RBCs. This corresponded to an infusion of 58% [CI: 46%-70%], 29% [CI: 17%-42%], and 27% [CI: 17%-38%] of the baseline BV for conventional, anaerobic, and AN+CO₂, respectively. Therefore, animals receiving conventionally stored RBCs had significantly higher hematocrit and plasma Hb concentration at the end of resuscitation (**Table 2.1**).

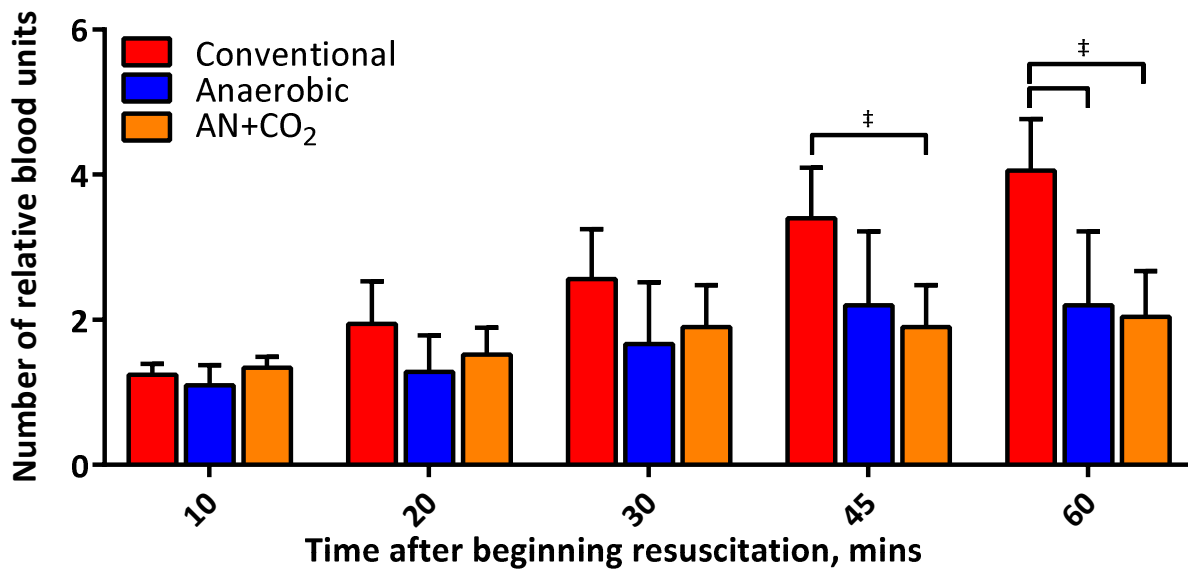


Figure 2.6: Volume of blood (in relative number of blood units) delivered to the animals throughout the protocol. Significantly more conventionally stored blood than anaerobically stored blood was transfused while attempting to reach the hemodynamic goal. †P<0.05. ‡P<0.01.

Blood chemistry. Shock induced systemic acidosis for all groups, and transfusion of conventionally stored RBCs worsened the acidosis (**Table 2.2**). Anaerobic and AN+CO₂ RBCs restored blood pH by the end of the experimental protocol. Potassium was significantly lower after transfusion of anaerobic and AN+CO₂ stored RBCs compared to conventionally stored RBCs. Calcium was significantly higher after transfusion of anaerobically stored RBCs than conventionally stored RBCs. Shock increased glucose levels, which remained elevated during resuscitation. Lactate also increased during shock. Resuscitation with conventionally stored RBCs initially increased lactate levels, while anaerobically stored RBCs slightly decreased lactate levels (**Figure 2.7A**). Lactate levels were elevated at the end of resuscitation. Animals transfused with conventionally stored RBCs had higher lactate compared to animals transfused with anaerobically stored RBCs. Metabolic reprogramming secondary to hemorrhagic shock has been extensively described in humans and animal models.^{31,33} Shock promoted significant metabolic changes (see

Supplemental Data Sets). Anaerobically stored RBCs was able to correct the metabolic derangements observed during shock compared to conventionally stored RBCs (**Figure 2.7**).

Table 2.2: Blood gasses and chemistry of rats during hemorrhagic shock/resuscitation protocol. Data are presented as median with 95% confidence interval. †P<0.05 vs conventional. ‡P<0.01 vs conventional. §P<0.05 vs anaerobic. ¶P<0.01 vs anaerobic.

		Conventional	Anaerobic	AN+CO ₂
pH	BL		7.43 [7.39-7.47]	
	Shock		7.25 [7.15-7.28]	
	Early Resus	7.05 [6.96-7.18]	‡7.23 [7.09-7.36]	‡7.25 [7.13-7.31]
	Late Resus	7.13 [6.97-7.32]	‡7.36 [7.27-7.41]	‡7.37 [7.33-7.42]
PCO₂, mmHg	BL		38.7 [35.7-44.0]	
	Shock		30.2 [27.8-33.1]	
	Early Resus	36.8 [32.8-43.7]	36.9 [34.1-64.8]	33.7 [31.4-39.7]
	Late Resus	40.4 [37.0-48.8]	41.2 [34.3-53.5]	37.9 [31.2-40.4]
PO₂, mmHg	BL		93.8 [87.0-101.0]	
	Shock		118.0 [106.0-128.0]	
	Early Resus	91.9 [87.5-96.3]	90.2 [69.0-108.0]	†¶109.0 [102.0-125.0]
	Late Resus	56.6 [44.0-68.1]	‡82.4 [65.9-89.7]	‡89.7 [78.9-114.0]
Glucose, mg/dL	BL		199 [176-230]	
	Shock		450 [328-556]	
	Early Resus	415 [267-450]	392 [344-577]	423 [170-529]
	Late Resus	315 [186-330]	294 [221-459]	350 [183-459]
K⁺, mEq/L	BL		4.3 [4.1-4.7]	
	Shock		5.1 [4.6-5.7]	
	Early Resus	6.0 [5.5-6.3]	‡5.2 [4.8-5.5]	‡4.5 [4.3-5.2]
	Late Resus	6.4 [5.4-7.1]	‡4.8 [4.6-5.3]	‡4.7 [3.9-5.1]
Na⁺, mEq/L	BL		138 [136-139]	
	Shock		134 [133-136]	
	Early Resus	136 [135-140]	134 [130-136]	136 [133-140]
	Late Resus	137 [136-141]	137 [134-139]	137 [134-138]
Ca²⁺, mEq/L	BL		2.03 [1.80-2.19]	
	Shock		2.36 [2.11-2.48]	
	Early Resus	1.94 [1.83-1.97]	1.95 [1.68-2.14]	2.12 [1.8-2.23]
	Late Resus	1.64 [1.57-2.00]	†2.01 [1.87-2.43]	†1.89 [1.80-2.35]
Cl⁻, mEq/L	BL		102 [101-103]	
	Shock		100 [99-104]	
	Early Resus	101 [99-103]	100 [95-103]	100 [98-103]
	Late Resus	103 [99-107]	103 [99-107]	102 [100-105]

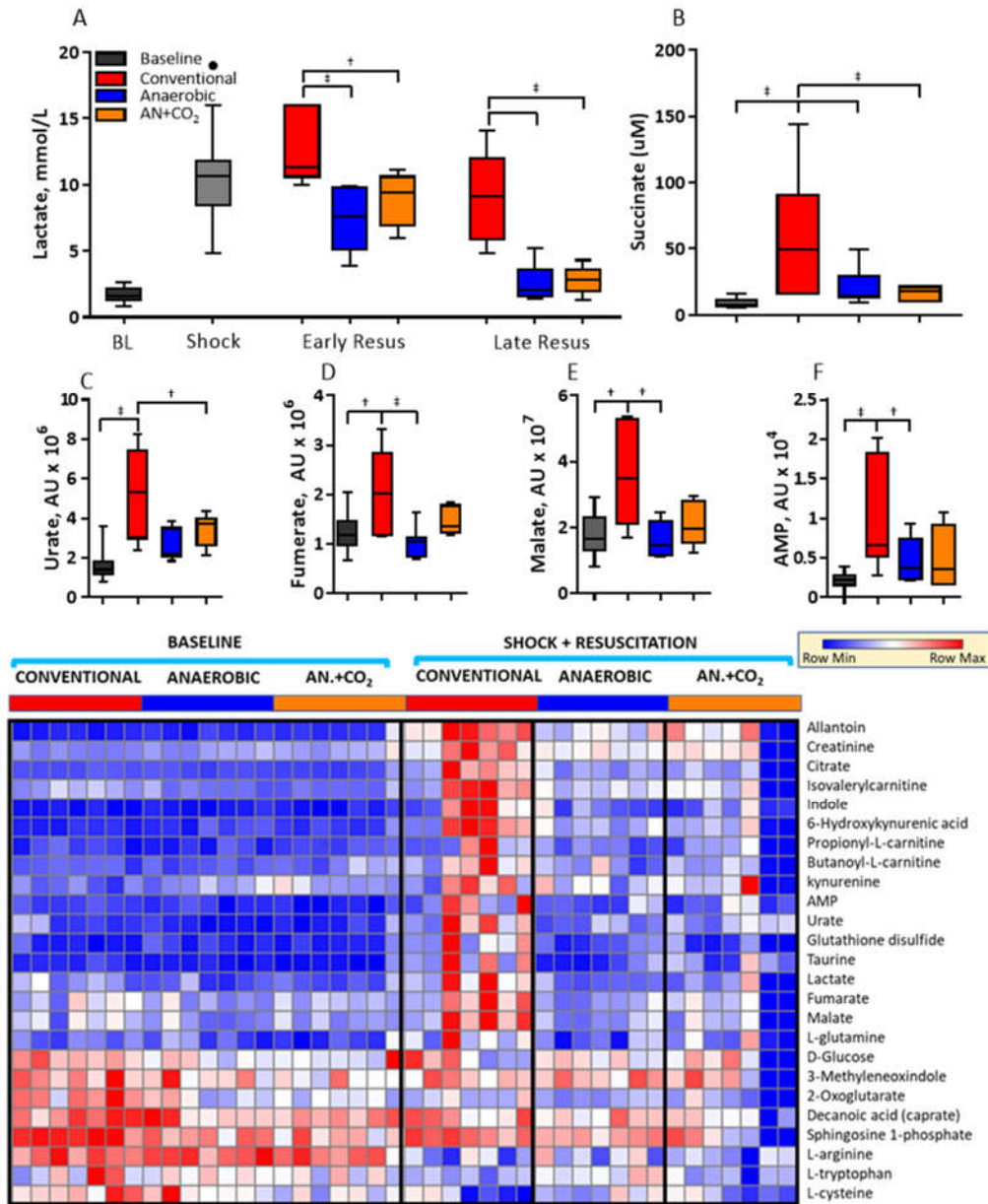


Figure 2.7: Blood chemistry and metabolomics during the hemorrhagic shock/resuscitation protocol. (A) Lactate increased during shock and increased further upon transfusion of conventionally stored cells. (B) Succinate was significantly higher for animal transfused with conventionally stored cells than anaerobically stored cells. (C), (D), (E), (F) Concentration of urate, fumarate, malate, and AMP in the plasma at baseline and the end of resuscitation for each group. (G) Heatmap of metabolites. †P<0.05, ‡P<0.01.

Oxygen delivery. Shock increased arterial Hb O₂ saturation. Resuscitation resulted in a decrease in arterial O₂ saturation. Conventionally stored RBC transfusion resulted in a dose-dependent decrease in arterial O₂ saturation (Figure 2.8A). Oxygen delivery (DO₂) decreased during shock and was partially restored upon transfusion in all experimental groups (Figure 2.8B).

Despite a decreased arterial O₂ saturation, conventionally stored RBCs restored DO₂ due to an increase in O₂ carrying capacity (hematocrit and Hb). No difference in DO₂ normalized by Hb was observed among resuscitation groups (**Figure 2.8C**). The ratio of DO₂ to oxygen consumption (DO₂/VO₂) increased after transfusion and remained increased by the end of protocol (**Figure 2.8D**).

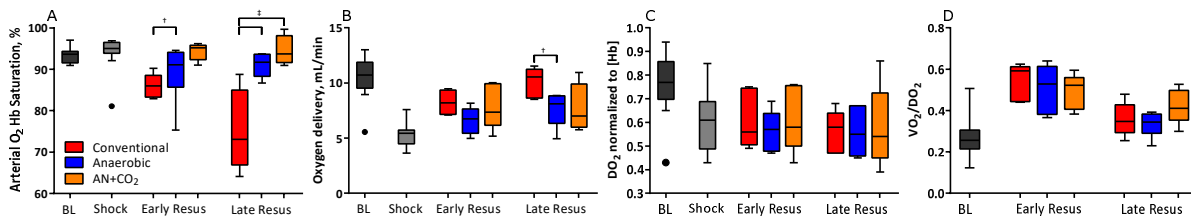


Figure 2.8: Oxygen delivery during the hemorrhagic shock/resuscitation. (A) Arterial Hb O₂ saturation decreased upon administration of conventionally stored cells, but not anaerobically stored cells. (B) Oxygen delivery increased after transfusion of stored RBCs. (C) When normalized to Hb content, there are no differences in oxygen delivery among groups. (D) A higher ratio of oxygen was extracted after resuscitation. †P<0.05, ‡P<0.01.

Organ damage, function, and hypoxia. Markers of organ injury, function, and inflammation are displayed in **Figure 2.9A-J**. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT), markers of liver damage, were elevated in animals transfused with conventionally stored RBCs compared to anaerobically stored RBCs. Serum creatinine and blood urea nitrogen (BUN), markers of kidney function, were slightly higher in the animals transfused with conventionally stored RBCs compared to anaerobically stored RBCs, but there were no significant differences in serum creatinine. Urinary neutrophil gelatinase-associated lipocalin (U-NGAL), a marker of acute kidney damage, was also elevated in animals given conventionally stored RBCs compared to anaerobically stored RBCs. CXCL1, a neutrophil activating protein was elevated in the liver, spleen, and lung for animals receiving conventionally stored RBCs compared to anaerobically stored RBCs. Other markers of inflammation, such as % of CD45⁺ neutrophils and interleukin-6 concentration, were also elevated in response to transfusion of conventionally stored RBCs compared to anaerobically stored RBCs. Organ

hypoxia, as measured by pimonidazole staining, is displayed in **Figure 2.9K**. All organs other than the kidney showed higher levels of hypoxia in animals given conventionally stored RBCs compared to anaerobic stored RBCs.

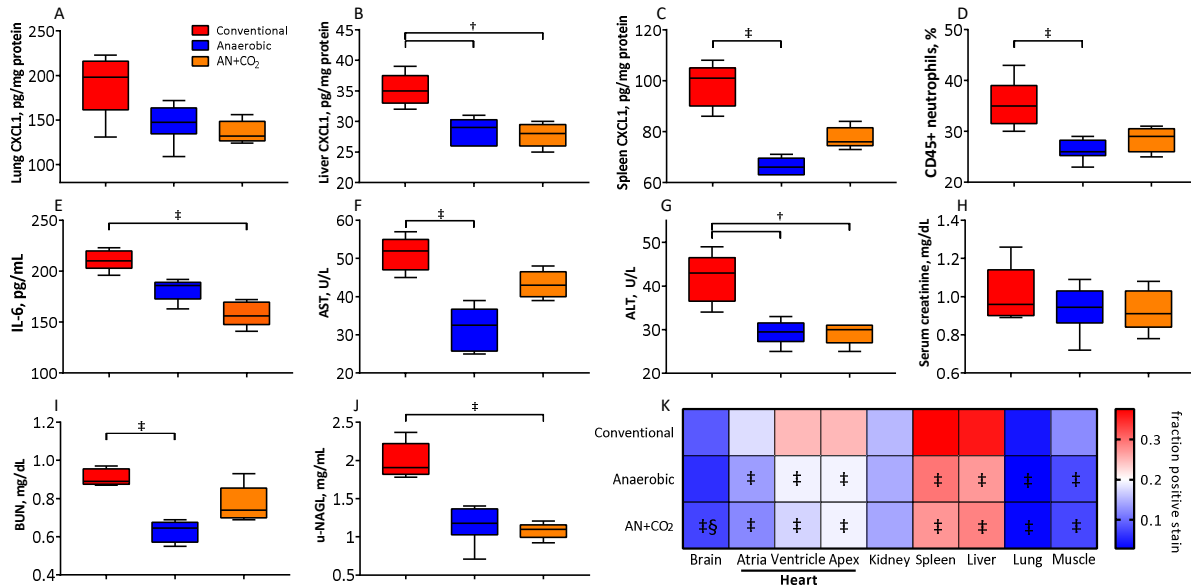


Figure 2.9: Markers of organ damage, function, inflammation, and hypoxia postmortem. All hemorrhaged animals showed significant organ damage, function, and inflammation relative to sham controls (not shown). ((A), (B), and (C)), are markers of neutrophil activation in individual organs. (D) Percentage of CD45+ neutrophils. (E) Interleukin-6 levels. (F) Aspartate aminotransferase (AST) and (G) Alanine aminotransferase (ALT) are markers of liver damage. (H) and (I) are markers of kidney function. (J) Urinary neutrophil gelatinase-associated lipocalin (u-NGAL) is a marker of acute kidney damage. (K) Organ hypoxia was quantified via pimonidazole staining 15 minutes before sacrifice and are expressed as a fraction of positive staining. †P<0.05 vs conventional, ‡P<0.01 vs conventional, §P<0.05 vs anaerobic.

2.5: Discussion

The principal finding of this study is that transfusion of stored RBCs under anaerobic and anaerobic/hypercapnic conditions after severe hemorrhage required lower transfusion volumes compared to conventionally stored RBCs. Anaerobically stored RBCs restored O₂ delivery and cardiac function, while improving systemic hemodynamics, and metabolism compared to transfusion of conventionally stored RBCs. Furthermore, transfusion of RBCs stored in anaerobic conditions produced a significantly smaller degree of organ and tissue damage compared to

transfusion with conventionally stored RBCs to arrive to the same clinically relevant hemodynamic goal.

RBCs stored under anaerobic and anaerobic/hypercapnia conditions were different in terms of blood pH, but no differences in pH were observed after transfusion. Conventionally stored RBCs did not fully achieve the resuscitation goals in all cases, whereas anaerobically stored RBCs met the resuscitation goals in all animals. Anaerobically stored RBCs reduced the volume necessary to resuscitate from HS compared to conventionally stored RBCs. Anaerobically stored RBCs restored MAP and vascular resistance compared to conventionally stored RBCs. Additionally, anaerobically stored RBCs decreased lactate levels, reduced the extent of the organ hypoxia, preserved organ function, and prevented vital organ injury relative to conventionally stored RBCs. Lastly, results indicate that the lack of O₂ in during storage of anaerobically stored RBCs did not have a negative impact in oxygen delivery and that preoxygenation of anaerobically stored RBCs before transfusion was not necessary.

In this study's protocol, the decision to administer additional volume was not based on transfusion triggers (e.g. Hb concentration), but on a hemodynamic goal (blood pressure). Thus, the volume transfused was defined by extent of injury due to the hemorrhagic shock, and the response of the cardiovascular system to the transfusion. Systemic hemodynamic parameters, such as MAP and HR, reflect the adherence to the experimental protocol. However, conventionally stored RBCs did not effectively restore MAP, suggesting that these RBCs produced a negative cardiovascular response compared to anaerobically stored RBCs, since shock was identical for all groups. Indices of cardiac function primarily dependent on blood volume (e.g. SW, CO, and dp/dt_{max}) improved after resuscitation independent of the storage condition. However, cardiac parameters dependent on active pressure generation, such as SVR and IEU, were corrected only

with anaerobically stored RBCs. The inability of conventionally stored RBCs to recover MAP after resuscitation is likely a consequence of differences in myocardial oxygen delivery and/or the low arterial O₂ saturation compared to transfusion of anaerobically stored RBCs. Although the recovery in MAP with anaerobically stored RBCs was due to an increase in SVR, no obvious vasoactive stimulus (such as hemolysis or increased blood viscosity) was associated with anaerobically stored RBCs (as hemolysis and blood viscosity were lower for anaerobically stored RBCs compared to conventionally stored RBCs).

During shock, lactate accumulated due to low perfusion, reduced oxygen delivery, and increased anaerobic cellular metabolism. Using blood lactate as a marker of the efficacy of transfusion, animals receiving conventionally stored RBCs showed a reduced recovery of O₂ delivery relative compared to anaerobically stored RBCs. Similarly, other important metabolic markers of hypoxia, such as succinate and carboxylic acids, were elevated in animals given conventionally stored RBCs. Conventionally stored RBCs did not restore aerobic metabolism after HS, either due to inappropriate O₂ delivery to tissues or the inability to restore microcirculatory perfusion.³⁴ The observed lactate levels after transfusion of conventionally stored RBCs alludes to poor cardiac microvascular perfusion, since the myocardium preferentially uses lactate as an energy source.^{35,36} Improper cardiac O₂ release in the conventionally stored RBCs relative to the anaerobically stored RBCs was also evidenced by the decreased left ventricle IEU.²⁹ As the left ventricle consumes nearly all O₂ delivered, IEU is an excellent indicator of appropriate O₂ delivery/consumption to/by the left ventricle.³⁷ Inability to preserve MAP after transfusion of conventionally stored RBCs can also be attributed to limited IEU, as elevated stroke volume is less energetically costly than high end systolic left ventricular pressure.³⁷ Suppressed dp/dt_{min} in the conventionally stored RBCs group is another indicator of cardiac ischemia.³⁸ However, this

suppression is also likely due to the high transfusion volumes and the increase in blood viscosity post transfusion of conventionally stored blood.

The larger (nearly double) transfusion volume given to the conventional storage group significantly increased hematocrit and consequently blood viscosity, which impaired the left ventricular ejection of blood. Additionally, results obtained with conventionally stored RBCs suggest poor coronary perfusion resulting from higher blood viscosity and reduced RBC deformability,²⁴ which reduces ventricular O₂ extraction by limiting coronary microcirculatory function.³⁴ The conventionally stored RBCs may have acted more as nitric oxide (NO) sinks than anaerobically stored RBCs, due to oxidative stress during storage, resulting in vasoconstriction and further exacerbating poor coronary perfusion posttransfusion.^{39,40} Despite only receiving approximately double the RBC volume, animals transfused with conventionally stored RBCs experienced more than four times the levels of plasma Hb. This indicates a higher degree of intravascular hemolysis, overwhelming the free hemoglobin and heme scavenging system, and further impacting NO bioavailability, in animals transfused with conventionally stored RBCs compared to anaerobically stored RBCs.³⁹ Higher hematocrit achieved after transfusion of conventionally stored RBCs increased DO₂ to levels exceeding those of animals transfused with anaerobically stored RBCs. When normalized to the Hb, all 3 groups had very similar DO₂. As the experimental design of the study had all animals breathing room air (21% O₂), it is possible to speculate that animals receiving conventionally stored RBCs could have benefited from a higher FiO₂ to increase post-transfusion arterial O₂ saturation and to prevent systemic vasorelaxation resulting from low arterial O₂ saturation.⁴¹ However, other evidence suggests that the transfused conventionally stored RBCs were less effective releasing O₂ to tissues, as confirmed by the

increased lactate and other metabolic markers of anaerobic metabolism, and the detected vital organ hypoxia with pimonidazole staining (**Figure 2.9K**).

Conventionally stored RBCs resulted in additional sequelae in the form of organ damage and elevated levels of organ and systemic hypoxia, as also shown by plasma levels of lactate, succinate, and other carboxylic acids. Organs with elevated levels of hypoxia also showed elevated inflammatory markers, but organ damage was not always associated with hypoxia. Conventionally stored RBCs resulted in elevated markers of kidney injury and impaired function, but no hypoxia was detected in the kidneys. However, hemolysis is known to affect kidneys and other vital organs.⁴² Systemic hemodynamic parameters do not reflect the increased vital organ hypoxia, organ injury, or increased inflammation. While conventionally stored RBCs passed the 24-hour recovery metric set in place to ensure safety of RBC units, they were only able to partially restore cardiac function, and their infusion resulted in additional sequelae.

The 24-hour posttransfusion recovery is the FDA approved metric for determining the life span of stored RBCs in additive solutions.¹⁵ The rationale underpinning this parameter is that RBCs removed from circulation do not transport and deliver O₂, so at least 75% of the transfused RBCs must circulate for at least 24 hours posttransfusion. This concept has been recently brought into question by the FDA,¹⁵ which is working to determine better parameters to assess the efficacy of transfusions. Posttransfusion 24-hour recovery studies were performed by infusing a small volume of radiolabeled cells and measuring the number of surviving labeled RBCs after 24 hours. The 24-hour recovery studies do not appear to correlate with the storage condition's ability to resuscitate from HS. Both anaerobically and conventionally stored RBCs had 24-hour posttransfusion recovery over 75%, but conventionally stored RBCs had obvious functional limitations in resuscitation from HS. The 24-hour posttransfusion recovery studies tend to

overestimate the real posttransfusion recovery, as the RBC labeling processes mechanically and biochemically challenge the cells and eliminate the most fragile and weak RBCs before infusion. In addition, posttransfusion recovery studies do not take into account the other materials accumulated in the blood during storage.²⁵ From a functional standpoint, defining “expiration dates” for stored RBCs in additive solutions based on 24-hour posttransfusion recovery is inappropriate, especially as RBC units age at different rates^{21,22} and 24-hour recovery does not correlate with other functional markers.

Storage under hypoxic conditions has been shown to prevent and reduce some irreversible storage lesions induced by oxidative stress.⁴³ However, one of the challenges of transfusing anaerobically stored RBCs is the risk of depleting O₂ from circulating blood by transfusing deoxygenated RBCs. However, our results indicate that transfused deoxygenated RBCs rapidly become oxygenated as they circulate, with no effect on arterial blood gasses or arterial O₂ saturation. One-hour post transfusion, the arterial O₂ saturation was significantly higher in animals transfused with anaerobically stored RBCs compared to conventionally stored RBCs. Anaerobically stored RBCs have been shown to have improved preservation of membrane deformability and decreased metHb formation, which can reduce damage of organs in the reticulo-endothelial system.^{24,25} Microvesiculation of the RBC membrane, which is known to cause adverse reactions,⁸⁻¹⁰ is also reduced during anaerobic storage.²⁶

Limitations. Studies in rats are not directly translatable to clinical scenarios, but the results of the study indicate that anaerobically storage of RBCs preserves cell viability and functionality compared to conventional storage. Rat RBCs likely have some metabolic differences from human RBCs during storage, but the ATP, 2,3DPG, and lactate at the end of storage are similar to those in humans, albeit the changes observed occur on a faster time scale.⁴⁴ Studies have recently pointed

out the advantages and caveats of focusing on circulating levels of lactate and carboxylates alone in rat models of shock and resuscitation. Furthermore, the results of the study are comparable to other animal models phylogenetically closer to humans (e.g. porcine and non-human primate models) as well as critically injured patients.^{45,46} As the study's resuscitation goal was aimed to replicate clinical scenarios, where transfusion is given until a defined hemodynamic goal is achieved, the differences in cardiac function among groups were not expected to be great, which limits the capability to discern functional differences between resuscitation groups. However, other primary outcomes of the study, like the volume infused during resuscitation, organ damage, and organ hypoxia, showed significant differences. To ensure there are no sequelae that come from transfusing anaerobically stored RBCs, primary outcomes would need to be observed over longer time periods and functional outcomes, such as neurological function, should be measured.

In summary, the results presented here show that anaerobically stored RBCs improved outcomes during resuscitation from HS. Anaerobically stored RBCs required significantly lower volume and resulted in fewer sequela than conventionally stored RBCs. Anaerobically stored RBCs clearly improved systemic metabolic parameters associated with HS and ameliorated/prevented the generation of pro-inflammatory cytokines, molecular mediators of some of the most common sequelae observed after trauma (e.g. acute lung injury).⁴⁷ Functionally, there are no discernable differences between storing blood anaerobically or anaerobically with CO₂, despite the metabolic differences of the two storage conditions.²⁶ Anaerobically stored RBCs appear to maintain viability for an extended duration, both from a 24-hour recovery, as well as from a functional point of view. These data suggest that storing RBCs anaerobically could result in better transfusion recipient outcomes. Additional prospective clinical trials for trauma patients may be needed to better understand the association between RBC storage conditions and outcomes.

2.6: Acknowledgements

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Chapter 2, in full, is accepted material as it appears in “Transfusion of Anaerobically or Conventionally Stored Blood After Hemorrhagic Shock.” by A. T. Williams, V. P. Jani, T. Nemkov, A. Lucas, T. Yoshida, A. Dunham, A. D’Alessandro, and P. Cabrales, in *Shock* Publish Ahead of Print (August 2019). The dissertation author was the primary investigator and author of this paper.

2.7: Appendix

2.7.1: Supplemental Methods

Animal preparation. Studies were performed in male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 200-250 g. Animal handling and care followed the NIH Guide for Care and Use of Laboratory Animals. The experimental protocol was approved by the UCSD Institutional Animal Care and Use Committee. Briefly, animals were anesthetized using isoflurane (5%/vol for initial induction, 2.5%/vol for maintenance, Drägerwerk AG, Lübeck, Germany) and prepared with: (i) left jugular vein and left femoral artery catheterization, (ii) tracheotomy (polyethylene-190 tube), and (iii) left ventricular (LV) conductance catheter introduced through the right carotid artery. Animals were placed in the supine position on a heating pad to maintain core body temperature at 37 °C. Animals were mechanically ventilated (TOPO ventilator, Kent Scientific, Torrington, CT), with a respiration rate of 70 breaths/min and peak inspiratory pressure of 8-12 cmH₂O. After tracheotomy, isoflurane (2.5%/vol) was administered

via the ventilator to preserve the depth of anesthesia during the experimental protocol. Isoflurane was reduced to 1.5%/vol after cessation of surgery. Depth of anesthesia was continually verified via toe pinch and if needed, isoflurane concentration was increased by 0.1%/vol to prevent animal discomfort.

Inclusion criteria. Animals were suitable for the experiments if: (i) Mean arterial blood pressure (MAP) was above 90 mmHg at baseline, (ii) stroke volume (SV) was above 100 μ L at baseline, (iii) systemic hematocrit was above 40% at baseline, and (iv) animals survived the shock period.

Blood collection and preparation. Briefly, male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 300-450 g were anesthetized with a ketamine/xylazine cocktail (ketamine: 100mg/kg, xylazine: 10 mg/kg) and a femoral artery catheter was implanted. Each donor bled freely into 1.4 mL of CP2D (Taken from an AS-3 blood preparation kit, Haemonetics Corporation, Braintree, MA) until 50% of blood volume was lost. Donor blood was then pooled, and CP2D concentration was adjusted to 14%. Pooled blood was then centrifuged at 1000 g for 7 minutes, and the supernatant was removed. AS-3 (22%/vol) was then added and the blood was mixed gently by inverting the bag for 1 minute. Pooled blood was then passed through a neonatal leukocyte reduction filter (Haemonetics Corporation, Braintree, MA). RBC units intended for conventional storage (**Conventional**) were then stored at 4°C. Units for anaerobic storage (**Anaerobic**) and Anaerobic + CO₂ storage (**AN+CO₂**) were then deoxygenated by filling the storage bag with nitrogen (or 5% CO₂, balance nitrogen), and mixing by rotation at approximately 30 RPM. The gas was replaced every 10 minutes until Hb O₂ saturation measured <8%. Anaerobically stored cells were then placed in a sealed container filled with nitrogen and O₂ sorbent packs and stored at 4°C. For *in vitro* and recovery studies, 2 pools (14 rats each) were split

3 ways and stored for up to 4 weeks. For *in vivo* hemorrhagic shock/resuscitation studies, 2 pools for each storage condition (total n = 6 pools, 10 rats each) were stored for 3 weeks before infusion.

Histology. Immunohistochemical staining for pimonidazole bound to hypoxic zones in vital tissues during hypoxia was completed via IV injection of the hypoxic marker Hypoxyprobe-1 (40 mg/kg pimonidazole, Hypoxyprobe, Burlington, MA) and 5 mg/kg Hoechst 33342 (Invitrogen, Carlsbad, CA) diluted in PBS (total volume 100 μ L) 15 minutes before the animal was sacrificed. Organs were immediately excised after euthanasia and frozen at -80°C . Three parasternal short axis (PSS, cross-sectional "slice") sections of the heart were created for analysis. 6 to 10 random areas per slide were analyzed for positive pimonidazole staining for each organ. Tissues were frozen and processed for cryosectioning followed by immunohistochemical analysis. Sections were fixed with 100% methanol for 20 minutes at -20°C , then blocked and permeabilized with 5% BSA, 5% goat serum, and 0.1% Tween 20 for 1-2 h at room temperature. Sections were then incubated with antipimonidazole (Hypoxyprobe, 1:100) antibodies overnight at 4°C . The sections were mounted in SlowFade DAPI (Invitrogen Corp. Carlsbad, CA) and imaged using a fluorescence digital microscope (VB-6000; Keyence, Osaka, Japan).

Aspartate transaminase (AST), alanine transaminase (ALT), and IL-6 levels were determined in serum samples using ELISA kits (KA1625 and KT-6104 from Abnova Corp, Taiwan, and BMS625 from Thermo Fisher, Waltham, MA). NGAL was determined in the urine using ELISA kits (ERLCN2, Thermo Fisher). Serum Creatinine and Blood Urea Nitrogen (BUN) were measured using colorimetric detection kits (KB02-H2 and K024-H5, Arbor Assays Inc., Ann Arbor, MI). Liver, Lung and spleen CXCL1 are performed on whole tissue homogenates by ELISA assay (ERCXCL1, Thermo Fisher, Waltham) and corrected for protein concentration using the PierceTM BCA assay kit (Thermo Fisher).

Positive CD45 neutrophils were quantified in the bronchoalveolar lavage (BAL) fluid collected by instilling sterile PBS into the lung. Percentage fraction of neutrophils in BAL was determined by flow cytometry (FACSCalibur; BD, Franklin Lakes, NJ). Neutrophils are identified by their typical appearance in the forward/side scatter and their expression of CD45 (554875, BD Biosciences).

Volume infused. Blood was infused via a flow-controlled syringe pump at 300 $\mu\text{L}/\text{min}$. Total blood volume infused was calculated as $V = Q * t$, where V is the volume infused, Q is the flow rate of the syringe pump, and t is the time that the syringe pump was active, as measured by a stop-watch. Resuscitation % was calculated as $R = \frac{V}{TBV}$ where R is the resuscitation % and TBV is the total blood volume of the rat, estimated as 7% body weight. Number of units infused was calculated as $N = \frac{R * [\text{Hb}]_{\text{donor}}}{([\text{Hb}]_{\text{human donor}} * V_{\text{blood bag}}) / \text{Human weight}}$ where $[\text{Hb}]_{\text{donor}}$ is the Hb concentration of the rat donor blood, $[\text{Hb}]_{\text{human donor}}$ is the Hb concentration of a typical unit of packed RBCs (assumed 20g/dL), $V_{\text{blood bag}}$ is the typical volume of a unit of packed RBCs (assumed 500mL) and human weight is the typical weight of a human (assumed 70kg), normalizing the mass of Hb given to a human on a per unit basis to the mass of Hb administered to the animal.

24-hour recovery. Blood was radiolabeled with Technetium-99 (Tc^{99}) as described by Zink et. al. (50). Briefly, RBC samples (1.0 mL) were added to a sterile reaction vial and gently mixed to dissolve the lyophilized UltraTag-RBC (UltraTag-RBC, Mallinckrodt, St. Louis, MO), and allowed to react for 5 to 7 minutes. Then, UltraTag pH buffers were added to adjust pH, by gently mixing them into the reaction vial by inverting the container. Samples were washed with sterile PBS twice and centrifuged to removed unreacted Tc^{99} , and labeled RBCs were injected. 200 μL of Tc^{99} radiolabeled blood (approximately 2% of blood volume) was delivered I.V. to male Sprague-Dawley rats (n=2 per pool, per storage condition; n=4 per storage condition) and 65 μL

samples were drawn at 5 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, and 24 hours post-injection via tail clip. Samples were all run for radioactivity on a Cobra II gamma counter (Packard Instrument Co., Meriden, CT) at the same time so counts reported are independent of sample time and only representative of the still-circulating radio-labeled RBCs.

ATP and 2,3DPG. Aliquots of RBCs were mixed with cold trichloroacetic acid (DiaSys Deutschland, Flacht, Germany) and vortexed for 60 seconds. Aliquots sat on ice for 5 minutes and were then centrifuged at 3600g at 4°C for 10 minutes. The supernatant was removed and frozen at -80°C for later analysis. Supernatants were assayed enzymatically with commercially available kits. DPG was measured with the Roche 2,3-Diphosphoglycerate kit (Roche Diagnostics, Indianapolis IN, USA) according to manufacturer's instruction. ATP was measured by DiaSys ATP Hexokinase FS kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany) according to manufacturer's instruction.

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CHAPTER 3: RESUSCITATION FROM HEMORRHAGIC SHOCK WITH FRESH AND STORED BLOOD AND POLYMERIZED HEMOGLOBIN

3.1: Abstract

BACKGROUND: Hemoglobin (Hb) based oxygen carriers (HBOCs) have been proposed as alternatives to blood for decades. Previous studies demonstrated that large molecular diameter HBOCs based on polymerized bovine Hb (PolybHb) attenuate Hb side-effects and toxicity. The objective of this study was to test the safety and efficacy of tense state PolybHb after long-term storage.

METHODS AND RESULTS: PolybHb was subjected to diafiltration to remove low molecular weight (< 500 kDa) species and stored for 2 years. PolybHb was studied in parallel with blood, collected from rats and stored leukodepleted under blood bank conditions for 3 weeks. Rats were hemorrhaged and resuscitated to 90% of the blood pressure before the hemorrhage with fresh blood, stored blood, fresh PolybHb, or two-year-stored PolybHb. Hemorrhagic shock impaired oxygen delivery and cardiac function. Resuscitation restored blood pressure and cardiac function, but stored blood required a significantly larger transfusion volume to recover from shock compared to fresh blood and PolybHb (fresh and stored). Stored blood transfusion elevated markers of organ damage compared to all other groups.

CONCLUSIONS: These studies indicate that large molecular diameter PolybHb is as efficacious as fresh blood in restoring cardiac function and confirm the lack of degradation of PolybHb's safety or efficacy during long-term storage.

3.2: Introduction

Allogenic red blood cell (RBC) transfusion is the gold standard treatment for reduced oxygen (O₂) delivery capacity that is induced by severe anemia and hemorrhagic trauma.¹ A large industry has developed around extending the time between blood donation and its transfusion, since fresh blood is rarely available. Despite recent advances in RBC storage, blood shortages are still common and the frequency of these shortages is expected to increase in the future based on current donation and rate of blood usage trends.² Elective surgeries can be delayed during blood shortages, but there is always an immediate need for RBC transfusions in anemia and trauma. Blood availability is even more severe and critical during emergency situations, such as natural disasters or in active war zones. Blood shortages can be mitigated by extending blood's shelf life, but a safe and reliable O₂-carrying alternative to blood for transfusion medicine remains a necessity for situations when blood is not available, or blood is not an option due to ethical or religious reasons.

The United States Food and Drug Administration (FDA) has approved the storage of human RBCs for up to 42 days at 4°C in appropriate additive solutions. This maximum *ex vivo* storage period was established based on two parameters: hemolysis during storage (<0.8%), and the 24 hour post transfusion survival of transfused RBCs (>75%).³ While neither of these parameters demonstrate the capacity of RBCs to transport and deliver O₂, they are the only standards currently used to assess stored blood efficacy. Recent evidence suggests that blood quality (and thus O₂ delivery capability) decreases with age due to RBC storage lesions.⁴ Recent clinical trials have failed to find differences between standard of care blood (typically 3 weeks old) and fresh blood (less than 1 week old), but these trials do not account for the underlying cause of hospital admission or the quality of the transfused RBCs.^{5,6} Extending the *ex vivo* storage period

of blood based on current FDA standards could be detrimental, as current standards do not accurately reflect the function of RBCs after storage.

Hemoglobin (Hb) based oxygen carriers (HBOCs) have been tested as an alternative to RBC transfusion in a multitude of studies. Some commercially developed HBOCs restored blood pressure and decreased the number of RBC units transfused in clinical trials, but caused more adverse effects than the control group.^{7,8} The side effects induced by early-generation HBOCs were likely due to their small molecular size.^{9,10} The small molecular diameter of early generations of HBOCs increased facilitated diffusion, favoring vascular hyperoxygenation and scavenging of endothelial cell derived nitric oxide (NO), and extravasated into the extravascular space, which caused vasoconstriction, hypertension, and oxidative tissue injury.¹¹ Additionally, small molecular diameter HBOCs can be filtered by the kidneys, affecting renal filtration rates and impacting renal proximal tubules, which are especially vulnerable to heme and iron toxicity.¹²

A new generation of HBOCs with increased molecular weight (MW) and concomitantly increased molecular size compared to previous generations of HBOCs is being developed to mitigate previously observed side-effects associated with low MW HBOCs. The increase in molecular size reduces vascular hyperoxygenation and NO scavenging by slowing the HBOC's diffusion.¹³ Large molecular diameter polymerized Hb (PolyHb) also prevents extravasation and kidney damage by size exclusion, as well as promoting clearance of PolyHb via normal Hb scavenging pathways.¹⁴ Increases in MW have no effect on HBOCs' benefits over blood, such as a lack of immunogenicity, universal blood type compatibility, and potentially indefinite storage. Lastly, polymerization of Hb remains the only valid scalable and cost-effective method of increasing HBOC MW/size. This study was designed to test the hypothesis that extended storage does not impact the safety and efficacy of high MW polymerized bovine Hb (PolybHb). To achieve

this objective, in this study we compared the ability to resuscitate from hemorrhagic shock (HS) and assessed the degree of organ damage following transfusion of fresh and stored rat blood, and fresh and stored high MW PolybHb.

3.3: Methods and Materials

Synthesis of PolybHb. PolybHb was synthesized in the low oxygen affinity tense (T) quaternary state at a 35:1 molar ratio of glutaraldehyde to bovine Hb, and then subjected to 8-9 cycles of diafiltration on a 500 kDa tangential flow filtration (TFF) module using a modified lactated Ringer's solution as described in the literature.¹⁵ This resulted in a PolybHb solution containing only polymerized Hb molecules bracketed between 0.2 μm and 500 kDa. Stored PolybHb was stored at -80°C until usage more than 2 years after it was first synthesized. Fresh PolybHb was stored at -80°C and was used within 3 months of generation. PolybHb was adjusted to a concentration of 10 g/dL with lactated Ringer's solution before transfusion.

Biophysical Properties of PolybHb. PolybHb equilibrium curves were measured using a Hemox Analyzer (TCS Scientific, Southampton, PA) at 37°C as previously described¹⁵. PolybHb viscosity was measured at 37°C using a computerized cone-plate (4 cm diameter, 2° cone angle) rheometer (Discovery HR-2, TA Instruments, New Castle, DE, USA).

Blood Collection and Preparation. Fresh blood. Blood removed from the animal during hemorrhage was centrifuged at 1500 g for 7 minutes, and the plasma and buffy coat were removed. The plasma removed was then added to the packed RBC pellet to target a Hct of 50%. Stored blood. Briefly, male Sprague-Dawley Rats (Harlan Laboratories, Indianapolis, IN) weighing 300-450 g were anesthetized with isoflurane (5%). Blood was collected via cardiac puncture into citrate phosphate double dextrose (CP2D, which was taken from an Additive Solution 3 [AS-3] blood preparation kit, Haemonetics Corporation, Braintree, MA). Donor blood was then pooled, and the

CP2D concentration was adjusted to 14%. Pooled blood was centrifuged at 1000 g for 7 minutes, and the supernatant was removed. AS-3 (22%/whole blood volume) was then added, and blood was mixed gently by inverting the bag for 1 minute. Pooled blood was then passed through a neonatal leukocyte reduction filter (Haemonetics Corporation, Braintree, MA). Leukodepleted rat blood was stored at 4°C for 3 weeks, which was previously shown to pass current FDA standards for stored blood.¹⁶

Animal Preparation. Studies were performed in 32 male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 200-250 g. Animal handling and care followed the NIH Guide for Care and Use of Laboratory Animals. The experimental protocol was approved by the UCSD Institutional Animal Care and Use Committee. Briefly, animals were anesthetized using isoflurane (5%/vol for initial induction, 2.5%/vol for maintenance, Drägerwerk AG, Lübeck, Germany) and prepared with: (i) left jugular vein and left femoral artery catheterization, and (ii) left ventricular (LV) conductance catheter introduced through the right carotid artery. Animals were placed in the supine position on a heating pad to maintain core body temperature at 37 °C and allowed to freely breathe from a nosecone. Isoflurane was reduced to 1.5%/vol after surgical preparation. The depth of anesthesia was continually verified via toe pinch and the isoflurane concentration was increased by 0.1%/vol to prevent animal discomfort if needed (**Figure 3.1A**).

Inclusion Criteria. Animals were suitable for experiments if: (i) mean arterial blood pressure (MAP) was above 85 mm Hg at baseline, (ii) stroke volume (SV) was above 90 μ L at baseline, (iii) systemic Hb was above 12 g/dL at baseline, and (iv) animals survived the shock period.

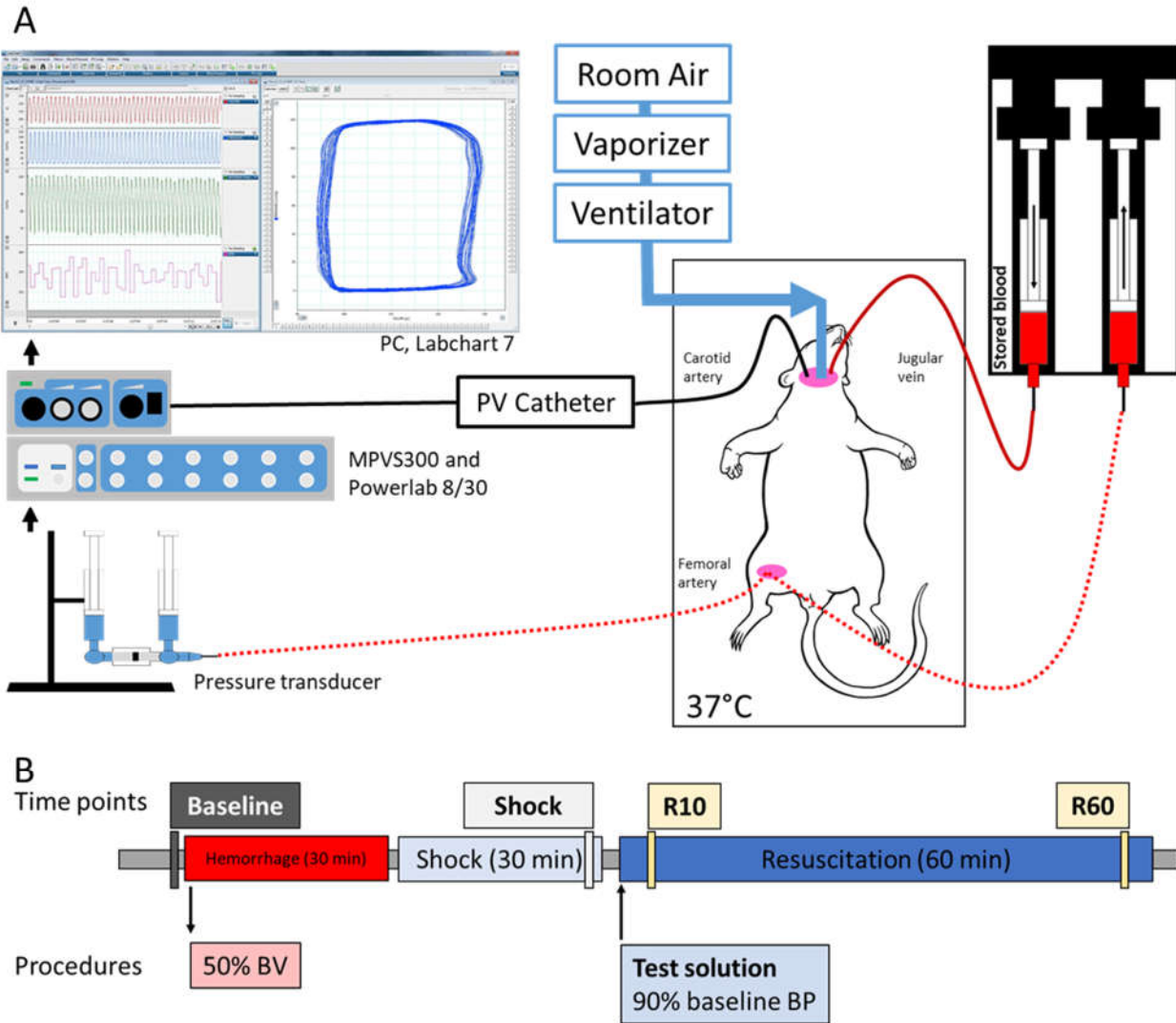


Figure 3.1: Animal preparation and timeline. **(A)** Animals were catheterized as depicted. Blood gas samples were taken from the carotid artery and jugular vein, cardiac function was measured via the PV catheter, and BP was measured via the femoral catheter. **(B)** The timeline describes procedures and timepoints shown in figures, as well as the general timing for the experiment.

Hemorrhagic Shock Resuscitation Protocol. After surgical instrumentation, animals were allowed to rest for 30 minutes to stabilize before baseline measurements were taken. Then the anesthetized rats were hemorrhaged by withdrawing 50% of the animal's blood volume (BV), estimated as 7% body weight, via the femoral artery catheter over 30 minutes into heparinized syringes. Hypovolemic shock was maintained for 30 minutes. Resuscitation was implemented by infusion of room temperature resuscitation solution through the jugular vein catheter until the

animal recovered to 90% of the MAP at baseline. If the animal's MAP fell below 80% of the MAP at baseline, additional resuscitation solution was infused. This resuscitation phase lasted for one hour (**Figure 3.1B**).

Volume Infused. Blood and PolybHb was infused via a flow-controlled syringe pump at 300 $\mu\text{L}/\text{min}$. Total blood volume infused was calculated as $V = Q * t$, where V is the volume infused, Q is the flow rate of the syringe pump, and t is the time that the syringe pump was active, as measured by a stop-watch. Resuscitation % was calculated as $R = \frac{V}{TBV}$ where R is the resuscitation % and TBV is the total blood volume of the rat, estimated as 7% body weight.

Cardiac Function. A 2F pressure-volume (PV) conductance catheter (SPR-858, Millar Instruments, TX) was inserted into the left ventricle (LV) using the closed chested method.¹⁷ Briefly, the PV catheter was inserted through the exposed right carotid artery and slowly advanced into the LV. Pressure and volume signals were acquired continuously (MPVS300, Millar Instruments, Houston, TX, and PowerLab 8/30, AD Instruments, Colorado Springs, CO). LV volume was measured in conductance units (relative volume unit, RVU) and converted to absolute blood volume (μL) at the end of the experiment.¹⁷ Parallel volume (the contribution of the myocardium to the measured LV volume) was calibrated via IV injection of 35 μL hypertonic saline (15% NaCl w/v).¹⁷

Systemic Hemodynamics Parameters. MAP and heart rate (HR) were recorded continuously from the femoral artery (PowerLab 8/30, AD Instruments, CO). Hematocrit (Hct) was measured via centrifugation from arterial blood collected into heparinized capillary tubes. Hb content was determined spectrophotometrically (B-Hemoglobin; Hemocue, Stockholm, Sweden). Arterial and venous blood were collected in heparinized glass capillary tubes (65 μL) and

immediately analyzed for oxygen partial pressure (pO₂), carbon dioxide partial pressure (pCO₂), and pH (Siemens 248, Munich, Germany).

Cardiac Pressure-Volume Indices. Cardiac function was analyzed using PowerLab software (PowerLab 7.8, AD Instruments, Colorado Springs, CO). Cardiac function parameters were calculated from 15 to 20 cardiac cycles at each time point. Stroke volume (SV), stroke work (SW), cardiac output (CO), and cardiac contraction and relaxation rate (dP/dt_{max} and dP/dt_{min}, respectively), were directly calculated in the PowerLab software. Systemic vascular resistance (SVR) was calculated as: $SVR = \frac{MAP}{CO}$. The internal energy utilization (IEU) was used as a measure of internal metabolism of the LV and was calculated as previously described.¹⁸

Oxygen Delivery. Oxygen delivery (DO₂) was calculated by multiplying CO and the arterial oxygen content. Oxygen consumption (VO₂) was calculated by multiplying CO and the arterio-venous difference in oxygen content.

Markers of Organ Damage and Inflammation. Aspartate transaminase (AST), alanine transaminase (ALT), and IL-6 levels were determined in serum samples using ELISA kits (KA1625 and KT-6104 from Abnova Corp, Taiwan, and BMS625 from Thermo Fisher, Waltham, MA). Neutrophil gelatinase associated lipocalin (NGAL) was determined in the urine using ELISA kits (ERLCN2, Thermo Fisher). Serum creatinine and blood urea nitrogen (BUN) were measured using colorimetric detection kits (KB02-H2 and K024-H5, Arbor Assays Inc., Ann Arbor, MI). Liver, lung and spleen CXCL1 were measured on whole tissue homogenates by ELISA (ERCXCL1, Thermo Fisher, Waltham) and corrected for protein concentration using the Pierce™ BCA assay kit (Thermo Fisher).

Positive CD45 neutrophils were quantified in the bronchoalveolar lavage (BAL) fluid collected by instilling sterile PBS into the lung. The percentage fraction of neutrophils in BAL was

determined by flow cytometry (FACSCalibur; BD, Franklin Lakes, NJ). Neutrophils were identified by their typical appearance in the forward/side scatter and their expression of CD45 (554875, BD Biosciences).

Preparation and Immunoprecipitation of Rat Tissue Extracts for Ferritin Determination.

Rat tissues were frozen and stored at -80°C until analyzed. Lysis buffer containing 1% deionized Triton X-100 and 0.1% sodium azide in 50 mM Tris-HCl pH 7.5 with Chelex-100 and Phenyl-methyl-sulfonyl-fluoride (0.25 mM) was added to the homogenized tissue, which was vortexed, sonicated for 1 min, and incubated on ice for 30 min, vortexing every 5-10 min. Aliquots were taken, centrifuged at 3,000 rpm for 15 min, and the supernatant analyzed for total protein (BCA, Pierce) and ferritin. The remaining suspension of homogenized tissue was incubated at 70°C for 10 min, cooled on ice and centrifuged at 10,000 g for 20 min. The supernatant was collected, and the pellet discarded. The total amount of ferritin in each extract was calculated using the results of the ELISA (Cat. no.MCA-155, Serotec, Oxford, UK). Saturating amounts of immune serum against rat tissue ferritin was then added to the extracts.

Epinephrine/Norepinephrine. Catecholamine plasma concentrations were determined using commercially available ELISA kits for catecholamines (Catecholamines ELISA kit - Ref: BA-E-6600, ImmuSmol, France). All ELISA samples were run in accordance with the manufacturer's instructions.

Statistical Analysis. Results are presented as the mean \pm SEM, as Tukey box-and-whisker plots, or as the median and 95% confidence interval. The values are presented as absolute values and relative to the baseline. A ratio of 1.0 signifies no change from baseline, whereas lower or higher ratios are indicative of changes proportionally lower or higher compared to baseline, respectively. Outliers are shown in the box-and-whisker plots as filled circles of the group's

respective colors. The Grubbs' method was used to assess closeness for all measured parameters at baseline and shock. Before experiments were initiated, sample size was determined based on $\alpha = 0.05$ and a power of 0.9 to detect differences in primary end points (MAP, contractility, and percent resuscitation) greater than 10%. Statistically significant changes between solutions and time points were analyzed using two-way analysis of variance (ANOVA), followed by *post-hoc* analyses using Tukey's multiple comparisons test when appropriate. All statistics were calculated using the Python Library StatsModels (ver. 0.9.0).¹⁹ Results were considered statistically significant if $P < 0.05$.

3.4: Results

PolybHb Characterization: Fresh PolybHb had lower Hb-O₂ affinity than stored PolybHb at the time of production ($P_{50} = 34$ mmHg and 30 mmHg, respectively), but similar Hb cooperativity, as indicated by the Hill coefficient of the Hb equilibrium curve (~ 1). Fresh and stored PolybHb both had high viscosity compared to rat blood (16.1, 16.6, and 4.2 cP at 100 s⁻¹, respectively).

In Vivo Studies. Thirty-two ($n = 32$) animals were entered into the hemorrhagic shock/resuscitation study. Animals were randomly assigned to the following groups: Fresh blood (**fBlood**, $n = 8$), stored blood (**sBlood**, $n = 8$), fresh PolybHb (**fPHB**, $n = 8$), and stored PolybHb (**sPHB**, $n = 8$). All animals survived the protocol and there were no statistical differences between animals at baseline.

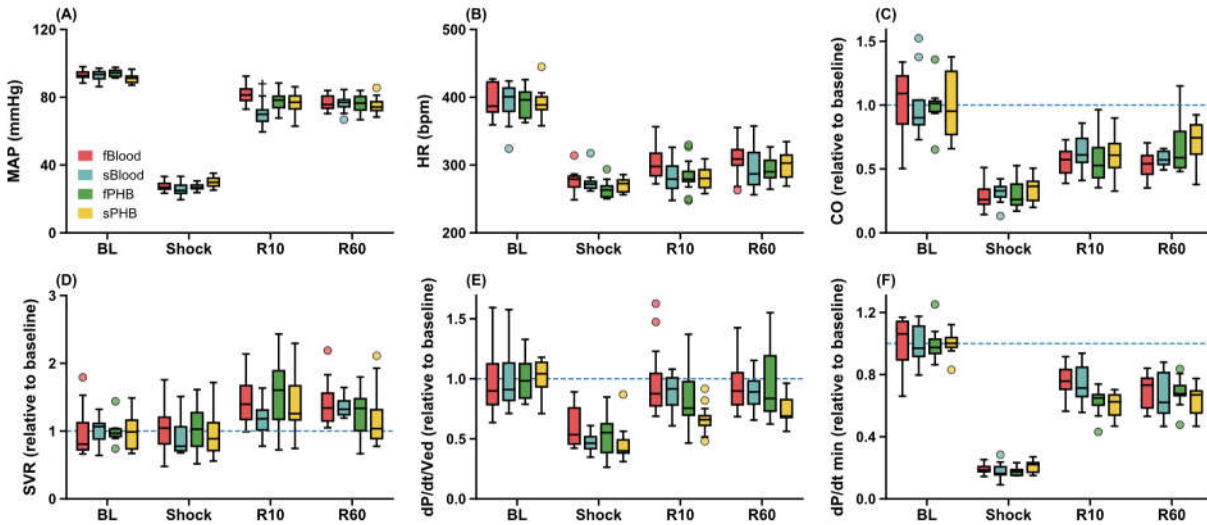


Figure 3.2: Hemodynamics and cardiac function pre-and-post HS/resuscitation with fresh and stored blood and T-state Polyhb. **(A)** MAP is not initially restored with sBlood, but fPHB and sPHB restored MAP to the same degree as fBlood. **(B)** HR is suppressed after shock and remained that way. **(C)** CO is decreased during shock and partially recovers for fPHB and sPHB. **(D)** SVR increases to a similar degree upon transfusion of all materials. **(E)** Isovolemic contraction relative to Ved is slightly lower upon transfusion of fPHB and sPHB. **(F)** Isovolemic relaxation is initially suppressed with fPHB and sPHB. N=8 per group. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

Systemic Hemodynamics. All animals had similar blood pressure at baseline and at the end of shock (**Figure 3.2A**). Restoration of blood pressure at 10 mins into resuscitation was similar for fBlood, fPHB, and sPHB, but statistically significantly lower for sBlood. With continued transfusion, all animals achieved the transfusion goal of recovering blood pressure and there were no statistically significant differences in MAP between groups 60 mins into resuscitation. Shock resulted in bradycardia relative to baseline, and the reduced HR was not resolved with transfusion of any test solution (**Figure 3.2B**). CO decreased from baseline during shock and remained statistically different from baseline during resuscitation for fBlood and sBlood, but CO was not significantly different than baseline level during resuscitation for fPHB and sPHB (**Figure 3.2C**). Resuscitation with fBlood and sBlood decreased CO 2% and 14% relative to shock, whereas fPHB and sPHB increased CO 14% and 22% relative to shock. SVR decreased during shock and was elevated relative to baseline for all groups after resuscitation (**Figure 3.2D**).

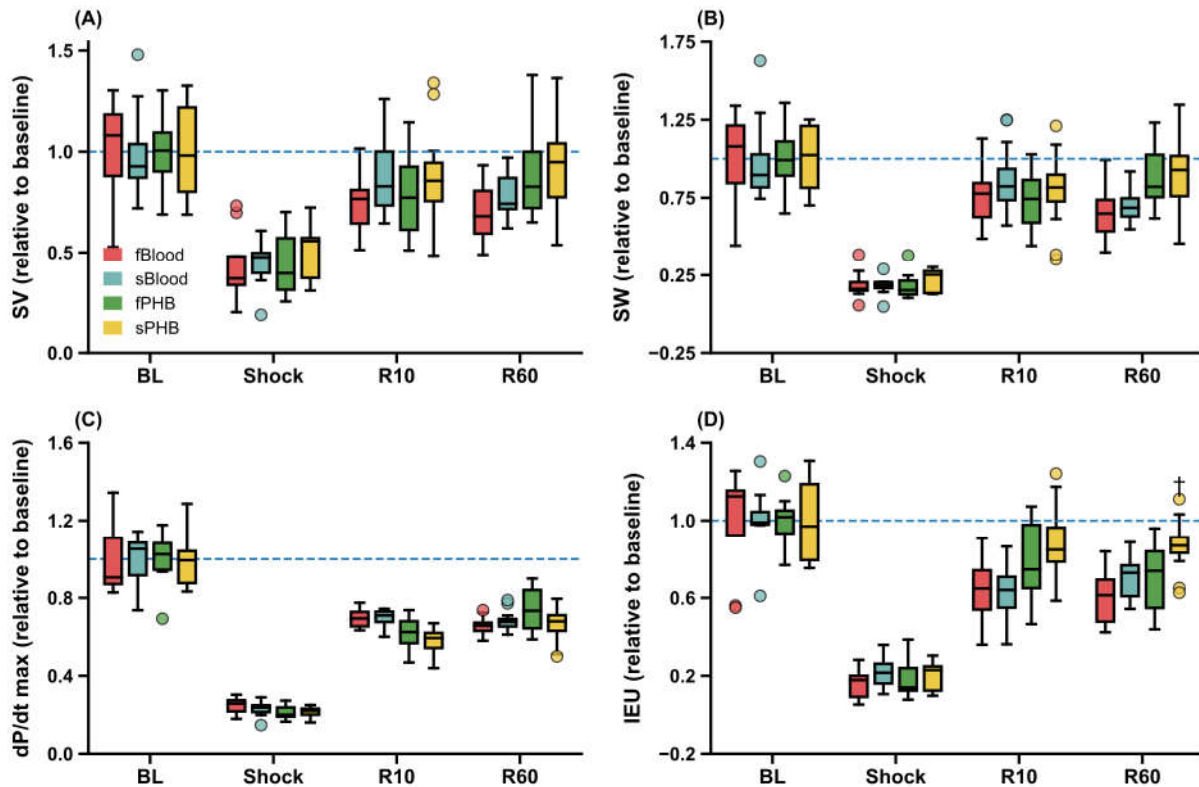


Figure 3.3: Additional parameters of cardiac function. **(A)** Stroke volume decreased slightly after HS but stayed close to baseline levels throughout the experiment. **(B)** Stroke work slightly decreases after hemorrhage and stays at approximately the same level throughout the protocol. **(C)** Isovolemic contraction decreases after hemorrhage and does not recover during the protocol. **(D)** Cardiac metabolism slows slightly during shock, and is recovered for sPHB. N=8 per group. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

Cardiac Function. SV decreased from baseline during shock. However, since resuscitation was implemented until 90% of the baseline MAP was reached, most hemodynamic and cardiac function parameters did not show statistical differences between groups after resuscitation. During resuscitation, sBlood, fPHB, and sPHB presented higher SV compared to fBlood, but differences between groups were not statistically significant (**Figure 3.3A**). Shock decreased SW and resuscitation partially restored SW in all groups, but fPHB and sPHB showed higher SW than fBlood and sBlood, likely due to their higher viscosity. (**Figure 3.3B**). Isovolemic contraction rate (dp/dt_{max}) decreased during shock and improved upon resuscitation but did not reach baseline values in any of the groups (**Figure 3.3C**). When normalized to end diastolic volume (V_{ed}), the isovolemic contraction rate ($dp/dt_{max}/V_{ed}$) did not change significantly from baseline, other than

the sPHB group, whose contractility was statistically significantly lower than baseline (**Figure 3.2E**). The isovolemic relaxation rate (dp/dt_{min}) decreased during shock and was restored upon resuscitation, but no group recovered to their baseline isovolemic relaxation rate (**Figure 3.2F**). IEU decreased during shock and increased upon resuscitation, and fPHB and sPHB showed slightly higher IEU than fBlood and sBlood (**Figure 3.3D**).

Table 3.1: Hematological parameters during the hemorrhagic shock/resuscitation protocol. pHb = plasma Hb. Data are presented as median [95% CI]. N=8 per group. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

Time point	Group	Hct, %	Hb, g/dL	pHb, g/dL
Baseline		43 [42-44]	13.6 [13.1-13.8]	
Shock		28 [27-29]	9.0 [8.7-9.1]	
R10	fBlood	35 [33-37]	10.4 [9.1-11.1]	0 [0-0]
	sBlood	38 [36-42] [†]	12.5 [11.9-12.7] [†]	0 [0-0]
	fPHB	27 [25-28] ^{†‡}	10.4 [9.1-11.1] [‡]	3.3 [1.3-3.9]
	sPHB	26 [24-27] ^{†‡}	9.7 [8.9-10.2] ^{†‡}	2 [1.3-3.5]
R60	fBlood	39 [36-42]	12.2 [11.1-13.1]	0 [0-0]
	sBlood	50 [41-52] [†]	16.3 [13.0-17.9] [†]	0.1 [0-0.1]
	fPHB	22 [19-28] ^{†‡}	10.8 [9.4-12.0] ^{†‡}	5.4 [1.2-7.2]
	sPHB	20 [18-21] ^{†‡}	10.4 [8.9-11.5] ^{†‡}	4.8 [3.1-6.8]

Hematology. A summary of hematological changes is presented in **Table 3.1**. The sBlood group required a statistically significantly greater transfusion volume for resuscitation than the fBlood, fPHB, and sPHB groups (**Figure 3.4**). The fBlood, fPHB, and sPHB groups all required similar transfusion volumes to resuscitate from HS. As a result of the higher transfusion volume, sBlood showed significantly higher Hct than fBlood. Resuscitation increased Hb (and oxygen carrying capacity), but the lower Hb concentration of PolybHb compared to transfused (fresh or stored) blood resulted in a lower overall total Hb level for fPHB and sPHB than fBlood and sBlood.

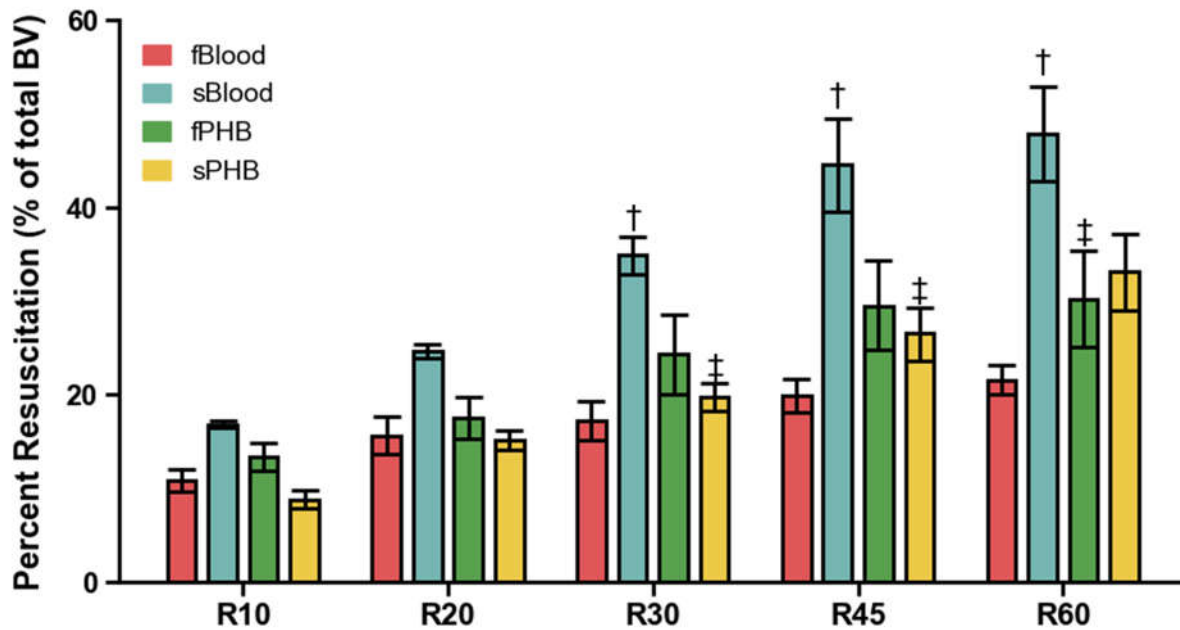


Figure 3.4: The same volume of fBlood, fPHB, and sPHB were needed for resuscitation. N = 8 per group. Data are presented as mean \pm SEM. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

Blood Chemistry. Shock resulted in acidosis, decreased arterial pCO₂, and increased arterial pO₂ for all groups (**Table 3.1**). Resuscitation did not correct systemic acidosis in any of the groups; however, arterial pO₂ was restored to baseline levels by the end of the protocol. fBlood and sBlood showed elevated arterial pCO₂ relative to baseline, while fPHB and sPHB returned to baseline pCO₂ levels.

Table 3.2: Blood gasses during the hemorrhagic shock/resuscitation protocol. Data are presented as median [95% CI]. N=8 per group. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

Time point	Group	pH	pCO ₂ , mmHg	pO ₂ , mmHg
Baseline		7.41 [7.40-7.42]	41.7 [39.8-44.0]	84.1 [80.2-88.2]
Shock		7.30 [7.26-7.32]	35.9 [33.1-37.6]	110.7 [106.9-116.6]
R10	fBlood	7.27 [7.15-7.36]	42.0 [40.0-50.9]	89.1 [73.2-102.6]
	sBlood	7.26 [7.11-7.3]	†39.1 [29.3-44.0]	102.6 [81.8-115.1]
	fPHB	7.29 [7.25-7.33]	42.2 [34.7-45.5]	90.8 [79.9-99.3]
	sPHB	7.32 [7.21-7.35]‡	37.8 [32.2-42.4]†	96.7 [86.0-122.5]
R60	fBlood	7.31 [7.28-7.39]	48.9 [42.9-57.1]	81.5 [54.4-87.6]
	sBlood	7.32 [7.20-7.40]	46.4 [39.2-54.3]	82.6 [70.8-99.7]
	fPHB	7.32 [7.27-7.35]	45.3 [40.4-49.8]	85.0 [70.8-89.9]
	sPHB	7.31 [7.18-7.39]	42.2 [38.8-47.4]†	83.9 [66.7-109.8]

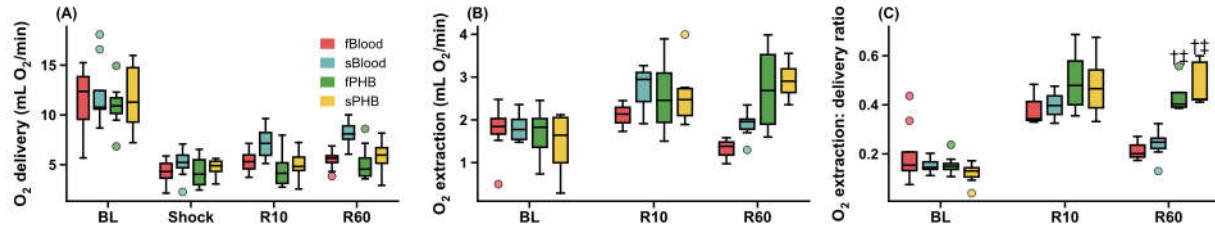


Figure 3.5: Systemic oxygen delivery parameters pre-and-post HS/resuscitation with fresh and stored blood and T-state PolyHb. **(A)** Oxygen delivery decreases with shock and stays suppressed, but oxygen delivery increases for sBlood due to the additional transfusion volume. **(B)** Oxygen extraction post-hemorrhage is increased. Oxygen extraction is slightly increased in the fPHB and sPHB groups due to the low oxygen affinity of T-state PolyHb. **(C)** The oxygen delivery: extraction ratio is increased post-shock but decreases to near-baseline for the fBlood and sBlood groups. N=8 per group. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

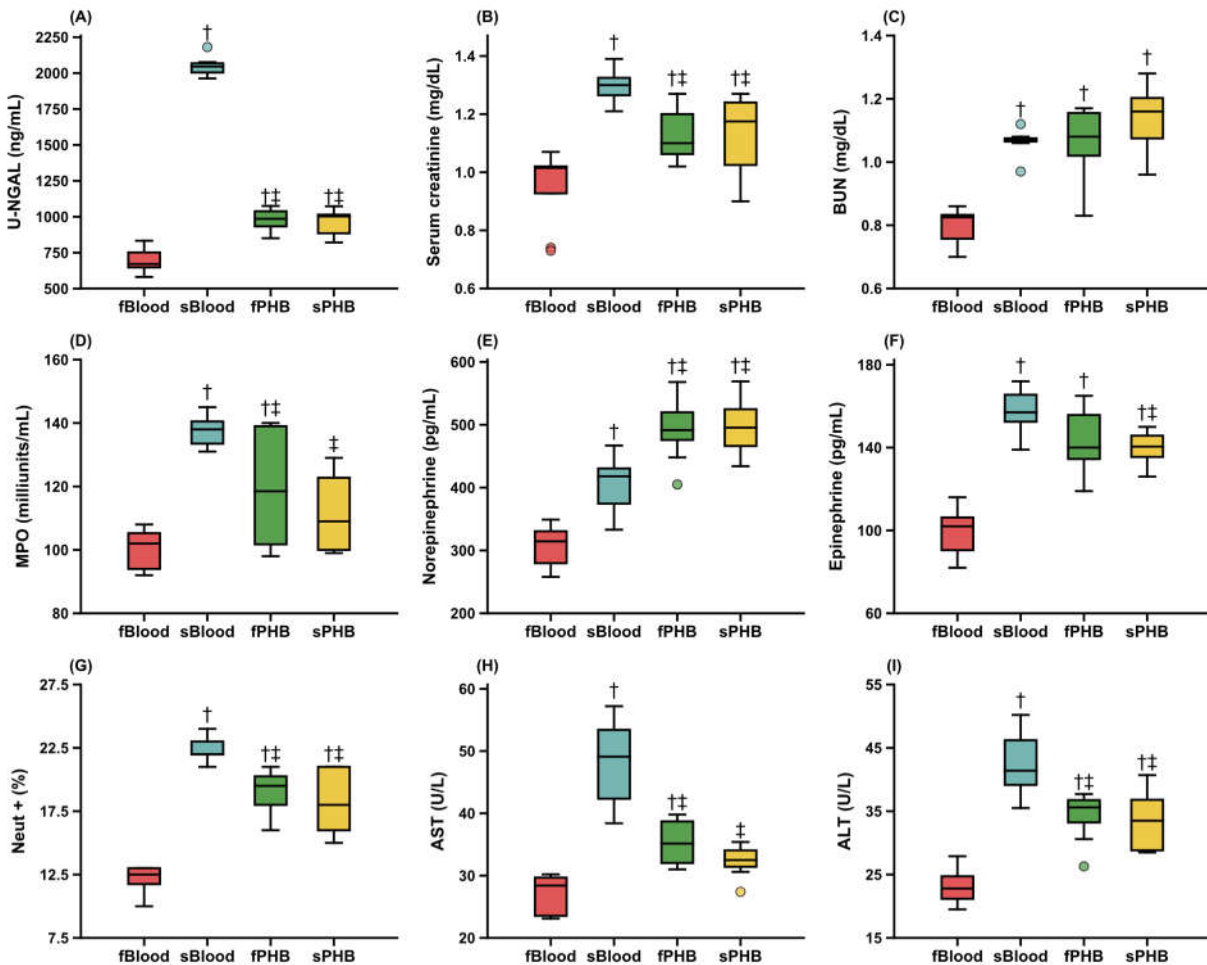


Figure 3.6: Markers of tissue damage and stress. **(A)** Significantly more kidney damage is observed in sBlood than fBlood, fPHB, or sPHB. **(B),(C)** Kidney function is suppressed for sBlood, fPHB, and sPHB. **(D)** Myeloperoxidase is most active in lungs for the sBlood group. **(E),(F)** sBlood, fPHB, and sPHB show higher levels of acute stress hormones. **(G)** Significantly more neutrophils are activated in the lungs for the sBlood group. **(H),(I)** More liver damage is observed in the sBlood group compared to fBlood, fPHB, sPHB. N=8 per group. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

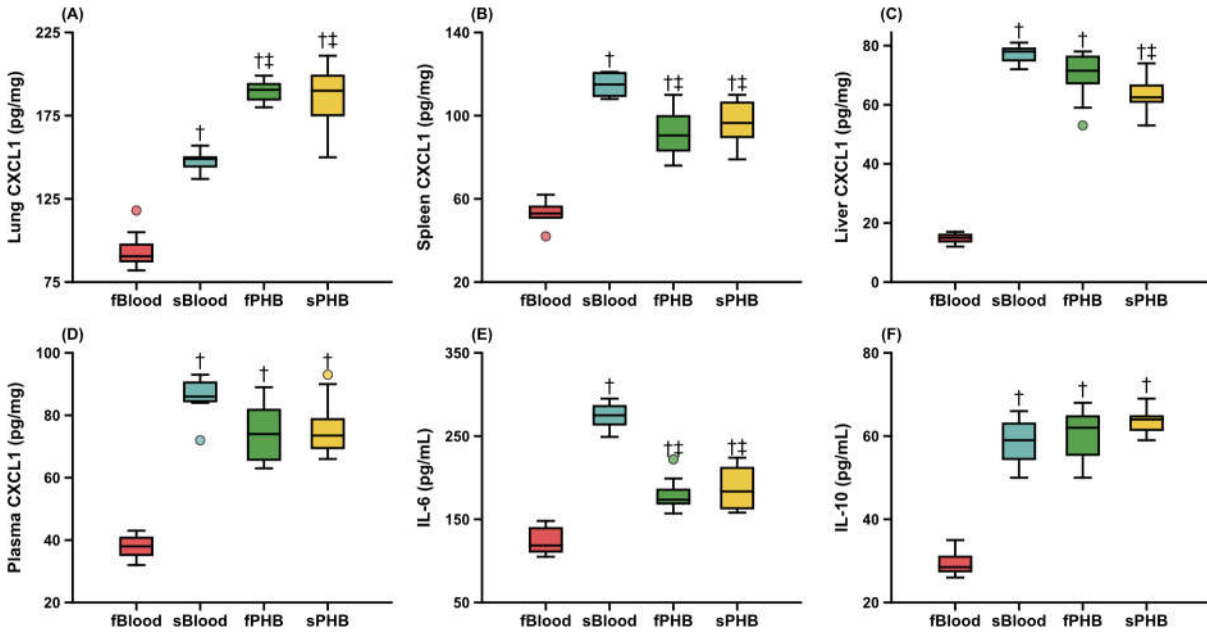


Figure 3.7: Cytokines post-hemorrhagic shock/resuscitation. **(A)** fPHB and sPHB show significantly higher lung neutrophil recruitment than fBlood. **(B),(C)** Significantly more reticuloendothelial inflammation is observed in sBlood than fBlood, fPHB, or sPHB. **(D)** Plasma CXCL1 is elevated at the end of resuscitation for sBlood, fPHB, and sPHB. **(E)** Significantly higher levels of the proinflammatory cytokine IL-6 are present in the sBlood group, but **(F)** similar levels of the anti-inflammatory cytokine IL-10 are present for the sBlood, fPHB, and sPHB groups at the end of resuscitation. N=8 per group. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

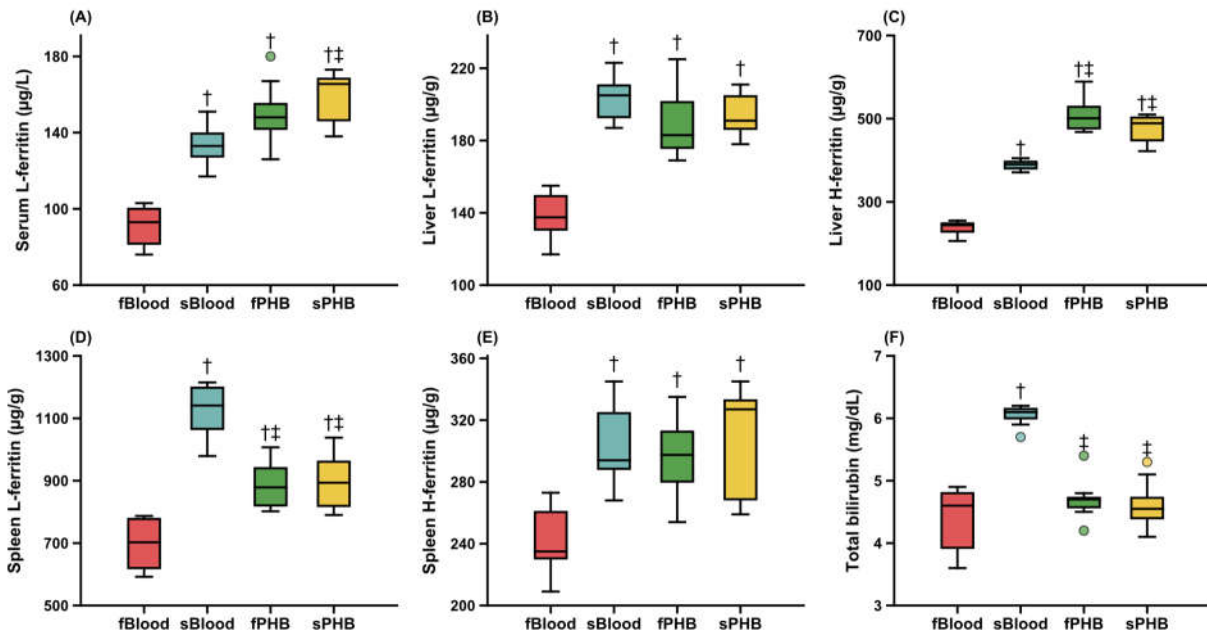


Figure 3.8: Iron transport post-transfusion. Ferritin is elevated in the reticuloendothelial system for sBlood, fPHB, and sPHB at the end of resuscitation. **(A)** Significantly higher levels of L-ferritin are present in the serum. **(B),(C)** Ferritin levels in the liver suggest that PolyHb is primarily metabolized in the liver. **(D),(E)** Ferritin levels in the spleen suggest that the spleen removes significantly more senescent RBCs than PolyHb. **(F)** Bilirubin is increased in the sBlood group. N=8 per group. †P<0.05 vs fBlood, ‡P<0.05 vs sBlood.

Oxygen Delivery. Oxygen delivery (DO_2) decreased during shock and was never fully restored during resuscitation with either blood or PolybHb. DO_2 in the sBlood group was higher than the DO_2 for the fBlood, fPHB, and sPHB groups, primarily due to a significantly larger transfused volume, and concurrently higher Hct and total Hb (**Figure 3.5A**). Oxygen extraction (VO_2) was increased 10 mins into resuscitation in all groups, but VO_2 was slightly lower at the end of resuscitation for the fBlood and sBlood groups compared to the fPHB and sPHB groups (**Figure 3.5B**). The O_2 extraction to delivery ratio (VO_2/DO_2) was significantly higher than baseline for fPHB and sPHB, but not for fBlood or sBlood (**Figure 3.5C**).

Markers of Organ Damage or Organ Function. The sBlood group showed significantly higher markers of organ injury compared to the fBlood, fPHB, and sPHB groups. Organs like the kidney and liver appear to be more affected by resuscitation with stored blood than PolybHb. This can be observed by differences in u-NGAL, serum creatinine, and BUN, markers of kidney injury, and by the differences in AST and ALT, markers of liver injury. Stress hormones were significantly lower for fBlood than sBlood, fPHB, and sPHB; however, norepinephrine was higher for the fPHB and sPHB groups than the sBlood group (**Figure 4**). CXCL1, a tissue inflammatory marker, was increased in sBlood, fPHB, and sPHB compared to fBlood in the lungs, spleen and liver. Additionally, lung neutrophil recruitment was significantly higher for fPHB and sPHB compared to sBlood. While the proinflammatory cytokine interleukin-6 was higher for sBlood than fPHB and sPHB, the levels of the anti-inflammatory cytokine interleukin-10 was not different among these 3 groups (**Figure 5**). Furthermore, ferritin levels increased for the sBlood, fPHB, and sPHB groups compared to the fBlood group. However, bilirubin was elevated only in animals transfused with stored blood (**Figure 6**). The fPHB and sPHB groups showed a significantly higher ratio of heavy-chain (H) ferritin to light-chain (L) ferritin compared to the sBlood group.

3.5: Discussion

The principal finding of this study is that resuscitation of rats from HS with fresh and stored PolybHb restores systemic hemodynamics, markers of cardiac function, and O₂ delivery to a similar extent as fresh blood. These results also demonstrate that PolybHb's capacity to resuscitate from HS does not change after 2 years of storage, while 3 weeks of storage can limit the capacity of blood to resuscitate from HS. Resuscitation by means of transfusions of fresh blood, and PolybHb, both fresh and stored, met the resuscitation goal of recovering MAP to 90% of baseline, within 10 minutes into resuscitation and maintained MAP during the observation period. On the other hand, there was a delay in reaching the resuscitation goal for animals receiving stored blood, and a larger volume was transfused than resuscitation with fresh blood and PolybHb. All groups recovered vascular resistance to similar degrees, suggesting that resuscitation with PolybHb does not result in vasoconstriction relative to resuscitation with blood.

Other studies have used total Hb to guide resuscitation from HS, but total Hb is not a positive indicator of recovery nor a direct indicator of tissue oxygenation.^{20,21} In this study, resuscitation was directed to achieve a target blood pressure, and the volume transfused was a primary metric to measure the efficacy of the resuscitation solution. The volume transfused to recover pressure accounts for the response of the cardiovascular system to correct effective blood volume, restore blood flow, and reinstate oxygen delivery. Systemic parameters, such as MAP and HR, suggest that the experimental protocol was followed closely. All animals reached the experimental goal, but animals transfused with stored blood experienced a slower recovery, demonstrating stored blood's limited ability to restore systemic blood flow, hemodynamics, cardiac function, and oxygen delivery compared to fresh blood.

During shock, limited blood flow and oxygen delivery causes cells to shift toward anaerobic metabolism, resulting in a buildup of lactic acid and creating metabolic acidosis. Through the Bohr effect, acidosis decreases RBC Hb's oxygen affinity, and increases oxygen offloading to tissues.²² The intrinsic low oxygen affinity of T-state PolybHb appears to promote oxygen offloading to tissues, which helps to re-establish oxygenation and minimizes the metabolic sequelae associated with HS. In the case of stored blood transfusion, metabolic acidosis may be exacerbated due to depletion of 2,3-bisphosphoglycerate (BPG) in the RBCs during blood storage,²³ which decreases the p50 and limits the Bohr effect. Since 2,3-BPG driven regulation of Hb O₂ affinity was first observed, the importance of RBC metabolism during blood storage has been pointed out under both physiological and pathological conditions. 2,3-BPG concentration in stored RBCs recovers slowly, and decreases O₂ extraction immediately following resuscitation.²⁴ Previous *in vivo* studies have demonstrated that increased Hb O₂ affinity impairs tissue oxygenation, thus preventing restoration of aerobic metabolism after resuscitation from HS with stored blood.¹⁶

One of the most significant results of this study, is the large transfusion volume of stored blood compared to both fresh blood and PolybHb during resuscitation. To reach the blood pressure goal, sBlood required reinfusion of the entire hemorrhaged volume (50% of the total blood volume), whereas fBlood required a substantially lower volume to be reinfused (21% of the total blood volume). Thus, blood usage can be potentially decreased by using fresh blood when possible. Moreover, the increased transfusion volume necessary to achieve hemodynamic stability with stored blood favors different types of transfusion-related adverse reactions that may occur.^{25,26} Animals in the sBlood group experienced systemic inflammation, as indicated by a significantly elevated IL-6 concentration in plasma, higher levels of markers of neutrophil recruitment in the

spleen and liver, and neutrophil activation in the lungs compared to the fBlood group and PolybHb groups. Elevated spleen inflammatory markers in the sBlood group is consistent with the spleen's function in clearing damaged or aged RBCs from the circulation. The RBC storage lesions and reduced deformability are likely responsible for increased in splenic inflammatory markers, as neutrophils are necessary for splenic hemolysis of stored RBCs through phagocytosis.²⁷ This hypothesis is further supported by the increased splenic ferritin and bilirubin for the sBlood group, both of which are markers of hemolysis.²⁸ Since PolybHb is acellular, the effect on the spleen is less pronounced for fPHB and sPHB than for the sBlood group. On the other hand, in the lungs, the acellular nature of PolybHb allows for direct interaction of the Hb with the lung resident macrophages and dendritic cells, upregulating recruitment of lung neutrophils (as indicated by lung CXCL1) in the fPHB and sPHB groups.²⁹ However, lung neutrophils are more active (as indicated by MPO activity and neutrophil activation) for the sBlood group than the fBlood and PolybHb groups, likely due to increased oxidized Hb (metHb) in stored blood.²⁹

Resuscitation from HS with PolybHb and stored blood appears to induce acute kidney injury, as detected by the increase in markers of kidney function (creatinine and BUN). Previous generations of HBOCs caused significant kidney damage,¹² but the fPHB and sPHB groups showed significantly less acute kidney injury (as reflected by low u-NAGL and serum creatinine) compared to sBlood. In addition, the sBlood group showed a statistically significant increase in markers of liver injury (AST and ALT) compared to the fBlood, fPHB, and sPHB groups. These elevated markers of organ injury in the sBlood group are likely due to storage lesions in RBCs, resulting in hemolysis and free heme. When the innate Hb scavenging system becomes saturated after transfusion of stored blood,³⁰ free Hb can be filtered through the kidneys, causing kidney damage.³⁰ Similarly, in the liver, extravasation of acellular Hb and release of heme and iron causes

oxidative stress, promoting acute liver injury.³¹ The Hb scavenging system also binds and degrades PolybHb, but at a slower rate than acellular Hb.¹⁴ Differences in bilirubin, a byproduct of heme catabolism, suggest that a significant amount of the iron stored following PolybHb transfusion was due to free iron present after transfusion, whereas the iron stored following stored blood transfusion was derived from heme. Without management of free iron post-transfusion, PolybHb could lead to iron toxicity, but previous studies have shown that supplementing HBOC transfusion with an iron chelator (deferoxamine) significantly reduced tissue toxicity.³² The higher ratio of H to L ferritin seen after transfusion of PolybHb compared to stored blood is indicative of low levels of oxidized heme in the transfused PolybHb.³³ Furthermore, the lower H-ferritin levels observed after transfusion of stored blood suggests that the acellular Hb released upon transfusion of stored RBCs oxidizes rapidly in circulation. This is also supported by aforementioned markers of lung neutrophil activation, as neutrophils are activated by metHb, but not non-oxidized Hb.²⁹

Limitations. Studies in rats are not directly translatable to clinical scenarios, but these initial results show promise regarding the efficacy of high MW PolybHb in resuscitation from HS. Furthermore, rats do not mimic the human response to oxidative stress since they innately produce ascorbic acid and upregulate ascorbic acid production in times of oxidative stress. Further testing in guinea pigs (a non-ascorbic acid producing animal species) is planned to assess the safety of this class of HBOCs. Finally, these experiments were designed to examine what happens during the ‘golden hour’ of resuscitation from HS and long-term studies with PolybHb are needed. To ensure there are no sequelae due to transfusion of PolybHb, and to examine whether the acute markers of injury continue to chronic injury, primary outcomes need to be observed over longer periods of time, and other functional outcomes should be assessed.

In summary, these results show that PolybHb possesses similar efficacy as fresh blood during resuscitation from HS. Furthermore, the efficacy and safety of PolybHb is preserved after 2 years of storage, while rat blood stored for 3 weeks showed reduced efficacy and increased resuscitation-associated sequelae compared to fresh blood and PolybHb. Although further studies are needed to ensure safety, these data support the use of high MW PolybHb as an initial oxygen bridge until blood is available for resuscitation in HS scenarios. In conclusion this study indicates that high MW PolybHb is efficacious in restoring markers of cardiac function after HS, and confirms PolybHb's efficacy after long-term storage.

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CHAPTER 4: BALANCE BETWEEN OXYGEN TRANSPORT AND BLOOD RHEOLOGY
DURING RESUSCITATION FROM HEMORRHAGIC SHOCK WITH POLYMERIZED
BOVINE HEMOGLOBIN

4.1: Abstract

BACKGROUND: Alternatives to blood for use in transfusion medicine have been investigated for decades. An ideal alternative to blood is expected to improve both oxygen (O₂) carrying capacity and O₂ delivery supporting microvascular blood flow. Previous studies have shown that large molecular diameter hemoglobin (Hb) based oxygen carriers (HBOCs) based on polymerized bovine Hb (PolybHb) reduce the toxicity and vasoconstriction of first generation HBOCs by increasing blood and plasma viscosity and preserving microvascular perfusion. The objective of this study was to examine the impact of PolybHb concentration, and therefore O₂ carrying capacity and solution viscosity on resuscitation from hemorrhagic shock in rats.

METHODS AND RESULTS: Large molecular diameter PolybHb was subjected to diafiltration on a 500 kDa tangential flow filtration (TFF) module to remove low molecular weight (MW) PolybHb molecules from the final product. Rats were hemorrhaged and maintained in hypovolemic shock for 30 minutes before transfusion of PolybHb at 10 g/dL (PHB10), 5 g/dL (PHB5) or 2.5 g/dL (PHB2.5) concentration, to restore blood pressure to 90% of the animal's baseline blood pressure. Resuscitation restored blood pressure and cardiac function in a PolybHb-concentration-dependent manner. Parameters indicative of the heart's metabolic activity indicated that the two higher PolybHb concentrations restored coronary O₂ delivery compared to the low concentration evaluated. Markers of organ damage and inflammation were highest for PHB10, while PHB5 and PHB2.5 showed similar expression of these markers.

CONCLUSIONS: These studies indicate that a concentration of ~ 5 g/dL of PolybHb may be near the optimal concentration to restore cardiac function, preserve organ function, and mitigate the toxicity large molecular diameter PolybHb during resuscitation from hemorrhagic shock.

4.2: Introduction

Hemorrhage is the leading cause of potentially salvageable trauma-related deaths.¹ In conjunction with hemostasis, survival of severe hemorrhage requires the restoration of blood volume (BV) to ensure proper cardiac function and tissue perfusion, which reinstates oxygen (O₂) delivery to tissues. Current fluid resuscitation strategies for class IV hemorrhages (any hemorrhage where more than 40% of blood volume is lost) are aggressive, necessitating implementation of a massive transfusion protocol (MTP).² MTP endpoints consider both physiological and laboratory criteria (mean arterial blood pressure and total hemoglobin [Hb] concentration) to minimize the volume transfused.³ Currently, patients are exposed to blood products from multiple donors due to blood component therapy, and the large volume infused during MTP, increasing the risk of disease transmission and transfusion-related immunomodulation (TRIM).^{4,5} Aggressive transfusion of crystalloids can cause fluid overload, leading to acute respiratory distress syndrome (ARDS), increasing morbidity and mortality.^{6,7} Furthermore, crystalloids ineffectively restore microcirculatory blood flow after resuscitation from trauma and decrease blood O₂ carrying capacity by diluting the remaining red blood cells (RBCs) in the circulation.⁸

Under normal conditions, only 25% of the O₂ delivered to tissues is extracted.⁹ During severe hemorrhagic shock (HS), regulatory mechanisms increase O₂ extraction and prioritize oxygenation of vital organs by redistributing blood flow to these tissues. As HS reduces O₂ carrying capacity and cardiac output, limited O₂ delivery compromises tissue oxygenation. The primary determinant of O₂ delivery is the total Hb concentration in the blood; if total Hb falls below a critical total Hb concentration, known as the “transfusion trigger,” then tissue O₂ demands will likely exceed O₂ delivery, which indicates that a blood transfusion is needed.² Typically, in healthy subjects, the transfusion trigger is equal to a total Hb concentration of 7 g/dL or less, but

more liberal transfusion triggers are applied to actively bleeding subjects.¹⁰⁻¹² Limited infusion of crystalloids is recommended.¹⁰ but when blood is not available, crystalloids are infused more freely to restore BV and maintain blood pressure and blood flow, at the cost of diluting the remaining RBCs in the circulation. Diluted blood has low O₂ carrying capacity and low blood viscosity, which prevents the restoration of microvascular blood flow during resuscitation from HS.¹³ Therefore, supplementing O₂ carrying capacity and restoring blood viscosity during fluid resuscitation is essential to improve recovery and overall survival. Prehospital RBC transfusion in subjects suffering from hemorrhage improves survival compared to pre-hospital crystalloid infusion, emphasizing the importance of rapid restoration of O₂ carrying capacity and blood viscosity.¹⁴ One method to restore O₂ carrying capacity and blood viscosity with minimal logistical constraints compared to blood are Hb based O₂ carriers (HBOCs), specifically large molecular diameter and high viscosity HBOCs.

HBOCs have been proposed as alternatives to blood, when blood is not available, or logistic constraints limit blood availability, such as on the battlefield and in emergency scenarios. HBOCs are not limited by blood type compatibility, they are non-immunogenic, and they can be easily stored for long periods under ambient environmental conditions without degradation of O₂ carrying capacity. Previous small molecular diameter HBOCs caused significant side effects upon transfusion that prevented their clinical acceptance. A new generation of HBOCs, based on polymerized Hb (PolyHb) with significantly larger molecular diameter, have shown promising results¹⁵. Preclinical testing of the larger molecular diameter PolyHb suggests that these PolyHbs prevent the side effects observed with small molecular diameter HBOCs.¹⁶

Additionally, the larger molecular diameter PolyHbs have high viscosity, which improves microvascular perfusion after hemorrhagic shock via endothelial mechanotransduction and the

production of endothelial derived nitric oxide (NO).^{17,18} By increasing O₂ carrying capacity and restoring blood viscosity, larger molecular diameter PolyHbs recovers microvascular blood flow and tissue oxygenation.^{16,19,20} The objective of this study was to examine the balance between O₂ carrying capacity and blood viscosity on resuscitation from HS with large molecular diameter PolyHbs. To achieve this objective, rats were subjected to a severe hemorrhage, followed by a hypovolemic shock, and resuscitated following the Advanced Trauma Life Support (ATLS) guidelines, which recommends maintenance of hemodynamic stability during resuscitation to a set blood pressure.² In this study, rats were resuscitated with large molecular diameter polymerized bovine Hb (PolybHb) at different concentrations. The animals' hemodynamics, cardiac function, blood chemistry was monitored during the protocol, and heme and iron metabolism, markers of stress, organ function, and organ damage were studied at the end of the protocol.

4.3: Methods and Materials

Synthesis of PolybHb. PolybHb was synthesized in the low O₂ affinity (tense, T) quaternary state at a 35:1 molar ratio of glutaraldehyde to bovine Hb, filtered through a 0.2 μm tangential flow filtration (TFF) module to remove molecules larger than 0.2 μm in size, and then subjected to diafiltration on a 500 kDa TFF module using a modified lactated Ringer's solution to remove molecules less than 500 kDa in size as previously described in the literature.²⁰ PolybHb concentration was adjusted to 10 g/dL, frozen and stored at -80°C until use. The 35:1 T-state PolybHb used in this study had the following biophysical properties: methemoglobin (metHb) level = 3.6%; P₅₀ (pO₂ at which Hb is 50% saturated with O₂) = 35 mmHg; cooperativity coefficient = 1; hydrodynamic diameter = 69 nm; and viscosity = 12.4 cP.

Viscosity and Colloid Osmotic Pressure of PolybHb. PolybHb viscosity was measured using a computerized cone-plate rheometer at 37°C (Discovery HR-2, TA Instruments, New

Castle, DE, USA). Colloidal osmotic pressure (COP) was measured using a 30 kDa cutoff filter at 22°C (Colloid Osmometer Model 4420, Wescor, Logan, UT, USA).

Animal Preparation. Studies were performed in male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN), weighing 200-250 g. Animal handling and care followed the NIH Guide for Care and Use of Laboratory Animals, and approved by the University of California San Diego Institutional Animal Care and Use Committee. Briefly, animals were anesthetized using isoflurane (Drägerwerk AG, Lübeck, Germany) in room air, and instrumented with a left femoral artery and a left jugular vein catheter, and a left ventricular (LV) pressure-volume (PV) conductance catheter introduced through the right carotid artery. The LV PV measurements were completed using the closed-chest method as previously described.²¹ Animals were placed on a heating pad at 37°C in the supine position and allowed to freely breathe from a nosecone delivering anesthesia. After surgical preparation, anesthesia was reduced and constantly monitored to decrease isoflurane cardio-suppression.

Inclusion Criteria. Animals were suitable for experiments if: (i) mean arterial blood pressure (MAP) was greater than 85 mm Hg at baseline, (ii) stroke volume (SV) was greater than 90 μ L at baseline, (iii) systemic Hb was greater than 12 g/dL at baseline, and (iv) animals survived the shock period.

Cardiac Function. A 2F conductance catheter (SPR-858, Millar Instruments, TX) was used to monitor LV PV loops. Pressure and volume signals were acquired continuously (MPVS300, Millar Instruments, Houston, TX; and PowerLab 8/30, AD Instruments, Colorado Springs, CO). LV volume was measured in conductance units (relative volume unit, RVU), and converted to absolute blood volume at the end of the experiment using calibrated cuvettes and blood collected

from the experiment. Parallel LV volume was calibrated via IV injection of 40 μL of 15% NaCl w/v.²¹ Indices of cardiac function and O₂ delivery were calculated as previously described.²²

Systemic Hemodynamic Parameters. MAP and heart rate (HR) were recorded continuously from the femoral artery (PowerLab 8/30, AD Instruments, CO). Hematocrit (Hct) was measured via centrifugation from arterial blood collected into heparinized capillary tubes. Total and plasma Hb content was measured spectrophotometrically (B-Hemoglobin; Hemocue, Stockholm, Sweden). Arterial and venous blood samples were collected in heparinized glass capillary tubes (65 μL) and immediately analyzed for pH, and partial pressures of O₂ and carbon dioxide (pO₂, and pCO₂; Siemens 248, Munich, Germany).

Hemorrhagic Shock and Resuscitation Protocol. Animals were allowed to rest for 30 minutes after surgical instrumentation before baseline measurements were taken. Anesthetized rats were then hemorrhaged by withdrawing 50% of the animal's blood volume (BV, estimated as 7% of body weight), via the femoral catheter over 30 minutes. Hypovolemia was maintained for 30 minutes. Resuscitation was implemented by infusion of room temperature PolybHb through the jugular vein catheter using a syringe pump at a flow of 300 $\mu\text{L}/\text{min}$, until the animal recovered to 90% of their baseline MAP. If the animal's MAP fell below 80% of their MAP at baseline, additional PolybHb was infused. This resuscitation phase lasted for one hour. The volume transfused was calculated based on the time the syringe pump was infusing test solutions. The percent resuscitation was calculated as the ratio of the volume infused relative to the animal's total BV.

Experimental Groups. Following HS, rats were randomly resuscitated with PolybHb at 10, 5 or 2.5 g/dL. PolybHb was diluted to 5 and 2.5 g/dL with a modified lactated Ringer's solution. PolybHb concentrations were measured using a B-Hemoglobin (Hemocue). Biophysical properties

of PolybHb 10, 5 and 2.5 g/dL are presented in **Table 1**. Fifteen (n = 15) animals were entered into the HS/resuscitation study. Animals were randomly assigned to the following groups: PBH10 (PolybHb concentration = 10 g/dL, n = 5), PBH5 (PolybHb concentration = 5 g/dL, n = 5), and PBH2.5 (PolybHb concentration = 2.5 g/dL, n = 5) based on the concentration of PolybHb transfused during resuscitation.

Markers of Organ Function, Damage and Inflammation. Aspartate transaminase (AST), alanine transaminase (ALT), and IL-6 levels were determined in serum samples using ELISA kits (KA1625 and KT-6104 from Abnova Corp, Taiwan, and BMS625 from Thermo Fisher, Waltham, MA). Neutrophil gelatinase associated lipocalin (NGAL) was determined in the urine using an ELISA kit (ERLCN2, Thermo Fisher). Serum creatinine and blood urea nitrogen (BUN) were measured using colorimetric detection kits (KB02-H2 and K024-H5, Arbor Assays Inc., Ann Arbor, MI). Liver, lung and spleen CXCL1 were measured on whole tissue homogenates by ELISA (ERCXCL1, Thermo Fisher, Waltham) and corrected for protein concentration using the PierceTM BCA assay kit (Thermo Fisher). Positive CD45 neutrophils were quantified in the bronchoalveolar lavage (BAL) fluid collected by instilling sterile PBS into the lung. The percentage fraction of neutrophils in BAL fluid was determined by flow cytometry (FACSCalibur; BD, Franklin Lakes, NJ). Neutrophils were identified by their typical appearance in the forward/side scatter and their expression of CD45 (554875, BD Biosciences).

Preparation and Immunoprecipitation of Rat Tissue Extracts for Ferritin. Rat tissues were frozen and stored at -80°C until analyzed. Lysis buffer containing 1% deionized Triton X-100 and 0.1% sodium azide in 50 mM Tris-HCl pH 7.5 with Chelex-100 and phenyl-methyl-sulfonyl-fluoride (0.25 mM) was added to the homogenized tissue, which was vortexed, sonicated for 1 min, and incubated on ice for 30 min, vortexing every 5-10 min. Aliquots were taken, centrifuged

at 3,000 rpm for 15 min, and the supernatant analyzed for total protein (BCA, Pierce) and ferritin. The remaining suspension of homogenized tissue was incubated at 70°C for 10 min, cooled on ice and centrifuged at 10,000 g for 20 min. The supernatant was collected, and the pellet discarded. The total amount of ferritin in each extract was calculated using the results of an ELISA kit (Cat. no.MCA-155, Serotec, Oxford, UK). Saturating amounts of immune serum against rat tissue ferritin were then added to the extracts.

Epinephrine/Norepinephrine. Catecholamine plasma concentrations were determined using commercially available ELISA kits for catecholamines (Catecholamines ELISA kit - Ref: BA-E-6600, ImmuSmol, France). All ELISA samples were run in accordance with the manufacturer's instructions.

Statistical Analysis. Results are presented as the mean \pm SEM, or as box-and-whisker plots, or as the median and 95% confidence interval. The values are presented as absolute values and relative to the baseline. A ratio of 1.0 signifies no change from baseline, whereas lower or higher ratios are indicative of changes proportionally lower or higher compared to baseline, respectively. The Grubbs' method was used to assess closeness for all measured parameters at baseline and shock. Before experiments were initiated, sample size was determined based on power calculations using $\alpha = 0.05$ and a power of $1-\beta=0.9$ to detect differences in primary end points (MAP, contractility, and percent resuscitation) greater than 10%. Statistically significant changes between solutions and time points were analyzed using two-way analysis of variance (ANOVA), followed by *post-hoc* analyses using Tukey's multiple comparisons test when appropriate. All statistics were calculated using the Python Library StatsModels (ver. 0.9.0).²³ Results were considered statistically significant if $p < 0.05$.

4.4: Results

Biophysical Properties of PolybHb. Viscosity and COP of PolybHb were both highly concentration dependent, as described in **Table 4.1**. Dilution of PolybHb from 10 g/dL to 5 g/dL substantially decreased viscosity, but additional dilution to 2.5 g/dL had a smaller impact on viscosity. COP decreased with a similar trend as viscosity.

Table 4.1: Biophysical properties of PolybHb and blood following protocol. Viscosity measured at 316 1/s at 37°C; COP measured at 22°C.

	PolybHb Viscosity (cP)	PolybHb COP (mmHg)	Whole blood viscosity (cP)	Plasma viscosity (cP)
Baseline			3.5	1.2
PHB10	12.4	5.3	3.1	1.9
PHB5	3.7	1.5	2.9	1.7
PHB2.5	3.0	0.8	2.3	1.3

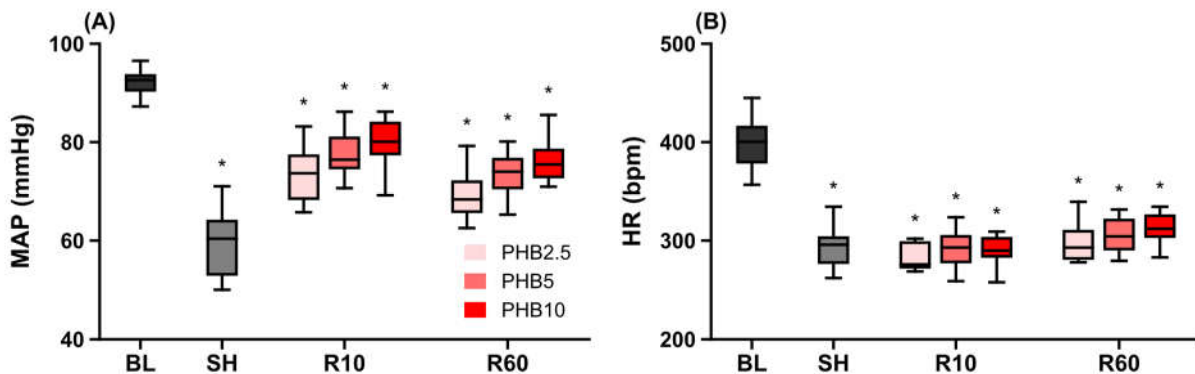


Figure 4.1: High concentration of PolybHb sustain central hemodynamics compared to low concentration. (A) MAP decreases during shock and is partially recovered during resuscitation. (B) Heart rate decreases during shock and does not fully recover in the 1 hour resuscitation. *P < 0.05 vs Baseline, †P < 0.05 vs PHB2.5, ‡P < 0.05 vs PHB5

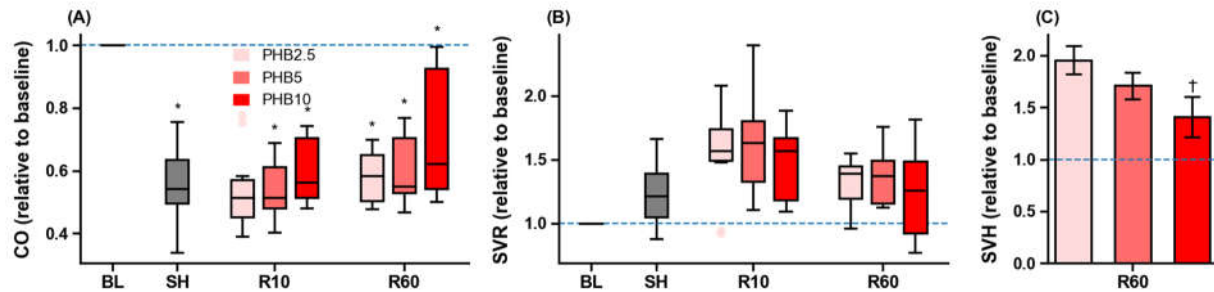


Figure 4.2: Central hemodynamics improve following transfusion of high concentration PolybHb. (A) Cardiac output recovers close to baseline values following PolybHb10 transfusion. (B) Systemic vascular resistance increases following resuscitation. (C) The high viscosity of PolybHb10 decreases vascular hinderance compared to PolybHb2.5 *P < 0.05 vs Baseline, †P < 0.05 vs PHB2.5, ‡P < 0.05 vs PHB5

Systemic Hemodynamics. All animals survived the entire experimental protocol, and there were no statistically significant differences between groups at baseline or shock. All animals had similar blood pressure at baseline and at the end of shock (**Figure 4.1A**). Blood pressure was restored similarly for all groups 10 minutes into resuscitation, but 60 minutes after resuscitation only PHB10 and PHB5 remained within the blood pressure target, while PHB2.5's blood pressure was below the target range (76% of baseline), despite continued infusion of PHB2.5. Shock induced bradycardia for all groups, and heart rate did not recover for any groups during resuscitation (**Figure 4.1B**). CO decreased from baseline during shock and was not restored to baseline levels after transfusion, however, CO recovered proportionally to PolybHb concentration (**Figure 4.2A**). Systemic vascular resistance (SVR) increased during shock, and further increased during resuscitation, but these changes were not statistically significant from baseline (**Figure 4.2B**). During resuscitation, however, systemic vascular hinderance (SVH), the contribution of blood vessel diameter to SVR, or the SVR independent of blood viscosity, decreased proportional to the concentration of PolybHb. This indicates vasoconstriction was inversely proportional to PolybHb concentration infused and circulating in the plasma (**Figure 4.2C**).

Cardiac Mechanoenergetics. Cardiac indices depicting the relationship between O₂ consumption and mechanical work of the left ventricle myocardium [such as SW, IEU, and the

work performed per volume ejected (SW/SV)], are presented in **Figure 4.3**. Shock significantly impaired SW, IEU, and SW/SV, and during resuscitation, cardiac mechanoenergetics were recovered depending on the concentration of PolybHb transfused. Early into the resuscitation, SW was restored to baseline levels for PHB10 and PHB5, but not for PHB2.5 (**Figure 4.3A**), but SW was restored to baseline for all groups by the end of the protocol. The energy required to eject an equivalent volume of blood (SW/SV), increased during resuscitation for all groups (**Figure 4.3B**), but SW/SV was lower for PHB2.5 compared to PHB10 or PHB5. Late into the resuscitation, PHB10 and PHB5 both maintained SW/SV at baseline levels, but SW/SV decreased for PHB2.5. IEU, a measure of the heart's metabolism, recovered to baseline values for PHB10 and PHB5, but never recovered for PHB2.5 (**Figure 4.3C**). Additionally, PHB2.5 had significantly lower IEU compared to PHB10 throughout resuscitation.

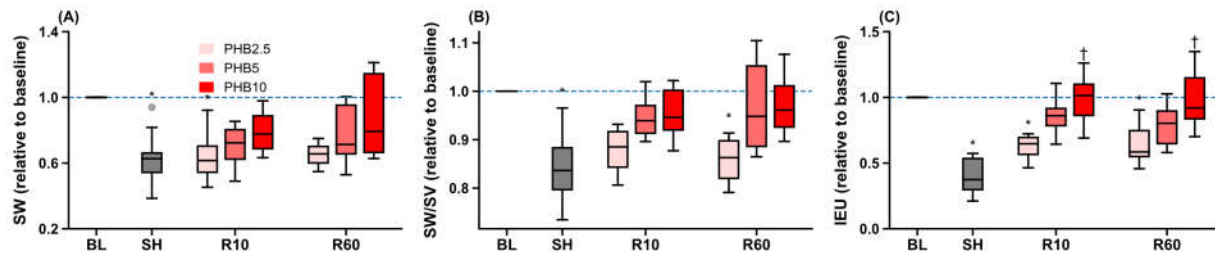


Figure 4.3: Cardiac mechanoenergetics following PolybHb transfusion. (A) Stroke work recovers to baseline levels. (B) Stroke work per volume ejected is elevated with PolybHb10 and PolybHb5, indicating better recovery of cardiac function and more pressure generation. (C) Internal energy utilization of the heart, an indicator of the heart's potential energy, is increased after transfusion of PolybHb10 relative to PolybHb2.5 *P < 0.05 vs Baseline, †P < 0.05 vs PHB2.5, ‡P < 0.05 vs PHB5

Hematology. A summary of hematological changes are described in **Table 4.1**. To achieve and maintain the blood pressure goal, significantly more PHB2.5 was transfused than PHB10 and PHB5 (**Figure 4.4**). Despite the different volumes given to all groups, hematocrit between the groups was similar at all time points. The total and plasma Hb was significantly higher for PHB10 compared to PHB5 and to PHB2.5, due to the higher concentration of PolybHb infused.

Resuscitation with PHB10 and PHB5 resulted in a significantly higher blood and plasma viscosity compared to PHB2.5 (Table 4.1).

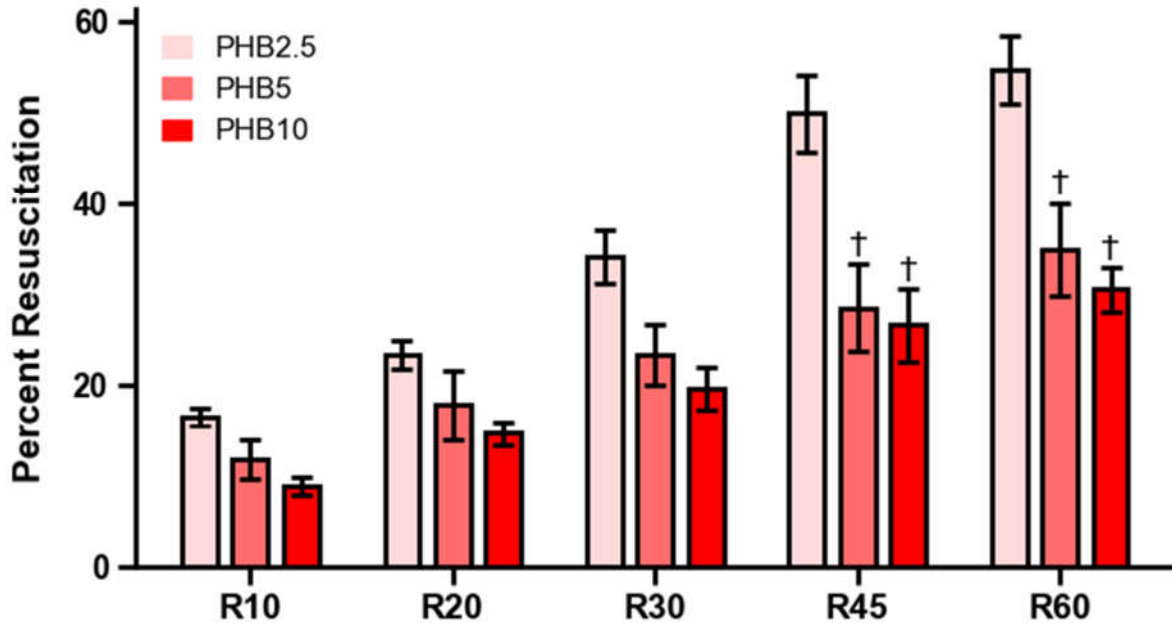


Figure 4.4: Significantly higher volume of PolybHb2.5 is required to reach the MAP target. *P < 0.05 vs Baseline, †P < 0.05 vs PHB2.5, ‡P < 0.05 vs PHB5

Table 4.2 Hematological parameters during the protocol. Data are presented as median [95% CI]. *P < 0.05 vs Baseline, †P < 0.05 vs PHB2.5, ‡P < 0.05 vs PHB5

	Baseline	Shock	Resuscitation 10 min			Resuscitation 60 min		
			2.5 g/dL	5 g/dL	10 g/dL	2.5 g/dL	5 g/dL	10 g/dL
Hct (%)	43 [42 - 44]	28 [27 - 29]	27 [25 - 29]	27 [26 - 28]	26 [24 - 28]	22 [21 - 24]	23 [21 - 25]	20 [19 - 21]
Total Hb (g/dL)	13.5 [13.3 - 13.7]	8.9 [8.7 - 9.1]	8.9 [8.2 - 9.6]	9.5 [9.0 - 10.0]	9.8 [9.6 - 10.0]†	8.8 [8.4 - 9.2]*	9.5 [9.3 - 9.7]†	10.1 [9.6 - 10.6]†
Plasma Hb (g/dL)			0.9 [0.7 - 1.1]*	1.7 [1.1 - 2.3]*	1.8 [0.8 - 2.8]*	2.2 [1.8 - 2.6]*	3.3 [2.2 - 4.4]*	4.1 [3.0 - 5.2]*†‡

Blood Chemistry. HS induced acidosis, increased arterial pO₂, and decreased arterial pCO₂ compared to baseline (Table 4.3). Acidosis was resolved by resuscitation with PHB5, but it was not fully resolved for PHB10 or PHB2.5. The base-excess, which describes the acid-base balance,

significantly decreased during shock. Acid-base balance never fully recovered to baseline, and animals remained slightly acidic during resuscitation. Acid-base balance was restored closer to baseline for PHB5 and PHB2.5 than PHB10, but there were no significant differences between groups.

Table 4.3: Blood gasses recover to near baseline values for all groups. Data are presented as median [95% CI]. *P < 0.05 vs Baseline, †P < 0.05 vs PHB2.5, ‡P < 0.05 vs PHB5

	Baseline	Shock	Resuscitation 10 min			Resuscitation 60 min		
			2.5 g/dL	5 g/dL	10 g/dL	2.5 g/dL	5 g/dL	10 g/dL
pH	7.41 [7.40 - 7.43]	7.29 [7.27 - 7.31]*	7.27 [7.21 - 7.32]*	7.33 [7.26 - 7.39]*	7.31 [7.27 - 7.34]*	7.33 [7.32 - 7.35]*	7.36 [7.34 - 7.39]	7.31 [7.26 - 7.36]*
pCO ₂ (mmHg)	40.9 [38.5 - 43.3]	35.0 [33.3 - 36.7]*	38.5 [36.3 - 40.7]	41.3 [33.2 - 49.4]	37.0 [33.6 - 40.4]	45.3 [43.1 - 47.5]	40.4 [33.7 - 47.1]	43.0 [40.8 - 45.2]
pO ₂ (mmHg)	86 [81 - 91]	113 [108 - 118]*	97 [84 - 110]	108 [91 - 124]	97 [84 - 110]	81 [72 - 90]	92 [75 - 109]	76 [57 - 95]
Base excess	1.8 [1.2 - 2.5]	-9.1 [-10.4 - -7.7]*	-8.5 [-11.9 - -5.1]*	-6.8 [-10.2 - -3.4]*	-7.0 [-8.5 - -5.5]*	-2.3 [-4.0 - -0.6]	-1.9 [-4.4 - 0.6]	-4.4 [-7.2 - -1.6]

O₂ Delivery and Extraction. O₂ delivery (DO₂) decreased during shock and did not fully recover during resuscitation (**Figure 4.5A**). There are slight concentration-dependent differences in DO₂, but these differences were not significant between groups. Late into resuscitation, DO₂ was highest for PHB10, and was approximately 1.3 times and 1.4 greater than DO₂ for PHB5, and PHB2.5, respectively. Animals maintained a similar degree of O₂ extraction (VO₂) throughout the experiment, but VO₂ remained higher for PHB10 after resuscitation, whereas the VO₂ decreased slightly for PHB5 and PHB2.5 during resuscitation (**Figure 4.5B**). All groups had very similar VO₂:DO₂ ratios, indicative that the low affinity PolybHb facilitates O₂ offloading independently of the concentration of PolybHb (**Figure 4.5C**). When broken down into the O₂ carrying components (RBCs and PolybHb), the DO₂ and VO₂ by PolybHb was determined by its' plasma concentration (**Figure 4.5D-I**). However, a significantly higher fraction of the total O₂ extracted was derived from RBCs compared to PolybHb, and the ratio of VO₂:DO₂ was significantly higher

for RBCs than PolybHb, suggesting that PolybHb acts as an intermediary of O₂ offloading to tissues.

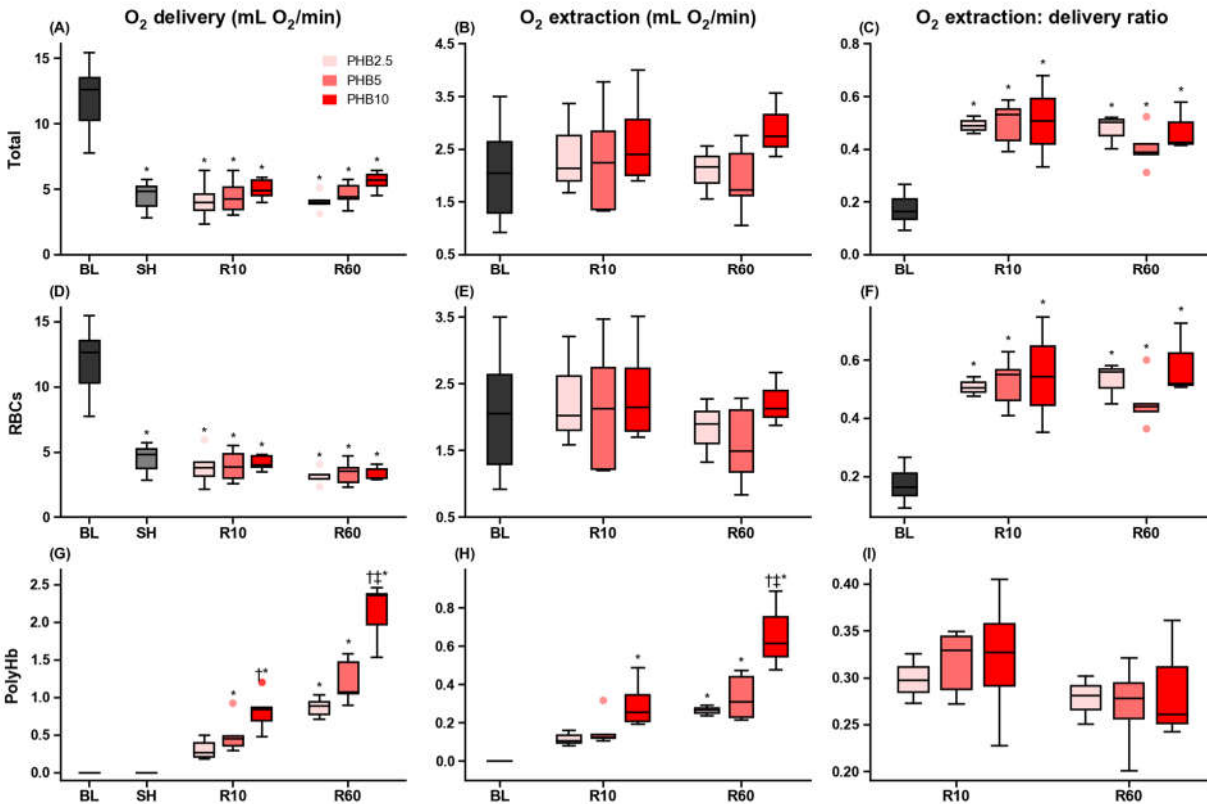


Figure 4.5: Parameters of O₂ transport. (A-C) O₂ transported by the whole blood. (D-F) O₂ transported by the RBCs. (G-I) O₂ transported by the PolybHb. *P < 0.05 vs Baseline, †P < 0.05 vs PHB2.5, ‡P < 0.05 vs PHB5

Markers of Organ Function, Damage, and Inflammation. In general, markers of vital organ damage and inflammation increased with the concentration of PolybHb infused during resuscitation (**Figure 4.6**). A marker of acute kidney damage (urinary neutrophil gelatinase associated lipocalin [u-NGAL]) was significantly higher for PHB10 compared to PHB5 and PHB2.5, as were molecules normally cleared by the kidneys, such as serum creatinine and blood urea nitrogen (BUN). Similarly, liver enzymes associated with liver damage, such as aspartate and alanine aminotransferases, showed higher plasma concentrations for PHB10 compared to PHB5 and PHB2.5. Catecholamine levels were similar for all groups. Markers of systemic and lung inflammation decreased with PolybHb concentration. Systemic inflammatory markers decreased

with the concentration of PolyHb transfused (**Figure 4.7**). Inflammatory markers of organs in the reticuloendothelial system were similar for PHB10 and PHB2.5 and were significantly higher than PHB5. Markers of iron transport post-resuscitation all increased in a PolyHb concentration-dependent manner (**Figure 4.8**), as expected.

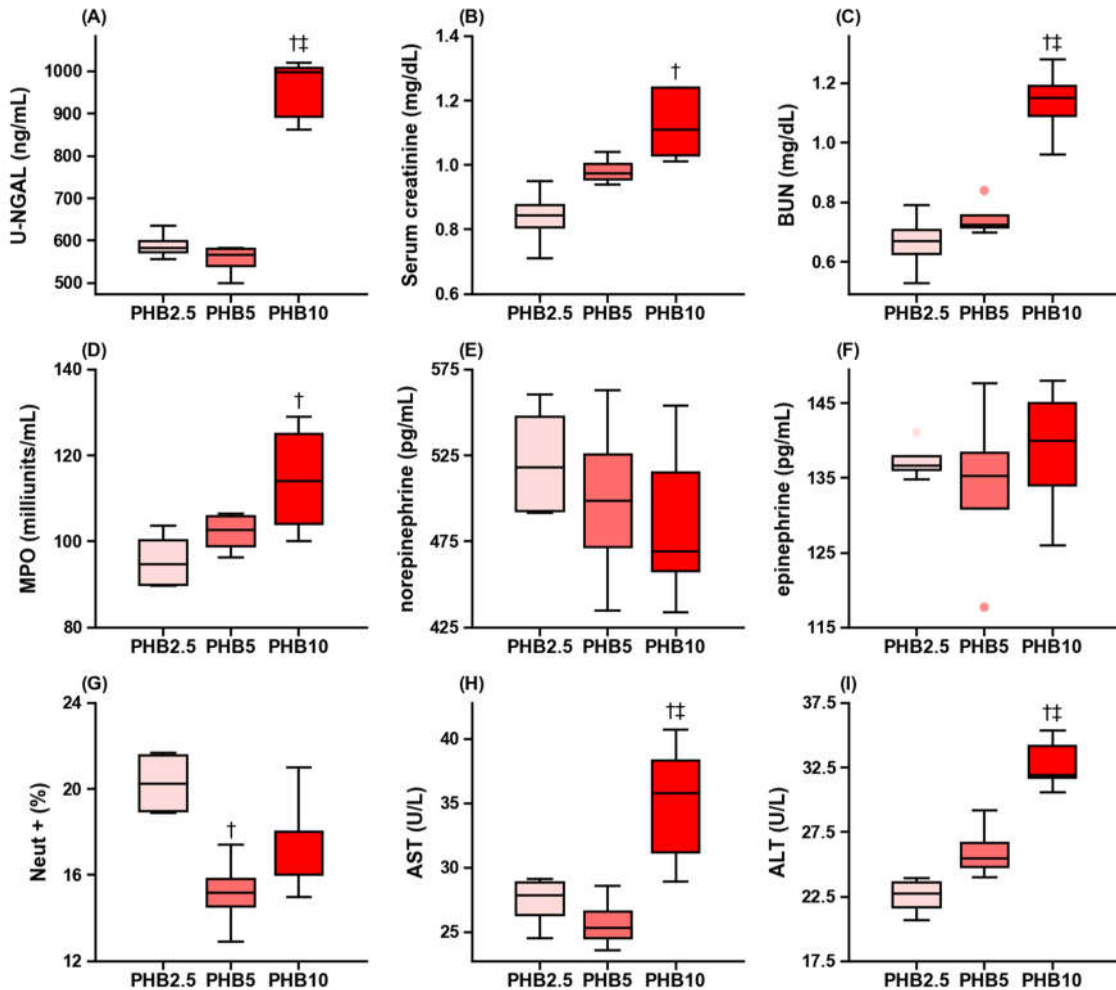


Figure 4.6: Markers of organ damage and function. (A-C) Kidney markers. (D,G) Lung markers. (E,F) Systemic Markers. (H,I) Liver markers. †P < 0.05 vs PHB2.5, ‡P < 0.05 vs PHB5

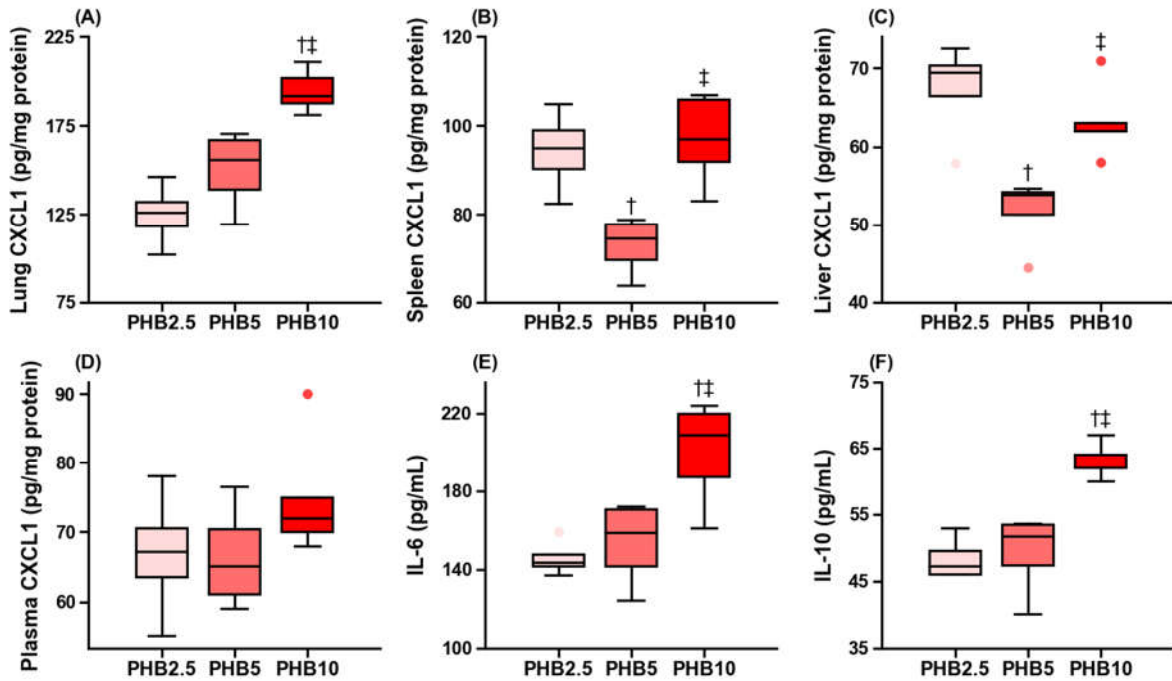


Figure 4.7: Markers of organ neutrophil recruitment and inflammation. (A) Neutrophil recruitment in lungs is dependent on the PolybHb concentration transfused (B-D) Neutrophil recruitment in the spleen, liver, and systemically appear lowest for PHB5. (E,F) The inflammatory and anti-inflammatory response appear to be equal and PolybHb concentration dependent. †P < 0.05 vs PHB2.5, ‡P < 0.05 vs PHB5

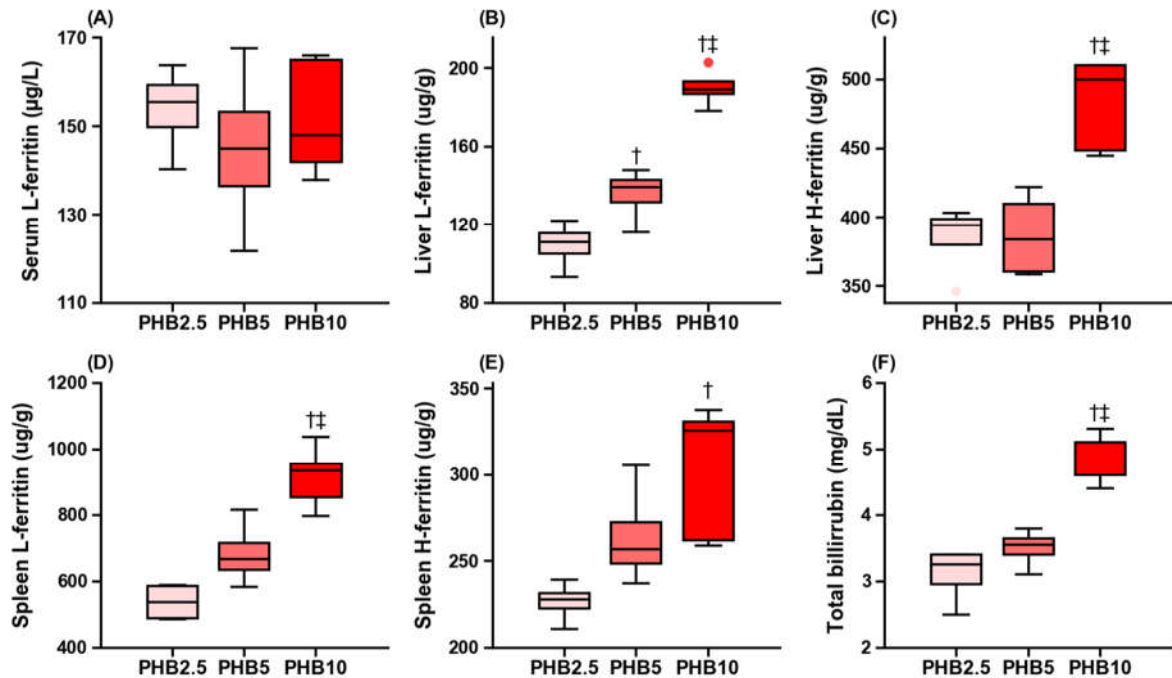


Figure 4.8: Markers of iron transport. (A) Plasma iron transport appear to be independent of the PolybHb concentration transfused (B-E) Markers of iron transport in the liver, spleen are dependent on the concentration of PolybHb transfused. (F) Heme metabolism is dependent on the concentration of PolybHb transfused. †P < 0.05 vs PHB2.5, ‡P < 0.05 vs PHB5

4.5: Discussion

The principal finding of this study is that resuscitation from HS with high concentrations of PolybHb (PHB10 and PHB5) restored cardiac function and O₂ delivery compared to the low concentration of PolybHb (PHB2.5). PolybHb improved CO, SW, and the heart's IEU as a function of the Hb concentration. Markers of organ function, organ damage, and inflammation, in general, also changed proportionally to the concentration of PolybHb. Therefore, the outcome of resuscitation from HS appears to be dependent on PolybHb concentration. These results demonstrate that higher concentrations PolybHb are effective in recovering intravascular volume, as a lower transfusion volume was necessary to restore MAP and cardiac function with PHB10. The low concentration of PolybHb (PHB2.5) had low intravascular retention, as confirmed by the larger transfusion volume needed to resuscitate compared to PHB10 and PHB5, and their similar final hematocrits after resuscitation. As the main differences between the resuscitation fluids were their O₂ carrying capacity and viscosity, the physiological mechanisms related to these two properties likely determined the recovery from HS.

The recovery of cardiac function in this study was proportional to PolybHb solution viscosity. Resuscitation from HS with high viscosity solutions improves the recovery of microvascular function.¹⁷ Research focused on the microcirculation has shown that restoration of microvascular perfusion is more effective than recovery of O₂ carrying capacity in restoring tissue oxygenation.²⁴ This research has also demonstrated that recovery of systemic markers such as blood pressure, O₂ saturation, or lactic acid concentration during resuscitation from HS are not indicative of effective tissue oxygenation, nor are they strong markers of focal ischemia or predictors of future multiorgan failure.²⁵ Transfusion of PHB10 and PHB5 provided an increase of 46% and 31% in plasma viscosity relative to resuscitation with PHB2.5. The differences in

viscosity may account for some of the differences observed in cardiac function, vascular hinderance, and the total volume transfused. In addition, differences in viscosity induced by PolybHb also affect the shear stress exerted by flowing blood on vascular endothelial cells, which influences vessel diameter and modulates the release of vasodilatory autocooids (prostacyclin, and NO). Given the heart's high metabolism, restoration and maintenance of coronary perfusion is essential to ensure cardiac function. The coronary microcirculation, much like the systemic microcirculation, is highly sensitive to endothelial shear stress resulting from blood flow,²⁶ thus NO mediated coronary vasodilation is then likely to have increased with PHB5 and PHB10, improving cardiac perfusion and O₂ delivery. While systemic arterioles are traditionally deemed to be the main regulators of systemic vascular resistance due to their smooth muscle mediated vasomotion, large arteries also have significant amounts of smooth muscle that regulate their stiffness through vasoactive NO mechanisms.²⁷ PHB10 and PHB5 also provided a 35% and 26% increase in whole blood viscosity relative to PHB2.5. While microvascular wall shear stress is primarily determined by plasma viscosity, whole blood viscosity plays a larger role than plasma viscosity in the NO-regulated vasoactive mechanisms of large arteries due the Poiseuille flow regime that decreases the contribution of the cell-free lubrication layer on wall shear stress. The changes in whole blood viscosity produced by the infusion of PHB10 and PHB5 compared to PHB2.5 (**Table 4.1**) increases shear stress in large systemic vessels, decreasing arterial stiffness which has been shown to improve coronary blood flow.²⁸ Combined, these viscosity driven mechanisms decrease afterload and increase coronary O₂ availability which helps to recover CO.²⁹

Traditional non-O₂ carrying resuscitation fluids increase BV and transiently recover CO during resuscitation from HS. However, they also dilute the remaining RBCs in the circulation, reducing the total O₂ carrying capacity of blood. On the other hand, PolybHb increases BV and

total O₂ carrying capacity simultaneously, which helps sustain tissue oxygenation. As blood flow is restored to peripheral tissues during resuscitation, O₂ demand increases and the heart is challenged to perform more work. In these experiments, total O₂ delivery was proportional to the concentration of PolybHb transfused. During shock, acidosis results in a rightward shift of the Hb-O₂ affinity curve, thus favoring O₂ extraction. The low O₂ affinity of PolybHb, and its presence in solution in plasma further enhances tissue O₂ delivery and extraction. The low Hb-O₂ affinity of PolybHb allows for facilitated release of O₂ from the RBCs and brings the PolybHb-bound O₂ closer to the tissue, which causes a steeper O₂ gradient between blood and tissues, thus increasing the O₂ flux leaving the microcirculation. This enhanced O₂ extraction behavior is demonstrated with the sustained rise in VO₂:DO₂ following resuscitation with PolybHb. Furthermore, PolybHb clearly facilitates the transfer of O₂ from RBCs to the tissues. At the end of resuscitation, the PolybHb in the PHB10 group accounts for 37% of the total DO₂, but only 22% of the total VO₂ (VO₂:DO₂ = 26%). A similar trend is observed with PHB5 and PHB2.5, where only 28% of the O₂ delivered by the PolybHb is actually offloaded. A much higher percentage of the O₂ is extracted from the RBCs, where a total of 52%, 44%, and 56% of O₂ delivered by the RBCs is extracted. Therefore, PolybHb acts as an intermediary O₂ carrying species, promoting the offloading of O₂ to tissues, apparently independent of concentration. However, the increase in total O₂ carrying capacity achieved by transfusion of PolybHb appears to be less critical than the facilitated O₂ offloading and the increased viscosity, as animals still had a significant reserve of O₂ available for delivery, as indicated by VO₂:DO₂.

Restoration of cardiac function is critical in ensuring proper O₂ delivery to peripheral tissues during resuscitation from HS. Catecholamines released during shock redistribute blood flow from peripheral tissues to vital tissues, such as the heart and brain, to sustain their

metabolism.³⁰ Thus, the heart must generate pressure to overcome vasoconstriction and restore capillary perfusion pressure to prevent hypoxia and the accumulation of byproducts of anaerobic metabolism of peripheral tissues. The restoration of capillary perfusion pressure following HS depends on a number of factors that are all significantly affected by blood viscosity.²⁴ The heart must overcome cardiac afterload and generate flow (CO) to exceed the metabolic demands of perfused tissues. In these experiments, CO recovered proportional to the concentration of PolybHb infused, with no improvement in CO from shock for PHB2.5. The CO, and the viscosity of the blood, generates shear stress on the vascular wall which regulates SVR. While SVR was not different between groups, SVH was significantly higher for PHB10 than PHB2.5; since PHB2.5 did not improve CO or increase viscosity, it is unlikely that SVH improved significantly from shock for this group. It was previously demonstrated in a hamster window chamber model that capillary perfusion pressure is primarily dependent on microvascular diameter (reported by SVH), not the viscosity of the solution.²⁴ As such, SVH is a sensitive indicator of capillary perfusion pressure, and thus microvascular O₂ delivery. Indeed, cardiac mechanoenergetic parameters (SW, IEU, SW/SV; **Figure 4.3**) suggest an increase in cardiac O₂ consumption for PHB10 and PHB5 compared to PHB2.5; these changes in cardiac O₂ consumption are satisfied by the changes in coronary O₂ delivery, as there were no significant differences in base-excess between experimental groups. These data suggest that PHB10 and PHB5 improve cardiac function following resuscitation from HS.

The O₂ delivery, plasma viscosity, and COP appear to be important than the total volume infused during fluid resuscitation in terms of recovering pressure, cardiac function, and blood flow (**Figure 4.4**). The volume requirement of PHB10 and PHB5 were significantly lower than PHB2.5, but they increased the total Hb concentration and plasma viscosity compared to PHB2.5. The

ability to restore proper tissue blood flow and cardiac function at lower infusion volumes is extremely beneficial, as it limits the potential toxicity of PolybHb. In fact, transfusion of PHB2.5 could prove deleterious compared to PHB5 and PHB10 as the large resuscitation volume needed dilutes native O₂ carrying capacity and can induce acute respiratory distress syndrome and edema. Additionally, lack of proper tissue perfusion resulting from the use of PHB2.5 could lead to downstream consequences, such as ischemia and multiorgan failure. The low COP of the PHB2.5 likely contributed to the large transfusion volume required to recover from shock, since approximately 24% of the PHB2.5 that was transfused was removed from the circulation (based on difference in the theoretical hematocrit following transfusion, and the measured hematocrit). Lastly, large volume infusions during resuscitation result in renal dysfunction³¹ and increases the likelihood of systemic or pulmonary edema, resulting in further complications following resuscitation from HS.

In this study, some markers of kidney function (serum creatinine, BUN), indicate that PolybHb suppressed kidney function, while other markers indicate that PolybHb resulted in acute liver injury (ALT), acute inflammation (Lung CXCL1, IL-6, and IL-10), and altered iron transport (liver L-ferritin, spleen L and H-ferritin, and total bilirubin), in a concentration dependent manner. Other markers of kidney (u-NGAL) and liver injury (AST), as well as markers of inflammation (spleen and liver CXCL1) did not demonstrate a clear concentration dependence. In fact, for markers that did not demonstrate a concentration dependence, these markers showed the lowest level in the PHB5 group. Furthermore, for markers that did show a concentration dependent relationship PHB2.5 and PHB5 expressed very similar levels of markers, often with PHB5 only showing marginally higher markers, and PHB10 showing substantially higher markers. This could be an important consideration when deciding the dosage of PolybHb. However, markers of acute

organ injury in this study were primarily driven by transfusion-associated toxicities, as the time scale of the study is too short to examine secondary injury caused by the different degrees of recovery experienced by the groups. Based on these data, and the cardiac function data presented earlier, the intermediate concentration of PolybHb (PHB5) seems to be optimal to restore cardiac function and hemodynamics to a comparable degree as PHB10, with similar transfused volume, and with less tissue inflammatory markers and acute tissue toxicity compared to PHB10.

Previous studies with small molecular diameter HBOCs demonstrated deleterious changes in both the heart and microvasculature following the transfusion of high concentrations of HBOCs.³²⁻³⁴ These studies found that a moderate dosage of HBOCs resulted in fewer complications, as animals experienced dose-dependent vasoconstriction which limited O₂ delivery and increased left ventricular afterload. Previous HBOCs contained >1% non-polymerized Hb and possessed significantly lower average molecular diameters than the PolybHb tested in this study. The differences between our study and previous studies are likely due to the drastically different biophysical properties of previous HBOCs and new large molecular diameter of the PolybHb evaluated in this study, in addition to the removal of most of the low molecular diameter PolybHb molecules by diafiltration on a 500 kDa TFF module. The molecular size and purity of PolybHb prevents tissue extravasation, separates PolybHb from the vascular endothelium via steric hindrance, and lastly increases plasma viscosity. Large molecular diameter PolybHb results in a more stable product with limited vascular smooth muscle NO consumption and increased endothelial mechanotransduction compared to previous HBOCs. The concentration dependent increase of cardiac function observed in this study confirms that the large molecular size of PolybHb prevents vasoconstriction and hypertension. Furthermore, these data suggest that large

molecular diameter PolybHb could be transfused more liberally than previous HBOCs without passing from an ideal ‘therapeutic range’ into a range where HBOC administration can be harmful.

Limitations. Studies in rats cannot be directly translated to clinical scenarios, but these results suggest that high viscosity O₂ carriers improve resuscitation from HS. It is also difficult to independently assess the effects of viscosity and O₂ carrying capacity following resuscitation with PolybHb. These two parameters are intrinsically linked, and modifications to the manufacturing process would ultimately result in a significantly different material that could change the COP, vasoactivity, and/or parameters of O₂ loading and offloading. As this study was aimed at replicating ATLS guidelines, which suggest hypotonic resuscitation, differences in cardiac function between groups were not expected to be large. However, the significant differences in the volume transfused indicate the differences in efficacy between concentrations of PolybHb, and a mild dose-dependence is apparent. Inverse to cardiac parameters, markers of acute organ damage suggest that low concentrations of PolybHb may be beneficial. Since this study was aimed at assessing the so-called ‘golden-hour’ of resuscitation, secondary organ injury resulting from insufficient resuscitation from HS are not examined and may further support the use of PHB10 than the acute parameters measured.

This study demonstrated that PolybHb restores cardiac function and MAP in a concentration dependent manner during resuscitation from HS. This concentration dependency was not seen in previous studies due to vasoactivity of previous HBOCs, that is significantly attenuated by the additional processing steps that results in a large molecular diameter PolybHb. These processing steps also cause PolybHb to have high viscosity, which has been shown to be a critical determinant in restoration of microvascular perfusion during resuscitation from shock¹⁷. Overall, the intermediate concentration of PolybHb evaluated restored cardiac function and

hemodynamics comparable to the highest concentration, and minimized tissue inflammation and injury associated with the higher PolybHb concentration. Thus, while the increased molecular size of PolybHb inhibits the pressor effects observed in previous generation HBOCs, the molecular size does not eliminate the intrinsic toxicity of Hb-based products. Overall, large molecular diameter PolybHb is a promising alternative to the transfusion of blood in austere environments, or after mass casualty events when type-O blood supply is insufficient, or where logistical constraints prevent the use of conventional blood transfusions.

4.6: Acknowledgements

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Chapter 4, in full, is currently being prepared for submission of the material as “Balance Between Oxygen Transport and Blood Rheology During Resuscitation from Hemorrhagic Shock with Polymerized Hemoglobin” by A. T. Williams, A. Lucas, C. R. Muller, C. Bolden-Rush, A. F. Palmer, and P. Cabrales. The dissertation author was the primary investigator and author of this paper.

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CHAPTER 5: POLYMERIZED HEMOGLOBIN WITH INCREASED MOLECULAR SIZE REDUCES TOXICITY IN HEALTHY GUINEA PIGS

5.1: Abstract

BACKGROUND: Hemoglobin (Hb) based oxygen (O₂) carriers (HBOCs) have been developed as an alternative to red blood cells (RBCs) for use in transfusion medicine. HBOCs have many benefits over RBCs; however, previous generations of HBOCs failed in clinical trials due to unanticipated cardiotoxicity. These problems likely originated from vasoconstriction, hypertension, oxidative stress, and the presence of low molecular weight (MW) Hb species in the HBOC formulation. Therefore, the objective of this study is to compare the toxicity of small MW PolyHb (**SPolyHb**) to large MW PolyHb (**LPolyHb**) in guinea pigs, since they lack the ability to synthesize vitamin C and are more sensitive to oxidative stress relative to other preclinical animal models.

METHODS AND RESULTS: Study groups were defined by study solutions, and the control group received 5% human serum albumin (HSA). Solutions were injected as a hypervolemic (topload) infusion of 10% of the blood volume into animals. A set of animals was monitored for 2 hours, in terms of mean arterial pressure, heart rate, and blood gases; and another set of animals was monitored for 24 hours for pharmacokinetics. SPolyHb caused a 50% elevation in MAP from baseline, while LPolyHb caused only a small increase in MAP. Both PolyHbs also increased markers of organ damage, and tissue and systemic inflammation compared to controls. SPolyHb caused significant changes in tissue function and vital organ toxicity markers compared to LPolyHb, specifically markers related to kidney, liver, and lung injury, as well as systemic inflammation and iron transport by the reticuloendothelial system. LPolyHb had a longer half-life

than SPolyHb, which correlates with the observations made in the reticuloendothelial and iron transport systems.

CONCLUSIONS: These studies indicate that the molecular size of PolyHb determines vasoactivity, circulation time, mechanism of elimination, toxicity and inflammation induced by its infusion.

5.2: Introduction

According to the American Red Cross, a blood transfusion occurs every two seconds in the United States (U.S.), resulting in approximately 36,000 units of red blood cells (RBCs) being transfused every day.¹ It is estimated that only 3% of age-eligible persons donate blood, resulting in a donation rate that falls short of current demand.^{1,2} In trauma, hemorrhage accounts for nearly 35% of pre-hospital deaths; moreover, nearly 20% of military casualties are in hemorrhagic shock (HS) on arrival to field hospitals and an additional 5% require urgent transfusion.^{3,4} Blood transfusion is a life-saving therapy in patients with HS, restoring blood volume, blood O₂ carrying capacity, cardiac output (CO), and vital organ perfusion. A recent review concluded that hemorrhage accounted for almost 90% of potentially survivable battlefield deaths⁵ - lives that could be saved with better hemorrhage control, field-ready blood, blood components, or blood substitutes. Blood transfusions can be logistically difficult; currently, the U.S. Food and Drug Administration (FDA) states that RBCs must be used within 42 days, and platelets within just 5 days. Furthermore, the volunteer-based blood supply system is particularly vulnerable to the effects of pandemics, as donors may donate less blood because of illness, the need to care for ill family members, or the fear of exposure to influenza. In addition there are other transfusion related-problems such as the risk of blood-type mismatch and disease transmission.⁶ In this context developing alternatives to blood products is necessary.

Hemoglobin (Hb)-based oxygen (O₂) carriers (HBOCs) have been proposed as an alternative to RBCs for transfusion medicine. HBOCs have many advantages over RBCs, including a lack of immunogenicity, prolonged storage life, and potential to use non-human Hb sources for production, among others. However, phase III clinical trials of previous generations of HBOCs were met with several serious issues, such as severe vasoconstriction, hypertension, and

myocardial infarction that were not detected in pre-clinical trials.⁷ These problems likely originate from low molecular weight (MW) Hb species in the HBOCs.⁸ The two major mechanisms to explain the occurrence of Hb-induced vasoconstriction are: 1) nitric oxide (NO) scavenging, and 2) oversupply of O₂ to the vascular smooth muscle cells. NO scavenging occurs since oxyHb rapidly and irreversibly reacts with NO, producing oxidized Hb (metHb) and nitrate, a reaction that is normally buffered by the RBC membrane. O₂ oversupply occurs due to radial diffusion of HBOC molecules, as they are less restricted to the center of the vessel, which results in facilitated O₂ transport.⁹ Both NO scavenging and oversupply of O₂ cause vasoconstriction and hypertension, which are further exacerbated by the extravasation of acellular Hb into the perivascular space.¹⁰ The vasoconstriction and hypertension observed with previous generations of HBOCs likely accounted for some of the injury observed in vital organs such as the kidney, liver and spleen.¹¹ Organ injury was also likely enhanced by oxidative stress, which can result in systemic and vital organ inflammation.¹²

The toxicity of acellular Hb is also enhanced by the release of heme, which elicits lipid peroxidation and mitochondrial damage and increases the production of reactive oxygen species (ROS)¹³. Heme selectively triggers pro-inflammatory receptors, and activates proteasomes¹⁴. Increased levels of plasma Hb result in Hb filtration in renal glomeruli and subsequent tubular injury¹⁵. ROS can induce cellular toxicity (via nucleic acid, lipid, and protein oxidation), and decrease the O₂ carrying capacity of Hb via the formation of metHb.^{12,13} Therefore, Hb in the perivascular space may induce oxidative tissue damage that can account for the cases of myocardial infarction observed in phase III clinical trials of small sized commercial PolyHbs such as Hemopure® and PolyHeme®.¹⁶ To address the desperate need for a lifesaving treatment for

limited O₂ carrying capacity that is free of severe side-effects, we developed a large MW polymerized Hb (LPolyHb).

An important aspect of pre-clinical toxicological evaluation of HBOCs is the selection of appropriate species and models that most closely reflect human physiology and pharmacology. In conjunction with RBCs and plasma, tissue ascorbic acid (AA) is the primary small molecule reductant that maintains the non-oxidized ferrous (HbFe²⁺) form of Hb/HBOCs and prevents accumulation of the non-O₂ carrying and heme unstable metHb (HbFe³⁺).¹⁷ The side effects observed in clinical trials of small sized commercial PolyHbs were not apparent in tests performed in animal models such as mice and rats. Rats, mice, and hamsters all produce ascorbic acid (AA) innately, and increase AA production in response to oxidative stress.¹⁸

Guinea pigs are potentially a more relevant small animal species for predicting HBOC safety in humans. The rationale for evaluating the guinea pig is based on several factors that suggest a similarity with humans in terms of overall antioxidant status. Guinea pigs, like humans, are incapable of endogenous production of ascorbic acid (AA) due to the evolutionary loss of functional hepatic L-gulonolactone oxidase (LGO).¹⁸ Guinea pigs, non-human primates, and humans have interestingly evolved in similar ways to distribute antioxidant capabilities within the circulation and tissue parenchyma to counteract the loss of AA.¹⁹ Therefore, guinea pigs are a more appropriate pre-clinical model to study the side effects of PolyHbs. Based on our previous findings, our hypothesis is that increasing the molecular size of PolyHb reduces nitric oxide scavenging and vascular hyperoxygenation, thus causing less toxicity and preventing hypertension. Thus, the objective of this study was to compare the toxicity of large MW PolyHb (LPolyHb) compared to small MW PolyHb (SPolyHb) in guinea pigs.

5.3: Methods and Materials

Polymerized Hemoglobin. PolyHb was synthesized in the low oxygen affinity (T) state with a 35:1 ratio of glutaraldehyde to Hb, and then subjected to 8-9 cycles of diafiltration with different MW cutoff membranes, as previously described.²⁰ This resulted in 2 PolyHb solutions: **SPolyHb**, containing polymerized Hb molecules with MW >100 kDa; and **LPolyHb**, containing polymerized Hb molecules with MW >500 kDa. To estimate the MW distribution of the Hb/PolyHb, we performed HPLC size exclusion chromatography (SEC) on all samples. HPLC-SEC was performed with an Acclaim SEC-1000 column (Thermo Scientific, Waltham, MA) on a Thermo Scientific Dionex Ultimate UHPLC system. The flow rate of the mobile phase (0.5 mM 7.4 phosphate buffer) was maintained at 0.35 mL/min for all runs. All quantitative measurements of PolyHb composition were performed via analysis of absorbance at 280 nm. To estimate the MW distribution of the PolyHb, we used the manufacturer provided calibration of the SEC column corrected for the holdup time in the tubing between system components.

Additionally, the calibration was validated in the laboratory with a protein ladder containing a variety of proteins ranging from 50 to 250 kDa. Polymerization order was estimated with a deconvolution algorithm on the resulting chromatograms. In brief, we assumed that each polymerization order elutes at a mean time equivalent to its approximate MW. We also assumed that the molecular distribution of each of the polymer size orders follows a Gaussian distribution. Because the Acclaim SEC-1000 has a separation range of 1 to 1000 kDa and an exclusion limit of 3 to 7.5 MDa, we only deconvolute the polymer size orders less than 2048 kDa. Due to the exclusion limit, the large MW species (> 1024 kDa) are too close to deconvolute and are instead treated as a single species that is assumed to contain all large MW species. This analysis identified

4 distinct PolyHb fractions within SPolyHb and LPolyHb: 0th order (64kDa, non-polymerized), 1st order (128 kDa), 2nd order (256 kDa), and 3rd order + (≥ 512 kDa).

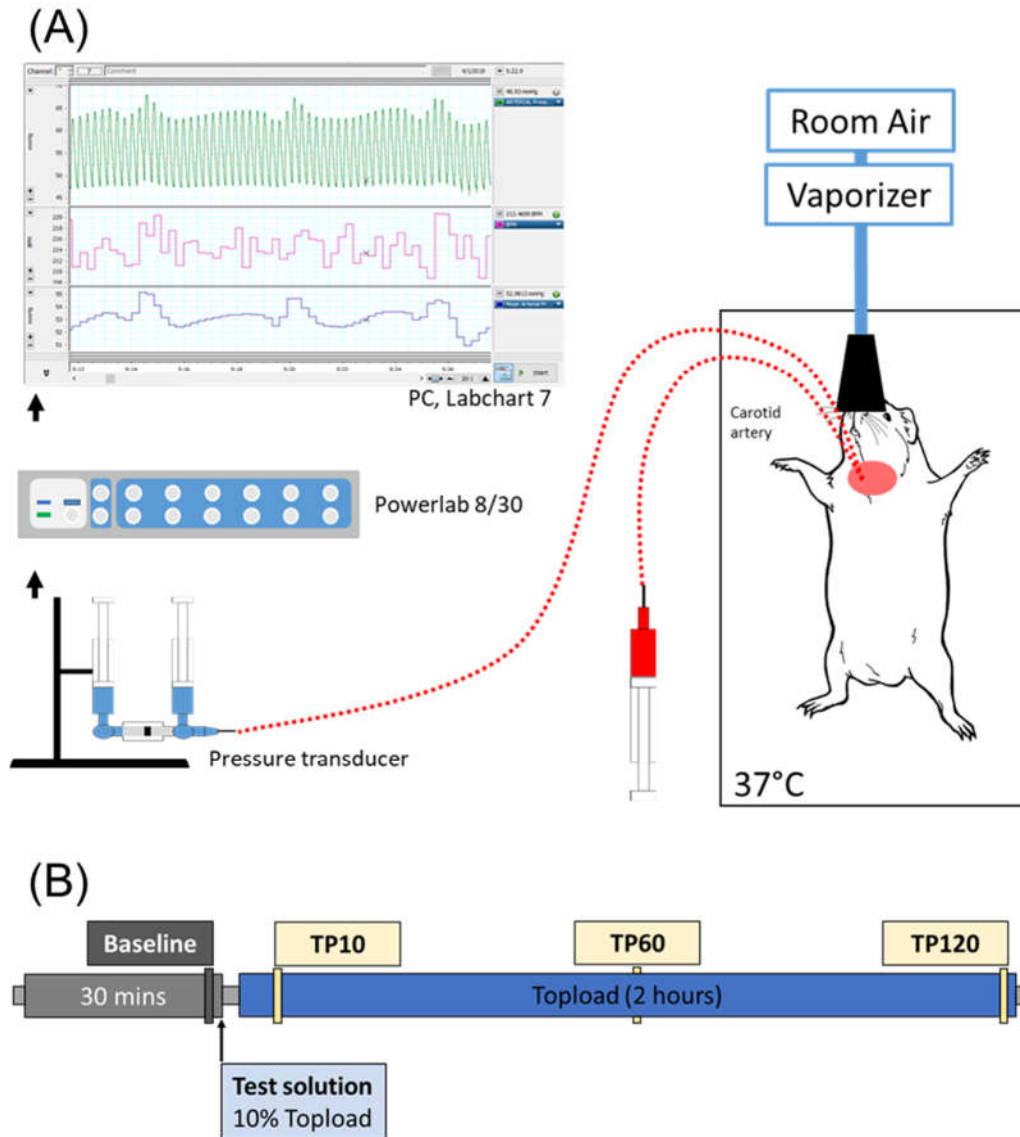


Figure 5.1. Depiction of experimental setup. (A) Guinea pigs were anesthetized with isoflurane, and blood pressure was continuously measured through a carotid artery catheter. (B) Timeline of the experiment. Blood samples were taken at baseline, and 10, 60, and 120 minutes following topload. Sham animals were not given a topload.

Animal Preparation. We followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals for animal handling and care, and the local Animal Care Committee approved the experimental protocol. Guinea pigs weighing between 350 and 400 grams were anesthetized with isoflurane (Drägerwerk AG, Lübeck, Germany) slowly, by increasing the

isoflurane dosage 0.4% every 3 minutes until a surgical depth of anesthesia was achieved, typically 3%. This ensured that the animals did not stop breathing due to airway irritation by isoflurane. Then the animals were placed on a heating pad to maintain core body temperature at 37 °C for the duration of the experiment. Animals were instrumented with a catheter in the right carotid artery to monitor mean arterial pressure (MAP) and heart rate (HR), withdraw blood to measure hematocrit and blood gases, and to deliver the study solution. A graphical representation of the experimental preparation is presented in **Figure 5.1A**.

Experimental Groups. Study groups were assigned based on the study solution given during the topload. Animals were randomly assigned to SPolyHb (n=8), LPolyHb (n=8), HSA (control) (n=8) or Sham (n=8). Sham animals were subjected to the surgical procedure, but no solution was infused. All measurements and time points were the same, and animals were under anesthesia for the same duration. A second set of animals were randomly assigned to SPolyHb (n = 5), or LPolyHb (n = 5) to study the pharmacokinetics, and rate of metHb formation in vivo.

Topload Protocol. After surgical preparation, isoflurane was reduced to 1.5% and animals were allowed to stabilize for approximately 15 minutes before baseline measurements. After baseline, study solutions were injected as hypervolemic infusions of 10% of the blood volume (estimated as 7.5% of the body weight), and animals were monitored for 2 hours, tracking mean arterial pressure (MAP), heart rate (HR), and blood gasses. A visual representation of the timeline is presented in **Figure 5.1B**. Animals were then sacrificed via sodium pentobarbital overdose (300 mg/kg). Blood and tissues were harvested from all groups and immediately frozen, and maintained at -80°C, to analyze markers of iron metabolism and vital organ function, toxicity, and inflammation. These analyses were performed by the UC San Diego Histology Core via ELISA analysis of tissue homogenates.

Systemic Hemodynamics Parameters. MAP and HR were recorded continuously from the carotid artery (PowerLab 8/30, AD Instruments, CO). Hematocrit (Hct) was measured via centrifuged arterial blood samples. Total and plasma Hb content was measured spectrophotometrically (B-Hemoglobin; Hemocue, Stockholm, Sweden). Arterial blood was collected into heparinized glass capillary tubes and immediately analyzed for partial pressures of oxygen (pO₂) and carbon dioxide (pCO₂), as well as pH (Siemens Rapidlab 248; Munich, Germany).

Pharmacokinetics Protocol. Study solutions were injected as a 5% hypervolemic infusion IV to awake guinea pigs. IV blood samples were drawn 5 minutes, 2 hours, 4 hours, 8 hours, 12 hours, and 24 hours following the initial infusion, and plasma Hb concentration was measured spectrophotometrically from centrifuged arterial blood samples. The metHb fraction of plasma was measured via absorbance at 630 nm.

Statistical analysis. Results are presented as the mean and standard error of the mean. The values are presented as absolute values and relative to baseline. A ratio of 1.0 signifies no change from baseline, whereas lower or higher ratios are indicative of changes proportionally lower or higher compared to baseline, respectively. The Grubbs' method was used to assess closeness for all measured parameters at baseline. Before experiments were initiated, sample size was determined via power calculations using $\alpha=0.05$ and $1-\beta=0.9$ to detect differences in primary end points greater than 10%. Statistically significant changes between solutions were analyzed using one-way analysis of variance (ANOVA), and when the parameters were followed through time, parameters were analyzed using two-way ANOVA, followed by *post-hoc* analyses using Tukey's multiple comparisons test when appropriate. All statistics were calculated using the Python Library StatsModels (ver. 0.9.0).²¹ Results were considered statistically significant if $p < 0.05$.

5.4: Results

Polymerized hemoglobin characterization. SPolyHb and LPolyHb were adjusted to a final Hb concentration of 10 g/dL before infusion. MetHb levels were approximately 5% for both solutions. UV-visible HPLC-SEC chromatograms, and the Hb size order distribution are shown in **Figure 5.2**. SPolyHb and LPolyHb had similar amounts of 0th (single Hb molecule) and 2nd (Hb polymer composed of 4 Hb molecules) order PolyHb molecules. However, SPolyHb had a significant fraction (approximately 11% by weight) of 1st (Hb polymer composed of 2 Hb molecules) order polymerized Hb molecules not present in LPolyHb.

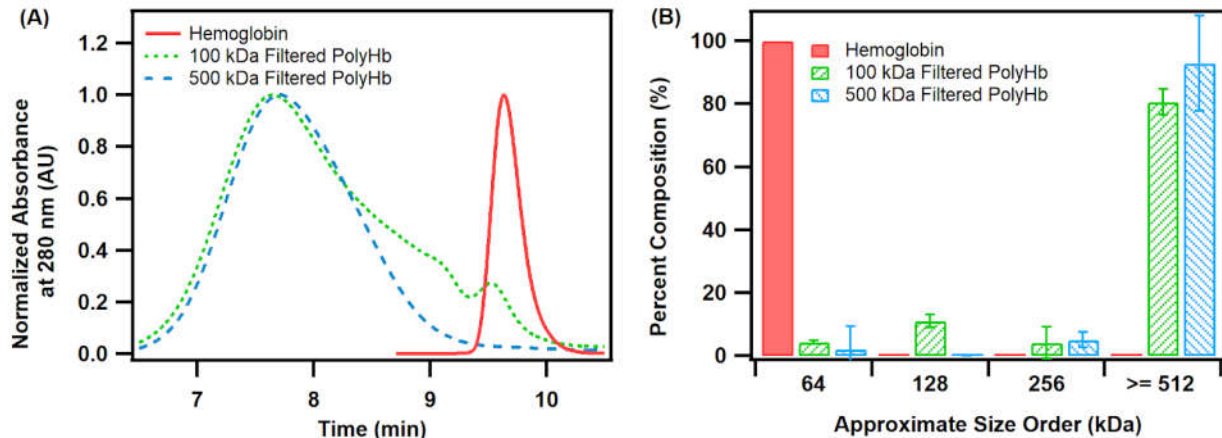


Figure 5.2: Size distribution of 100 kDa Filtered PolyHb (SPolyHb) and 500 kDa Filtered PolyHb (LPolyHb) were determined by HPLC size exclusion chromatography (SEC). (A) HPLC-SEC chromatogram of absorbance at 280nm. (B) Hb size order distribution based on deconvolution of HPLC-SEC chromatograms. 0th-order (64kDa, non-polymerized), 1st order (128 kDa), 2nd order (256 kDa), and 3rd order (\geq 512 kDa).

Systemic hemodynamics. Ten minutes post-topload, SPolyHb showed significantly increased MAP compared to all other groups (**Figure 5.3**). This increase in MAP was not a result of hypervolemia, as the topload control group (HSA) experienced only an 8% increase in MAP from baseline, whereas SPolyHb experienced a 50% increase in MAP from baseline. This effect persisted for one hour, but after 2 hours there were no statistically significant differences in MAP between groups. Moreover, there were no differences in the heart rate at these times.

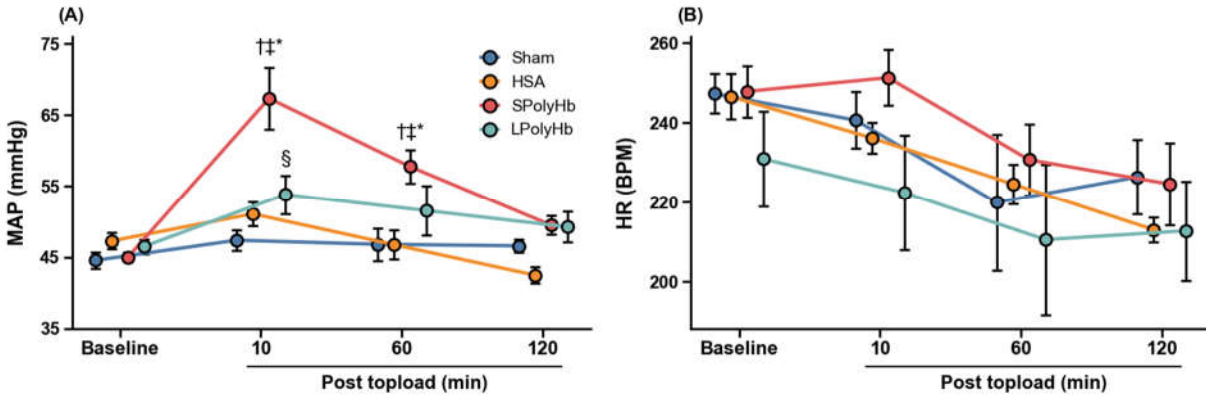


Figure 5.3: Central hemodynamics in anesthetized guinea pigs following topload. (A) SPolyHb causes a significant increase in MAP for up to 1 hour, but hypertension is resolved by 2 hours. (B) Heart rate decreases slightly for all groups throughout the protocol. N = 8 per group. Data are presented as mean \pm SEM. * $p < 0.05$ vs baseline, $\dagger p < 0.05$ vs Sham, $\ddagger p < 0.05$ vs HSA, $\S p < 0.05$ vs SPolyHb

Table 5.1: Hematological parameters did not change significantly following topload. N = 8 per group. Data are presented as mean \pm SEM. * $p < 0.05$ vs baseline, $\dagger p < 0.05$ vs Sham, $\ddagger p < 0.05$ vs HSA, $\S p < 0.05$ vs SPolyHb

		Sham	HSA	SPolyHb	LPolyHb
Total Hb (g/dL)	Baseline	12.6 \pm 0.2	12.6 \pm 0.3	12.9 \pm 0.2	13.1 \pm 0.3
	10 mins	12.6 \pm 0.3	11.3 \pm 0.2*	13.5 \pm 0.3 [‡]	13.7 \pm 0.3 [‡]
	1 hr	12.5 \pm 0.4	11.2 \pm 0.2*	13.1 \pm 0.2 [‡]	13.4 \pm 0.3 [‡]
	2 hrs	12.1 \pm 0.3	11.3 \pm 0.2*	12.7 \pm 0.2	12.9 \pm 0.3 [‡]
Hct (%)	Baseline	43 \pm 1	42 \pm 1	43 \pm 1	44 \pm 1
	10 mins	42 \pm 1	38 \pm 1* [†]	42 \pm 1	42 \pm 1 [‡]
	1 hr	41 \pm 1	38 \pm 1*	41 \pm 1	42 \pm 1
	2 hrs	40 \pm 1	38 \pm 1*	40 \pm 1	41 \pm 1
PolyHb (g/dL)	10 mins			1.7 \pm 0.1* ^{†‡}	1.7 \pm 0.1* ^{†‡}
	1 hr			1.4 \pm 0.1* ^{†‡}	1.6 \pm 0.1* ^{†‡}
	2 hrs			1.3 \pm 0.1* ^{†‡}	1.5 \pm 0.1* ^{†‡}

Table 5.2: Blood gasses were largely unaltered by the topload, but pO₂ trended lower for animals transfused with PolyHb.

		Sham	HSA	SPolyHb	LPolyHb
pH	Baseline	7.21 \pm 0.03	7.30 \pm 0.01	7.23 \pm 0.02	7.29 \pm 0.02
	10 mins	7.21 \pm 0.04	7.32 \pm 0.02	7.25 \pm 0.02	7.29 \pm 0.03
	1 hr	7.20 \pm 0.08	7.33 \pm 0.02	7.25 \pm 0.03	7.27 \pm 0.04
	2 hrs	7.25 \pm 0.06	7.32 \pm 0.02	7.28 \pm 0.02	7.27 \pm 0.04
pO ₂ (mmHg)	Baseline	70 \pm 8	82 \pm 6	74 \pm 6	84 \pm 8
	10 mins	73 \pm 10	87 \pm 9	67 \pm 6	67 \pm 12
	1 hr	73 \pm 11	74 \pm 7	62 \pm 6	60 \pm 9
	2 hrs	75 \pm 8	74 \pm 8	50 \pm 6	58 \pm 10
pCO ₂ (mmHg)	Baseline	55 \pm 6	51 \pm 2	54 \pm 2	58 \pm 5
	10 mins	61 \pm 7	54 \pm 3	58 \pm 2	63 \pm 7
	1 hr	72 \pm 13	58 \pm 3	67 \pm 4	70 \pm 10
	2 hrs	65 \pm 9	60 \pm 4	68 \pm 4	70 \pm 8

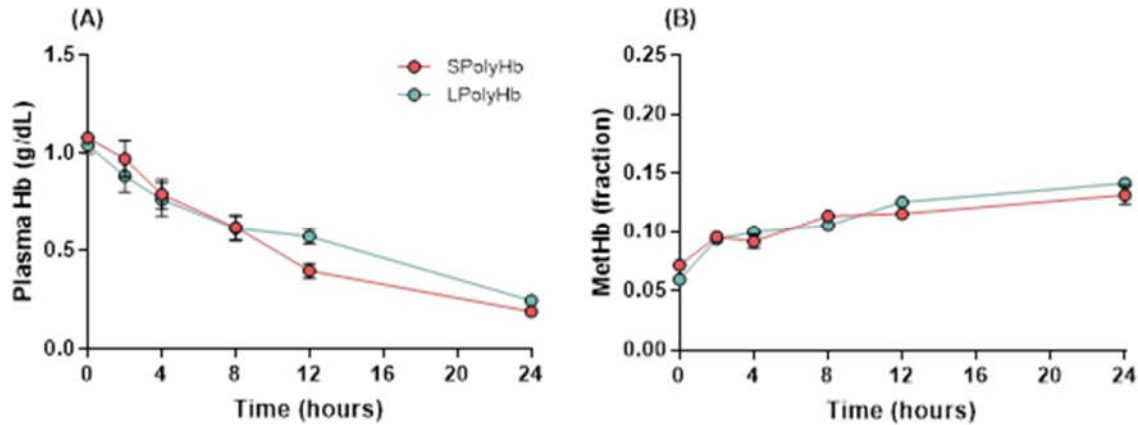


Figure 5.4: Pharmacokinetics of LPolyHb and SPolyHb. (A) LPolyHb possesses longer half-life, approximately 12.6 hours vs the 8.9 hours of SPolyHb (B) LPolyHb and SPolyHb possess similar MetHb formation rates.

Hematological parameters and blood gasses. As expected, SPolyHb and LPolyHb increased Total-Hb and plasma Hb (pHb) during the experimental protocol compared to Sham and HSA. One and two hours after topload, LPolyHb showed a slightly higher pHb concentration compared to SPolyHb (**Table 5.1**). Pharmacokinetic data, shown in **Figure 5.4**, demonstrate the extended half-life of LPolyHb (12.6 hours) compared to SPolyHb (8.9 hours). No remarkable differences between groups were found in the hematocrit (**Table 5.1**), arterial pH, pO₂, or pCO₂ (**Table 5.2**), however, arterial pO₂ tended to decrease whereas pCO₂ tended to increase during the protocol, independent of infusion, though these changes were not statistically different from baseline. While not statistically significant, animals given PolyHb tended to have pO₂'s 10-15 mmHg lower than Sham and HSA.

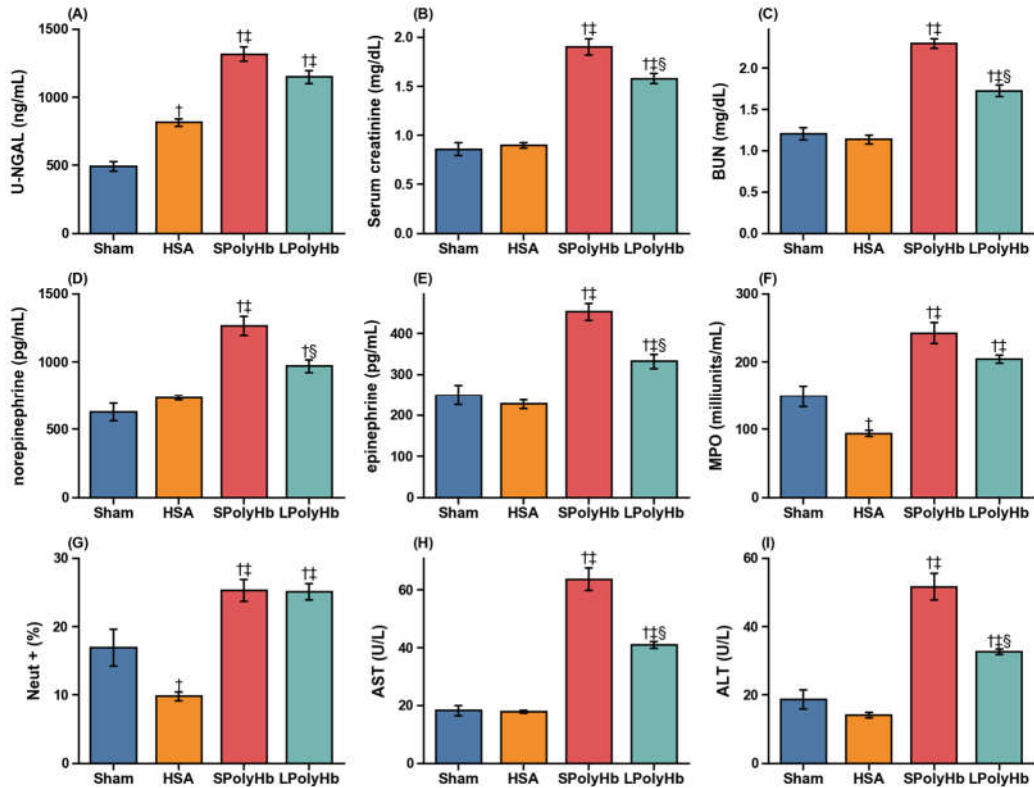


Figure 5.5: Markers of organ damage and function following topload. (A) Neutrophil gelatinase associated lipocalin in the urine, an acute marker of kidney damage. (B) and (C), Serum creatinine and blood urea nitrogen, markers of kidney function. (D) and (E), Catecholamine markers of stress. (F) and (G), Myeloperoxidase and CD45+ neutrophils in the lungs, markers of lung neutrophil activity. (H) and (I), Aspartate and alanine aminotransferase, markers of liver injury. N = 5 per group. Data are presented as mean \pm SEM. †p < 0.05 vs Sham, ‡p < 0.05 vs HSA, §p < 0.05 vs SPolyHb

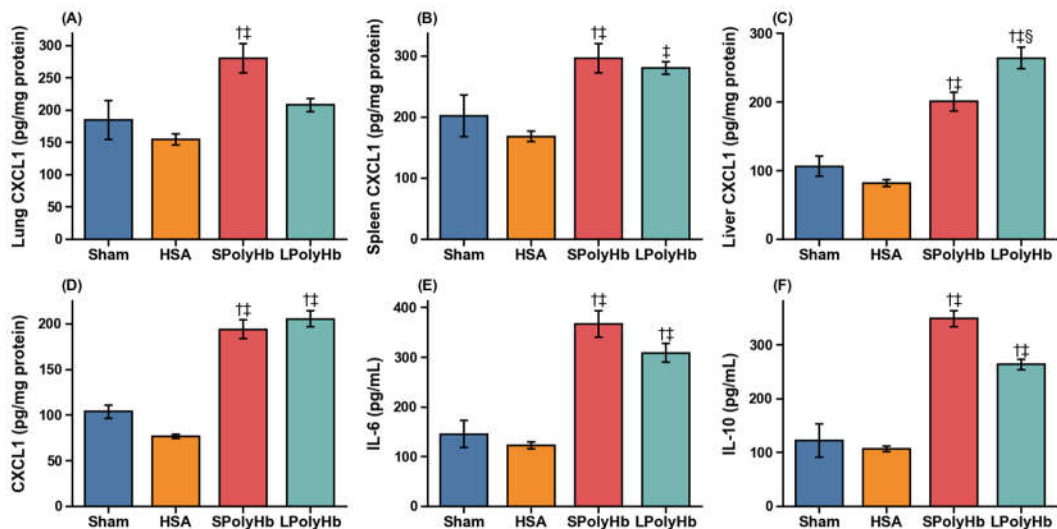


Figure 5.6: Markers of inflammation. CXCL1, a neutrophil recruiting cytokine, activity in (A) lungs, (B) spleen, (C) liver, and (D) systemically. (E) and (F) Interleukin-6 and interleukin-10 concentration in the plasma. N = 5 per group. Data are presented as mean \pm SEM. †p < 0.05 vs Sham, ‡p < 0.05 vs HSA, §p < 0.05 vs SPolyHb

Organ function, damage, and inflammation. Markers of organ damage and function are shown in **Figure 5.5**. Both SPolyHb and LPolyHb caused an increase in a marker of acute kidney damage (urine derived neutrophil gelatinase associated lipocalin, u-NGAL) compared to Sham and 5% HSA. Serum creatinine and blood urea nitrogen (BUN) was increased in both SPolyHb and LPolyHb groups; however, LPolyHb presented lower concentrations compared to SPolyHb for both parameters. HSA alone increased elevated u-NGAL compared to Sham, but no differences were found in serum creatinine or BUN, also markers for kidney function, between the Sham and HSA groups. AST and ALT, markers of liver damage, were increased in both PolyHb groups, but the LPolyHb group presented lower AST and ALT compared to SPolyHb. A similar response was observed in systemic catecholamines. **Figure 5.6** shows systemic and tissue-specific markers of inflammation and neutrophil recruitment. Surprisingly, liver CXCL1 expression was elevated in the SPolyHb group compared to Sham and HSA, but LPolyHb presented higher liver CXCL1 expression than SPolyHb. Infusion of both PolyHbs also resulted in systemic inflammation, as indicated by the increase in IL-6 and plasma CXCL1. Furthermore, there was an increase in myeloperoxidase (MPO) expression, and higher neutrophil activation for both PolyHb groups compared to Sham and HSA, indicating lung inflammation. The anti-inflammatory marker, IL-10, was elevated in both PolyHb groups compared to Sham and HSA, and interestingly LPolyHb presented lower IL-10 concentration than SPolyHb. Spleen CXCL1 was increased similarly in SPolyHb and LPolyHb compared to Sham and HSA. Furthermore, lung CXCL1 expression was increased in both PolyHb groups compared to Sham and HSA, however LPolyHb showed lower expression compared to SPolyHb.

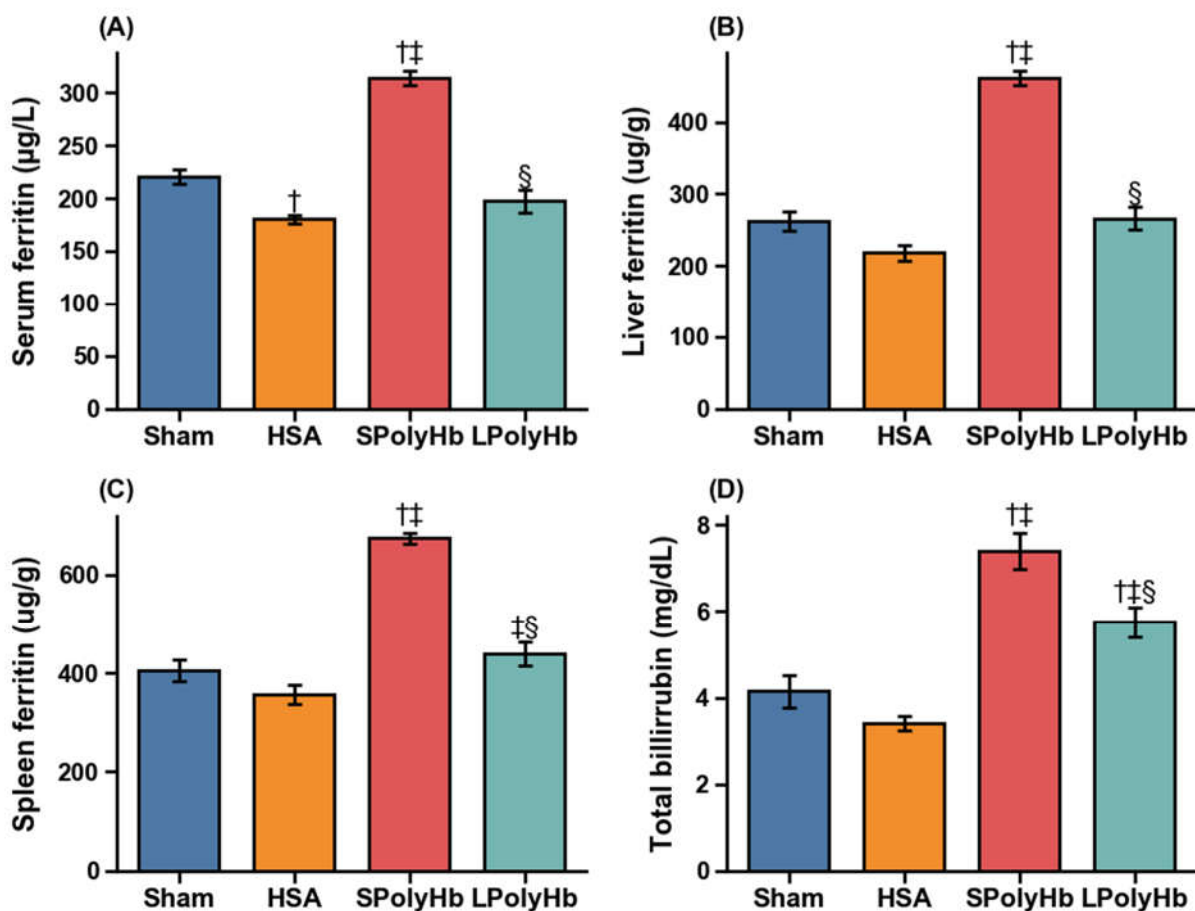


Figure 5.7: Markers of iron transport and storage. Ferritin in the (A) plasma, (B) liver, and (C) spleen, markers of iron storage. (D) Plasma bilirubin concentration, a byproduct of heme catabolism. N = 5 per group. Data are presented as mean \pm SEM. †p < 0.05 vs Sham, ‡p < 0.05 vs HSA, §p < 0.05 vs SPolyHb

Iron transport. Markers of iron transport and storage following infusion of PolyHb are shown in **Figure 5.7**. Infusion of HSA did not change markers of iron transport compared to Sham. Infusion of SPolyHb and LPolyHb increased total bilirubin compared to Sham and HSA, but there was less of a response for the LPolyHb group compared to SPolyHb. LPolyHb did not cause increased serum, spleen, or liver ferritin compared to Sham, whereas SPolyHb elevated serum, spleen, and liver ferritin compared to all other groups.

5.5: Discussion

The principal finding of this study is that the vasoactivity and toxicity of PolyHb and their acute implications are determined by the molecular size of the PolyHb infused. This study also

demonstrates that acute markers of kidney and liver injury are attenuated by increasing the molecular size of polymerized Hb. Similarly, increasing molecular size of the polymerized Hb decreases lung inflammatory responses, but does not attenuate lung neutrophil activity. However, liver and spleen appear to be equally sensitive to polymerized Hb, independent of molecular size. Both SPolyHb and LPolyHb have significantly higher MW and lower concentrations of tetrameric Hb than previous HBOCs. As such, previous HBOCs were likely even more vasoactive than SPolyHb, which resulted in hypertension and cardiac events during clinical testing, and ultimately lead to their failure in clinical trials.

While SPolyHb infusion did not produce cardiac chronotropic effects, it resulted in severe systemic hypertension, probably from the presence of 0th (Hb) and 1st (Hb polymer composed of 2 Hb molecules) order PolyHb molecules at low concentrations. These Hb fractions have small enough diameters to extravasate into the sub-endothelial space and scavenge NO²². The extravasated Hb and Hb breakdown products results in vasoconstriction, hypertension, and oxidative stress until they are cleared by tissue resident macrophages²³, or possibly catabolized by endothelial cells²⁴. Furthermore, 0th and 1st order PolyHb fractions flow near the vessel wall and scavenge NO from within the blood vessel, independent from extravasation. Indeed, previous work has demonstrated that lower-order PolyHb molecules (<500 kDa) are significantly more vasoactive than the higher-order PolyHb molecules (>500 kDa), independent of the degree of polymerization²⁵. This study demonstrates that, in addition to free Hb, 1st order PolyHb molecules (128 kDa) contribute significantly to the vasoactive nature of previous generations of glutaraldehyde-polymerized HBOCs, as similar amounts of free Hb were present in SPolyHb and LPolyHb, but only SPolyHb caused hypertension.

Increased catecholamine secretion in the plasma with SPolyHb does not explain the hypertension observed, as catecholamines were elevated at the end of the protocol, but the hypertension was resolved by that time point. Previous HBOC formulations, such as alpha-alpha cross-linked Hb, caused hypertension for up to 5 hours after infusion. There are two main factors contributing to the observed short-term hypertension: i) 0th and 1st order PolyHb account for a relatively small mass in the overall composition of SPolyHb; and ii) lower-order PolyHb molecules are more readily sequestered by haptoglobin, or taken up by hepatocytes via CD163 mediated endocytosis than higher-order PolyHb molecules.^{14,26} These two factors cause faster clearance of SPolyHb. Enhanced clearance of SPolyHb is suggested by the differences in plasma Hb concentration 1 and 2 hours post-topload, and confirmed by PK studies.

Hematological parameters were relatively stable for all groups, with only the HSA group experienced a significant change from baseline, as expected due to the higher colloidal osmotic pressure of the HSA solution. However, the plasma Hb concentration, or the PolyHb concentration in-vivo, showed slight differences between SPolyHb and LPolyHb groups. Initially, the 10% topload infusion resulted in a plasma Hb concentration of 1.7 g/dL for both small and LPolyHb. After 1 hour, the plasma Hb concentration was slightly lower for SPolyHb than LPolyHb. As mentioned previously, this is likely due to enhanced clearance of the small MW fractions, extravasation, or distributed catabolism of these smaller Hb molecules.²⁴ As a result, SPolyHb has a much shorter half-life than LPolyHb, limiting its functionality as an oxygen therapeutic.

There were no remarkable differences in blood gases between groups; pO₂ tended to decrease while pCO₂ tended to increase throughout the protocol, likely due to extended anesthesia. Guinea pigs are susceptible to respiratory irritants, and have been used in the past to study respiratory irritation²⁷. Furthermore, other researchers have noted similar airway irritation and

irregular breathing in guinea pigs under isoflurane²⁸, so these findings are expected. While not statistically significant, both PolyHb groups ended the experimental protocol with lower pO₂ than control groups, possibly due to pulmonary vasoconstriction,²⁹ a known consequence of HBOC transfusion that can be alleviated with inhaled NO,³⁰ or due to the enhanced lung neutrophil activity observed (**Figure 5.5F and G**).

All organs collected at the end of the protocol were impacted an infusion of PolyHb. Furthermore, most parameters were more elevated by SPolyHb than LPolyHb. Systemically, catecholamines, and markers of enhanced inflammatory (plasma CXCL1, IL-6) and anti-inflammatory (IL-10) responses were higher for the PolyHb groups. Catecholamines can act as free radical scavengers, and are likely released in response to oxidative stress from PolyHb transfusion³¹. This suggests that SPolyHb causes more oxidative stress than LPolyHb, as indicated by their differences in catecholamine concentration. Additionally, HBOCs, and free Hb in general, are known to activate the innate immune system¹³, as they are seen as foreign agents, which would elevate inflammatory markers.

Lungs from animals transfused with LPolyHb and control animals had similar levels of CXCL1 (**Figure 5.5A**), a chemoattractant cytokine that recruits neutrophils, whereas SPolyHb experienced increased levels of CXCL1 in the lungs. However, SPolyHb and LPolyHb both increased neutrophil activation and activity (as indicated by MPO) in the lungs (**Figure 5.5F and G**). In vitro, neutrophils are not typically activated by HBOCs³². However, guinea pig plasma is a poor reductive environment, as much of their reductive capacity has shifted from the plasma to tissues, similar to humans¹⁷. As such, free Hb in guinea pig blood readily oxidizes into metHb in vivo¹⁷, which is a known activator of neutrophils³³. Markers of spleen and liver inflammation and damage were also increased following infusion of PolyHb. However, there were no differences in

splenic inflammation between SPolyHb and LPolyHb. This is expected, as a large portion of splenic iron uptake is from phagocytosis of senescent RBCs³⁴, so the cell-free nature of PolyHb would shift uptake more toward the liver. Therefore, in the lungs, neutrophil recruitment is strongly influenced by hypertension, possibly due to ischemia-reperfusion injury³⁵, whereas neutrophil activity in the lungs, and CXCL1 expression in the spleen, results from the presence of free Hb, but not necessarily hypertension.

As previously mentioned, any foreign agent is expected to increase neutrophil recruitment as part of the innate immune response. Interesting, liver CXCL1 was higher for LPolyHb than SPolyHb. Uptake of 0th and 1st order PolyHb molecules by Kupffer cells occurs much more quickly than the uptake of higher-order PolyHb molecules²⁶. This, and the increased half-life of LPolyHb likely increased CD163 expression relative to SPolybHb²³, which is positively correlated with CXCL1 expression.³⁶ However, the increased presence of AST and ALT, which only enter the plasma following liver or muscle injury, indicate that SPolyHb results in significantly more acute liver and/or muscular injury, possibly due to oxidative stress following heme exposure or due to local ischemia.³⁷ On the other hand, these markers are likely only acute, as Baek et al. demonstrated no significant liver toxicity in guinea pigs following PolyHb transfusion, independent of the size of the polymers.²⁶

Urine of animals transfused with 5% HSA and PolyHb contained higher levels of NGAL than sham animals (**Figure 5.5A**). U-NGAL is commonly used as a marker of acute kidney injury, so it is likely that albumin and some fraction of PolyHb molecules were filtered by the kidneys, as both are known to cause renal injury.^{15,38} However, markers of kidney function, plasma creatinine and BUN, were only elevated for the 2 PolyHb groups, indicating that the injury was less severe for HSA (**Figure 5.5B,C**). Furthermore, SPolyHb impaired kidney function more than LPolyHb,

based on these parameters. While kidney filtration of Hb likely played some role in the kidney injury observed in these experiments, there was only a small percentage of low-MW fragments in SPolyHb that could cause injury via filtration. Baek et al. observed significant tubular damage following transfusion of a low-MW PolyHb, most of which was excreted renally as Hb tetramers.²⁶ The 10:1 PolybHb used in that study possessed a similar MW as pure bovine Hb. In contrast, the SPolyHb in this study only contained a small mass of tetrameric Hb (i.e. 0th order Hb polymer) which could contribute to renal injury via filtration. Another hypothesis for this degree of kidney injury is ischemia following afferent vasoconstriction. NO contributes significantly to the tone of afferent arterioles, but does not impact the tone of efferent arterioles³⁹. As such, NO scavenging decreases blood pressure and flow within the glomeruli. This may have also played a role in the cardiac lesions seen in certain animal models after HBOC transfusion: changes in renal vascular resistance due to vasoconstriction could activate the juxtaglomerular (JG) apparatus, upregulating the renin angiotensin aldosterone system (RAAS), which is linked to myocardial necrosis following NO inhibition.²⁵ In fact, it is well established that the primary source of renin (the first enzyme of the RAAS) in the circulation is the kidney. Renin expression and secretion are tightly regulated at the JG apparatus by two distinct mechanisms: a renal baroreceptor, and sodium chloride (NaCl) delivery to the macula densa resulting in increased levels of renin in plasma when homeostasis is disrupted.⁴⁰

Levels of serum, liver, and spleen ferritin, as well as plasma bilirubin suggest faster catabolism and storage of SPolyHb, due to the aforementioned mechanisms. Differences in IL-10 between SPolyHb and LPolyHb further corroborate improved hepatic uptake of SPolyHb, as endocytosis of Hb:haptoglobin complexes via CD163 releases IL-10, and induces heme oxygenase-1 synthesis to improve clearance of free Hb.⁴¹ Ferritin was not elevated for LPolyHb,

likely a consequence of the length of the experimental protocol. However, the elevated plasma bilirubin level for SPolyHb and LPolyHb suggests that heme catabolism occurred in this time period, albeit significantly more catabolism of SPolyHb occurred.

Limitations. This study has several limitations that should be noted. For one, the guinea pigs were held under isoflurane anesthesia for the length of the experiment. This likely contributed to differences in baseline pH between groups, as well as an overall trend toward respiratory acidosis, and suppressed heart rate for all groups. Additionally, anesthesia in guinea pigs is known to severely depress their cardiovascular system,²⁸ resulting in sub-physiological baseline blood pressure. However, since all groups were subjected to the same experimental conditions, the controls are robust and this study provides interesting results comparing the effects of PolyHb molecular size on vasoactivity. Next, the volume used in these experiments is relatively small, only approximately one unit. This was to ensure survival of the animals, and to distinguish toxicity and vasoactivity of the materials themselves, and isolate those effects from large shifts in O₂ carrying capacity or biophysical properties of blood. These data are also over a relatively short period of time, so they lack information regarding the long-term consequences of HBOC transfusion. However, acute injuries often inform chronic outcomes, so improving the safety profile of HBOCs via acute studies can improve patient quality of care. Finally, these data are in healthy animals, so these results are not representative of the safety profile in patients with increased vascular permeability, such as during endothelial dysfunction. To address this, future studies with guinea pigs fed a high-fat high-sucrose diet are underway.

This study indicates that the side effects seen upon PolyHb transfusion are diminished by increasing the molecular size of PolyHb molecules. LPolyHb also likely has the advantage of a longer circulatory half-life than smaller MW PolyHb, indicated by the differences in markers of

systemic and reticuloendothelial iron transport. Further studies in guinea pigs are warranted, as their mechanisms for potentiating oxidative stress are similar to those of humans, thus making the safety profiles of HBOCs tested in guinea pigs more translatable than safety profiles established in rats, swine, and other ascorbic-acid producing species.

5.6: Acknowledgements

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CHAPTER 6: SAFETY EVALUATION OF HIGH MOLECULAR WEIGHT POLYMERIZED HEMOGLOBIN

6.1: Abstract

Hemoglobin (Hb)-based oxygen (O₂) carriers (HBOCs) have been developed as an alternative to red blood cells (RBCs) when RBCs are not available. HBOCs possess numerous advantages over RBCs and blood, but clinical trials of early HBOC formulations revealed unforeseen side effects including hypertension, vasoconstriction, and myocardial lesions. These side effects were not revealed in pre-clinical testing likely because classical preclinical models possess increased antioxidant capabilities relative to humans. Large molecular size polymerized bovine Hb (PolybHb) is a newly developed HBOC with promising pre-clinical results, but with limited studies using animal models that mimic human antioxidant capacity, such as guinea pigs. Thus, the aim of this study is to evaluate the implication of exchange transfusion with PolybHb in guinea pigs. This study compares the changes in indices of cardiac function, inflammatory markers, and organ function markers, 24 hours after exchange transfusion with PolybHb with high (R-State) and low (T-State) O₂ affinity. PolybHb was synthesized in the low O₂ affinity (T-State), or in the high O₂ affinity (R-State), and then diafiltrated on a 500 kDa hollow-fiber filter. Guinea pigs were subjected to an exchange transfusion of 20% of animal's blood volume (estimated as 7.5% of body weight) with PolybHb. Additionally, hamsters instrumented with a dorsal window chamber were subjected to an exchange transfusion of similar volume to assess the impact of PolybHb O₂ affinity on microhemodynamics and O₂ delivery and O₂ extraction. Both T-State and R-State PolybHb slightly altered cardiac function, increasing the developed pressure and decreasing the isovolemic relaxation compared to Sham. PolybHb did not cause chronic organ injury or inflammation. T-State PolybHb induced acute, but very mild vasoconstriction shortly

after transfusion, while R-State did not have any acute effect in microvascular tone. Microvascular O₂ delivery and extraction were unaltered by PolybHb transfusion. In conclusion, these results suggest that both T-State and R-State PolybHb did not cause significant tissue damage, systemic inflammation, or microhemodynamic aberrations when transfused in healthy animals, but their impacts in more vulnerable patient populations still need to be explored.

6.2: Introduction

The need for an alternative to allogeneic red blood cells (RBCs) for transfusion has been recognized for more than a century due to blood shortages, blood disease transmission, and pandemics.^{1,2} Hemoglobin (Hb) based oxygen (O₂) carriers (HBOCs) have been developed as an alternative to RBCs to restore O₂ carrying capacity when RBCs or blood are not available, but progress in HBOC development has slowed down after failed phase III clinical trials.^{3,4} These trials revealed side effects, such as hypertension, vasoconstriction, and myocardial lesions, that were not detected in pre-clinical trials.⁵⁻⁷ Mechanistic analysis of the origin of these problems demonstrated that they are driven by the small molecular size of HBOCs., Small molecular size HBOCs reside closely to the blood vessel wall where they scavenge nitric oxide, facilitate O₂ diffusion, promote vascular hyperoxygenation, and can move from the vessel lumen into the surrounding tissue.^{8,9} Vasoconstriction can result in organ injury due to mechanical damage or impaired O₂ delivery,¹⁰ but the extravasation of small molecular size HBOCs into tissues, can ultimately release the protein heme and free iron, which have prooxidant and proinflammatory properties.¹¹

High molecular size Polymerized bovine Hb (PolybHb) has been recently developed and tested with promising results, which demonstrate that PolybHb preserves microvascular hemodynamics, does not induce vasoconstriction, and effectively restores O₂ delivery and utilization by tissues.¹² However, it is clear that before clinical trials can proceed for new HBOC formulations, new studies, in animal models that better replicate human Hb-associated toxicity, are necessary to better test the safety and efficacy of HBOCs. The clinical trial failures with certain HBOCs suggest that preclinical animal testing may not have been sufficiently predictive of safety in humans. In this case, a guinea pig model may be more representative of human HBOC toxicity,

because like humans, they are incapable of endogenous production of ascorbic acid (AA), which stems from the evolutionary loss of functional hepatic L-gulonolactone oxidase (LGO).¹³ AA has direct antioxidant effects, and also it is a substrate for the redox enzyme ascorbate peroxidase, which is particularly important in oxidative stress resistance.¹⁴ Previous studies have demonstrated that guinea pigs, unlike rats, presented a compromised plasma antioxidative status after a HBOC exchange transfusion.¹³

In addition to increased molecular size, it has been shown that PolybHb can be locked in the T-State (deoxygenated, low O₂ affinity) or R-State (oxygenated, high O₂ affinity) to control and target O₂ delivery.¹⁵ Low O₂ affinity PolybHb favors pre-capillary O₂ delivery to tissues, which can increase the O₂ flux across the vessel wall, triggering vascular autoregulatory mechanisms to prevent O₂ toxicity.¹⁶ Oversupply of O₂ by low affinity PolybHb could also favor the formation of reactive oxygen species (ROS), further emphasizing the importance of guinea pigs' antioxidant status in assessing the safety profile of HBOCs. On the other hand, high O₂ affinity PolybHb tends to mostly release O₂ at the capillary level, where tissue pO₂ is low, and where the O₂ is needed, thus preventing the vascular hyperoxygenation and decreasing vasoconstriction and ROS production.⁸

Microvascular hemodynamic responses to infusion of PolybHb is crucial in understanding the implications of PolybHb transfusion on vital organ perfusion and consequently oxygenation.¹⁷ A variety of animal models have been used to assess the microcirculation, however, the hamster dorsal chamber window model has the benefits of direct analysis of the intact tissue without anesthesia, which allows for characterization of autonomic regulation. The objective of this study was to evaluate the cardiac, inflammatory, and functional implications transfusion with high (R)

and low (T) O₂ affinity high molecular size PolybHb, as well as their effects on microvascular hemodynamics and O₂ delivery.

6.3: Methods and Materials

6.3.1: Guinea Pig Toxicity

Animal Preparation. We followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals for animal handling and care, and the local Animal Care Committee approved the experimental protocol. Guinea pigs (GP) weighing between 350 and 400 grams were used. The animals were placed on a heating pad to maintain core body temperature at 37 °C for the duration of the experiment under anesthesia. A graphical representation of the experimental procedure is shown in Figure 1.1.

Polymerized Hemoglobin. PolybHb was synthesized in the low O₂ affinity (T) state, with a 35:1 ratio of glutaraldehyde to Hb, or in the high O₂ affinity (R) state, with a 30:1 ratio of glutaraldehyde to Hb, and then both were subjected to 8-9 cycles of diafiltration on a 500 kDa hollow fibre filter. This resulted in a PolybHb solution containing only polymerized Hb molecules with MW greater than 500 kDa.

Exchange Transfusion: GP were anesthetized with ketamine/xylazine (k/x 40/5 mg/kg), and supplemented with 1.0% isoflurane (Drägerwerk AG, Lübeck, Germany) to maintain anesthetic depth. Animals were instrumented with a catheter in the right carotid artery and left jugular vein. Anesthetized GP underwent a 20% exchange-transfusion (blood volume estimated as 7.5% of body weight) with the study solutions at 500uL/min. The Sham group was subjected to the same procedure, but blood was not exchanged. Animals were randomly assigned to one of the 3 experimental groups: Sham, T-State (10g/dL), and R-State (10g/dL). Mean arterial pressure (MAP), heart rate (HR), hematocrit and blood gasses were measured before, and 15 after

exchange-transfusion. After that the catheters were removed, the surgical site was closed, and the animals were allowed to recover.

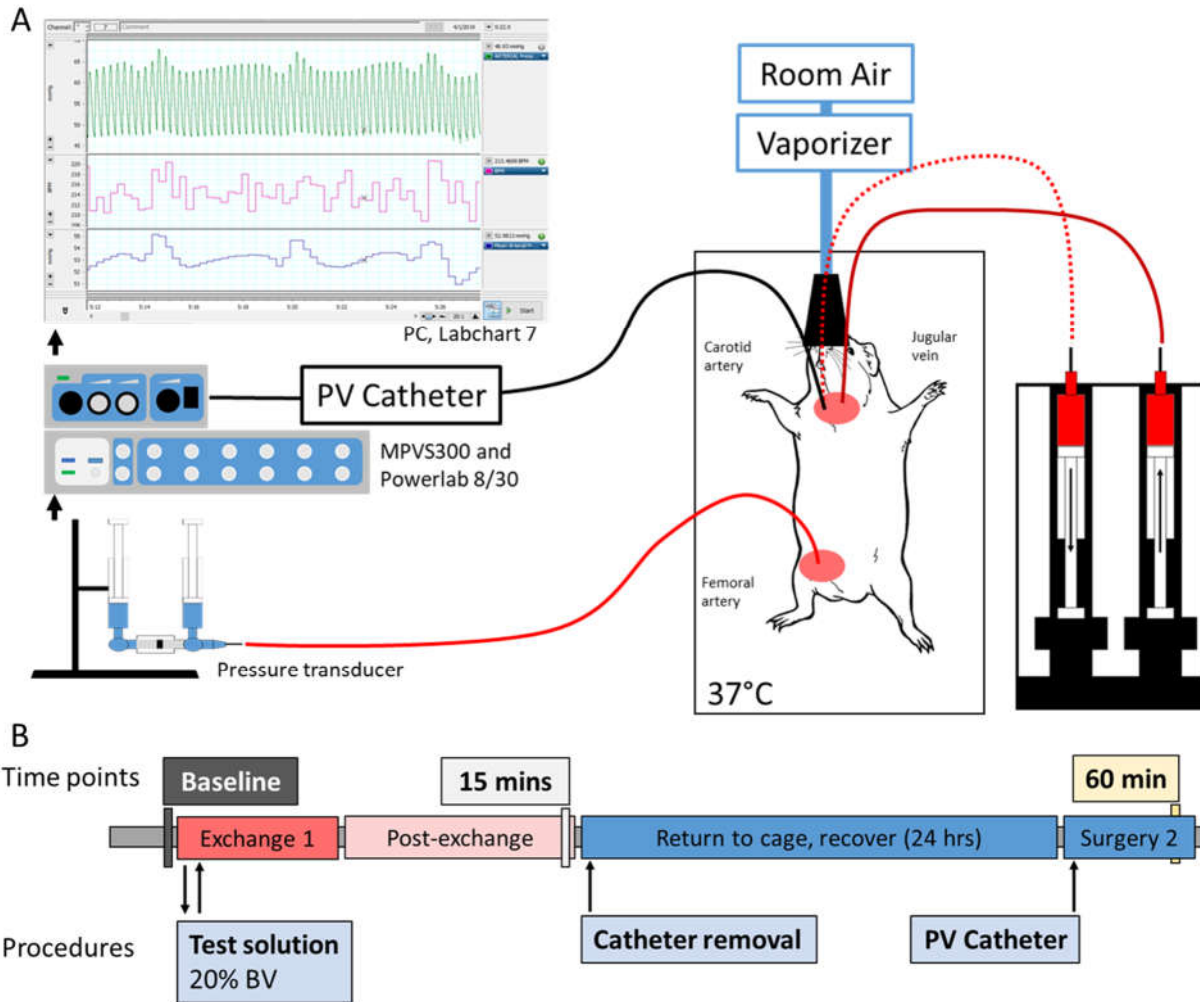


Figure 6.1: Representation of the guinea pig experimental setup. On day 1, guinea pigs were instrumented in their carotid artery and jugular vein. On day 2, the carotid artery was catheterized with the pressure-volume catheter, and the femoral vein was catheterized for mean arterial pressure and heart rate.

Pressure-Volume Conductance Catheter: 24 hours after the exchange-transfusion, GP were anesthetized with isoflurane (Drägerwerk AG, Lübeck, Germany) slowly, by increasing the isoflurane 0.4% every 3 minutes until a surgical depth of anesthesia was achieved, typically 3%. This ensured that the animals did not stop breathing due airway irritation by isoflurane and prevented variations in HR. Animals were instrumented with a catheter in the right femoral artery

to monitor mean arterial pressure (MAP) and heart rate (HR), and left ventricular (LV) conductance catheter introduced through the right carotid artery to monitor cardiac function.

Cardiac Function. A 2F pressure-volume (PV) conductance catheter (SPR-858, Millar Instruments, TX) was inserted into the left ventricle (LV) using the closed chested method [20]. Briefly, the PV catheter was inserted through the exposed right carotid artery and slowly advanced into the LV. Pressure was acquired continuously (MPVS300, Millar Instruments, Houston, TX, and PowerLab 8/30, AD Instruments, Colorado Springs, CO).

Harvesting tissues. Post cardiac function analysis, blood was collected and centrifuged to separate the plasma. The animal sacrificed with Fatal Plus® (sodium pentobarbital, 300 mg/kg), urine was collected, kidney, liver spleen and lung were harvested. Markers of inflammation function and organ injury were evaluated. These analyses were performed by the UC San Diego Histology Core via ELISA analysis of tissue homogenates.

6.3.2: Hamster Oxygen Distribution After 20% Exchange Transfusion

Window Chamber Animal Preparation. Animal handling and care followed the NIH Guide for the Care and Use of Laboratory Animals and the experimental protocol was approved by the local animal care committee. Studies were performed in 55-65g male Golden Syrian Hamsters (Charles River Laboratories, Boston, MA) fitted with a dorsal skinfold window chamber. The hamster window chamber model is widely used for microvascular studies without anesthesia. The complete surgical technique has been described previously.¹⁸ 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 O₂ partial pressure (paO₂) > 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, New Hyde Park, NY). 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. Locations of arterioles and venules were noted within the visible region of the chamber for further analysis with hyperspectral imaging.

Vascular Responses - Isovolumic Exchange Protocol. Hamsters underwent a 20% exchange of R-State or T-State PolybHb at 10 g/dL, at 100 μL/min. Vascular response to PolybHb was quantified 15 minutes, 2 hours, and 24 hours after the exchange.

Hematological Parameters. Hematocrit was measured from centrifuged arterial blood samples taken in heparinized capillary tubes. Hemoglobin content was determined spectrophotometrically (B-Hemoglobin, Hemocue, Stockholm, Sweden). Arterial blood was collected in heparinized glass capillaries (50 μL) and immediately analyzed for pO₂, pCO₂, and pH (ABL90; Radiometer America, Brea, CA). Arterial Hb saturations were 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)². The measured centerline velocity (V) was corrected according to blood vessel size to obtain the mean RBC velocity³. A video image-shearing method was used to measure blood vessel diameter (D)⁴. Blood flow (Q) was calculated from the measured values as

$$Q = \pi \times V \left(\frac{D}{2} \right)^2.$$

Functional Capillary Density (FCD). Functional capillaries, defined as capillary segments that have RBC transit of at least one RBC in a 60 second period in 10 successive microscopic fields, were assessed in a region of 0.46 mm². FCD (cm⁻¹) is calculated as the total length of RBC perfused capillaries divided by the area (0.46 mm²).

Hyperspectral Imaging. After chamber mapping and microhemodynamic measurements, the animal dorsal window was imaged using a Pika L hyperspectral imaging system (Resonon Inc, Bozeman, MT) from which hyperspectral images (HSI) of the microcirculation were acquired. The white reference reflectance spectra (R_{ref}) of the system was calibrated using a white Teflon slab. The animal was allowed to rest in the tube environment for 20 minutes before imaging. Images were taken at baseline, 15 minutes, 2 hours, and 24 hours after 20% exchange-transfusion.

Each HSI contained wavelength bands from 450nm to 600nm with a spacing of 5nm between adjacent bands. Vessels were segmented from the background using a Gabor filter bank ranging from 0 to 360 degrees in the resulting image of the ratio between band 575 to band 485, which previous experiments have shown has maximum Hb contrast, independent of saturation. The saturation of Hb in the vessel was determined through a pixel-by-pixel least-squares spectral fit as

$$\epsilon(\lambda) = b_0 + b_1\epsilon_{Hb}(\lambda) + b_2\epsilon_{HbO_2}(\lambda)$$

Where $\epsilon(\lambda)$ is the absorbance spectra of any given pixel, given by $\epsilon(\lambda) = \log \log \left(\frac{R_{ref}(\lambda)}{R(\lambda)} \right)$, where R_{ref} is the white reference reflectance spectra, and R is the reflectance spectra from the HSI. ϵ_{Hb} corresponds to the standard absorbance of deoxyhemoglobin and ϵ_{HbO_2} to the standard absorbance of oxyhemoglobin. The b_0 , b_1 and b_2 are the coefficients determined by least-squares, where b_0 accounts for any offset, and b_1 and b_2 are proportional to the amount of deoxy- and oxyhemoglobin respectively. Once the coefficients were determined, the saturation was calculated as

$$SO_2 = \frac{b_2}{b_1 + b_2} \times 100\%$$

Statistical Analysis. All values are expressed as mean \pm SE. Data within each group were analyzed using one-way analyses of variance (ANOVA) or Two-Way analysis of variance for repeated measurements. When appropriate, post hoc analyses were performed with the Tukey or Dunnett's multiple comparisons test. Microhemodynamics data are presented as ratios relative to baseline. 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 6, GraphPad Software, Inc., San Diego, CA). Changes were considered significant if $p < 0.05$.

6.4: Results

In this study, healthy guinea pigs were evaluated to assess the influence of PolyHb on cardiac function and markers of organ injury and inflammation. Awake, intact hamsters

instrumented with the dorsal window chamber were also evaluated to examine the effect of PolyHb transfusion on microvascular hemodynamics and microvascular O₂ delivery. All animals survived the procedures and animals were similar at baseline.

6.4.1: Guinea Pig toxicity

Blood Pressure and Cardiovascular Parameters. Exchange transfusion did not alter mean arterial pressure (MAP) or heart rate (HR) compared to baseline, however, all animals trended toward a slightly lower MAP 15 minutes post-exchange. 24 hours post-exchange, the MAP for T-State and R-State trended back toward baseline, while MAP for Sham slightly decreased further. As a result, T-State and R-State possessed 39% and 27% higher MAP compared to Sham at 24 hours, respectively. HR was significantly higher at 24 hours than at baseline or 15 minutes for all groups, likely due to the difference in anesthesia utilized on day 2 (Time course data not shown; 24 hour time point shown in Figure 6.1).

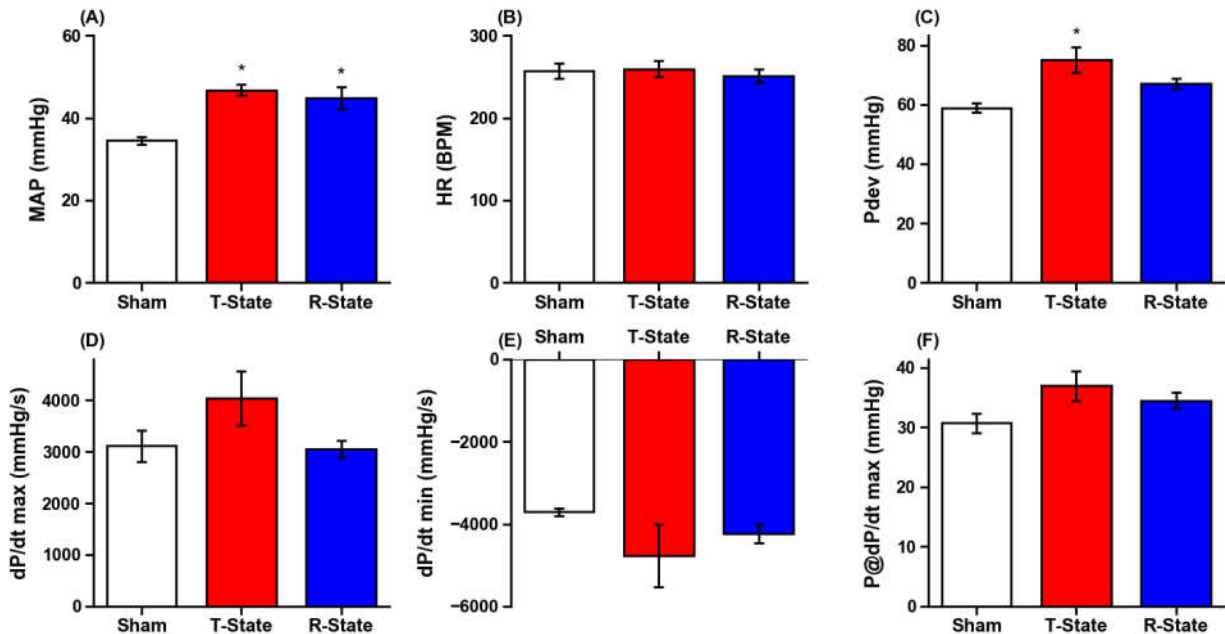


Figure 6.2: Cardiovascular parameters of guinea pigs 24 hours post-exchange transfusion. (A) Mean arterial pressure. (B) Heart rate. (C) Developed pressure. (D) Isovolemic contractility. (E) Isovolemic relaxation. (F) Pressure at max contraction rate. *p < 0.05 vs Sham.

Indices of cardiac function were only minorly affected by exchange transfusion with PolybHb. The T-State PolybHb increased cardiac contractility in terms of the maximum rate of pressure development, and increased the isovolemic relaxation rate compared to R-State PolybHb and Sham. T-State PolybHb also increased the left ventricular total developed pressure compared to Sham. The R-State PolybHb presented no significant changes in cardiac function compared to Sham (Figure 6.3).

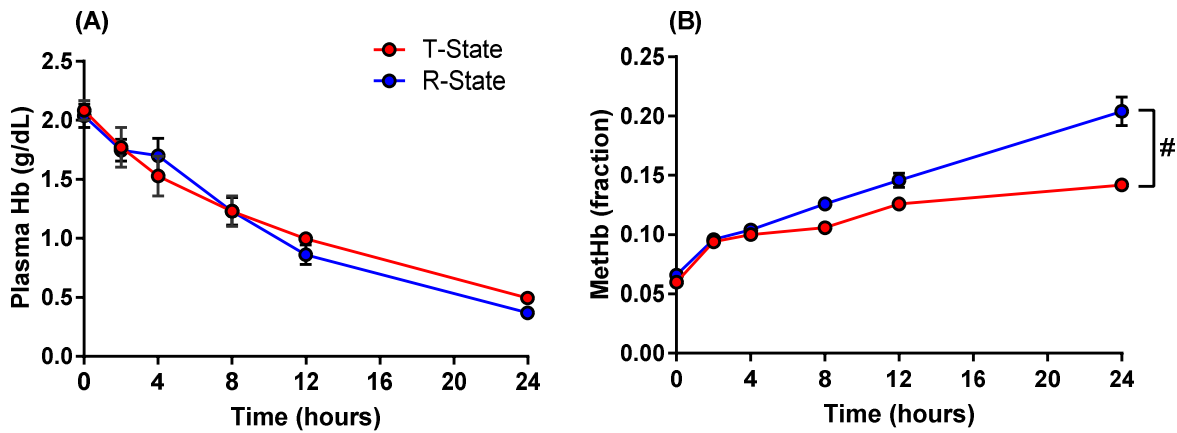


Figure 6.3: (A) Pharmacokinetics and (B) MetHb fraction of PolybHb in guinea pigs after 24 hours. # $p < 0.05$

Metabolic Parameters. As expected, exchange transfusion reduced the hematocrit for both T-State and R-State PolybHb compared to Sham, but the total Hb was unaffected. After 24 hours, hematocrit remained low for both T-State and R-State PolybHb compared to sham. Additionally, at 24 hours, total Hb decreased significantly for both PolybHbs compared to sham. T-State and R-State PolybHb possessed similar half-lives, (approximately 11 hours), but R-State PolybHb oxidized much more rapidly *in vivo*, resulting in 20% of the remaining PolybHb circulating in the metHb form at 24 hours, whereas only 14% of T-State PolybHb was oxidized at 24 hours. PolybHb, independent of the O_2 affinity, did not induce any differences in pH, pCO_2 or pO_2 compared to sham. Electrolytes, glucose, and lactate in blood were also similar between groups as shown in Figure 6.4.

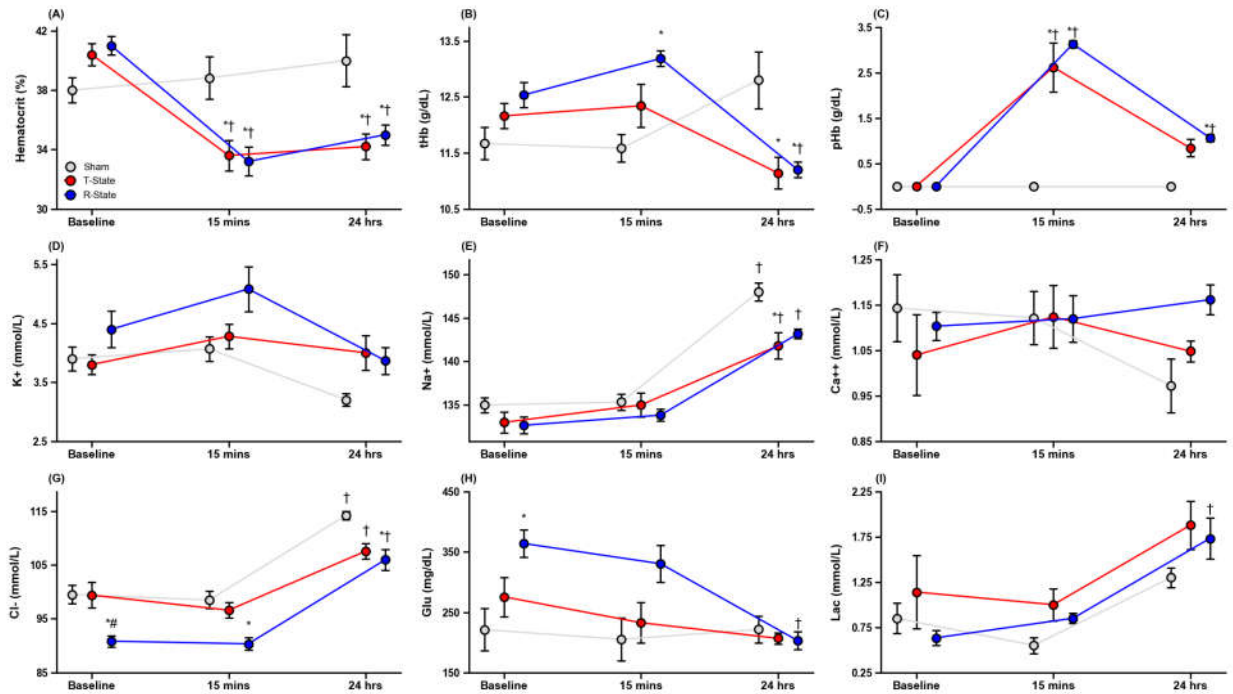


Figure 6.4. Hematological parameters, electrolytes and metabolic parameters of guinea pigs throughout the procedure. (A) Hematocrit. (B) Total hemoglobin concentration. (C) Plasma hemoglobin concentration. (D) Potassium. (E) Sodium. (F) Calcium. (G) Chloride. (H) Glucose. (I) Lactate. * $p < 0.05$ vs Sham, † $p < 0.05$ vs Baseline.

Inflammation and Tissue Injury. To elucidate the safety of PolybHb following transfusion in guinea pigs, common markers of inflammation and tissue injury and function were assessed. PolybHb did not cause any remarkable change in markers of liver injury, as evaluated through a lack of change in aspartate and alanine transferase (AST, ALT) in plasma relative to sham. However, both PolybHbs increased CXCL1, a neutrophil recruiting cytokine, in the liver compared to the Sham group, suggesting an acute hepatic inflammatory response (Figure 4). Neutrophil gelatinase associated lipocalin (NGAL) was elevated in the urine of animals transfused with PolybHbs, suggesting acute renal injury compared to sham animals. Additionally, there was an increase in both plasma creatinine and blood urea nitrogen concentration in R-State PolybHb compared to Sham. (Figure 4). Systemic CXCL1 and IL-6 were increased equally in both R-State and T-State PolybHb compared to the Sham group. Moreover, IL-10 increased for R-State PolybHb compared to sham (Figure 6.5). Guinea pigs transfused with PolybHb showed minimal

increases in CXCL1 in the spleen and lungs. Furthermore, PolybHb transfusion did not impact epinephrine, but interestingly norepinephrine was increased for R-State PolybHb compared to T-State (Table 6.1). Serum, Liver, and spleen ferritin, and total bilirubin were evaluated to characterize the iron transport in the animals submitted to a 20% exchange transfusion, but there were no observable changes in iron transport compared to sham (Table 6.2).

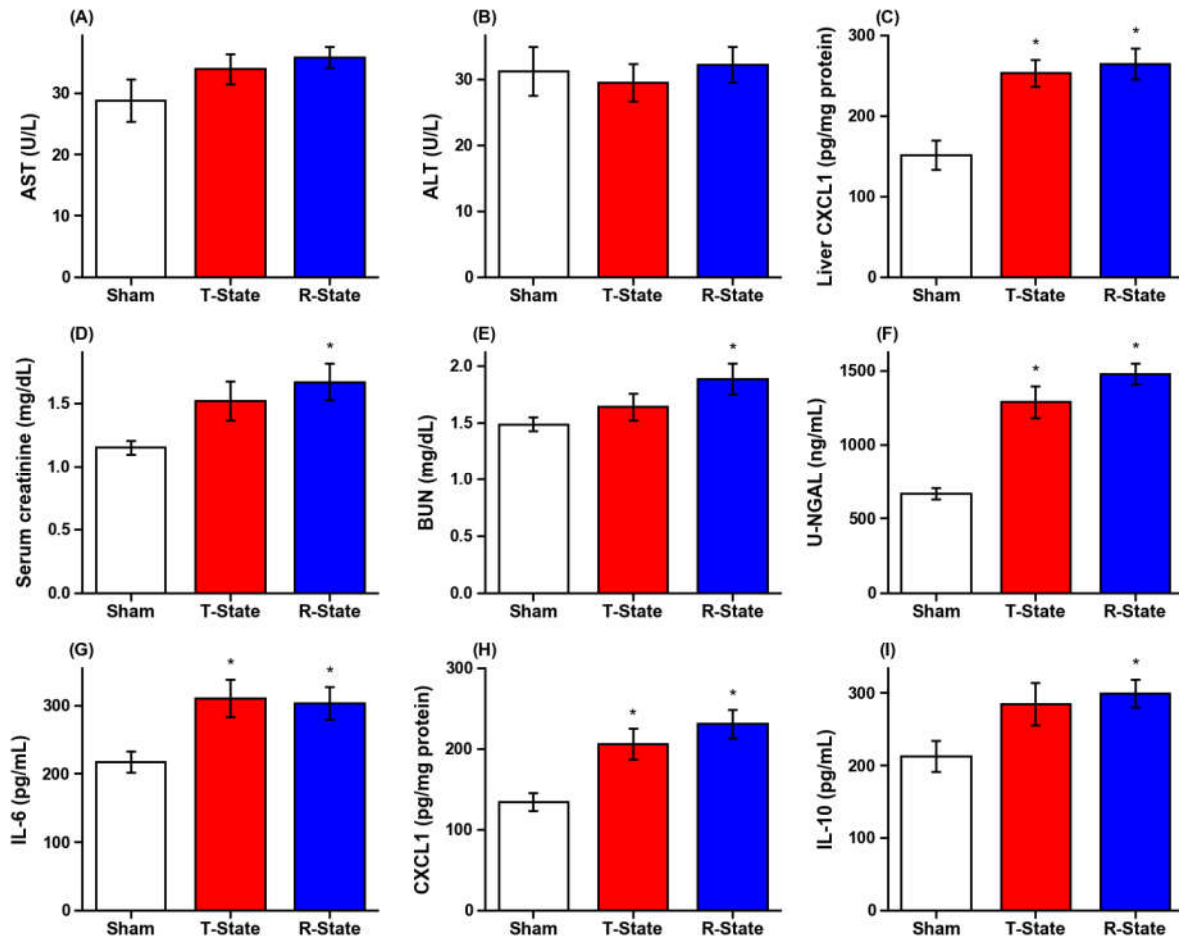


Figure 6.5: Parameters of organ injury and inflammation 24 hours post-exchange. (A) Aspartate transferase. (B) Alanine transferase. (C) Liver CXCL1. (D) Serum creatinine. (E) Blood urea nitrogen. (F) Urinary neutrophil gelatinase associated lipocalin. (G) Systemic IL-6. (H) Systemic CXCL1. (I) Systemic IL-10. * $p < 0.05$ vs Sham.

Table 6.1: Catecholamines and tissue inflammation. Data presented as mean±SE. * p < 0.05 vs Sham, #p < 0.05 vs. T-State.

Parameters	Sham	T-State	R-State
Norepinephrine (pg/mL)	858.3±36.0	1064.3±95.8	1312.1±44.1*#
Epinephrine (pg/mL)	337.8±12.5	316.6±29.9	349.8±23.4
CXCL1 Spleen (pg/mg protein)	249.8±14.8	236.1±6.1	285.8±16.0
CXCL1 Lung (pg/mg protein)	252.7±11.7	250.7±20.4	273.6±13.9
MPO (milliunits/mL)	198.7±16.5	198.9±16.3	216.5±10.1

Table 6.2: Iron Transport. Data presented as mean±SE.

Parameters	Sham	T-State	R-State
Serum Ferritin (ug/L)	254.3±11.4	277.8±14.0	300.3±18.8
Serum Total bilirubin (mg/dL)	5.5±0.4	5.5±0.6	5.9±0.5
Spleen Ferritin (ug/g)	488.8±22.8	456.3±28.0	480.8±43.4
Liver Ferritin (ug/g)	297.1±66.7	302.8±26.8	345.3±22.5

6.4.2: Oxygen Distribution in the Microcirculation After 20% Exchange Transfusion

Systemic Parameters. Hamsters did not experience any significant changes in MAP or HR in response to exchange transfusion with PolyHb (Table 6.3). While there were no changes in arterial pCO₂ after transfusion with PolyHb, the R-State PolyHb increased pO₂ after the exchange relative to baseline, but this change in pO₂ was resolved 24 hours post-exchange (Table 3). Neither T-State or R-State changed arterial pH following exchange transfusion. However, after 24 hours, T-State saw a significantly higher pH than at baseline or at 15 minutes, likely due to the marginally lower pH at baseline.

Table 6.3: Systemic parameters of hamsters. Data presented as mean±SE. †p < 0.05 vs baseline, &p < 0.05 vs 15 minutes.

	T-State								
	MAP (mmHg)	HR (BPM)	tHb (g/dL)	Hct (%)	pHb (g/dL)	SO ₂ (%)	pH	pCO ₂ (mmHg)	pO ₂ (mmHg)
Baseline	117±4	421±30	14.9±0.5	49±1	0	88.8±0.9	7.34±0.02	57.0±1.7	58.7±3.5
15 mins	126±9	406±19	13.7±0.4	38±1	2.3±0.1	85.4±1.6	7.33±0.02	59.5±1.6	60.4±2.4
24 hrs	118±11	442±6	10.9±0.5	34±1	0.3±0.1	86.2±1.7	7.41±0.02†&	60.9±1.8	56.3±4.2
	R-State								
	MAP (mmHg)	HR (BPM)	tHb (g/dL)	Hct (%)	pHb (g/dL)	SO ₂ (%)	pH	pCO ₂ (mmHg)	pO ₂ (mmHg)
Baseline	116±1	429±10	14.5±0.9	46±3	0	89.7±1.8	7.38±0.02	55.4±1.7	60.1±3.1
15 mins	127±4	426±24	13.6±0.9	37±2	2.3±0.1	92.1±0.9	7.39±0.02	51.1±2.3	76.7±4.1†
24 hrs	122±7	422±19	11.1±0.8	37±3	0.4±0.1	89.5±2.2	7.39±0.02	56.8±2.5	57.4±5.2&

Microhemodynamics. Arteriolar diameter very slightly, but statistically significant decreased from baseline 15 minutes after exchange transfusion with T-State PolybHb. After 24 hours, arteriolar diameter was marginally higher than at baseline, and statistically significantly increased compared to 15 minutes, and marginally higher than at baseline. No changes in arteriolar diameter were observed in R-State group. Velocity was similar for both R-State PolybHb and T-State PolybHb; it initially decreased by more than 50% relative to baseline, but by 24 hours, arterial velocity was marginally higher than baseline, and significantly higher than immediately following the exchange. As a result of changes in velocity and diameter, arteriole flow decreased for T-State PolybHb 15 minutes post-exchange, but increased after 24 hours, relative to baseline. R-State PolybHb did not show any statistically significant changes in arteriole flow, but it was slightly lower at 15 minutes than it was at baseline, and but returned to baseline after 24 hours. T-State showed statistically significantly greater flow at 24 hours than R-State (Figure 6.6). Venule diameter, velocity, and flow were not changed in any experimental group, as shown in Figure 6.7.

Functional Capillary Density (FCD). There was a slightly, but not statistically significant, decrease in FCD for hamsters exchanged with T-State PolybHb, which had slightly recovered to baseline after 24. No changes in FCD were observed in the animals exchanged with R-State PolybHb (Figure 6.6D). *Oxygen Saturation using Hyperspectral Imaging.* Arteriole HbO₂ saturation decreased approximately 10% following exchange transfusion with both R-State and T-State PolybHb, but after 2 hours arteriolar O₂ saturation returned to baseline. After 24 hours, arteriolar O₂ saturation remained at baseline for both R-State and T-State PolybHb. Venule Hb-O₂ saturation decreased 30% relative to baseline for both T-State and R-State PolybHb 15 min following exchange. T-State's venular O₂ saturation recovered to baseline after 2 hours, and stayed

at baseline at 24 hours, whereas R-State's venular O₂ was slightly lower than baseline both 2 and 24 hours after transfusion (Figure 6.8).

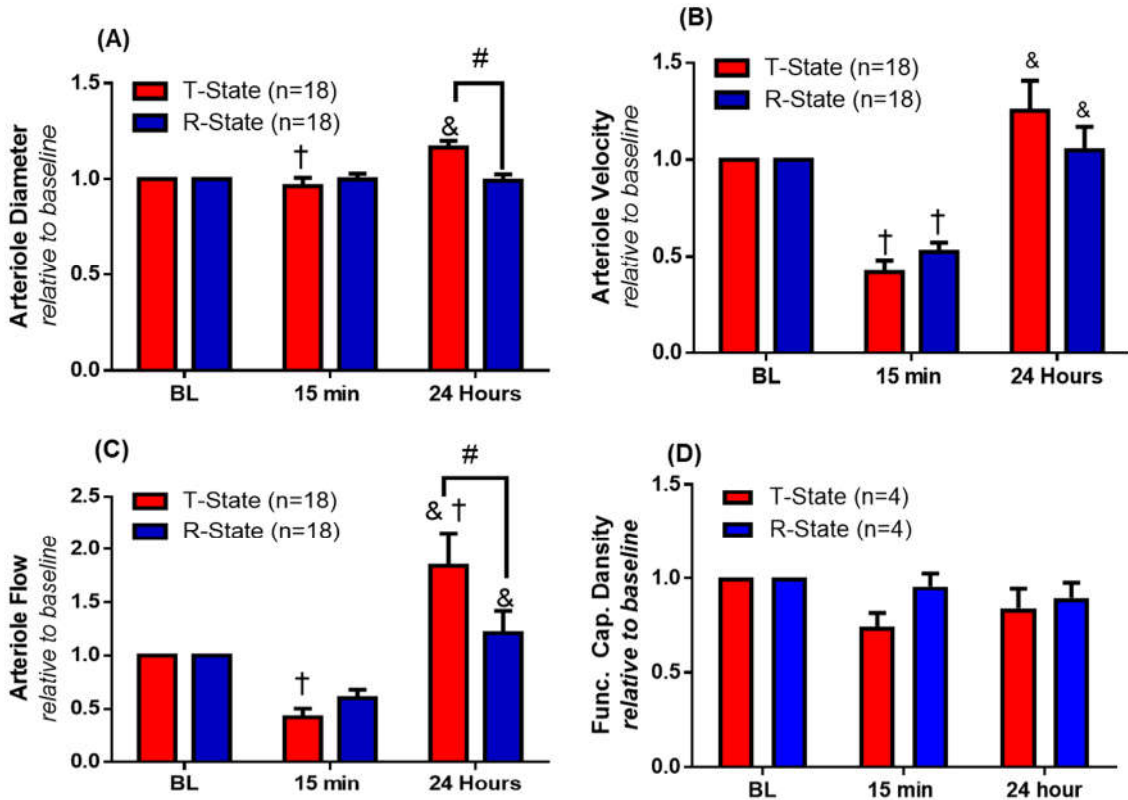


Figure 6.6: Arteriolar hemodynamics in the hamster chamber window. (A) Arteriole diameter, (B) Arteriole velocity, (C) Arteriole Flow, and (D) Functional capillary density of the hamster window chamber (before exchange), 15 min, and 24 hours after 20% exchange transfusion. †p < 0.05 vs Baseline, &p < 0.05 vs 15 min, #p < 0.05 vs T-State.

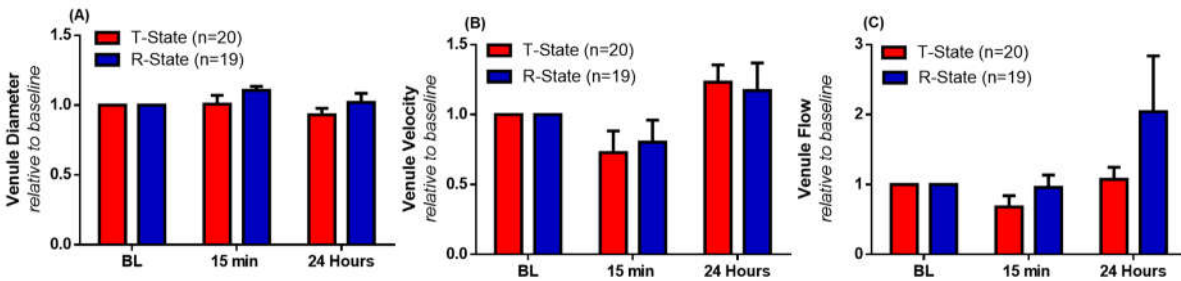


Figure 6.7: Venular hemodynamics are unaffected by exchange transfusion with PolybHb.

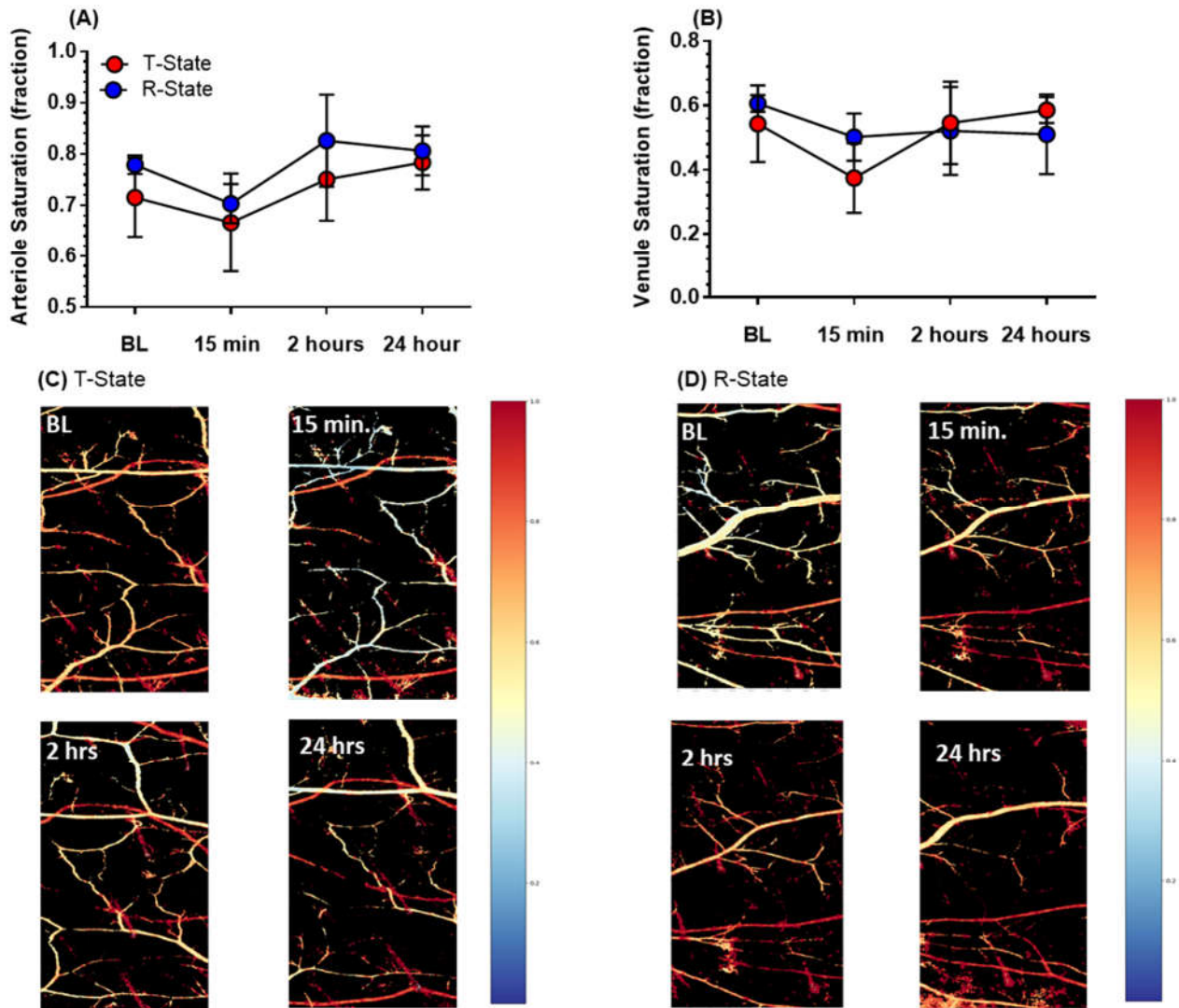


Figure 6.8: Changes in microvascular O₂ distribution following exchange transfusion. (A) Arteriole SO₂, and (B) Venule SO₂. Panels (C) and (D) are representative hyperspectral images the microcirculation following exchange with T and R-State PolyHb, respectively.

6.5: Discussion

The main finding of this study is that PolyHb produced mild microhemodynamic aberrations that recovered less than 24 hours post-transfusion, and caused mild inflammation in an animal model relevant to human safety of HBOCs. PolyHb caused inflammation independent of the quaternary state, but this inflammation was mild, with no observable liver damage, spleen inflammation, or lung inflammation. Since guinea pigs do not produce AA, they have certain evolutionary antioxidant adaptations that are similar to humans. As such, assessing the safety

profile of PolybHb in guinea pigs provides a much clearer understanding of how these antioxidant mechanisms affect toxicity, and provides a clearer picture of PolybHb safety in humans.

Phase III clinical trials of previous generations of HBOCs were met with several side effects, such as severe vasoconstriction, hypertension, and myocardial infarction that were not detected in pre-clinical trials, likely due the animal species used to test these HBOCs. In this study, a new generation of PolybHb possessing significantly higher molecular size was tested, comparing the effects of quaternary state on cardiac function, microhemodynamics, and toxicity. Increasing MW decreases NO scavenging by preventing HBOC extravasation, which interrupts endothelial smooth muscle NO transmission.¹⁹ NO acts as a potent vasodilator following binding to guanylyl cyclase in smooth muscle cells.²⁰ Therefore, maintaining smooth-muscle NO concentration helps prevent vasoconstriction and thus hypertension. As previously shown by our group, the size/molecular mass of the PolybHb molecule has a direct impact on microhemodynamics, with smaller MW causing significantly more vasoconstriction and hypertension than large MW fractions.¹² However, excessive delivery of O₂ to the vascular endothelium also causes vasoconstriction.^{8,16,21} As such, by controlling the quaternary state (and thus O₂ affinity) of PolybHb, the autoregulatory response to endothelial oxygenation can also be controlled. While slight alterations in microvascular diameter in response to the low O₂ affinity were observed, these changes were relatively mild in the hamster model used (<10%).

The hamster window chamber model allows us to evaluate O₂ delivery and microhemodynamics in the awake, unanesthetized animal. Microcirculatory function is the main prerequisite for adequate tissue oxygenation and thus organ function. The microcirculation transports O₂ and nutrients to tissue, and ensures adequate immunological function, thus making it a point of research to understand blood substitute efficacy. In experiments assessing the

microhemodynamics, we observed that T-State PolybHb provoked transient vasoconstriction, but R-State PolybHb did not elicit such a response. The quaternary state of PolybHb does not affect its NO dioxygenation rate, and the glutaraldehyde:bHb ratios used to generate PolybHb results in similar molecular diameters for T-State and R-State PolybHb.²² Furthermore, T-State PolybHb possesses slightly higher viscosity than R-State PolybHb (14cP vs 10cP at 100s⁻¹), therefore the lack of vasoconstriction for R-State PolybHb is not due to elevated endothelial mechanotransduction. As such, differences in O₂ delivery, are likely responsible for changes in microhemodynamics observed following exchange transfusion. However, after 24 hours no vasoconstriction was observed, and vessels were vasodilated, likely due to a reduction in total O₂ carrying capacity. We also observed a small (non-significant) decrease in FCD for hamsters transfused with T-State PolybHb, but no such decrease was observed with R-State PolybHb. FCD indicates the degree of tissue perfusion, and a higher FCD ensure proper tissue homeostasis. Since we saw no significant changes in FCD, that indicates that PolybHb preserves tissue homeostasis, independent the quaternary state.

Through HSI, the hamster window chamber model also allows us to evaluate microcirculatory O₂ distribution with high spatial resolution, as demonstrated in Figure 6. These experiments demonstrated very little changes in microvascular O₂ distribution following exchange transfusion, independent of the quaternary state. The exchange transfusion model implemented requires minimal perturbation of homeostasis, so maintenance of microvascular O₂ distribution is expected. In different models, such as hypoxia²³ or severe anemia, the O₂ distribution is disrupted such that HbO₂ affinity can have a larger impact on O₂ distribution.

In our study T-State PolybHb caused slight vasoconstriction, while R-State PolybHb avoided this effect, however, the vasoconstriction observed with T-State was very mild compared

to previous studies in hamsters.¹² On the other hand, in guinea pigs, MAP was higher independent of the quaternary state of PolybHb, which indicates that other mechanisms could be involved in the increased MAP at 24 hours, as arteriolar diameter in hamsters had actually increased from baseline. This suggests that long term mechanisms that increase blood pressure after more than 24 hours may be active in guinea pigs, but further studies are needed to better understand this. Regardless of the precise mechanism, these inter-species differences are likely due to guinea pigs' higher sensitivity to ROS compared to AA-producing species.²⁴ Our results suggest that the mechanisms involved in the increased MAP could be different between R and T-State; R-State presented increased norepinephrine, which may be associated with the increased blood pressure for this group due to activation of α_1 and α_2 receptors in blood vessels. However the T-State did not present this increase in norepinephrine, suggesting a different mechanism for T-State's increase in blood pressure relative to sham. Previous guinea pig studies have demonstrated that R-State PolybHb oxidizes faster in vivo than T-State PolybHb,²⁵ which were confirmed in these data (Figure 6.3). Once oxidized, Hb can no longer react with NO. As such, a higher fraction of T-State is scavenging NO 24-hour post-transfusion than R-State. This, along with the important role that NO plays in regulating cardiac contractility and vascular tone,²⁶ may explain the differences in cardiac function observed between Sham, T-State PolybHb, and R-State PolybHb. However, oxidized Hb is heme-unstable, often resulting in dissociation of heme from the surrounding globulin structure.

Heme, which is mainly derived from hemoglobin (Hb), is a strong oxidant and has potent pro-inflammatory properties.²⁷ These properties become apparent if the intricate intra-erythrocytic compartmentalization of heme is compromised after the destruction of erythrocytes, or transfusion of acellular Hb solutions.²⁸ In addition to disrupting NO signalling, extravasation of low MW

HBOCs and unmodified acellular Hb increases the oxidative potential of free heme. Extravasation removes heme from the reductive environment of plasma, and has been shown to lead to pancreatic/liver enzyme elevation, kidney injury, and inflammation.¹⁶ In these experiments, we observed that both states of PolybHb caused some systemic and liver inflammation, but there was no indications of chronic liver damage, spleen, or lung inflammation associated with the PolybHb exchange-transfusion. Additionally, markers of kidney injury were more strongly associated with R-State PolybHb, however the kidney injury could be an acute and transient effect. The kidney injury is likely associated with kidney exposure to oxidized Hb, since, as previously mentioned, R-State PolybHb oxidizes faster in vivo than T-State PolybHb. As such, these data suggest that the high MW of PolybHb helps prevent oxidative reactions and chronic injury that was associated with previous generations of HBOCs.

Limitations. It is important to highlight that these exchange transfusions were made in healthy guinea pigs and hamsters with the goal of understanding possible side effects of these high MW PolybHb. To better understand differences in efficacy between T-State and R-State PolybHb, further studies in animals under pathological conditions are necessary, since high or low O₂ affinity might be beneficial depending on the type of pathological model used. Other HBOCs have been tested and show that HBOCs improve recovery from septic shock, hemorrhagic shock, and myocardial infarction.^{29–33} Additionally, healthy animals do not suffer from endothelial dysfunction, which increases vascular permeability, and may compromise safety of HBOCs. Further evaluations in models that are representative of vulnerable patient populations are currently underway to alleviate this limitation.

In summary, we can conclude that although PolybHb transfusion increases markers indicating acute organ damage and inflammation, increasing the MW and controlling the

quaternary state of PolybHb helps partially prevent the toxicity of these HBOCs. Moreover, more experiments are needed to better understand the efficacy of both, low and high O₂ affinity in pathological conditions such as severe anemia and hemorrhagic shock.

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CHAPTER 7: CONCLUSIONS

The work presented in this dissertation was performed to achieve 3 aims, all with the overarching goal of reducing the dependence on blood transfusion in an age where the quality of *ex vivo* stored blood is being questioned, and where shortages of blood occur frequently. The first aim assessed the efficacy of a novel blood storage technique during resuscitation from hemorrhagic shock. The second aim analyzed the efficacy of a high molecular diameter hemoglobin (Hb)-based oxygen (O₂) carrier (HBOC) during resuscitation from hemorrhagic shock, and determined the biophysical properties of this HBOC that contribute to its efficacy. Finally, the third aim evaluated the safety of this class of HBOCs in a human-relevant pre-clinical model, examining both transient, and chronic effects. Finally, 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: Assessment of a Novel Blood Storage Technique

Our study in a clinically relevant (i.e. goal-guided) model of resuscitation from hemorrhagic shock demonstrated that the quality of stored red blood cells (RBCs) has a substantial impact on their efficacy and efficiency in restoring hemodynamics following hemorrhagic shock. Numerous assays have been utilized to show that anaerobically stored RBCs possess high quality than conventionally stored RBCs, a result that is also demonstrated in this study. The number of RBCs surviving in circulation after 24 hours is the primary metric by which the FDA approves new RBC storage techniques and solutions. The rationale underpinning this metric is that, at a minimum, RBCs that are transfused must survive in circulation long enough to transport O₂. While this rationale is fair, this assay fails to assess how RBC storage lesions impact O₂ transport in manners other than pure O₂ carrying capacity, such as changes in microhemodynamics that occur

following stored RBC transport. Furthermore, to perform this assay, RBCs are subjected to chemical and physical stressors during the radiolabeling process. This artificially increases the “quality” of the RBC pool due to hemolysis of RBCs that cannot survive the process, and thus could explain why the literature commonly finds that human RBC quality is severely diminished after only 3 weeks, despite the FDA approving *ex vivo* storage of RBCs for up to 6 weeks.

Despite the weakness in this assay, it demonstrated that a significantly higher number of anaerobically stored RBCs survived after 24 hours compared to conventionally stored RBCs, which translated into better outcomes after resuscitation from hemorrhagic shock with significantly less blood transfused. These preliminary data support the use of anaerobically stored RBCs in place of conventionally stored RBCs as they could reduce the number of RBC units required due to their improved quality. However, the 24-hour recovery assay demonstrated that anaerobic storage does not appear to significantly improve the effective storage life of RBCs, so alternatives with longer storage lives are necessary.

7.2: Efficacy of Polymerized Hemoglobin During Resuscitation from Hemorrhagic Shock

It is well established that *ex vivo* storage of RBCs impairs their functionality, which was further demonstrated in this aim. More importantly, however, was a lack of storage-associated degradation of PolybHb, despite two years of storage. This represents a 20-fold increase in storage lifetime, which is vital for emergency preparedness, as units can be stocked and stored without fear of expiry. Furthermore, this aim demonstrated that PolybHb displayed similar efficacy as freshly collected RBCs during resuscitation from hemorrhagic shock, as rats reached the goal blood pressure at the same time, with similar volume requirements, and maintained this goal for the duration of the observation period. Additionally, many parameters also demonstrated that PolybHb resulted in less organ injury than stored RBCs, albeit this injury was greater than fresh

RBCs. Clearly, fresh RBCs are ideal, but fresh RBCs are rarely available, so a large blood banking industry has developed. Since PolybHb was more efficacious, and resulted in less toxicity, than end-of-storage RBCs, the development of PolybHb could urge the blood banking community to focus efforts on improving the quality of RBCs, rather than extending the storage life, particularly because polymerized human Hb could be created from expired blood bags.

This aim also demonstrated that PolybHb has certain biophysical properties that result in it effectively resuscitating from hemorrhagic shock. Namely, the high viscosity of PolybHb improved myocardial O₂ delivery, as evidenced by markers of cardiac mechanoenergetics, likely due to NO-mediated vasodilation. The low O₂ affinity of this PolybHb formulation also increased O₂ extraction, which helps restore tissue oxygenation and aerobic metabolism following hemorrhagic shock. PolybHb's oxygen carrying capacity, while important, seemed less vital in the animals' recovery, since only approximately 20-30% of the O₂ delivered by PolybHb was utilized by tissues, whereas RBCs saw O₂ extraction:O₂ delivery ratios upwards of 60%. This is an important consideration in the design of future HBOCs, as developing them with high viscosity, but lower O₂ carrying capacity, could both improve recovery from HS and decrease Hb-associated sequelae. Both of these studies demonstrate the efficacy of PolybHb as an alternative to RBCs, and could reduce the overall need of RBC units.

7.3: Safety of Polymerized Hemoglobin in a Human-Relevant Animal Model

The third aim of this dissertation evaluated the safety of PolybHb in guinea pigs, a human-relevant animal model. Guinea pigs do not produce ascorbic acid, which is the primary small-molecule reductant of Hb. Much of Hb's toxicity comes from its pro-oxidant properties. Therefore, evaluating the safety of PolybHb in guinea pigs elucidates its safety profile in humans. This aim demonstrated that the molecular size of PolybHb plays a large role in its toxicity, as the lower

molecular weight variant tested resulted in significant vasoconstriction, and increased markers of end-organ injury and inflammation. Furthermore, the molecular size of PolyHb also regulates its catabolism, with the higher molecular weight variant surviving in circulation significantly longer. To avoid repeat transfusions, HBOCs should survive in the circulation for as long as possible, otherwise they do not act as effective blood volume expanders or O₂ carriers. Previous HBOC formulations possessed significantly lower average molecular size than even the sPolyHb variant tested in this aim, typically < 500 kDa. As such, the vasoactivity and tissue injury seen in this study from sPolyHb is likely mild in comparison to previous HBOC formulations.

Another critical finding is the lack of tissue injury and inflammation 24 hours after PolyHb exchange-transfusion in guinea pigs. This indicates that, while there may be some mild acute injury, the injury does not progress to chronic tissue damage. This result is also independent of the O₂ affinity of the PolyHb. This finding is interesting because it was theorized that the low O₂ affinity of T-State PolyHb would result in higher reactive oxygen species (ROS) production compared to the high-affinity R-State PolyHb, as a significant portion of O₂ is released from T-State PolyHb before the capillaries. These data also demonstrated that neither T nor R-State PolyHb impaired microvascular O₂ delivery and utilization, an important determinant in the efficacy of a blood substitute. These data overall strongly support the use of PolyHb, and suggest that this HBOC formulation has a better safety profile than previous HBOCs. These data, combined with the data from the previous aim, suggest that PolyHb can reduce the dependence on RBCs due to its storage life, efficacy, and safety.

7.4: Strength of the Study

Aims 1 and 2 were performed with a goal blood pressure in mind. This is similar to Advanced Trauma Life Support® (ATLS) recommendations, as the ATLS recommends a mild

hypotonic resuscitation in hemorrhagic shock patients that have not been surgically stabilized, such as patients during the “golden hour” of resuscitation. This animal model helped to elucidate the efficacy of RBCs and HBOCs, as the volume required to achieve and maintain hemodynamic stability is multifactorial, but very dependent on the cardiovascular system’s response to the fluid transfused. Furthermore, we were able to very tightly monitor left ventricular cardiac function using a miniaturized pressure-volume catheter. Monitoring and understanding how the heart responds to resuscitation is vital, since the heart is responsible for delivering blood, and thus O₂ to the entirety of the organism. Finally, we implemented guinea pigs as an animal model of HBOC safety. Since guinea pigs have similar anti-oxidant profiles as humans, the safety of HBOCs in guinea pigs is similar to the safety of HBOCs in humans.

7.5: Limitations of the Study

This dissertation was performed primarily in isoflurane anesthetized animals. The response of anesthetized animals is not always consistent with the awake-response. Furthermore, isoflurane is a known cardio protectant, so the awake response to hemorrhagic shock could be more severe than we demonstrated. Additionally, since many of these studies were aimed at only assessing the “golden hour”, longer-term effects of resuscitation were not evaluated, and these longer-term outcomes could be different from those observed here. For both rat and guinea pig studies, evaluation of left ventricular function requires precise placement of the pressure-volume catheter. This positioning can be very difficult, particularly in the guinea pigs, which could weaken the significance of the cardiac function results. Finally, the interventions implemented in the last aim were quite small, only on the order of 1-2 “blood bags” worth of volume. Larger interventions could contribute more to injury and inflammation in a non-linear manner, so safety after transfusion of larger volumes is necessary to establish a proper safety profile of PolyHb.

7.6: Future Works

Future works are aimed at evaluating PolyHb in guinea pigs that have been fed a high-fat, high-sucrose diet. These animals develop endothelial dysfunction, which is underdiagnosed in humans, but has severe consequences on the safety of PolyHb. Endothelial dysfunction increases vascular permeability, and could possibly allow extravasation of even the LPolyHb formulation tested. Additionally, since the safety of R-State and T-State PolyHb appear to be different, future works will explore the use-scenarios of each PolyHb. For instance, during anemia, the low affinity of T-State PolyHb could promote O₂ utilization by tissues, but R-State PolyHb could prevent this. In contrast, during hypoxic-hypoxia R-State PolyHb could improve O₂ delivery to tissues by promoting O₂ loading in the lungs, whereas T-State PolyHb could hinder blood O₂ uptake in the lungs.