University of Alabama at Birmingham
UAB Digital Commons

All ETDs from UAB | UAB Theses & Dissertations

2014

Effects Of Storage On The Red Blood Cell And Its Implications For Transfusion

Ryan D. Stapley
University of Alabama at Birmingham

Follow this and additional works at: https://digitalcommons.library.uab.edu/etd-collection

Recommended Citation

Stapley, Ryan D., "Effects Of Storage On The Red Blood Cell And Its Implications For Transfusion" (2014). *All ETDs from UAB*. 3026.
https://digitalcommons.library.uab.edu/etd-collection/3026

This content has been accepted for inclusion by an authorized administrator of the UAB Digital Commons, and is provided as a free open access item. All inquiries regarding this item or the UAB Digital Commons should be directed to the UAB Libraries Office of Scholarly Communication.

EFFECTS OF STORAGE ON THE RED BLOOD CELL AND ITS IMPLICATIONS FOR TRANSFUSION

by

RYAN D. STAPLEY

RAKESH P. PATEL, COMMITTEE CHAIR
SCOTT BALLINGER
JEFF KERBY
JACK LANCASTER
MARISA MARQUES
ROGER WHITE

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2014

EFFECTS OF STORAGE ON THE RED BLOOD CELL AND ITS IMPLICATIONS FOR TRANSFUSION

RYAN D. STAPLEY

MOLECULAR AND CELLULAR PATHOLOGY

ABSTRACT

Transfusion with red blood cells (RBC) remains a frontline therapy in critically ill patients. Recent studies have documented an association between storage duration and an increased incidence of transfusion-related toxicities. This transfusion-related toxicity is characterized by both a pro-inflammatory state and a microcirculatory dysfunction and is associated with changes that occur to the RBC and its media during storage referred to as the storage lesion. These changes that occur to the RBC include an increase in cell-free hemoglobin, heme, free iron, microparticles, as well as changes to the RBC itself. This thesis explores how these elements could contribute to transfusion-related toxicity. Erythrocytic hemoglobin can rapidly scavenge nitric oxide (NO), thereby inhibiting NO signaling leading to inflammation and microcirculatory dysfunction. We show that storage-related changes in RBC lead to increased NO-scavenging kinetics with RBC stored for 42 days compared to those freshly isolated; an affect that translated to a greater inhibition of NO-dependent vasodilation. We also have developed a mouse model of storage lesion that involves a first hit of trauma/hemorrhage followed by a second hit caused by transfusion with stored blood. Using this model we show that acute lung injury can be attenuated and survival increased by i) washing RBC to remove storage media, ii) nitrite administration, and iii) inhibition of the TLR4 receptor by TAK-242.

DEDICATION

This thesis is dedicated to my wife, Danella,

and my sons, Vance, Caleb and Bennett.

ACKNOWLEDGMENTS

I would like to acknowledge the help of my family and friends who have supported me during my time at graduate school. I am grateful for the patience and understanding of my wife who has helped me through difficult times.

I would like to thank all of my classmates during my first year of graduate school who help me study and understand material presented.

I would also like to thank my lab mates who helped me out in difficult situations and gave of their time to help my understanding.

I would like to thank my committee who has given me much support and feedback to help me in my research.

Finally I would like to thank Rakesh for being supportive and being an example of hard work and determination.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER 1

RED BLOOD STORAGE AND TRANSFUSION

Introduction

The majority of my research at UAB has been to determine mechanisms by which blood storage negatively affects transfusion outcomes. While transfusion is important in a number of disease states, recent data show that there is an association between the storage age of red blood cells (RBC) and increased morbidity and mortality in transfusion recipients. In particular, my focus has been on what is known as the blood storage lesion. The term storage lesion has been used indiscriminately to denote both changes that occur to RBC during storage and reflect tissue injury post transfusion with older RBC. In this thesis I define storage lesion as the biochemical and morphological changes that occur to the RBC and storage media during cold storage. The storage lesion has been associated with increased complications that occur after transfusion by means of increased inflammation and microcirculatory dysfunction. In this first chapter I will give a short introduction on the current state of blood banking and the biochemical and morphological changes of the RBC during storage. I will then explain how these changes in the RBC could contribute to the increased inflammation and microcirculatory dysfunction that are associated with transfusion of these stored RBC. In addition, I will provide background into current animal models used to investigate storage lesion, specifically how these pertain to my studies.

Evolution of Blood collection and storage practice

The concept of blood transfusions started as early as the 17th century when physicians experimented by transfusing human or animal blood into human volunteers. The results were usually poor and in some cases resulted in death, but proved transfusion was possible. Dr. James Blundell is credited for the first successfully usage of human blood transfusion to treat postpartum hemorrhage in 1818 [1, 2]. Subsequent transfusions for hemorrhage met with mixed results until the discovery of blood groups by Karl Landsteiner, who would eventually be awarded the Nobel Prize in Physiology and Medicine in 1930 [2]. The knowledge of blood groups significantly reduced mortality in transfusion patients and led to blood transfusions becoming a lifesaving treatment. In the early 1900s, with the aid of refrigeration and anticoagulants, blood was able to be stored and given in transfusions at later dates. This increased the utility and frequency of the procedure. The advent of World War I and the subsequent need of blood transfusions to the injured brought about a rapid development in blood storage and transfusion and initiated the need for blood banks. The amount of blood being stored and its need during war time necessitated regulations for governing the storage time of blood. A condition was set that at 24 hours post transfusion, 70% of the RBC transfused must be viable in the circulation; this criterion was arbitrarily selected as a means to avoid hemoglobinuria. This condition was modified to a 75% survival in the 1980s [3] and it should be noted that 75% survival of RBC is an average and not a minimum. Though there are a variety of storage solutions available, all use a solution consisting of dextrose, adenine, citrate, mannitol and sodium chloride with some slight variations in ratios. Dextrose and adenine allow RBC to maintain glycolysis and ATP production during storage. Sodium

chloride is used to maintain osmolarity and mannitol has been shown to help maintain the stability of the RBC membrane and decrease hemolysis. The RBC are then stored at a temperature of 4 degrees Celsius, for up to 42 days. As blood banking technology continued to develop, blood was eventually divided into components: plasma, platelets and RBC, for storage and usage. In separating these blood products, white blood cells (WBC) were removed to varying degrees. Studies showed that products in which WBC were removed, (leukoreduced), had decreased risk of human leukocyte antigen (HLA) alloimmunization and platelet refractoriness observed in multi-transfused patients, and transmission of leukotropic viruses after transfusion [4-9]. Several institutions have instituted a leukoreduction protocol to improve removal of the WBC component. While several methods of leukoreduction have been established, the most effective method of leukoreduction currently implemented utilizes WBC filters. Filters which are used in the U.S and Europe result in a 99.99% removal rate, however it should be noted that in some areas, especially those with limited resources, less efficient methods are used or not done at all [10]. Leukoreduction has been shown to decrease mortality in patients and is generally seen as an accepted practice to decrease complications in transfusions [11, 12].

Transfusion Toxicity

Blood typing and leukocyte-reduction of stored blood has decreased complications associated with transfusion. However, an increase risk of lung injury, renal injury, and mortality is associated with transfusion of older RBC as compared to those who received no transfusion [13-16]. The idea that transfusion of RBC can cause damage has prompted a reassessment of when to use transfusion. Current FDA guidelines advise

that a hemoglobin (Hb) concentration of 10 g/dL is sufficient to maintain oxygen delivery. However it was noted during the 2012 National Summit of Overuse, where overuse is defined as "the use of health services where, in circumstances where the likelihood of benefit is negligible and therefore the patient faces only the risk of harm" that RBC transfusion is the 5th most overused procedure in the US [17]. Studies have shown that maintaining a Hb concentration of 7-9 g/dL has no different outcome as compared 10 g/dL [18-20] leading to ongoing discussion about what the transfusion trigger should be i.e. at what hemoglobin level should a transfusion be indicated. Despite the overuse of transfusion, demand is on the rise especially as the population ages. This coupled with a decrease in blood provided by donors, experts predict a blood shortage in the near future [21-24]. To meet the demands of RBC transfusion in the future we need to better understand blood storage and improve the process by which blood is stored and transfused.

As mentioned previously, transfusion of RBC is associated with an increased risk of adverse outcomes post transfusion. This association has prompted research to determine whether the duration of RBC storage is a factor contributing to increased toxicity. Retrospective and prospective studies have shown a positive correlation between storage time and increased risk of adverse outcome in the context of ICU patients, [25-27] trauma patients, [28-30] and cardiac patients [31-34]. These adverse outcomes include increased mortality, prolonged hospital stay, multi-organ failure, acute lung injury, and nosocomial infections. However it should be noted that other studies investigating RBC storage duration as a factor to toxicity have been inconclusive or seen no effects of storage age on transfusion toxicity [35-38]. The Age of Red Blood Cells in

Premature Infants (ARIPI) study that compared transfusions of young RBC (<7 days) to old RBC (>7 days) in premature neonates recorded no difference between to transfusions with young RBC. Studies from both sides of the issue of storage age have limitations such as a bias toward more severely ill patients receiving older RBC, patients receiving multiple transfusions of both young and old RBC and insufficient contrast between young and old RBC [39, 40]. Three prospective clinical trials are currently underway that will expand our knowledge of the transfusion toxicity of longer stored RBC prior to transfusion. The Red Cell Storage Duration Study (RECESS) is a multicenter prospective study that compares the effects of complex cardiac surgery patients receiving fresh units of less than 10 days of storage to those receiving older units of 21 days or greater. The change in the composite multiple organ dysfunction score (MODS) from the pre-operative baseline will be measured as the primary outcome [41]. The Age of Blood Evaluation (ABLE) is another multicenter study that will enroll 2500 severely ill ICU patients that will either receive exclusively fresh blood (less than 7 days of storage) compared to those who have will receive blood of uncontrolled length of storage. These transfusions will be of the oldest blood available as is the common practice of hospitals. Primary outcome is determined by 90 day all-cause mortality with secondary outcomes of organ failure, and serious nosocomial infections [42]. The third study is being done in Australia, New Zealand and possibly Europe and is referred to as the Standard Issue Transfusion Versus Fresher Red Blood Cell Use in Intensive Care – A Randomized Controlled Trial (TRANSFUSE). In this study ICU patients will receive a transfusion of either the freshest blood available or receive blood using the current hospital standard which is the oldest blood available. This study plans to enroll 5000 patients and will

measure mortality at 90 days post transfusion as well as persistent organ dysfunction. The results of these studies will be made available in the next few years and will greatly influence current blood banking practices.

That said, several recent experimental studies, including those shown in Chapter 3 demonstrate that transfusion with stored RBC does promote tissue injury. It is also well established that several changes occur to RBC and storage media during storage. If it is shown that transfusion with older RBC does increase risk of injury, it becomes important to better understand how changes to the RBC during storage could contribute to toxicity as well as possible strategies to improve transfusion outcomes. If there no association is found, there still remains a need to improve blood storage techniques to meet future demand. In the following section I review several of the well-known changes that occur during storage as well their effects upon transfusion.

Biochemical changes of the RBC during storage

During the storage of RBC a dysfunction in the allosteric mechanisms that regulate oxygen affinity of hemoglobin are observed leading to a mismatch between oxygen delivery and tissue requirements. These include decreased concentrations 2,3-bisphosphoglycerate (2,3-BPG) and ATP which decrease oxygen affinity. The loss of 2,3-BPG in RBC during storage is rapid decreasing from 5mM to undetectable levels within 1 week of storage [43]. Upon transfusion, de novo synthesis of 2,3-BPG will restore levels to normal within 48 hours, however it is important to note that over the initial 48 hours after delivery, and depending on amount of blood transfused, the overall oxygen affinity will remain elevated and can cause tissue hypoxia [44]. Similar changes

occur in ATP, which in addition to affecting oxygen affinity will also compromise other RBC functions related to regulating active transport, antioxidant reactions and oxidative stress defense mechanisms, membrane stability and phospholipid distribution and other energy requiring reactions. This increases the vulnerability of RBC to cell stresses encountered in the circulation system resulting in increased lysis [45]. To help compensate for the loss of ATP, erythrocytes are stored in a media that contain dextrose which repletes ATP levels by glycolysis. Solutions also include adenine to enable the synthesis of ATP. These two components will ameliorate the loss of ATP levels but will not completely restore them. Presently there are no storage solutions that directly limit the loss of 2,3-BPG.

Currently employed storage solutions have been developed with the primary goal of preventing hemoglobin oxidation and slowing the loss of allosteric effectors. Despite these efforts, emerging evidence indicate that other changes in the RBC still occur that could lead to dysregulated oxygen delivery. For example, expression of the RBC membrane protein anion exchange 1 (AE1, or band 3), which is important in ion homeostasis in the blood and mediating the exchange of chloride and bicarbonate ions across the RBC membrane, decreases by ~50% over a 6 week storage time [46]. Loss of AE-1 appears to be a result of proteosomal activity and the precise functional consequences of such a dramatic loss of AE-1 on RBC function after resuscitation is not clear, but underscores the concept that multiple biochemical changes during storage will compromise regulated oxygen delivery.

Many studies have documented increased oxidative stress in various RBC components during storage time and with different storage solutions [47-50]. This is

typically characterized by an increase in markers of oxidative stress (e.g. increase in protein carbonyls, thiol and lipid oxidation products) and by a loss of reductants, antioxidants and antioxidant enzyme activities (e.g. glutathione, glutathione peroxidase, peroxiredoxin). Redox cycling reactions between hemoglobin and lipid hydroperoxides and/or hydrogen peroxide are central in mediating oxidative damage to RBC components [51, 52] with the consequences being manifest depending on the target, extent and nature of the oxidative event. One example pertinent to oxygen carrying capacity is loss of NAD(P)H reducing equivalents which compromises the methemoglobin reductase system and thereby leads to an accumulation of methemoglobin. Similarly, a RBC with compromised antioxidant defenses is also more susceptible to damage by exogenous reactive species that may be encountered in the circulation, especially in patients who have vascular inflammation which likely encompasses many groups who would require transfusions. Moreover, a common property of stored RBC is increased fragility which can arise from lipid peroxidation (and subsequent compromised membrane integrity) and/or oxidative modification of cytoskeletal proteins.

In addition to changes to the RBC during storage there are changes to the media it is stored in. Storage media has an increase in cell free hemoglobin and non-transferrin-bound iron [53-55]. These are products from RBC that hemolyzed that buildup during storage. Unless the blood is washed prior to transfusion, which is only done in limited circumstances, the patient receives these products in addition to the stored RBC. Each of these products has been shown to cause injury upon transfusion. Cell-free hemoglobin has a well-known toxicity and is associated with acute hypertension, vascular injury, and

kidney dysfunction [56-60]. Non-transferrin bound iron has been shown to inhibit cellular immune function of both monocytes and neutrophils [55, 61-63].

Another component that increases during storage is heme, and it oxidized form hemin. It is not currently known what levels of cell free heme are seen in stored RBC. Heme has recently been shown to activate the TLR4 receptor resulting in an increase of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [59, 60, 64]. Activation of the TLR4 receptor has been shown in animal models of sickle cell anemia and infection to promote inflammation of tissue and also leads to acute chest syndrome and sepsis respectively.

Red Blood Cell changes in Shape and Structure during storage

The biconcave disc shape of RBC is essential for function as it increases the surface area-to-volume ratio facilitating the transport of oxygen across the membrane. This discoid shape also allows deformability and thus transit through smaller capillaries. As RBC age during storage, its discoid shape changes to a crenellated form referred to as an echinocyte. "Echino" is a prefix denoting spines and indicate the small protrusions that appear on older RBC. Echinocytes also exhibit a decrease in volume but a relative increase in surface area (Figure 1-1). This increase in surface area could increase the diffusion of nitric oxide (NO) into the RBC. Echinocytes have also been shown to be less deformable and thus more fragile [65-68].

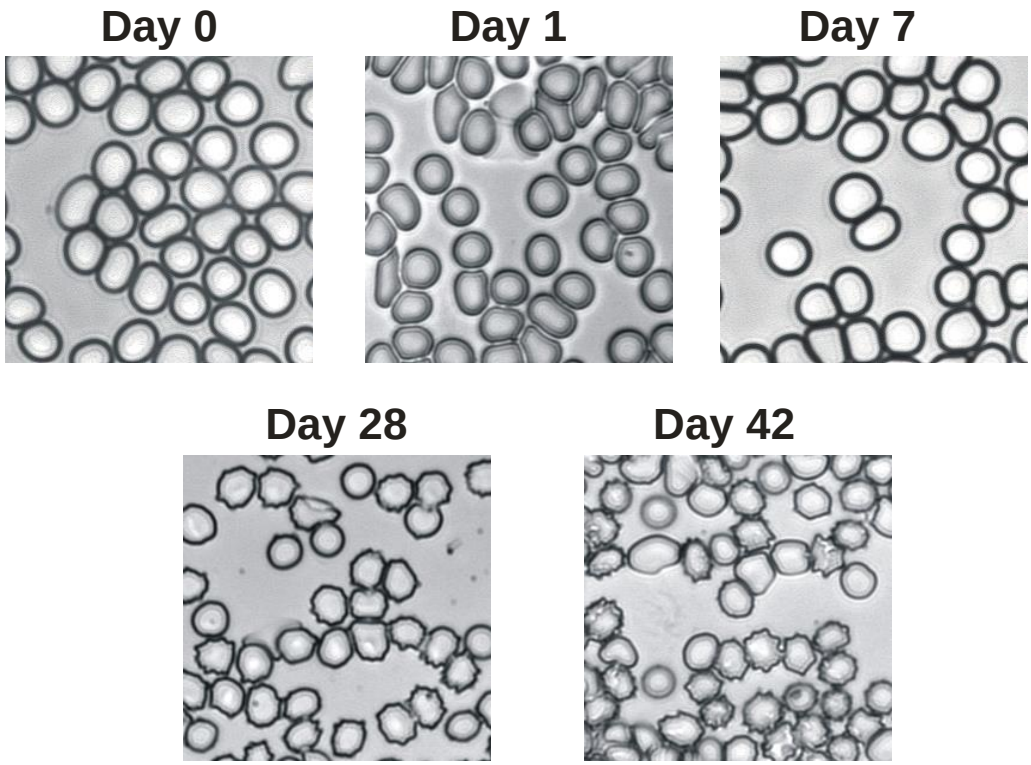

Figure 1-1 Representative light microscope images of RBC stored for 0-42d.
Unwashed blood from segments was obtained from UAB blood bank. Blood from days 0, 1, 7, 28, 42 were prepared on microscope slides and fixed with methanol. Fresh red blood cells as seen in Day 0 are of relative uniform shape consisting of discoid structure. As RBC age they transition to a smaller denser cell. Additionally some cells begin to develop spine like extrusions as seen above in day 28 and day 42.

These changes to the shape of the RBC are important for the expression of epitopes that target the RBC for removal by the immune system but also results in a higher probability for lysis both during storage and after transfusion. In fact hemolysis during storage varies between 1-5.5% depending on the storage medium and length. This is important because cell-free hemoglobin resulting from RBC lysis is a potent oxidizing agent and scavenger of NO in the vascular compartment which is discussed later.

It has been shown that as RBC age there is a loss of sialic acid. Sialic acid contributes a negative charge to the membrane which causes a repulsion of other erythrocytes. It has been hypothesized that the loss of charge would have a negative impact on the RBC's membrane deformability as well as increase interactions between RBC enhancing erythrocyte aggregation. Aggregation of RBC affects blood fluidity and has been found to be increased in patients exhibiting hypertension [69].

It has been discovered recently that RBC will bleb small vesicles during storage [70]. These vesicles, commonly referred to as microparticles, primarily contain hemoglobin and are defined as vesicles expressing glycophorin A protein, a glycoprotein found in all RBC. Vesicles can be differentiated from RBC by size with microparticles being of 50-100nm whereas RBC are of 6-8μm in size. Recent studies have shown that microparticles increase inflammation. This is caused by decreased chemokine binding by Duffy antigen which normally would remove chemokines from circulation [71]. Additional investigation into microparticles has noted a high rate of NO consumption similar to that of cell free hemoglobin [72, 73].

Two Hit Hypothesis of transfusion toxicity

It should be noted that if a healthy individual is given a transfusion of aged RBC, minimal damage occurs [74]. It is only when patients with an underlying disease are given a transfusion of aged RBC that an increase in transfusion toxicity is observed. Though there are several adverse complications that are associated with the storage lesion, two factors seen in common among the storage lesion toxicity is microcirculatory dysfunction and increased inflammation [75]. Current theory suggests that the

complications seen in the storage lesion is a two hit process where the first hit is provided by the underlying disease state that generally necessitates the use of transfusion. The second hit is given with transfusion of older RBC.

The second hit from the blood storage lesion could occur via multiple mechanisms. One mechanism that has received recent attention recently is the vasoconstrictive, pro-oxidative and pro-inflammatory effects of cell free hemoglobin that arises from the lysis of older fragile RBC. As mentioned previously, the concentration of cell-free hemoglobin within the storage solution of the RBC increases as it is stored; additionally the RBC being transfused are fragile and less deformable which could result in lysis upon or shortly after transfusion. Hemoglobin reacts with nitric oxide (NO) in both an oxygenated and deoxygenated state at a high rate (4–8 $X10^7$ $L \cdot mol^{-1} \cdot s^{-1}$ and 3–$6X10^7$ $L \cdot mol^{-1} \cdot s^{-1}$ respectively) [76, 77]. Normally, hemoglobin is encapsulated in the RBC which is estimated to decrease the rate of NO scavenging approximately 1000-fold (Figure 1-2). This is accomplished by a combination of factors including a diffusion barrier generated by the RBC membrane as well as an unstirred layer that surrounds the RBC which also decreases diffusion. It has been shown that RBC will amass at the center of the vessel during flow. The space where the RBC is not normally found is referred to as the cell free zone and is adjacent to the endothelial cells thus increasing diffusion distances for NO [78-81]. However, cell free hemoglobin is not affected by these factors and will cause an increase in NO scavenging until it is captured by haptoglobin and removed by the mononuclear phagocyte system. Another product of RBC storage is microparticles that have NO scavenging rate similar to cell-free

hemoglobin and not the RBC [73]. In this next section I review the production regulation and signaling effects of NO.

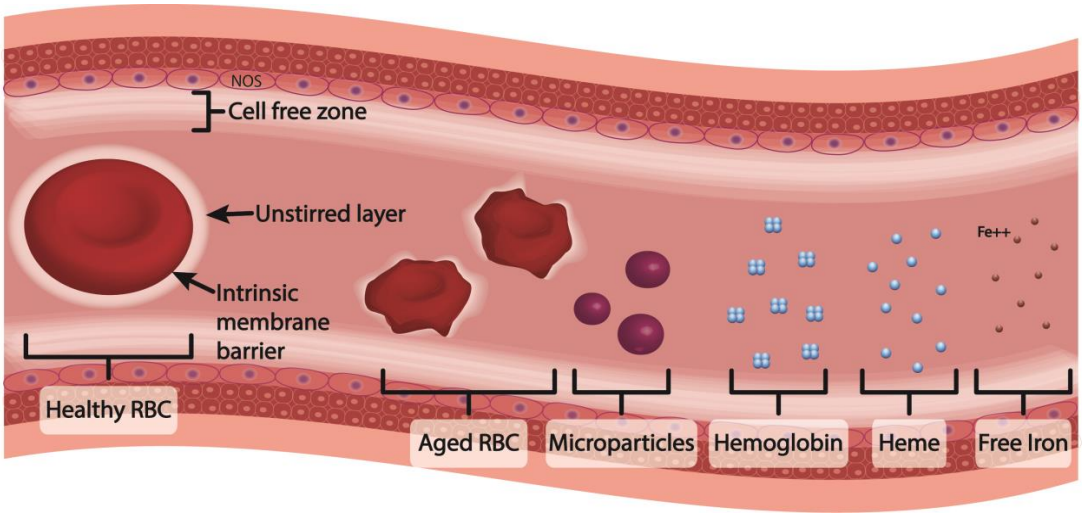

Figure 1-2 Methods of RBC NO scavenging
RBC have an approximate 1000 fold decrease in NO scavenging compared to cell-free hemoglobin due to a cell free zone adjacent to endothelial cells, an unstirred layer that surrounds the RBC and membrane proteins that decrease NO diffusion into the cell. A transfusion of aged RBC will include aged RBC, microparticles, cell-free hemoglobin, heme and free iron.

Nitric Oxide

To better understand storage lesion toxicity it becomes important to understand what effects nitric oxide (NO) has physiologically and what would occur if NO concentrations were decreased during transfusions. It has been noted that NO is produced both enzymatically by nitric oxide synthases (NOS) as well as non-enzymatically by the reduction of nitrate and nitrate to NO. For this section I will focus on the enzymatic production and will address nitrate and nitrite as substrates of NO in the following

section. There are three isoforms of NOS known as endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). All three isoforms will catalyze the hydroxylation of L-arginine from oxygen and NADPH to produce L-citrulline and NO [82]. iNOS is associated with cells of the immune system and is transcriptionally regulated in response to inflammation and cytokines. Once activated iNOS will produce large quantities of NO that are used by the inflammatory system as a means of defense by destroying evasive pathogens. Conversely, eNOS and nNOS are constitutively expressed and will produce small quantities of NO. In small doses NO acts as a signal in decreasing aggregation of platelets, decreased leukocyte adhesion, antioxidant effects as well as interacting with soluble guanylyl cyclase in the smooth muscle layer to produce cyclic guanosine monophosphate (cGMP) which in turn activates the cGMP-dependent protein kinase or Protein Kinase G (PKG). PKG eventually leads to a decrease in cellular calcium levels resulting in relaxation of vascular smooth muscle. In studies it has been shown that an inhibition of NO production by endothelial NOS will result in decreases in vessel flow ranging from 20-40% [13, 14, 83, 84].

NO diffuses randomly from the endothelial layer, so not only will NO diffuse to the smooth muscle to interact with soluble guanylyl cyclase but it will also diffuse to the luminal side of the vessel [80]. This luminal side contains a high concentration of hemoglobin that scavenges NO very quickly, however this hemoglobin is predominantly encapsulated in the RBC and thus its scavenging rate is decreased by the factors mentioned previously.

Nitrite reduction to nitric oxide

The inorganic anions nitrate and nitrite were once considered inert end products of nitric oxide (NO) metabolism and dietary constituents. However it has been shown that both anions can act as storage pool for NO in a non-enzymatic production of NO. Nitrate anion will reduce to nitrite by symbiotic bacteria in the oral cavity [85]. During hypoxia nitrite can function as a substrate for NO through a one electron reduction. Many proteins have been shown to reduce nitrite to NO including myoglobin, neuroglobin cytochrome C oxidase, cytoglobin, hemoglobin, and xanthine oxidase.[64, 86, 87] Hypoxia and low pH facilitate nitrite reduction. In my studies with the RBC we focus on the reaction of nitrite and hemoglobin.

The RBC or more specifically the hemoglobin within the RBC, will scavenge NO, but in hypoxic conditions hemoglobin will reduce nitrite to NO. Ferrous heme mediates NO scavenging, specifically, oxyhemoglobin reacts with NO to form nitrate and methemoglobin (metHb) whereas deoxyhemoglobin reacts with NO forming nitrosylhemoglobin (HbNO):

$$HbO_2^{(2+)} + {}^{\bullet}NO \rightarrow Hb^{3+} + NO_3^-$$

$$Hb^{2+} + {}^{\bullet}NO \rightarrow HbNO$$

Both of these reactions will remove NO and thereby inhibit vasodilation and other effects of NO.

The RBC will also reduce the anion nitrite by deoxyhemoglobin to produce NO according to the following mechanism:

$$NO_2^- + H^+ + Fe^{II}Hb \rightarrow {}^{\bullet}NO + Fe^{III}Hb + OH^-$$

Nitrite can also be oxidized by oxyhemoglobin to produce nitrate in the following mechanism:

$$2[HbO_2]^{2+} + 2NO_2^- + 2H^+ \rightarrow 2Hb^{3+} + 2NO_3^- + H_2O_2$$

On one hand we have the RBC reducing nitrite to NO and stimulating NO signaling, on the other hand the RBC will scavenge NO and oxidize nitrite to nitrate leading to inhibition of NO signaling (see Figure 1-3). This balance is maintained in homeostasis, however during disease states this balance could be upset leading to an increase in inhibition of NO signaling by the RBC.

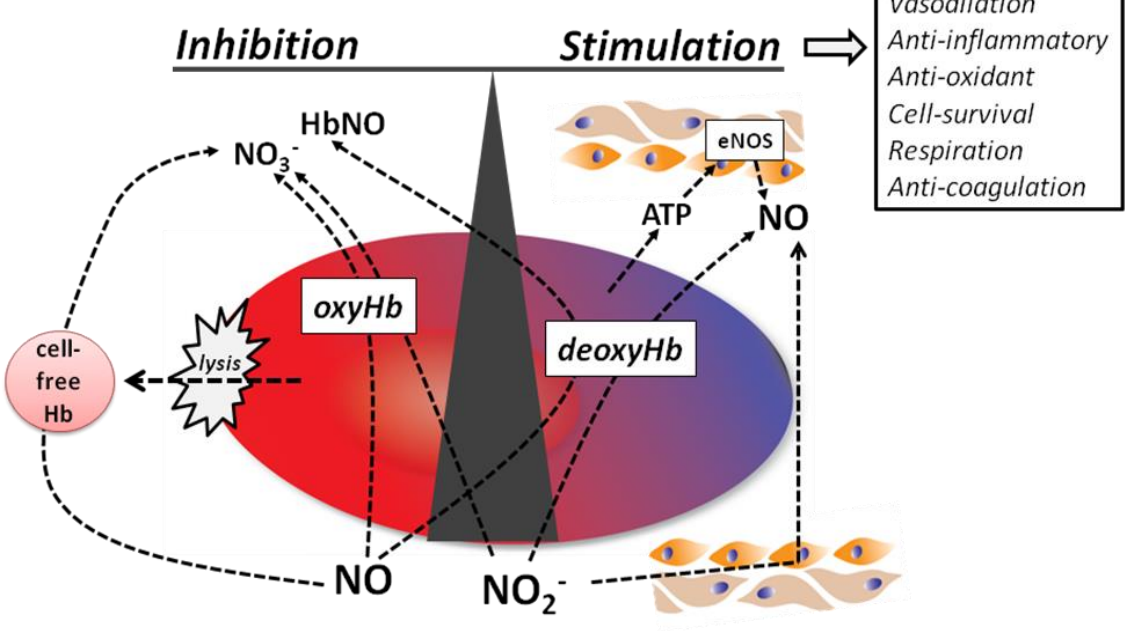

Figure 1-3 Current models for how RBC can inhibit and stimulate NO-signaling.
Shown is how hemoglobin oxygen sensing may be coupled to NO-scavenging (by intact or cell-free hemoglobin), or formation (from deoxyhemoglobin mediated nitrite-reduction or ATP release and subsequent eNOS activation) to control NO-bioactivity in the vasculature. Also shown is the concept that nitrite reduction to elicit NO-signaling during hypoxia may occur by tissues independent of RBC, but RBC can affect this process by controlling nitrite concentration via oxidation to nitrate.

Nitrite as a substrate for nitric oxide

An increase in NO signaling could right this balance and restore homeostasis. The idea to increase NO signaling through therapeutic means is not new and has been explored via different avenues. One of the first methods to restore NO is through inhaled NO. Currently, inhaled NO is approved by the US Food and Drug Administration as a treatment for newborns with hypoxic respiratory failure. It is also being tested as a treatment in a variety of conditions including perioperative pulmonary hypertension, cardiac transplantation, acute respiratory distress syndrome, and ischemia-reperfusion injury [10]. In addition to inhaled NO, nitrite has also been suggested as therapeutic means to increase NO signaling. As mentioned before, nitrite can be reduced to NO and has been shown to be a reservoir for NO in ischemia [72, 88, 89]. Studies have shown nitrite levels to be depleted after ischemia injury [15] and the addition of therapeutic nitrite protects against ischemic injury. Several other studies have demonstrated that nitrite therapy can protect against a variety of conditions including hypertension, hemorrhagic stroke, and inflammatory injury in ischemic tissues [85, 90, 91]. Our lab has used nitrite as a therapeutic agent in decreasing acute lung injury (ALI) of chlorine exposed rats [16] and attenuating hypertension in a trauma/hemorrhage model resuscitated with HBOC-201, a blood substitute consisting of purified cell-free, gluteraldehyde cross-linked and polymerized bovine hemoglobin [92].

Transfusion Toxicity Therapies

There is a loss of NO signaling that occurs with transfusion of stored RBC. In this thesis, I will test to approaches to i) avoid loss of NO by washing stored RBC

immediately prior to transfusion to remove low molecular weight components that scavenge NO and ii) administer nitrite to replete endogenous NO-signaling. With respect to washing, this process will remove RBC degradation products including cell-free hemoglobin and microparticles, leaving only intact RBC for transfusion. Washing of the RBC is used in limited circumstances but has shown to be effective in decreasing inflammation in cardiac surgery patients [93] and in animal models of transfusion [53, 55]. Another therapy is to increase NO bioavailability by administration of nitrite as outlined above.

Animal Models of the Storage Lesion

Key tools in understanding the storage lesion toxicity are animal models that recapitulate toxicities observed after transfusion with stored RBC. Several animal models for stored RBC toxicity have been developed recently using rat, mouse, and dog [30, 81-84], and supporting the two hit model of transfusion injury. The 'first hit' may vary and include hemorrhage, lipopolysaccharide and pneumonia infection. In our studies we use a murine model of trauma/hemorrhage as a first hit because trauma patients reeive significant amounts of stored RBC and, retrospective analysis have shown storage lesion toxicity in this patient population[75, 94, 95]. Trauma is described as serious and body altering physical injury. Due to its nature, trauma is often associated with loss of blood and these patients therefore are primary recipients of transfusions, accounting for 10-15 % of RBC transfusions in the US [18]. To assess transfusion injury, out focus will be acute lung injury (ALI) which is one of the co-morbidities that increase with stored RBC transfusion compared younger RBC [96-99].It needs to be specified here that the

phenomenon we are concerned with is transfusion related injury and not transfusion related acute lung injury (TRALI). The factors that are associated with TRALI are debated but TRALI is characterized by increased pulmonary edema, neutrophil accumulation, and alveolar injury which combines to cause respiratory stress and hypoxemia that occurs within 6 hours of transfusion. It is indistinguishable from ALI except for the incidence of transfusion [100] Originally TRALI was thought to be caused by immune responses of MHCI and MHCII complexes and is independent of the age of the RBC that are transfused. Recently the storage lesion has been linked to TRALI in a pathway independent of MHCI and MHCII. Whether these two pathway are linked is a subject of current research [101]. Our model focuses on the storage lesion and not the immune responses of MHCI and MHCII and should not be confused with TRALI.

Introduction Summary

There have been many studies investigating the storage lesion and how the storage lesion could lead to injury upon transfusion. It is known that the RBC changes morphologically and biochemically, however little attention has been given to how these changes could affect NO scavenging. Additionally, it has recently been discovered that heme increases inflammation by activation of the TLR4 receptor. In this thesis we investigate how these two components could contribute to transfusion toxicity. We also show new strategies to improve transfusion by washing RBC and nitrite therapy.

CHAPTER 2

ERYTHROCYTE STORAGE INCREASES RATES OF NO AND NITRITE SCAVENGING: IMPLICATIONS FOR TRANSFUSION-RELATED TOXICITY

Introduction

Previous studies have demonstrated the association of *in vivo* toxicity with transfusion of older packed red blood cells (pRBC) despite leucoreduction [102] , suggesting that mechanisms intrinsic to the RBC may also play a role in storage-lesion-dependent pathology. In this context, an emerging and unifying hypothesis to explain compromised tissue perfusion and exacerbation in inflammatory responses is that aged pRBC cause a loss in NO signaling. NO plays important roles in vascular homoeostasis with a decrease in its bioavailability leading to hypertension, coagulation and inflammation. Specific proposed mechanisms for decreased NO signaling with transfusion of older pRBC include a storage-dependent loss of RBC-dependent stimulation of NO signaling [via loss of S-nitrosoHb (hemoglobin) or ATP release] [43, 103, 104] and/or increased rates of NO scavenging by Hb in RBC-derived microparticles or after hemolysis. This is underscored by the similarity between the RBC storage-lesion toxicities with the pathogenic effects of acellular Hb-based oxygen carriers.

The NO scavenging reactions of Hb discussed above refer to oxy-ferrous heme-dependent oxidation of NO to nitrate and deoxyferrous heme binding of NO to form nitrosylHb (Hb-Fe2+-NO). With cell-free Hb, both reactions occur with rate constants between 3 and 8×10^{7} $M^{-1}\cdot s^{-1}$ [105-107] With erythrocytic, and hence encapsulated, Hb,

the rate constant for NO scavenging is decreased by ~500–1000-fold, a property proposed to be key in allowing endothelial-derived NO to regulate signaling processes in the vasculature [78, 79, 108-110]. The exact mechanism for the decreased NO scavenging rate of RBC Hb compared with cell-free Hb remains debated and involves diffusion barriers created by an unstirred layer immediately adjacent to the RBC and/or membrane-based structures, which slow down NO reactions. Interestingly, these diffusion barriers are regulated by RBC size, shape and surface area [108] biophysical properties that are known to change during RBC storage. However, the effects of these changes on reactions with NO have not been considered previously and are tested in the present study.

In addition, we also tested if reactions between RBC and nitrite were altered during storage. Nitrite reactions with Hb are regulated by the Hb fractional saturation such that under oxygenated conditions, nitrite is oxidized to nitrate, but when deoxygenated, nitrite reduction to NO can occur to mediate hypoxic NO signaling [88, 89, 111, 112]. The balance between nitrite oxidation compared with nitrite reduction is important in regulating NO signaling [113, 114] and could play a role in affecting inflammatory tissue injury, since lower nitrite levels predispose to, whereas nitrite supplementation protects against, ischaemic and inflammation-dependent tissue injury [115]. Since stored RBC have decreased p50 (oxygen partial pressure producing 50% saturation.) values and altered membrane properties which could affect nitrite transport, we reasoned that altered nitrite metabolism and NO formation by stored RBC could also play a role in transfusion-related toxicities.

Experimental Procedures

Materials

All materials were purchased from Sigma–Aldrich (St Louis, MO) except MahmaNONOate and L-NMMA, which were obtained from Axxora Platform (San Diego, CA). Male Sprague–Dawley rats (200–250 g) were purchased from Harlan (Indianapolis, IN). All animal studies were performed following Institutional Animal Care and Use approved procedures.

Light microscopy of RBC

RBC (5µl of packed cells) were smeared onto a glass slide and fixed using methanol and then stained with Wrights stain. Slides were imaged at 40X magnification using a Leica DM600 microscope (Leica Microsystems, Bannockburn, IL). Images were adjusted to control for contrast.

Ex Vivo Aorta Vasodilation

Thoracic aortas were isolated from male Sprague-Dawley rats (200-250g) and divided into approximately eight 5-mm wide sections. Rings were suspended between two hooks connected to a force transducer and placed within a vessel bath chamber containing Krebs-Henseleit (KH) buffer as described previously in [113]. After two rounds of KCl-induced contractions followed by washing and 30min equilibration, vessels were equilibrated with 21% or 1% oxygen in KH buffer at 37°C in the presence of 5% CO_2. Vessels were pretreated with indomethacin (5 µM) and N-monomethyl-L-arginine (L-NMMA; 100µM) and precontracted with phenylephrine (200nM at 21%

oxygen tension and 400nM at 1% oxygen tension) before the addition of RBC (0.3% hematocrit final concentration). Once vessels had reached a stable tone, vasorelaxation was elicited by the addition of either sodium nitrite (3 and 10 μM) or the NO donor Mahma-NONOate (10nM and 30nM). A dose-dependent protocol was used to limit the time of experiment to <10min post RBC addition; preliminary studies determined that longer durations resulted in significant RBC hemolysis which would preclude assessment of RBC-dependent effects on NO- or nitrite dependent vasodilation. Vasorelaxation was determined in the absence and presence of RBC, and the percent inhibition of nitrite- or NO-dependent vasodilation by the RBC calculated using stable tensions at the end of vasodilation. Also, at the end of each experiment, the concentration of cell free and RBC heme in the vessel bioassay chamber was measured.

Heme concentration measurement

Hemglobin was measured by deconvolution of visible spectra for oxyhemoglobin, deoxyhemoglobin and methemoglobin as previously described [113] or by Drabkins assay when sample size was limited or below the limit of detection of deconvolution.

Fractional saturation calculation

RBC were lysed in deionized water at a ratio of 1:5 and then diluted to 20μM heme in PBS. This resultant suspension maintained the ratio of hemoglobin to allosteric effectors whilst allowing measurement of visible spectra (450-700nm) without scattering associated with intact RBC. 3ml of hemolysate was deoxygenated in tonometer and equilibrated at 34°C. Wavelength scans were taken after the sequential addition of 1ml of

air until complete oxygenation of the hemoglobin was achieved. OxyHb and deoxyHb concentrations were measured by spectral deconvolution as described [113] and deoxyhemoglobin vs. pO_2 plotted to calculate P_{50}. P_{50} values for hemolysates collected from freshly isolated (day 0) RBC were 28.9 ± 1 mmHg (mean ± SEM, n=4), a value which falls within the range of P_{50} reported for intact freshly isolated RBC at pH7.4, 37°C (27-30mmHg). Validation of the approach used is further provided by the magnitude of storage age dependent decrease in P_{50} being similar to recent studies in which oxygen binding affinity of stored RBC was determined by a Hemox analyzer[116].

Kinetic analysis of NO scavenging by RBC

The rate of NO-dioxygenation induced by RBC was determined using competition kinetics as previously described by Vaughn and coworkers [117] with slight modifications. Three experimental conditions were used, PBS + 0.5%BSA containing either, oxyHb, oxyHb with spermine NONOate (SpNO, an NO donor), or a suspension of RBC plus oxyHb and SpNO. Final concentrations were 7μM for oxyHb, 7%Hct for RBC (RBC were added after washing (3 times at 1500 x g, 10min) to remove any hemolysis derived products that may have accumulated during storage). Experiments were started by addition of SpNO (10μM, preliminary studies established these conditions to result in linear rates of oxyHb oxidation over 60min, data not shown). Samples were placed in six-well tissue culture plates at room temperature and rocked gently on a rocking platform. Prior to the addition of SpNO samples were taken to assess free hemoglobin concentrations. After addition of SpNO, 0.5mL samples were taken every 10min for 60min, immediately centrifuged (20 s at 2,000 ×g) to separate the RBC, and the

supernatant collected and concentration of oxyHb and metHb determined by visible spectroscopy. The relative rate of cell-free oxyHb oxidation to metHb by SpNO, in the presence or absence of RBC allows determination of kinetics of RBC-dependent NO-dioxygenation reactions. In separate experiments evaluating the effects of butylated hydroxytoluene (BHT, 100μM), superoxide dismutase (SOD) or SOD + catalase (both 100 U/ml),reagents were added and incubated for 5 minutes prior to addition of SpNO.

Relative rate constants for RBC (k_{RBC}) vs. cell-free oxyHb (k_{Hb}) were calculated as described [117] using the following equation 3:

$[metHb]_c - (1\text{-}Hct)[MetHb]_{ex} / [oxyHb]_{RBC} = (K_{RBC}/K_{Hb}) \ln([totalHb]_{ex} / [oxyHb]_{ex})$ Equation 3

where $[metHb]_c$ is the concentration of cell-free metHb in the preparation containing cell-free oxyHb and SpNO (no RBC), Hct is the RBC hematocrit, $[oxyHb]_{RBC}$ is the concentration of total Hb in RBC, k_{RBC} and k_{Hb} are the rate constants for RBC and Hb-dependent NO-dioxygenation reactions respectively, $[totalHb]_{ex}$ and $[oxyHb]_{ex}$ are the total cell free and oxyHb concentrations respectively in preparations containing RBC. At each time point sampled, $[metHb]_c - (1\text{-}Hct)[MetHb]ex / [oxyHb]_{RBC}$ the term (y-axis) was plotted against ln([totalHb]ex / [oxyHb]ex) term (x-axis) and k_{RBC}/k_{Hb} determined by the gradient. Initial studies indicated that significant hemolysis occurred during the experiment when assessing RBC stored for more than 14d. The protocol was modified therefore to collect samples every 3 min for 12 min, a time period over which the above described plot remained linear and cell-free hemoglobin concentration changed by <6%. An increase in cell-free hemoglobin concentration beyond 6% would also not allow a linear plot of the previously mentioned values

Microparticle measurement

Stored RBC were left unwashed (60%Hct) or washed (3 times) and brought to 60% Hct in PBS + 0.1% BSA. RBC were then incubated with anti-glycophorin A-FITC conjugated antibody (0.17µg/ml) for 30min in the dark at room temperature [70]. Samples were then analyzed by flow cytometry using a Becton Dickinson FACSCalibur (BD Biosciences, Franklin Lakes, NJ) and events acquired using CellQuest software. Approximately 100,000 events were collected per measurement. All analyses were done with FlowJo software (Tree Star, Inc., Ashland, OR).

Nitrite Consumption by RBC

Nitrite consumption by RBC (washed to remove microparticles and cell-free heme) was determined as described [118] at different oxygen tensions in a controlled-atmosphere chamber (Plas Labs, Lansing, MI) using atmospheric gas combined with nitrogen gas to produce 21% or 2% oxygen tensions. RBC suspensions at 5% Hct in Tris-buffer containing 0.1%BSA, pH 7.4 were equilibrated for 30min in six-well tissue culture plates with gentle rocking. To initiate experiments, nitrite (100µM) was added (nitrite stock solutions were prepared in deoxygenated PBS) and aliquots removed at 0, 5 and 15 minutes. Aliquots were taken out of the chamber and immediately centrifuged at 2,000 g for 30 s. The extracellular fraction (supernatant) was collected and vortex mixed with equal volumes of methanol then frozen in liquid nitrogen. In all experiments, parallel incubations of nitrite alone in Tris-BSA buffer were included. Nitrite consumption was determined as the difference between its concentration in the samples with and without RBC.

Trauma Patient studies

Patients admitted in to the trauma intensive care unit at UAB with orders to receive 1 packed red blood cell (pRBC) unit transfusion were enrolled into the study. Study protocol, patient enrollment, exclusion criteria, and demographics were recently described.[119] Approval for this study was granted by the University of Alabama at Birmingham Institutional Review Board. Blood samples (400µl) were collected immediately before RBC transfusion and 1h after the completion of transfusion and immediately processed for whole blood nitrite measurements by methanol extraction as previously described[120] . Methanolic extracts were stored (-80°C) prior to nitrite measurement.

Nitrite and Nitrate measurements

Methanolic extracts were thawed on ice in the dark and then centrifuged (15,000 x g, 5 min, 4 °C). The supernatant volume was measured and nitrite and nitrate concentrations determined using triodide based chemiluminesence as previously described [120] on a Sievers NOanalyzer and comparison to respective standard curve.

Statistical Analysis

Storage time-dependent changes were analyzed by 1-way repeated measures ANOVA with Tukey's post-test or by 2-way ANOVA when also assessing effects of pO_2. Changes in circulating nitrite levels in trauma patients before and after RBC transfusion were analyzed by unpaired Student's t-test. P-values less than 0.05 were

considered significant. All analyses used GraphPad Prism Software (San Diego, CA, USA).

Results

Validation of storage-dependent changes in RBC

RBC segments of pRBC of different storage ages were collected from the UAB blood bank or RBC isolated from healthy volunteers and leukodepleted to standard blood banking conditions at our institution were used. Figure 2-1 shows blood bags stored at UAB blood bank. The RBC segments are tubing that are seen at the top of each blood bag. These segments contain the same solution of RBC as found in the blood bag but are sealed off from the blood bag to allow easy removal for testing of RBC by blood bank technicians.

2-1 Blood bags and associated segments

Shown is an example of blood bags stored at UAB blood bank. Attached to the top of each blood bag are tubing referred to as segments. Segments contain small amount of stored RBC that are sealed away from the blood bag.

After leukodepletion by filter blood was centrifuged in either 15 mL or 50mL BD falcon conical tubes to isolate RBC. RBC were then suspended in Adsol 1 storage media at 60% Hct and stored in 50mL BD falcon conical tubes. Hemoglobin concentrations in the supernatant were measured over time from either preparation. (Figure 2-2A). A significant increase in cell free hemoglobin was seen in RBC centrifuged in 50mL tubes as compared to RBC centrifuged in 15mL tubes. The hemoglobin concentrations during storage seen in RBC centrifuged in 15mL tubes is consistent with previous studies [121] and this method was used to prepare RBC for all data shown afterwards. The increase in

hemolysis seen between different preparation protocols was surprising and is addressed in the discussion section. RBC P_{50} was also shown to decrease (Figure 2-2B) consistent with previous studies [122].

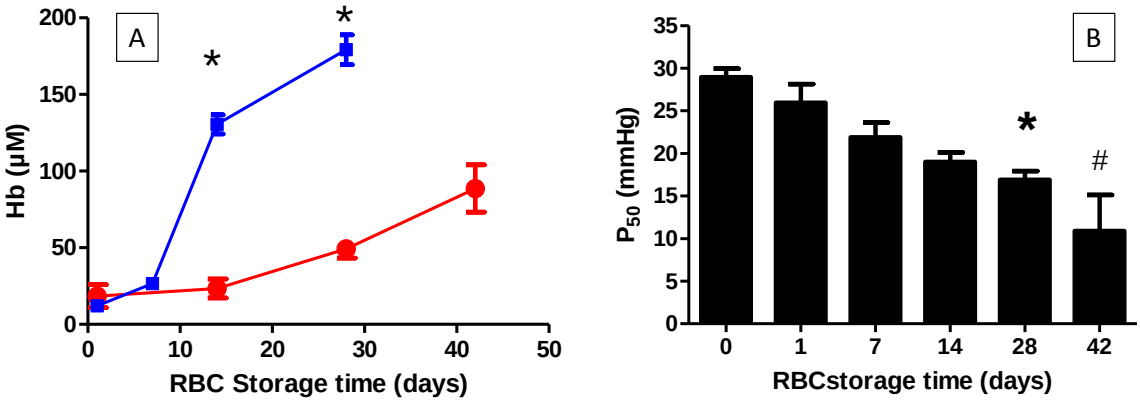

Figure 2-2 RBC storage increases oxygen affinity.
Panel A: Blood was collect from volunteers and leukodepleted by filtration. After filtration blood was centrifuged for 10 minutes at 1500g in either 15 mL(red) or 50mL (blue) BD falcon conical tubes. After centrifugation, plasma is removed and packed RBC are brought to a 60% Hemaocrit using Adsol-1 storage media. RBC are then stored at 4° Celsius in 50mL BD falcon conical tubes. At 1, 7, 14, 28 and 42 days aliquots of stored blood are assessed for hemolysis by measuring hemoglobin concentration in supernatant. Data show mean ± SEM (n=3) *P < 0.05 vs 50mL conical tubes. Panel B: RBC were collected from healthy volunteers, leukodepleted, stored for the indicated times and P_{50} values assessed. Data show mean ± SEM (n=3-4) *P < 0.05 vs day 0; $^{\#}$P < 0.05 vs days 0, 1, 7 by 1-way ANOVA with Tukey's post test.

RBC storage increases NO scavenging and inhibits NO-dependent vasodilation.

The rate constant for NO-reactions with oxyhemoglobin in RBC can be determined using a competition assay, in which the NO-donor SpNO is added to a mixture of cell-free oxyhemoglobin and RBC, and then following the time dependent formation of cell-free methemoglobin. At non-limiting SpNO concentrations, an increase in the rate of RBC-hemoglobin reactions with NO is reflected as a decrease in the rate of cell-free methemoglobin formation. Figure 2-3A shows representative kinetic traces and

demonstrates that RBC-dependent inhibition of cell-free methemoglobin formation increases with the length of RBC storage. The transition from linear to curved kinetic traces for metHb formation in the presence of RBC is similar to previous studies and reflects the competition between RBC and cell-free hemoglobin for reaction with NO. Figure 2-3 B plots the calculated ratio of rate constants for NO-dioxygenation reaction between erythrocytic and cell-free hemoglobin (k_{RBC} / k_{Hb}) and demonstrates that RBC-dependent NO-scavenging increases ~40-fold over 42 days of storage. Significance by 1-way ANOVA was observed with 42d old RBC relative to all other ages. A trend towards increasing NO-scavenging rates is also noted by 14-28d which was not significant due to variance within any given RBC age especially from day 14 and onwards (likely reflecting donor to donor differences). Exclusion of outlier data (n=1 each from 14d and 28d data set) detected by Grubbs' test resulted in an observation of a significant increase in NO-scavenging kinetics between both day 14 and 28 RBC relative to day 0 ($P < 0.05$ by t-test, not shown). Addition of SOD, SOD + catalase or BHT, had no effect on NO-dioxygenation rates by 42d old RBC (not shown) suggesting that increased superoxide or lipid alkoxyl / peroxyl radicals are not responsible for accelerated NO-consumption.

NO-dependent dilation of isolated rat thoracic aorta was used to test if storage dependent increased NO scavenging by RBC translates to a greater degree of inhibition of NO-signaling. Aortas were pre-constricted with PE and L-NMMA, the latter to inhibit endogenous NO-formation from endothelial nitric oxide synthase. RBC of different ages were then added, and followed by addition of MNO (10nM and 30nM).

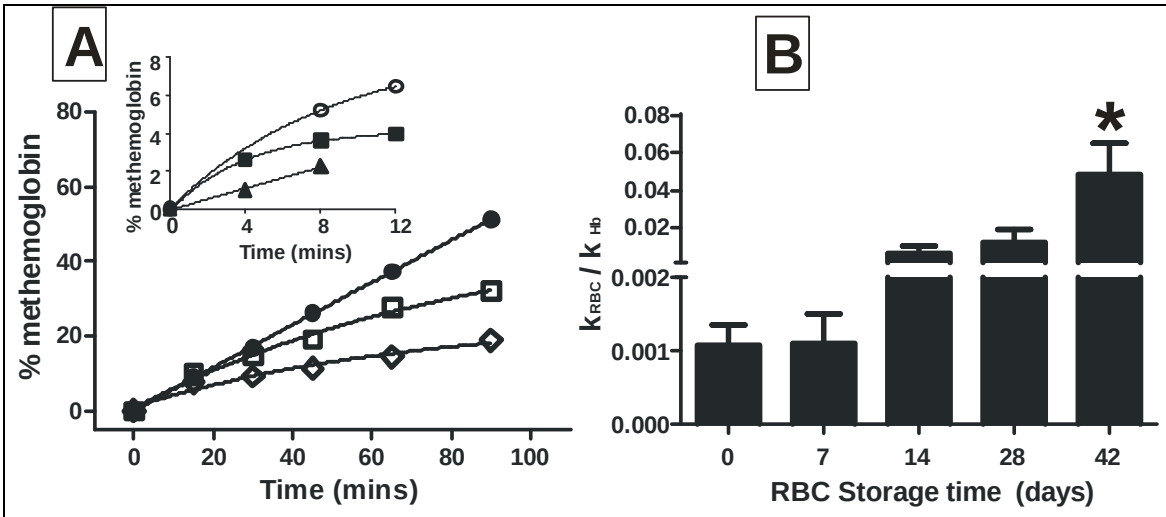

Figure 2-3 Effects of RBC storage time on NO-scavenging kinetics
Panel A shows representative traces for SperNO dependent formation of cell-free metHb from oxyHb (7μM) in the presence and absence of RBC (7% Hct) of different storage ages (●, cell free oxyhemoglobin alone; □, day 0 RBC, ◊, day 7 RBC). Inset shows data with older RBC (○, day 14; ■, day 28; ▲, day 42) where kinetics were determined over shorter times due to hemolysis (as described in methods). Panel B shows calculated ratio of rate constants for NO-dioxygenation by RBC relative to cell-free Hb. RBC of different ages were collected from UAB blood bank. For day 0 samples, RBC were collected from healthy volunteers, leukodepleted and processed as described in methods. Experiments were performed in PBS, pH 7.4 at 20°C. Data represent mean ± SEM, n = 3-7. *P < 0.05 relative to day 0,7,14, 28 by 1-way ANOVA (P < 0.01) and Tukey's post test.

Figure 2-4 A shows representative vessel tension traces showing that MNO (30nM) stimulated ~40% dilation from when vessels reached stable tension, which was inhibited in the presence of day 0 and day 42 RBC; the latter having a greater inhibitory effect. Figure 2-4B shows the percent inhibition of MNO-dependent vasodilation by RBC as a function of storage time. 42d RBC inhibited MNO-dependent vasodilation at both 21% and 1% O_2 consistent with increased NO-scavenging kinetics. No significant effect of oxygen tension (P = 0.1, by 2-way ANOVA) was observed.

As shown in Figure 2-2B, RBC P_{50} decreases with storage time. Since the rate of NO-scavenging by oxyhemoglobin is slightly (~1.5-2-fold) faster compared to deoxyhemoglobin [100, 105, 106, 108] the increased inhibition of NO-dependent vasodilation observed with older RBC (~2-3 fold for 0d vs. 42d RBC), could reflect the presence of more oxy- vs. deoxyhemoglobin. This is unlikely however since the rate of NO-consumption by RBC is zero order with respect to hemoglobin[108]. Another possibility is that when deoxygenated, the RBC membrane permeability to NO is increased contributing in part, to differential NO-scavenging kinetics by RBC compared to cell-free hemoglobin.[105] Inhibition of MNO-dependent vasodilation was replotted as a function of oxygen fractional saturation of RBC in vessel bioassay chambers (Figure 2-4C). At 1% O_2, fractional saturation was higher for 42d vs. 0d RBC ($P<0.05$ by 1-way ANOVA with Tukey's post test) and inhibition of MNO-dependent vasodilation paralleled storage time (Figure 2-4B) suggesting that higher concentrations of oxyhemoglobin in older RBC mediate increased inhibition of MNO-dependent vasodilation. At 21% O_2 however, no difference in fractional saturation was observed ($P = 0.21$ by 1-way ANOVA) between RBC of different age. This suggests that increased inhibition of NO-scavenging, and increased rate of NO reactions are due to storage dependent changes in RBC morphology or biochemistry that are independent of oxygen affinity.

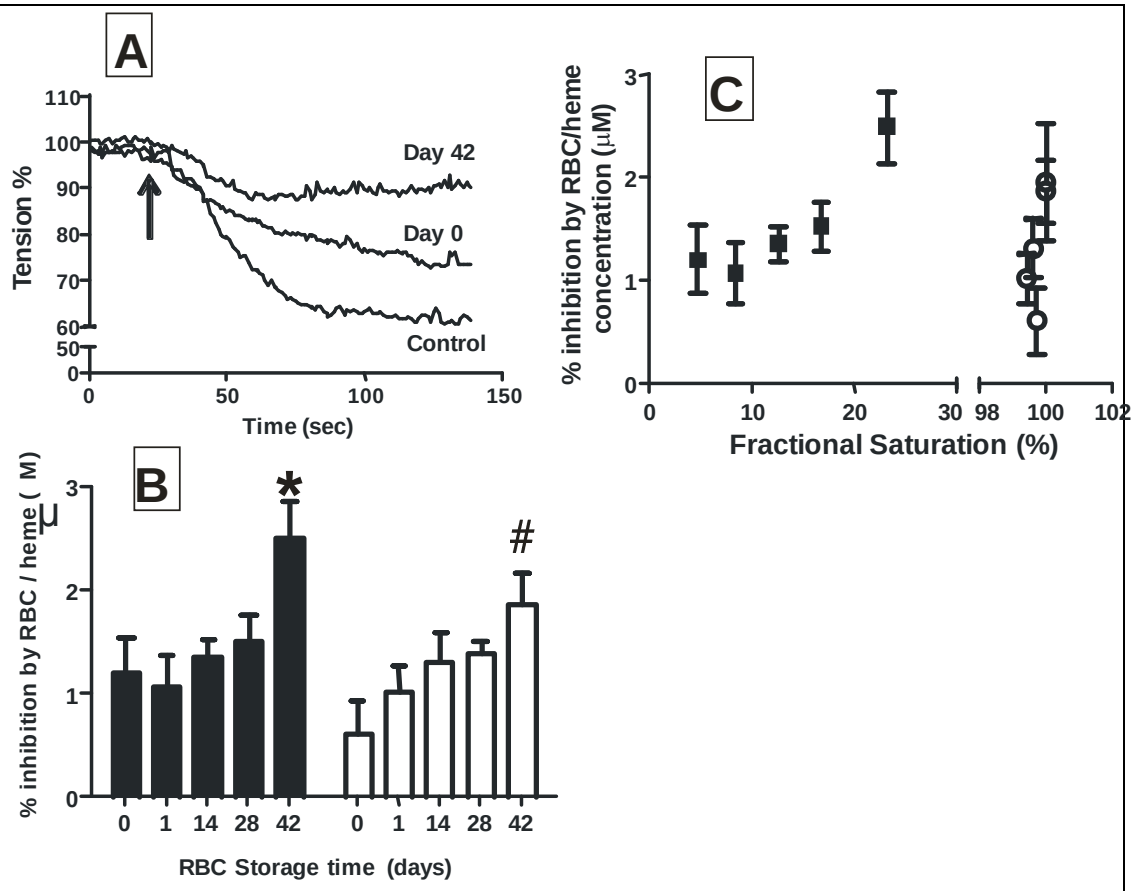

Figure 2-4 Effects of RBC storage time on NO-dependent vasodilation.
RBC (0.3%Hct) of different ages were added to vessel bioassay chambers followed by addition of MNO to rat aortic segments. Experiments were performed at 21%O_2 and 1% O_2. Panel A shows representative vessel tension vs. time traces (21%O_2). RBC were added at time 0 and MNO (30nM) addition indicated by arrow. Panel B shows percent inhibition of MNO-dependent vasodilation by RBC of different storage ages at 1% O_2 (■) and 21% O_2 (□). Data are normalized to the RBC heme concentration in the vessel bioassay chamber measured at the end of each experiment and are mean ± SEM (n=5-6). *P < 0.05 relative to day 0, 1, 14 and $^{\#}$P < 0.05 relative to day 0 by 1-way ANOVA with Tukey's post test. Panel C plots percent inhibition of MNO-dependent vasodilation by RBC as a function of calculated oxygen fractional saturation of RBC. ■, data collected at 21%O_2, ○, data collected at 1%O_2.

Stored RBC are more sensitive to hemolysis and produce more hemoglobin containing microparticles, both of which exhibit increased NO-scavenging kinetics.[70] Microparticle formation and hemolysis was measured before and after washing. Microparticles increased with storage age, but washing removed them to ≤ 0.5% microparticles which are determined as glycophorin A positive events (Figure 2-5 A-C). Moreover, washing also decreased cell-free hemoglobin to <0.5μM (not shown). Since hemolysis may occur during the time over which vessel relaxation is assessed, cell-free hemoglobin was measured in vessel bioassay chambers at the end of each experiment. No significant correlation between the concentration of cell-free heme and the extent of inhibition of MNO-dependent vasodilation was observed (Figure 2-5D). Collectively, these data suggest that using the described protocols, neither microparticles nor cell-free hemoglobin contributed to the enhanced NO-scavenging observed by stored RBC.

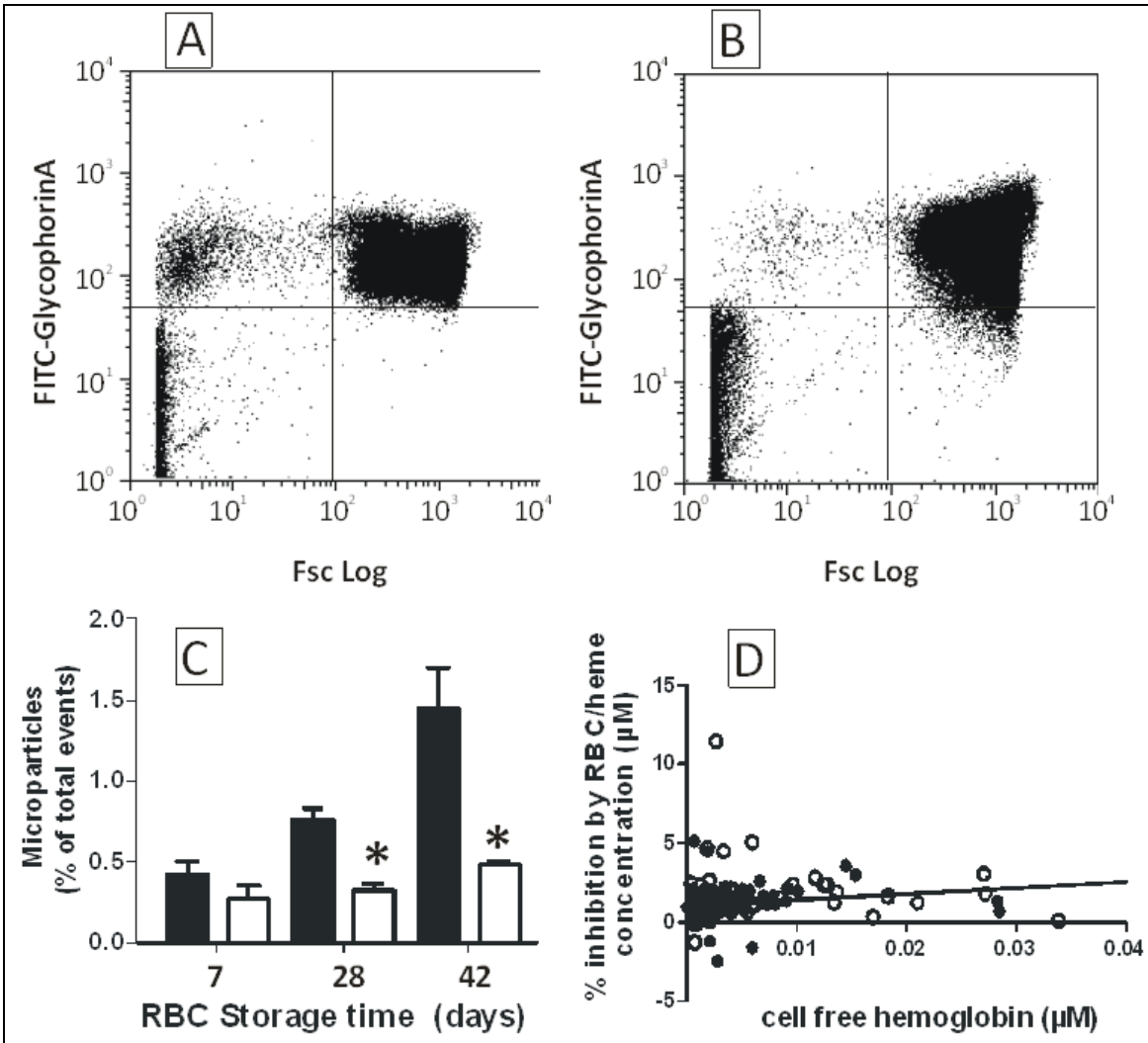

Figure 2-5: Testing a role for microparticles or cell-free hemoglobin in enhanced stored RBC dependent inhibition of NO-signaling.

Panel A and B show representative histograms for microparticle analysis in 42d RBC by FACS before and after washing respectively. Events in upper left quadrant represent microparticles and upper right quadrant intact RBC. Panel C shows changes in microparticle levels during storage before (■) and after (□) washing. Data are mean ± SEM (n=3). *P<0.02 relative to before washing by t-test. Panel D shows cell-free hemoglobin concentrations measured in vessel bioassay chambers plotted against the % inhibition of MNO-dependent vasodilation (●, data from 21% O_2 experiments, ○, data from 1%O_2 experiments). No significant correlation was observed by linear regression analysis (P = 0.49) shown by line.

Effects of RBC storage on nitrite metabolism

Nitrite was added to RBC of different storage ages pre-equilibrated at either 21% O_2 or 2% O_2. Figure 2-6 shows how RBC storage affects nitrite consumption and nitrate formation profiles. Key results from these experiments were i) consistent with our previous data [118], nitrite consumption was faster under deoxygenated conditions compared to oxygenated conditions with freshly isolated (day 0) RBC (Figure 2-6A), ii) Figure 2-6A-E show that as a function of storage time, the difference between low and high oxygen-dependent nitrite consumption kinetics increases up to 14d, and then decreases thereafter, with no oxygen-dependent difference evident with 42d old RBC. iii) comparison of how storage age affected nitrite consumption at each pO_2 showed that at low O_2, rates of nitrite consumption were higher with RBC stored for 14d or longer compared to 0d RBC.

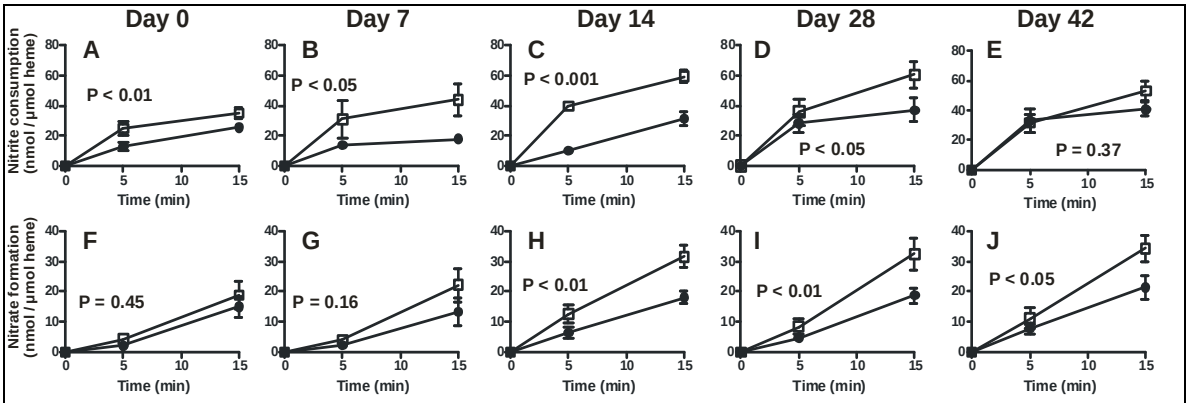

Figure 2-6 RBC storage age effects on nitrite metabolism.
Nitrite (100µM) was added to RBC (5%Hct) of different storage ages in PBS + 0.1% BSA pre-equilibrated at either 21% O_2 (●) or 2% O_2 (□). At 5 and 15 min after nitrite addition, RBC were pelleted and nitrite and nitrate levels measured in the extra-erythrocytic fractions. Data show nitrite consumption (Panels A-E) and nitrate formation (Panels F-J) normalized to heme. P-values indicated on each panel demonstrate effects of pO_2 on nitrite consumption or nitrate formation rates determined by 2-way ANOVA. Calculated oxygen fractional saturations for 2% O_2 condition for storage times 0, 7, 14, 28, 42 were respectively 0.27, 0.46, 0.56, 0.66, 0.69.

Similarly, significant increases in rates of nitrite consumption under oxygenated conditions were observed by stored RBC compared to d0 RBC (Figure 2-7), iv) Both low and high oxygen conditions resulted in similar rates of nitrate formation from nitrite with d0 RBC (Figure 2-6F). As a function of storage time however, an oxygen dependent effect became apparent with nitrate formation being greater at the lower relative to the higher oxygen tensions with RBC stored for 14d or longer (Figure 2-6F-J).

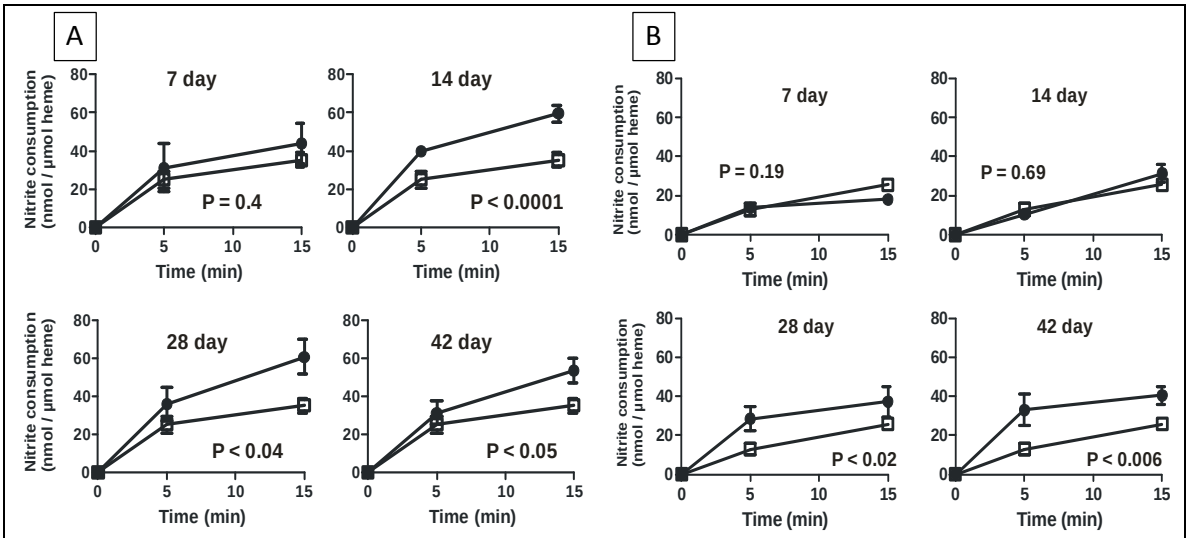

Figure 2-7 Storage age effects on nitrite consumption kinetics by RBC at 2% and 21% O_2

Panel A. At 2% O_2, nitrite (100µM) was added to RBC (5%Hct) of different storage ages in PBS + 0.1% BSA pre-equilibrated at 2% O_2. At 5 and 15 min after nitrite addition, RBC were pelleted and nitrite levels measured in the extra-erythrocytic fractions. Each panel shows effect of indicated RBC storage age (●) relative to day 0 RBC (☐). P-values indicated on each panel demonstrate effects of storage age on nitrite consumption determined by 2-way ANOVA. Calculated oxygen fractional saturations for 2% O_2 condition for storage times 0, 7, 14, 28, 42 were respectively 0.27, 0.46, 0.56, 0.66, 0.69. Panel B. At 21% O_2 nitrate was measure consumption was measured in the same manner as 2% O_2. Each panel shows effect of indicated RBC storage age (●) relative to day 0 RBC (☐). P-values indicated on each panel demonstrate effects of storage age on nitrite consumption determined by 2-way ANOVA.

Importantly nitrate formation increased as a function of storage time at both high and low oxygen conditions; Figure 2-7 B shows rates at 21% O_2.

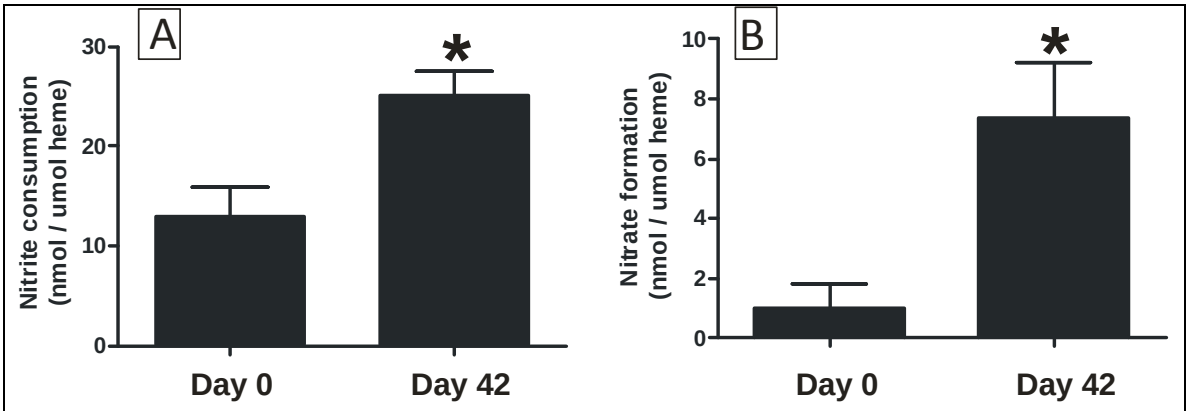

Figure 2-8 RBC storage increases nitrite consumption and nitrate formation under oxygenated conditions.
Nitrite was added to RBC at 21% O_2 as described in Figure 2-6 legend and nitrite consumption (panel A) and nitrate formation (panel B) measured at 5 min. Data show mean ± SEM (n=4-5). * $P < 0.03$ by t-test.

Effects of RBC storage on nitrite-dependent vasodilation

Nitrite dependent vasodilation was assessed in the presence and absence of RBC and at 21% and 1% O_2 Figure 2-8A-B show representative tension vs. time traces. Consistent with previous studies, nitrite alone is a more potent vasodilator at low oxygen tensions [114, 123, 124]. RBC inhibited nitrite dependent vasodilation when oxygenated, but promoted vasodilation when deoxygenated. Figure 2-8C shows how storage duration affected RBC-dependent inhibition or potentiation of nitrite-dependent vessel relaxation. Low oxygen tensions resulted in a relative potentiation of nitrite dependent vasodilation compared to high oxygen tensions consistent with a deoxygenation-dependent nitrite-reductase activity of RBC. This relative effect was not affected by RBC storage age.

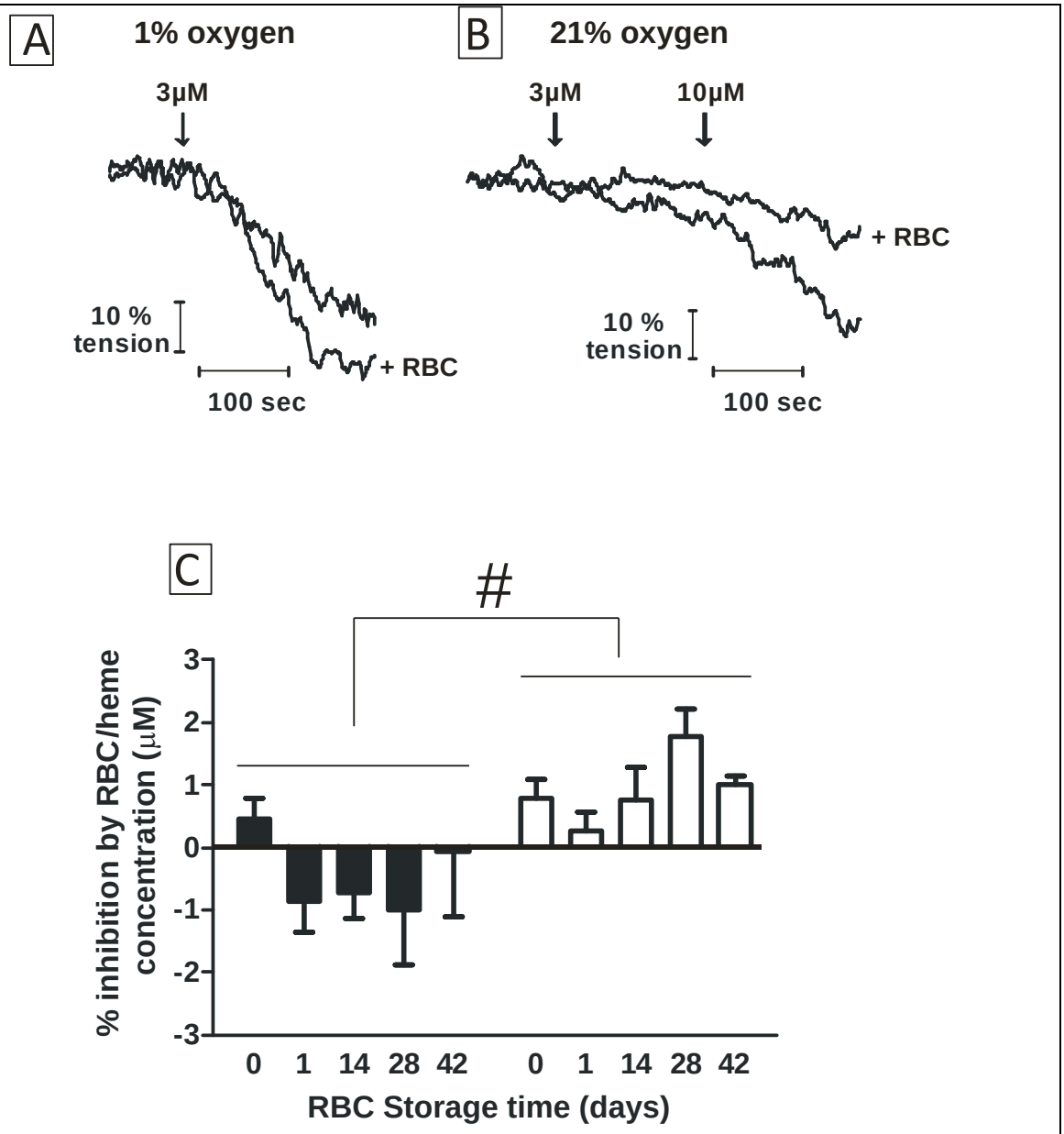

Figure 2-9 Effects of RBC storage on nitrite-dependent vasodilation of rat thoracic aorta.

Nitrite (3 and 10µM) was added to aortic baths containing Krebs buffer with or without RBC (0.3%Hct) of different storage ages and at 1% O_2 or 21% O_2. Panel A-B show representative vessel tension traces. Arrows indicate nitrite addition. Panel C shows percent change in vasodilation elicited by RBC relative to nitrite alone at 1% O_2 (■) and 21% O_2 (□) and storage age. Data are normalized to the concentration of RBC heme in each vessel bioassay chamber. A positive value denotes inhibition, and negative value indicates potentiation of nitrite-dependent vasodilation. Data are mean ± SEM (n = 3-6). $^{\#}P < 0.005$ by 2-way ANOVA for effects of oxygen.

Effects of RBC storage and transfusion on nitrite levels in trauma patients

Figure 2-10A shows that whole blood nitrite levels decreased significantly after transfusion of stable trauma patients with 1pRBC unit. Each unit transfused occurred over ~60-90min and resulted in ~60% decrease in blood nitrite levels. Moreover, the decrease in nitrite levels was greater when transfusion occurred with RBC stored for >25d compared to <25d (Figure 2-9B). Since the same volume (1unit, ~500ml) of RBC were transfused, these data also suggest that decreased nitrite levels are not due to resultant dilution of blood (~10%).

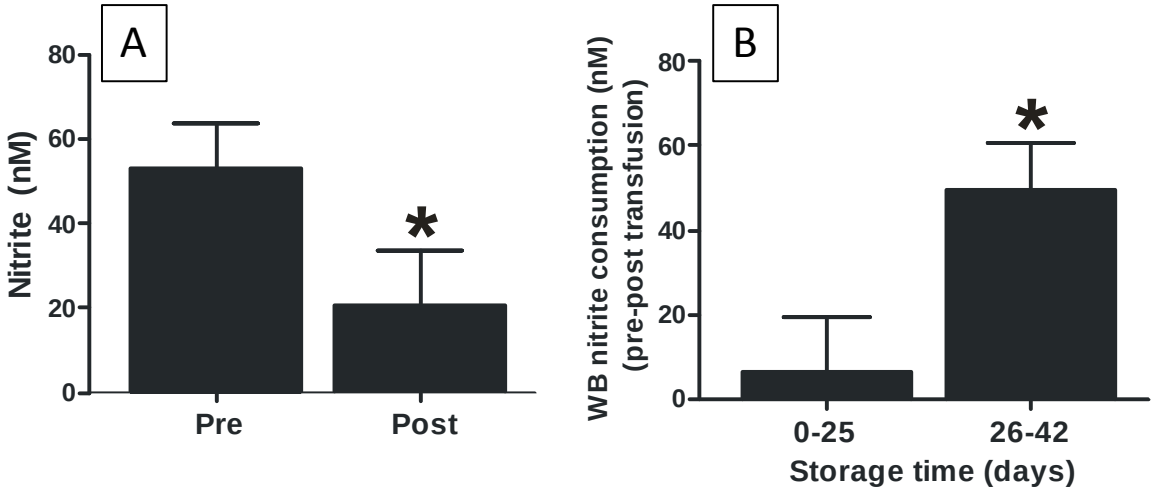

Figure 2-10 RBC transfusion decreases circulating nitrite levels in stable Trauma patients

Panel A: Whole blood nitrite was measured pre- and post-transfusion with 1 unit of RBC in stable trauma patients. Data show mean ± SEM (n =31), *P<0.01 by paired t-test. Panel B: Data were separated by the storage age of transfused RBC (0-25d, n = 14 or 26-42 d, n = 17) and changes in whole blood nitrite consumption (pre – post transfusion) plotted for these groups. *P<0.02 by unpaired t-test.

Discussion

When preparing blood for storage it was noted that hemolysis rate was affected by centrifugation container where the larger container caused an increase. What is occurring during centrifugation in 50mL volumes that causes an increase in hemolysis is unknown but could be from differences in centrifugal pressure resultant from different volumes during centrifugation. A recent study investigated the effects of centrifuge time on hemolysis rate [125]. In processing RBC prior to storage, blood is centrifuged to separate RBC and plasma. This centrifuge time can vary. This study showed that RBC centrifuged for a longer period of time had a small but significant increase in cell free hemoglobin levels at 5 weeks of storage. We speculate that centrifugal pressure could stress the RBC and result in more fragile RBC. These more fragile RBC would initially remain intact, but as storage time increased, these cells would lyse resulting in an increase in hemolysis. Though we have not fully investigated this issue, it did put us in the mindset that small alterations during collection could result in drastic changes to the RBC during storage.

Decreased NO-signaling has emerged as a central mechanism underlying the RBC storage lesion [75, 126-128]. This hypothesis is further supported by data presented herein, that show RBC-dependent scavenging of NO and nitrite is enhanced during storage. We propose that this contributes to an overall deficit in NO-bioavailability and predisposition to circulatory dysfunction and tissue inflammatory injury, two features emblematic of transfusion related toxicity [129-131].

Decreased RBC dependent stimulation of NO-signaling and increased NO-scavenging (secondary to hemolysis and increased microparticle formation) with stored

cells, have been reported to contribute to a deficit in NO-bioactivity [43, 70, 104, 132, 133]. How the storage time dependent changes in these processes translate to the clinical manifestation of storage lesion related toxicity is difficult to ascertain directly. Retrospective studies indicate adverse effects are observed with transfusion of RBC stored for >14d [31]. Consistent with this, ATP release and subsequent activation of eNOS is diminished with deficits observed with 7-14d old RBC and increased hemolysis and microparticle formation occurs in a similar storage time dependent manner with significant increases and effects on NO-scavenging evident after ~14-21d (Figures 2-2A and 2-5). Reported deficits in SNOHb bioactivity occur much faster and within a day of storage [43, 104] leading to suggestions that the RBC lesion occurs even earlier than expected or that there is no direct cause and effect relationship in this case [134]. How intact stored RBC-interactions with NO are affected by storage has received little attention. Data presented herein indicate that significant increases in NO-scavenging occur with RBC stored for 42d but with trends being observed by 14-28d. How intact RBC affect NO-homeostasis is an important consideration since i) biophysical and biochemical properties of the RBC are key in controlling NO-diffusion [73, 109, 117] and slowing NO-scavenging by encapsulated heme, and ii) RBC storage leads to smaller, less flexible cells with greater surface area to volume ratio. The latter is predicted to decrease NO-diffusion barriers and thus increase NO-scavenging rates (see below). To test this hypothesis, we utilized *in vitro* competition kinetic assays to determine the rate of NO-dioxygenation by RBC stored for different times. The k_{RBC}/ k_{Hb} increased ~40-fold over 42 days which was reflected by an enhanced inhibition of NO-dependent vasodilation. Importantly, although experimental conditions that allowed exclusion of

contributions from storage dependent hemolysis and microparticles were used, we note that while washing decreased microparticles to control levels, complete removal was not achieved, precluding a definitive exclusion of these species in contributing to the increased NO-dioxygenation kinetics or inhibited MNO-dependent vasodilation observed. Although the rate constant of RBC-dependent scavenging of NO increased during storage, this remained significantly less than cell-free hemoglobin (by ~20 fold, Figure 2-3B). However, in a unit of stored RBC with a 60% Hct, the concentration of erythrocytic hemoglobin is ~150-fold greater compared to cell-free hemoglobin (~12mM RBC heme vs. ~80μM for cell-free hemoglobin, see Figure 2-2A. Since the rate of NO-scavenging is the product of the rate constant and concentration, this suggests that intact stored RBC may effectively contribute to NO-scavenging. According to current blood banking guidelines, at least 75% of transfused RBC are expected to be present in the circulation 24h post-transfusion. This suggests that RBC with enhanced NO-scavenging properties may be persistent for many hours post transfusion. The potential role for intact RBC dependent effects on NO-function is further underscored by the fact that we used leukodepleted RBC, which may decrease (although not completely prevent) hemolysis and microparticle formation. We also note that at least in trauma patients, evidence for storage lesion dependent toxicity still remains despite the use of leukodepleted pRBC [102]. It is difficult to ascertain the relative contributions of erythrocytic vs. microparticle vs. cell-free hemoglobin towards inhibited NO-signaling since steady states of the above species are unlikely in a transfusion setting. Many factors will affect the concentrations of intact RBC, microparticles and cell-free hemoglobin, including RBC turnover, variable hematocrit, number of pRBC units transfused, and the balance between formation of

microparticles and hemolysis vs. clearance of these species (e.g. cell-free hemoglobin is cleared by haptoglobin and CD163-dependent pathways) [56, 135, 136]. The calculation presented above also does not account for differences in NO-scavenging that occurs due to the RBC-free zone. Irrespective of the relative contributions, we posit that intact RBC are important partners with microparticles and cell-free hemoglobin that collectively inhibit endogenous NO-dependent signaling. In addition, our data suggest that therapeutic strategies aimed at limited NO-scavenging should also target intact RBC-dependent NO-reactions.

How storage increases RBC dependent scavenging of NO is unclear. The effects of storage include changes RBC size, shape, membrane permeability and extracellular diffusion; all of which can regulate the kinetics of NO-scavenging [73, 78, 105, 108, 109, 117, 137]. Moreover, the rate limit may be controlled by distinct factors as exemplified by recent experimental and modeling studies that indicate the unstirred layer is the primary component regulating NO-scavenging by RBC, whereas membrane permeability is the key controlling factor for NO-reactions with microparticles [73]. Although it has been proposed that an additional factor is an intracellular diffusion barrier due to the very high hemoglobin concentration [110, 137], this has been challenged on experimental and theoretical grounds. In addition, the fact that NO consumption by RBC is zero order with intracellular hemoglobin concentration (which was actually the initial observation that prompted the concept of an extracellular diffusion barrier) is also inconsistent with this conclusion. The size of RBC decrease in certain storage conditions. The half-life of NO ($t_{1/2}$) in the presence of RBC has been modeled previously [108] according to equation 4:

$$t_{1/2}=(\ln 2)/[4N\pi(D_{NO}\, r)] \quad \text{(Equation 4)}$$

where N = number of cells/ml, D_{NO} = aqueous diffusion constant for NO, and r = cell radius. According to this equation, as the cell radius increases there will be proportional decrease in the NO half-life or an increase in RBC scavenging kinetics. This is consistent with the concept of an unstirred layer, where at non-limiting hemoglobin concentrations, the rate of NO-dioxygenation is proportional to the RBC surface area. During storage RBC change from a biconcave disc to echinocytes with spiculated membranes. Although the cells become smaller, these morphologic changes are likely to increase the total surface area (comparing like volumes of old RBC to young RBC), which would result in increased NO-consumption rates. Another consideration is that the internal cell volume of RBC decrease during storage. However, hemoglobin concentration does not change (not shown) indicating that during storage, hemoglobin packaging is altered. How this may affect the rate of NO-dioxygenation can be derived from equations 5-6.

$$V_i/V_T = N \times 4/3 \times \pi r^3 \qquad \text{(equation 5)}$$

$$\text{or} \qquad N = (V_i/V_T) / (4/3)\,\pi r^3 \qquad \text{(equation 6)}$$

where the ratio of internal volume of the cell (V_i) to total volume occupied by all cells (V_t) is equal to the number of cells / ml (N) multiplied by the volume of a sphere (where r is expressed in cm).

Substituting equation 4, the half-life of NO in the presence of RBC with different volumes is shown in equation 7:

$$t_{1/2} = (\ln 2) r^2 / [3(V_i/V_T)\, D_{NO}] \qquad \text{(equation 7)}$$

Thus, if we compare a suspension of RBC of equal total hemoglobin concentration but different sizes similar to our experimental protocol to assess reaction kinetics, the rate of NO consumption by the smaller cells will be greater in proportion to

the square of the ratio of the radii. Thus, RBC with smaller volumes provide another possible explanation for why stored RBC reacted faster with NO. A limitation of this modeling is that it assumes spherical RBC while storage induces a spectrum of cells with different shapes and sizes. Thus, it is likely that individual RBC possess heterogenous NO-scavenging potential in stored pRBC units.

We also investigated the effects of storage time on reactions between RBC and nitrite under oxygenated and deoxygenated conditions to test whether deoxyhemoglobin dependent nitrite-reduction and NO-formation is altered. Storage age had no effect on how RBC modulated nitrite-dependent relaxation. At high oxygen tension, RBC of all storage ages inhibited, whereas at low oxygen, no change or a potentiation of relaxation was observed reflecting the balance between NO-scavenging by oxyheme and nitrite-reduction to NO by deoxyheme. However, as shown in Figure 2-4, 42d old RBC inhibited NO-dependent vasodilation more than 0d RBC. Since nitrite-dependent vasodilation occurs via NO-formation we speculate that to observe no storage age effect on nitrite-dilation at low oxygen, increased rates of nitrite-reduction must be occurring to counter increased NO-scavenging. Indeed, increased rates of nitrite consumption were observed at low oxygen by RBC stored for greater than 14d. Another consideration in these experiments is that the initial rates of nitrite reduction follow a bell-shaped dependence with respect to hemoglobin oxygen fractional saturation, with maximal rates achieved close to the P_{50}. Due to decreasing RBC P_{50}, calculated oxygen fractional saturations for RBC in nitrite consumption studies (at 2% O_2) indicated that maximal rates were observed with 14d RBC which were also closest to the P_{50} (see Figure 2-6). These data underscore the fact that the precise RBC oxygen fractional saturation, a

product of local pO_2 and RBC oxygen affinity will be key determinants of how quickly nitrite may be reduced to NO.

RBC storage also increased rates of nitrate formation under oxygenated conditions (Figure 2-6F-J), which was paralleled by increased rates of nitrite consumption (Figure 2-7). A higher concentration of oxyhemoglobin (due to lower P_{50}) may also underlie increased nitrate formation kinetics observed with stored RBC at 2% oxygen. Oxyhemoglobin mediated nitrite oxidation to nitrate is an autocatalytic reaction that proceeds via intermediate formation of hydrogen peroxide and nitrogen dioxide radical. Therefore antioxidant enzymes or reductants that can scavenge these reactive species slow down nitrite oxidation. Oxidative damage concomitant with loss of antioxidants has been reported in RBC during storage. Thus the increase in nitrite oxidation to nitrate observed with stored RBC is likely the result of lower endogenous reductant systems. The lower blood nitrite levels in stable trauma patients transfused with an older vs. younger pRBC unit may reflect increased rates of nitrite consumption by deoxygenated and/or oxygenated RBC. However, since P_{50} decreases with RBC storage (implying greater oxyhemoglobin concentrations) we speculate that RBC-dependent oxidation of nitrite predominates over reduction pathways.

In summary, we show that in addition to increased NO-scavenging by hemolyzed cell-free hemoglobin and microparticles, changes to the RBC itself that occur during storage can lead to decreased NO-bioavailability. We posit that the combination of stored RBC dependent increased NO-scavenging and nitrite oxidation dispose tissues to inflammatory stress during and following transfusion and underscores the potential

therapeutic benefit for NO-repletion strategies with recent studies showing promise in this concept.

CHAPTER 3

TAK-242 (A TLR4 INHIBITOR), NITRITE THERAPY AND WASHING OF OLDER RED BLOOD CELLS ATTENUATE LUNG INJURY AND INCREASE SURVIVAL IN A MURINE MODEL OF TRANSFUSION INJURY

Introduction

Transfusion with packed red blood cells (pRBC) is the front line therapy for critically ill patients. However, recent studies have documented positive associations between the number of RBC units transfused, the age of the RBC unit being transfused, and increased transfusion related morbidities and mortality [138]. For example, our studies have shown increased incidents of acute lung injury, acute kidney injury, pneumonia infection and mortality in trauma-hemorrhage patients, a population that receives a significant portion of the stored blood in primary care-centers [29, 94, 139]. Importantly, storage lesion toxicity is observed with diverse patient populations suggesting common mechanisms related to gain of toxic functions for stored RBC. This understanding has fuelled numerous research efforts aimed at elucidating the mechanisms by which stored RBC may elicit injurious responses after transfusion [140]. Current thinking suggests these are related to microcirculatory dysfunction, exacerbation of underlying inflammation, increased oxidative stress and increased disposition to nosocomial infections [54, 131, 139, 141-144]. During storage several structural, biochemical and metabolic alterations occur to the RBC. Referred to as the RBC storage lesion, these changes include loss of metabolites (e.g. ATP), loss of RBC volume with

accompanying formation of echinocytic RBC and hemoglobin-containing microparticles, RBC degradation and release cell-free hemoglobin (hemolysis), iron and cellular debris [65]. While potential toxic effects of each of these aspects of RBC storage have been studied (see below), little attention has been given to the potential of heme released during storage as a mediator of transfusion injury. Notably, recent studies suggest that heme is a potent inducer of inflammatory tissue injury in sickle cell disease and sepsis [60, 145-147].

Loss of nitric oxide (NO) homeostasis has emerged as a key mechanism underlying many of features of transfusion toxicity associated with stored RBC including microcirculatory dysfunction and inflammation. Stored intact RBC which have altered morphologies and contain hemoglobin containing microparticles and hemolysis derived cell-free hemoglobin all result in significant increases in ferrous heme dependent NO-scavenging kinetics compared to freshly isolated RBC [121, 148, 149]. This biochemical property also translates to a greater inhibition of NO-dependent signaling ex vivo and in vivo [121,148, 150]. In addition to scavenging NO, older RBC may also be less effective at stimulating endogenous NO-formation. Stored RBC display faster rates of nitrite oxidation in vitro, and transfusion of trauma patients with older RBC, but not younger RBC, results in lower circulating nitrite levels [148]. Since nitrite is a putative substrate for NO-formation in hypoxic tissues, this reactivity would lower an endogenous substrate for NO-formation, thereby lead to inhibition of NO-signaling. Moreover, RBC–derived ATP-dependent activation of endothelial nitric oxide synthase is lost with stored RBC [103], and RBC-S-nitrosothiols have also been discussed as potential mechanisms for loss of NO-homeostasis [43, 104]. Collectively, these data provide mechanistic insights

into how transfusions with stored RBC inhibit endogenous NO-signaling in the vasculature and suggest that NO-repletion strategies, or approaches that remove components in stored RBC that inhibit NO-signaling will be beneficial. In the latter context, washing of RBC immediately before transfusion to remove microparticles, cell-free hemoglobin and iron, prevented many of the injurious effects of stored RBC in a canine model of transfusion and infectious lung injury [55] and cytokine levels in a mouse model of trauma-hemorrhage [53] . In the current study we show similar protective effects of RBC washing towards acute lung injury and lethality in a murine model of trauma-hemorrhage. In addition, we show NO-repletion therapy using nitrite protects against stored RBC toxicity. Finally we provide evidence that heme released during storage is a key effector of injury in this model suggesting that heme toxicity is an additional and important consideration on storage lesion toxicity mechanisms.

Experimental Procedures

Animal Model

Male C57BL/6 mice weighing 22g to 30g were purchased from Harlan Laboratories (Indianapolis, Indiana) aged 8-10 weeks. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

Trauma/Hemorrhage

C57Bl/6 male mice were anaesthetized by inhalation of 5% isofluorane in air. The concentration of isofluorane was then reduced to the minimal concentration for

maintenance (<1%). The abdomen and groin were shaved and washed with 10% povidone-iodine. A 2 cm midline laparotomy was performed to induce soft-tissue trauma. The incision was closed in two layers (fascia/muscle and skin) and bathed in 1% lidocaine for analgesia. Both femoral arteries were cannulated with catheters (Braintree Scientific). Systemic arterial pressure was continuously monitored through one arterial line while hemorrhage and resuscitation was performed via the other. Mice were bled over 30 min to a mean arterial pressure (MAP) of 25±5 mmHg. This blood pressure was maintained for a further 60 min by additional bleeding as required. At the end of the hemorrhagic shock period, if animals were receiving nitrite treatment, 100µl of nitrite dissolved in saline was administered intravenously in a bolus form (nitrite stock solutions were 0.1 mM, or 1 mM) prior to transfusion. All animals were resuscitated over 30min with either 100µL or 300µL of RBC and saline at 4x volume shed during hemorrhage. All experiments were carried out 4 hours post-resuscitation whereupon the mouse was sacrificed.

Human or mouse RBC collection

Human RBC stored for up to 42d were collected from segments attached to blood bags from the UAB blood bank, and processed according to UAB Institutional Review Board approved protocols. All human RBC used in this study were leukoreduced and stored in Adsol-1. At the time of collection, RBC were collected by centrifugation (1500 x g, 10min, 4°C). Indices of storage related changes (described below) were determined on the resultant supernatant fraction. For mouse RBC, blood (~800µL) was collected from healthy male C57BL/6 mice via cardiac puncture in 50µl citrate buffer (trisodium

citrate (22.0g/L), citric acid (8.0g/L), dextrose (24.5g/L)). Blood was filtered through neonatal Sepacell filters or through Sephadex G25 microcellulose columns to remove leukocytes by gel filtration. Columns were washed with 10 times the volume of PBS or Adsol-1 with no differences in hemolysis during processing observed (not shown). The eluent was centrifuged at 1500 x g for 5 min, 4°C. The erythrocyte pellet was washed 3 times with cold Adsol-1 and concentrated to a hematocrit of 60% with Adsol-1 and stored in 0.7mL Eppendorf tubes with a head space of approximately 300µL. All solutions and procedures were performed under sterile conditions. RBC were stored at 4° C in the dark, for up to 10 days. LPS measurement in stored RBC using the Limulus Amebocyte lysate assay (Cambrex) indicated levels were below detection limit (not shown).

Washing RBC

RBC were washed when specified in the following manner. RBC were centrifuged at 1500g for 10 minutes. The supernatant was then removed and the RBC pellet resuspended in Adsol-1 at a volume of approximately 7 times the volume of packed RBC. The sample is centrifuged again at 1500g for 10 minutes, then the supernatant is removed and Adsol-1 is added as previously mentioned. This process is repeated 3 times. If washed RBC were used in transfusion, washing occurred 1 hour prior to transfusion and were kept on ice until transfusion occurred.

Loss of RBC during washing

The volume of RBC lost during washing was calculated using the Hb concentration in the RBC and in the supernatant. By measuring the Hb concentration and

volume of the supernatant prior to washing and during each wash, we can calculate the total moles of Hb removed in the washing process. Assuming that all Hb removed comes directly from the hemolysis of the RBC, we can use the Hb concentration of the RBC to calculate the volume of cells lost during hemolysis and the washing process. Washing at 5 and 10 days resulted in a 1% and 3% loss of RBC respectively.

Acute Lung injury assessment

Mice were euthanized with intraperitoneal ketamine and xylazine (100 and 10 mg/kg body weight respectively), a final blood sample was taken by cardiac puncture and an incision was made at the neck to expose the trachea and a 3mm endotracheal cannula inserted in the tracheas. Lungs were lavaged with 1 ml of PBS, flushing three times. Recovered aliquots of lavage fluid were kept on ice and centrifuged immediately at 300g for 10 minutes to pellet cells. Supernatants were removed and stored on ice for protein analysis using the Bio-Rad Protein Assay Reagent Kit and compared to BSA standards. Cells were re-suspended in 100µl of normal PBS solution and cells were counted using a Neubauer hemocytometer. Cells were then placed on slides using a cellspin (Tharmac) and stained using Wright's stain. Differential counts (specifically neutrophils, and macrophages) were then performed on slides via light microscopy.

Flow Cytometry

RBC preparations before and after leukoreduction were labeled with FITC conjugated anti CD45 antibody (0.17µg/ml) and white blood cells measured by FACS. Microparticles were measured by adding FITC conjugated TER-119 (a protein associated

with glycophorin A in mice and specific to the RBC) antibody to washed or unwashed RBC. Both preparations were incubated for 30min in the dark at room temperature. Samples were then analyzed by FACS using a Becton Dickinson FACSCalibur (BD Biosciences, Franklin Lakes, NJ) or a Beckman Coulter 3500 and events acquired using CellQuest or Beckman Coulter native software. Approximately 100,000 events were collected per measurement. All analyses were done with FlowJo software (Tree Star, Inc., Ashland, OR) or Kaluza. Microparticles were assessed as being TERR-119 positive events that were below 1μm in size.

Free heme measurement

We developed a spectral deconvolution approach to simultaneously measure oxyhemoglobin, methemoglobin and free heme in solution. This approach has been used extensively to determine concentrations of different oxidation and ligation states of hemoglobin [112, 151] and relies on individual species within a complex mixture having distinct absorbance spectra, and availability of spectra of a known concentration of each species alone. Concentrations of each individual species within a mixture are determined by deconvolution of experimental spectra against standards by multi-linear regression fitting. While hemoglobin can exist in a number of ligation and oxidation states, in the context of cell-free hemoglobin arising during RBC storage, only oxyhemoglobin and methemoglobin are detectable. Less is known on the oxidation or ligation state of any free heme that may arise. However, free heme is readily oxidized to the ferric oxidation state and bound by chloride (hemin) or hydroxide (hematin). We assumed any free heme present would be in the ferric oxidation state and used hemin to generate standard

spectra. Since spectra are pH and buffer composition sensitive, all standards were generated in Adsol-1 buffer, pH 7.4 to best mimic RBC storage conditions. We therefore used 3-component standard spectra (oxyhemoglobin, methemoglobin and hemin) for deconvolution. To test and validate the method, hemin (0-50μM) was mixed with different concentrations of oxyhemoglobin (oxyHb) and/or methemoglobin (metHb) (over ranges expected after storage induced hemolysis) and spectra measured (450-700nm, Figure 3-1). Each panel in Figure 3-1 shows spectra, respective residuals for spectral fits by multiple-linear regression and the measured hemoglobin (oxy or met) and hemin relative to the amount of each added (gradients indicating recovery across concentrations tested are shown on each graph). In every condition, the measured hemin concentration was directly proportional to the amount added with recovery varying from 94-108% indicated by gradients from linear regression fits. Moreover, addition of hemin had no effect on the ability of spectral deconvolution algorithm to measure oxyHb or metHb; all gradients for fits for oxyHb or metHb vs hemin were not significantly different from zero.

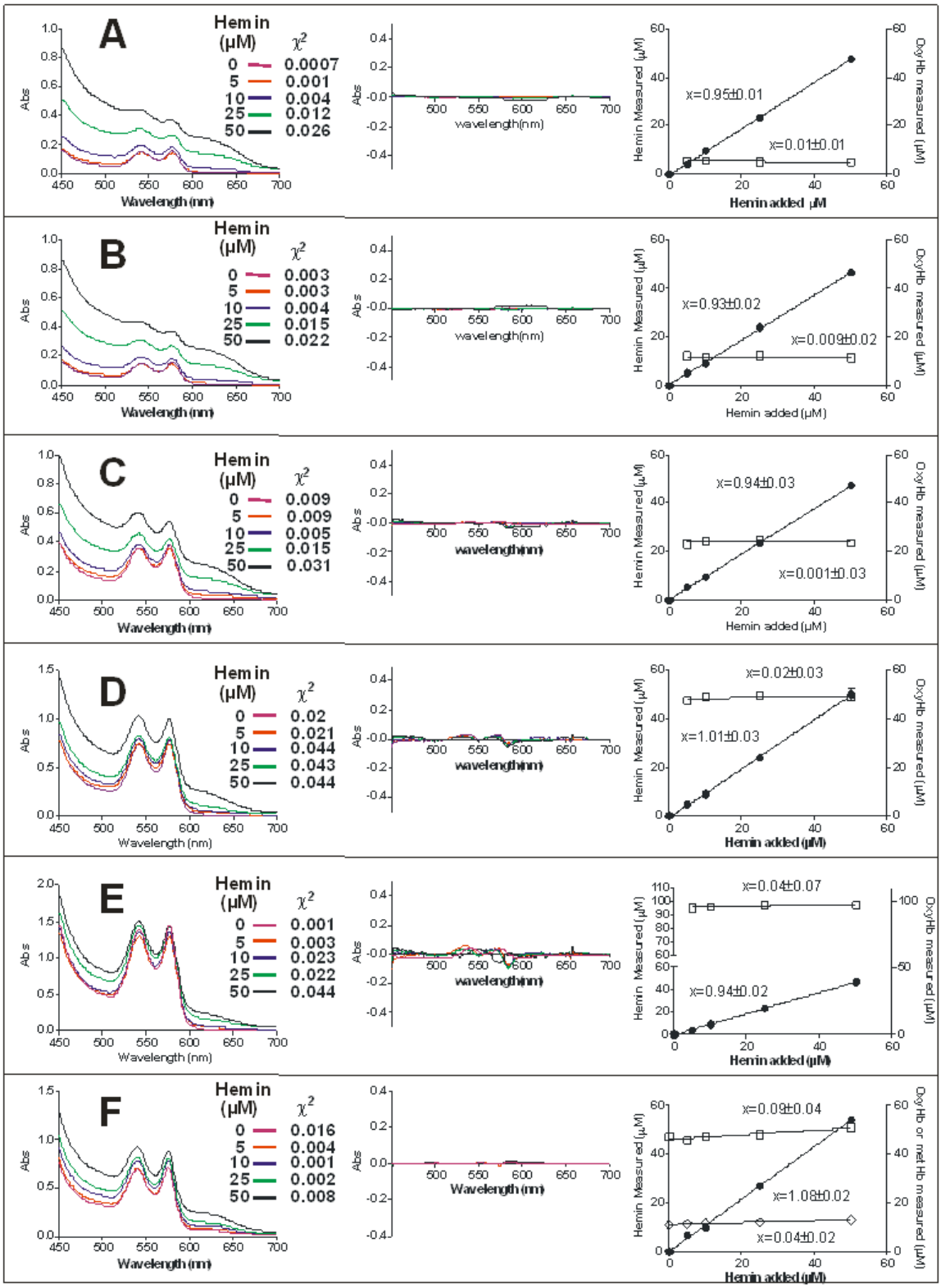
A
Hemin (μM)
χ²
0 0.0007
5 0.001
10 0.004
25 0.012
50 0.026
Abs
Wavelength (nm)
wavelength(nm)
Hemin Measured (μM)
OxyHb measured (μM)
x=0.95±0.01
x=0.01±0.01
Hemin added μM
B
Hemin (μM)
χ²
0 0.003
5 0.003
10 0.004
25 0.015
50 0.022
x=0.93±0.02
x=0.009±0.02
Hemin added (μM)
C
Hemin (μM)
χ²
0 0.009
5 0.009
10 0.005
25 0.015
50 0.031
x=0.94±0.03
x=0.001±0.03
D
Hemin (μM)
χ²
0 0.02
5 0.021
10 0.044
25 0.043
50 0.044
x=0.02±0.03
x=1.01±0.03
E
Hemin (μM)
χ²
0 0.001
5 0.003
10 0.023
25 0.022
50 0.044
x=0.04±0.07
x=0.94±0.02
F
Hemin (μM)
χ²
0 0.016
5 0.004
10 0.001
25 0.002
50 0.008
x=0.09±0.04
x=1.08±0.02
x=0.04±0.02
OxyHb or met Hb measured (μM)

Figure 3-1 Hemin measurement by deconvolution

Hemin (0-50μM) was mixed with oxyHb 5μM (Panel A), oxyHb 10μM (Panel B), oxyHb 25μM (Panel C), oxyHb 50μM (Panel D), oxyHb 100μM (Panel E) or oxyHb 50μM + metHb 10μM (Panel F) and spectra measured between 450-700nm. Deconvolution analysis was performed using standard spectra. In each panel shown are spectra, followed by residual fits (χ^2 values indicated goodness of fits for each spectra shown) and plots of measured hemin vs. added concentrations. Data were fitted by linear regression and are mean ± SEM (n=3-6).

Nitrite Oxidation by OxyHb

Nitrite oxidation was determined using hemolysates (prepared by lysing 20μl pRBC with 80μl ice-old water) and then diluting hemolysate in PBS, pH7.4 + 100μM DTPA to 25μM hemoglobin final concentration. Nitrite (1mM) was added to hemolysates in PBS (37°C) and methemoglobin formation measured as a function of time. Nitrite oxidation rates were compared by measuring lag times for methemoglobin formation (lower lag times indicating high rates of nitrite oxidation) as described [152].

Materials

TAK-242, a small molecule inhibitor of TLR4, was purchased from InvivoGen and dissolved in intraplipid (Sigma). All other materials were purchased from Sigma–Aldrich (St Louis, MO) except MahmaNONOate which was obtained from Axxora Platform (San Diego, CA). Sodium nitrite (Sigma) used in resuscitation was dissolved in saline. Male C57BL/6 mice weighing 22g to 30g were purchased from Harlan Laboratories (Indianapolis, Ind) of 8-10 weeks of age.

Results

Characterization of Murine RBC storage

Recent studies indicate that storage of murine RBC for approximately 2 weeks is equivalent to human RBC storage for 42 days [53]. This is based on comparing RBC stability and recovery post transfusion. Our goal was to determine the relative contribution of intact RBC or RBC degradation products in mediating transfusion toxicity in trauma, and test the role of inhibition of NO-bioavailability. C57bl/6 RBC were leukoreduced using either microcellulose sephadex G25 columns or by neonatal sepacell filters (Figure 3-2 A); both significantly decreased white blood cells (WBC) as compared to unfiltered blood, but no difference was seen between the two filtration methods. All RBC used in our studies were filtered with microcellulose sephadex G25 unless specified.

RBC were stored in Adsol-1 storage media for up to 10 days and markers of storage induced damage which include hemolysis, P_{50}, rates of nitric oxide dioxygenation, nitrite oxidation and microparticle formation were measured (Figure 3-2). Nitric oxide dioxygenation measurements were unobtainable at 10 days of storage time due to increased fragility and hemolysis of the RBC at this storage time. All parameters increased with time, except P_{50} which decreased; consistent with our previous reports of human RBC storage [148].

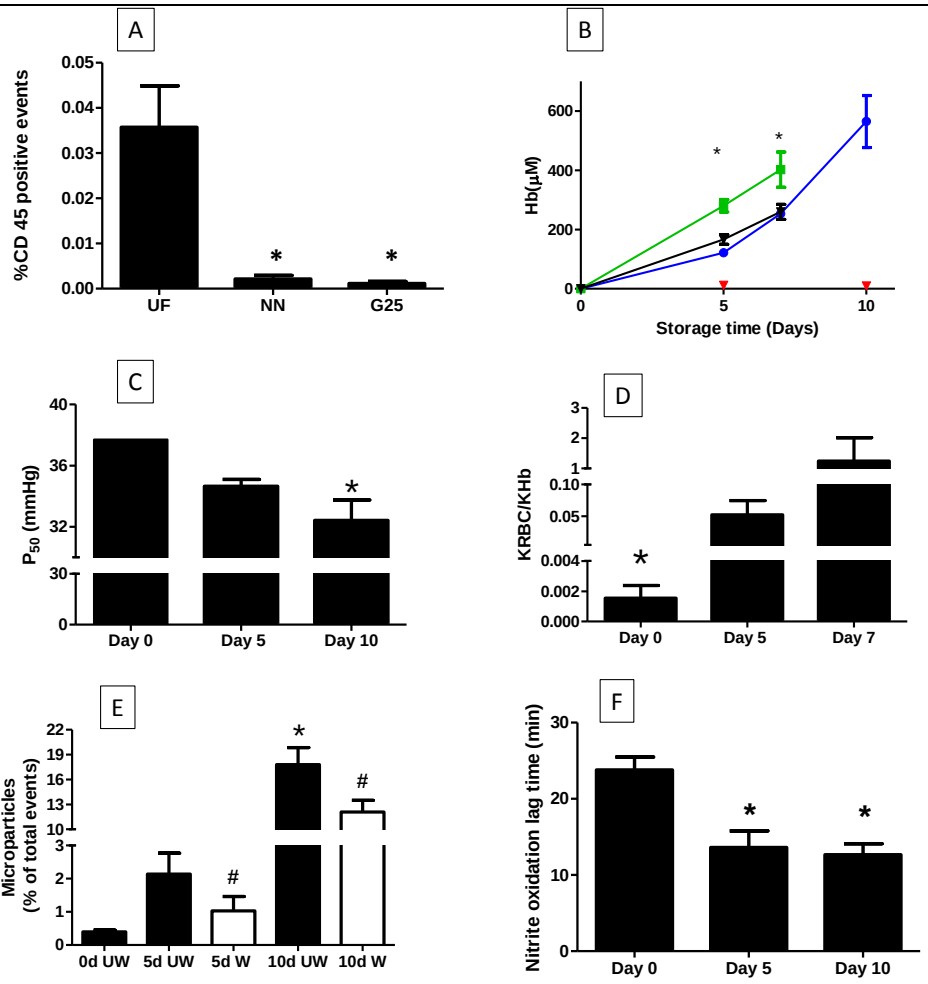

Figure 3-2 Characterization of murine RBC storage.
Panel A: C57BL/6 blood was filtered by either Purecell NEO Neonatal High Efficiency Leukocyte Reduction Filter (Pall corporation) (NN) or by sephadex G25: microcellulose column. (G25). Leukocyte content was determined by FACs and staining for the surface antigen CD45. * P< 0.05 compared to unfiltered (UF) by 1-way ANOVA with Tukey's post test (n=3). **Panel B**: Storage time dependent hemolysis in control (non leukoreduced) RBC (green), leukoreduced (LR) RBC (blue) or LR RBC after washing (red), change indicated by dashed arrows *P<0.01 by 2-way ANOVA with Bonferroni post test (n = 3-9). $^{\#}$P<0.02 by paired t-test (n=3-6). **Panel C:** Storage time dependent changes in P_{50}. *P<0.05 relative to day 0 (n=3) by 1-way ANOVA with Tukey's post test. **Panel D:** Relative rate constants for NO-dioxygenation by intra-erythrocytic (K_{RBC}) hemoglobin vs. acellular hemoglobin (K_{Hb}) as a function of storage age. *P<0.05 relative to day 0 by 1-way ANOVA with Tukey's post test (n=3-6). **Panel E:** Storage time dependent formation of TER-199 positive microparticles in LR mRBC before and after washing. *P<0.01 by 1-way ANOVA with Tukey's post test relative to day 0 unwashed RBC. $^{\#}$P<0.04 by paired t-test relative to respective unwashed RBC (n=3-6). **Panel F**: Lag times for hemolysate mediated nitrite oxidation. *P<0.01 by t-test (n=5).

Hemin measurements of stored human and murine RBC

We investigate heme as a contributor to storage lesion toxicity and needed to obtain accurate measurements of heme levels that accumulate in storage media over time. Heme measurement kits purchased through commercial means could not distinguish between hemoglobin and heme. We developed a means to measure hemin concentrations in Adsol-1 using deconvolution of visible wavelengths scans. Since the majority of cell-free hemoglobin arising from hemolysis is in the oxyferrous state, and free heme is likely to be in the ferric oxidation state, we reasoned that the distinct visible absorbance spectra of each species would allow for their measurement by spectral deconvolution. This method allowed us to measure heme concentrations in the presence of oxyhemoglobin and methemoglobin. Figure 3-3A shows that after 10d of murine RBC (mRBC) storage, significant increases in free heme are observed which are significantly lowered after washing. Figure 3-3B-D compares free heme, free oxyhemoglobin and free methemoglobin levels measured in the same samples of human RBC stored for 7d or 35d. Free heme increased ~10-fold from 1.97 ± 0.63μM to 17.5 ± 1.4μM (mean ± SEM) with storage. Total cell-free hemoglobin also increased with storage, with this being exclusively mediated by increases in oxyhemoglobin (Figure 3-3 C); no changes in methemoglobin were observed (Figure 3-3 D).

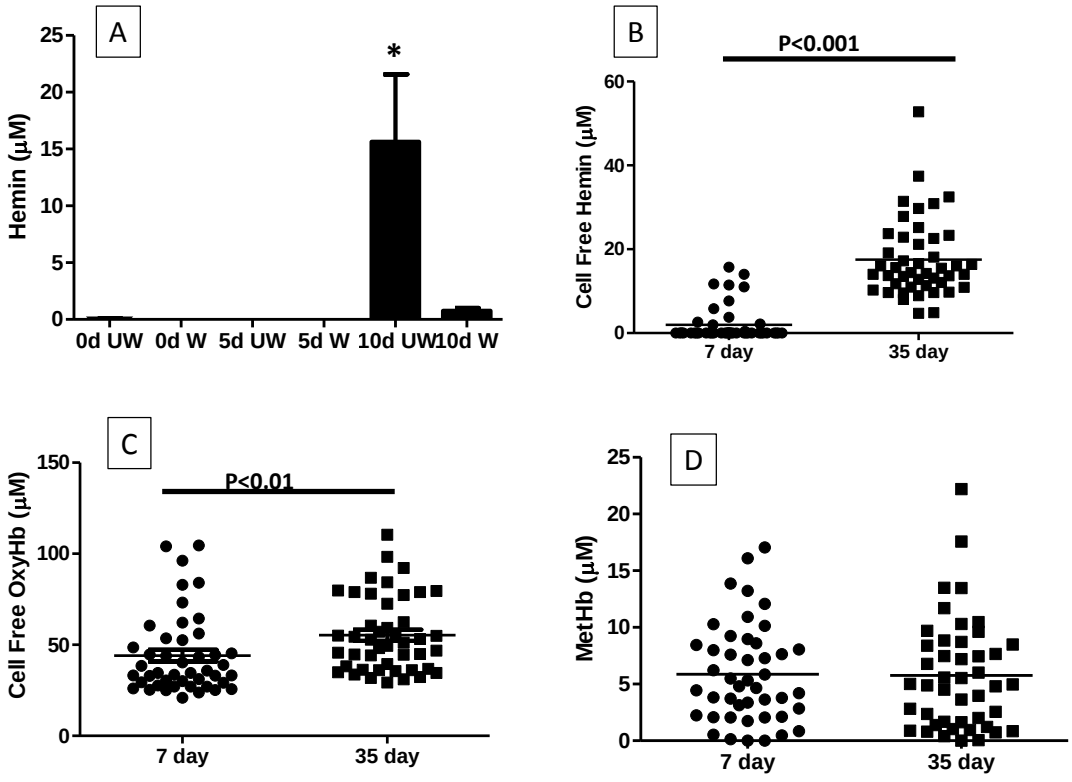

Figure 3-3 Hemin and Hemoglobin concentrations in stored blood
Panel A: Hemin concentration was measured in both washed and unwashed RBC supernatant of 0, 5 and 10 days of storage. Data show mean ± SEM (n=3-8). *P<0.05 vs. all other groups by 1-way ANOVA with Tukey's post test. Panels C-D: Supernatant was measured from segments collected from the UAB blood bank at 7 and 35 days of storage. Shown are the concentrations of hemin, oxyHb and metHb (Panel B, C and D respectively). N=45 *P<0.008 #P<0.0001 and by paired t-test.

Transfusion of 5 day old murine RBC increases protein concentration and neutrophil count in BAL

Trauma was induced by a midline incision and cannulation of the femoral arteries. Hemorrhage was induced until a MAP of approximate 30 mmHg was reached and was maintained for a period of one hour. . At the end of hemorrhage, mice are transfused with saline at 4x blood shed volume and either 100µl or 300µl RBC. Control mice were transfused with saline alone or received sham surgery. Mice were allowed 4 hours of

recovery at the end of which the mice were sacrificed and a bronchoavelolar lavage (BAL) was performed. Initial experiments saw no significant difference between transfusions of 100µl or 300µl RBC, so a focus was made on 100µl transfusion and the data presented here are from mice transfused with 100µl of RBC. Acute lung injury is characterized by increased pulmonary edema, neutrophil accumulation, and alveolar injury. The BAL taken at the end of recovery was assessed for protein concentration, WBC and neutrophil count (Figure 3-4). Mice receiving 100µl of 5 day RBC saw significant increases in protein concentration, WBC count, and neutrophil count when compared to mice receiving saline alone, fresh non-autologous leukoreduced blood (0d) as well as mice experiencing sham surgery (Panels A, E and I respectively). Mice receiving nitrite treatment in conjunction with transfusion of 5 day old RBC saw no significant decrease in protein concentration but did see significant decrease in WBC count and neutrophil count when compared to mice receiving no treatment (Panels B, F and J respectively). When 5 day old RBC were washed according to the procedure stated in methods and used in transfusion, no significant difference was seen in protein concentration, WBC count, or neutrophil count compared to transfusion of unwashed 5 day RBC (Panels C, G and K respectively). Mice treated with the TLR4 inhibitor, TAK-242, prior to transfusion with 5 day old RBC saw a significant decrease in protein concentration, WBC count, and neutrophil cell count as compared to mice receiving no treatment, whereas mice treated with vehicle alone saw no significant difference from untreated mice (Panels D,H and L respectively).

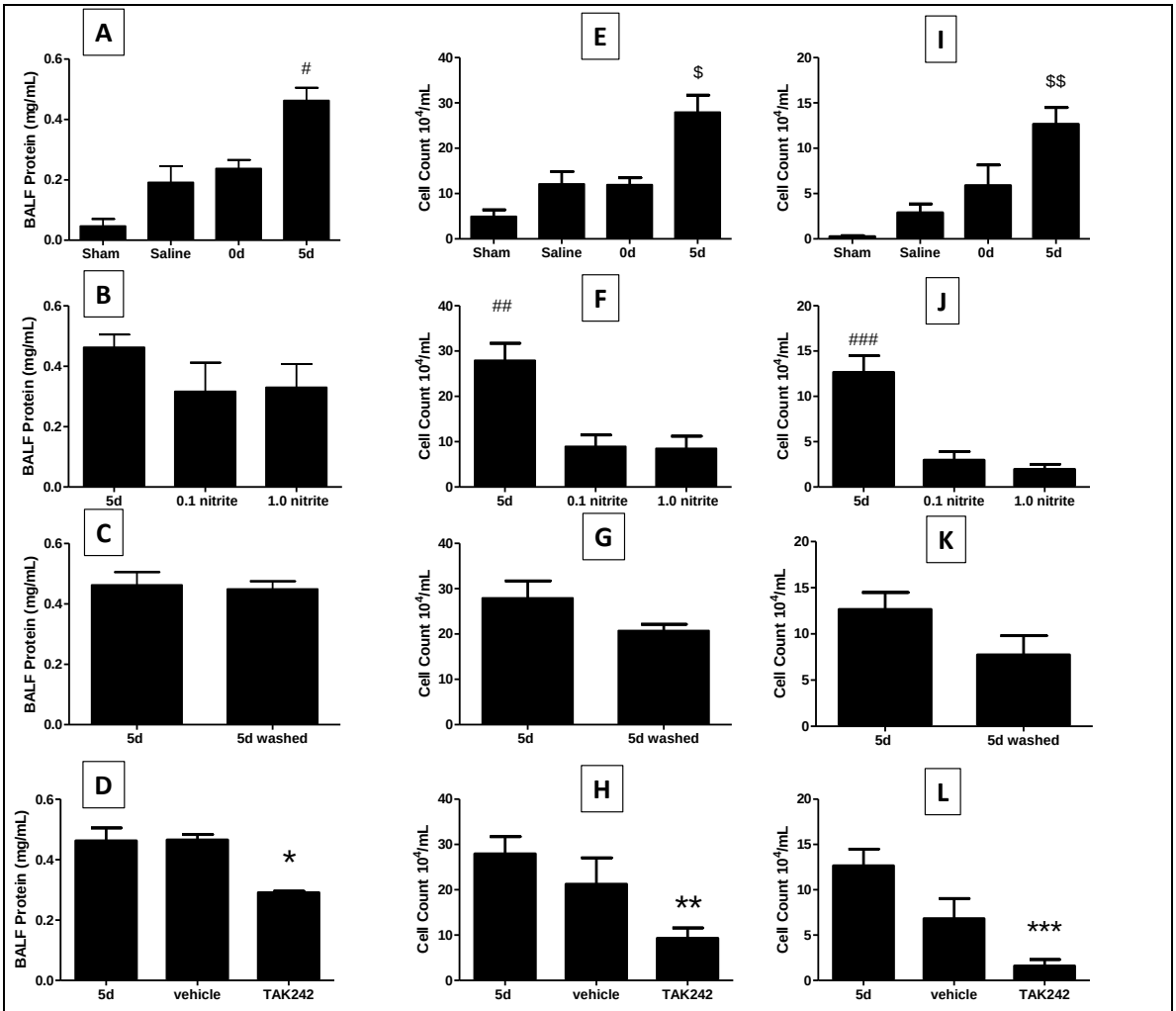

Figure 3-4 Bronchoalveolar Lavage of Transfused Mice

A BAL was performed on mice 4 hours post resuscitation. From this BAL, protein concentration was measured (Panels A-D), as well as a WBC cell count (Panels E-H) and neutrophil count (Panels I-J). Panels A, E and I are mice that have been transfused with Saline, 0 day blood (0d), 5d day blood (5d) or did not experience hemorrhage/transfusion (sham). #P<0.05 compared to all others by 1 way ANOVA Tukey's post test, N=4-7. Panels B, F, J show mice treated with nitrite therapy 0.1mM bolus (0.1 nitrite) or 1.0mM bolus (1.0 nitrite) 5 day blood is include again for comparison. *P<0.05 compared to all others by 1 way ANOVA Tukey's post test, N=4-7. Panels C, G, K show mice receiving washed 5 day blood with 5 day blood included for comparison. N=3-7. Panels D, H, L show mice treated with TAK-242 or vehicle and 5 day blood included for comparison. $P<0.05 compared to 5 day blood by 1 way ANOVA Tukey's post test, N=3-7.

Survival rate of mice transfused with 10 day old murine RBC

In addition to 5 day stored RBC, mice were also transfused with 10 day stored RBC. Using the trauma/hemorrhage procedure as previously outlined, transfused mice received 100µL of 10 day stored RBC and were compared to mice receiving the following treatments i) washed 10 day old RBC, ii) mice treated with 100µL of 1.0mM or 0.1mM bolus of nitrite prior to transfusion, and iii) mice administrated the TLR4 inhibitor TAK-242 prior to hemorrhage (Figure 3-5). When mice were transfused with 10 day stored blood, only a 13% survival rate was seen. However, when RBC were washed three times with Adsol-1 and used for transfusion, the survival rate significantly increased to 100%. Additionally, mice receiving a 100µl bolus of 1mM nitrite prior to transfusion of 100µl of 10 day RBC saw a significant increase in survival (80%) as compared to untreated (13%) and mice treated with only 0.1mM nitrite (20%). Mice treated with the TLR4 inhibitor TAK-242 also saw a significant increase in survival (80%) as compare to those untreated.

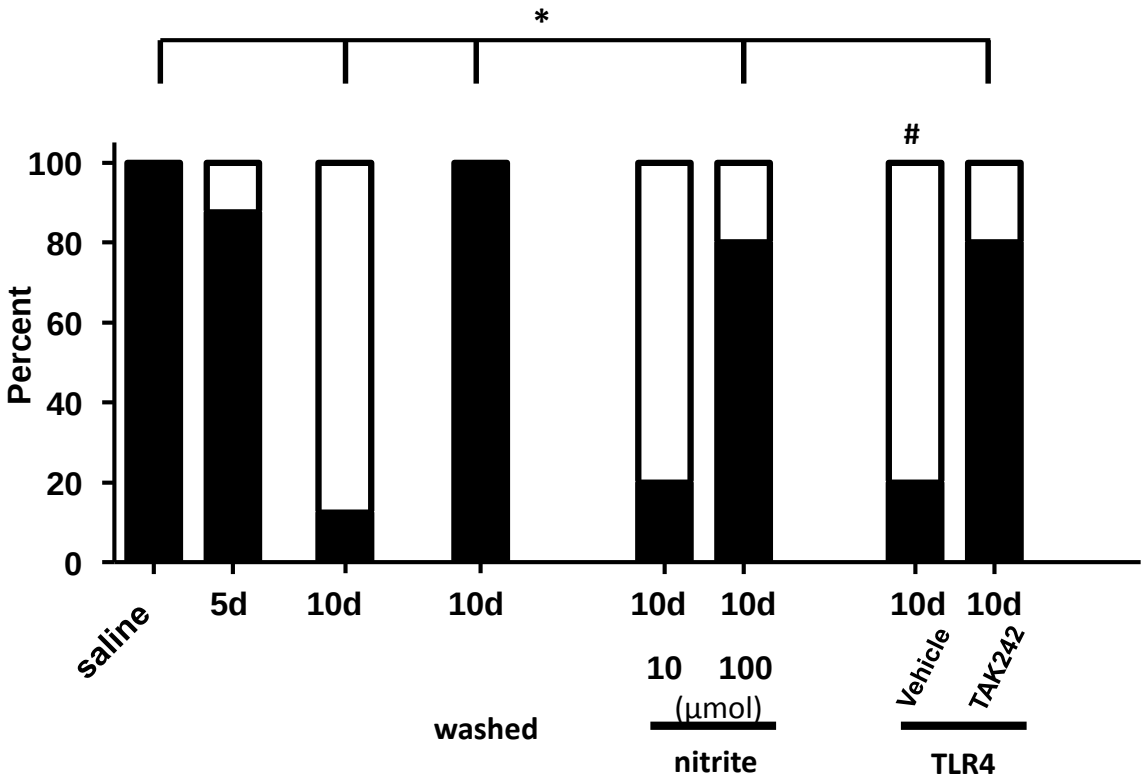

Figure 3-5 Mouse survival 4 hours post transfusion

Shown is the percent of mice that survived or died during trauma-hemorrhage resuscitation. Lethality was only observed post resuscitation. (☐ = dead; ■ = live)
N=3-9 *P<0.01 and #P<0.02 as compared to 1 unit 10 day unwashed RBC by N-1 Two Proportion test.

Discussion

Our goals in this study were to determine mechanisms by which transfusion with stored RBC mediate toxicity and test potential therapeutics. A mouse model of trauma hemorrhage coupled with transfusion with freshly isolated or leukoreduced stored RBC was used. Consistent with recent studies, storage of mouse RBC for 10d was sufficient to promote significant biochemical and functional changes in RBC observed in human RBC stored for 42d. These changes include hemolysis, microparticle formation and formation

of RBC that displayed increased rates of NO-dioxygenation and nitrite oxidation [53, 131, 153]. In addition, we show that significant increases in free heme also occur during storage of both mouse and human RBC underscoring its potential role in storage lesion related toxicities. We developed a spectral deconvolution method for measuring cell-free heme levels that also simultaneously measured cell-free hemoglobin and showed that during storage, both significantly increase, but the relative increase was higher for free heme. In other words the relative contribution of free heme to total extracellular heme increases with storage. This may reflect either direct heme release from RBC and/or heme release from degradation of cell-free hemoglobin. We speculate the latter, since our recent studies (not shown) suggest that cell-free hemoglobin undergoes more oxidative damage relative to erythrocytic hemoglobin. Moreover, we speculate that free heme levels may be underestimated as its hydrophobicity is likely to lead to membrane localization; we only measured heme that was dissolved in the extracellular fraction. A limitation in our measurements is that for human RBC, we quantitated storage-dependent free heme and hemoglobin formation from segments that are attached to RBC storage bags. Recent studies have shown segments have higher levels of hemolysis relative to the paired bag, and our data demonstrated differences in complement levels also [154, 155]. Further studies evaluating cell-free heme formation in bags vs. segments is required. To evaluate mechanisms and potential therapies for stored RBC toxicity, we employed a mouse model of trauma-hemorrhage to model both the two-hit concept of transfusion toxicity and to allow comparison with insights of the storage lesion gained from trauma patients. Transfusion with stored RBC, but not fresh RBC significantly induced acute lung injury or mortality with the severity of injury being proportional to the age of the

transfused RBC; these associations are similar to our studies with trauma patients in which poorer microcirculatory function and tissue oxygenation, higher incidents of acute lung injury and mortality were noted if they received older vs. younger RBC [119, 139, 156]. Our data also support the two hit model. Injury was exacerbated with stored, but not freshly isolated RBC transfusions. This is consistent with several recent experimental studies that show increased lung injury or infection following transfusion with stored RBC or stored RBC-derived microparticles [131, 144, 157, 158]. The general applicability of the two-hit hypothesis is also refuted by the fact that first hit comprised diverse stimuli (LPS injection, infection with pneumonia, high fat diet or trauma hemorrhage) and was observed in different animal models (mice or dogs).

We tested three potential therapies to attenuate stored RBC toxicity. Washing of RBC immediately prior to transfusion has been discussed with the concept that a single wash will remove smaller RBC degradation products (hemolysis, microparticles) or other potential pro-inflammatory effectors (e.g. cytokines, lipid peroxidation products) prior to transfusion [53, 55, 159]. Recent data have shown that washing stored RBC protects against hypertension, lung injury and infection. However, washing of younger RBC increased injury, consistent with concerns of RBC sensitization to subsequent hemolysis due to washing [55]. In addition to the rationale just discussed, we also tested washing to evaluate potential longer-term toxicity of intact stored RBC that remain after washing. Our previous data showed that stored intact RBC inhibited NO-signaling more so than fresh RBC, and prior studies have shown that intact stored RBC are less able to bind chemokines and inhibit inflammation [131, 148]. Since these RBC are likely to have a longer circulatory half-life than cell-free hemoglobin or microparticles, and be present at

higher concentrations, we reasoned that they may sustain an inhibition of endogenous NO-signaling and mediate a persistent pro-inflammatory stimulus. Interestingly, the effects of washing were varied. Mortality induced by 10d RBC was clearly prevented by washing RBC, consistent with a toxicity mediated by lower molecular weight components. However, under sub-lethal conditions, washing had no effect on stored RBC dependent increases in BAL protein or inflammatory cells, although trends were noted. This suggests that washing may be more effective, the more severe the injury caused by stored RBC transfusion. Another factor is the potential differential effect of washing on younger vs. older RBC. Our data suggest that transfusion of washed d0 RBC increased BAL cells to levels that were equal or higher relative to washed 5d RBC, suggesting a detrimental effect of washing on younger RBC, a conclusion similar to a recent study using canine RBC [55]. Moreover, the method of washing may also differentially affect RBC sensitivity to hemolysis [159]. Collectively, these data underscore the need for detailed understanding of how washing effects RBC and subsequent stability post-transfusion. In summary, our data suggest that while washing protects against injury, this is only evident when the injury is severe. Moreover, we hypothesize that while washing RBC does remove mediators of transfusion toxicity, older and intact RBC that remain may still provide a second hit to exacerbate the inflammatory component of acute lung injury.

We also tested nitrite therapy as means to attenuate storage lesion toxicity. Nitrite is a substrate for NO-formation with many studies showing therapeutic efficacy against circulatory and inflammatory diseases including trauma hemorrhage, sepsis and acute lung injury [92, 160, 161]. Moreover, stored RBC decrease nitrite levels *in vivo* which

was associated with poorer microcirculatory function, suggesting that nitrite repletion may protect against storage dependent toxicities [119, 148]. Interestingly, inhaled NO also attenuated stored RBC induced oxidative damage and inflammation, an affect that may be due to an increase in circulating nitrite [158]. Nitrite therapy prevented neutrophil accumulation in the BAL and a trend towards protection was observed with BAL protein also; we have previously noted different sensitivities of BAL protein and cell accumulation towards nitrite therapy. Exactly how nitrite can mitigate stored RBC-dependent toxicity is unclear. One mechanism is oxidation of cell-free oxyhemoglobin that would prevent NO-scavenging. However, the resultant methemoglobin could mediate oxidative stress. Other potential mechanisms include improving tissue blood flow and preventing cell death. While further studies are required to better elucidate the mechanisms by which nitrite protects, its potential as a therapeutic to limit stored RBC toxicity is indicated and also warrants further testing.

Finally, we tested TLR4 inhibition to evaluate the potential for cell-free heme to mediate tissue inflammation and injury. This pathway has been demonstrated to mediate multiple organ dysfunction in sickle cell disease and sepsis [60, 145]. TLR4 inhibition completely protected against all injury end points elicited by RBC stored for both 5d and 10d. This was a surprising result since TLR4 inhibition is not expected to affect inhibition of NO-signaling mediated by cell-free hemoglobin, microparticles or intact RBC. Moreover, there was no detectable cell-free heme in mRBC stored for 5d. The potent effects of TLR4 inhibition suggest ongoing generation of cell-free heme and/or other TLR4 ligands after transfusion which then mediates toxicity. Studies have identified several endogenous ligands of TL4 that are released from damage tissues such

as heat shock protein, fibronectin, heparan sulfate and nonhistone chromatin-binding protein high-mobility group box 1 (HMGB1) [162-165]. A recent study documenting that in the absence of eNOS and NO-signaling, the severity of TLR4 dependent necrotizing enterocolitis was increased [166]. This suggests an intriguing scenario in stored RBC toxicity, whereby loss of NO-signaling may synergize with TLR4 activation to promote tissue injury. The potential interaction between NO-inhibition and TLR4 activation by distinct components of stored RBC is currently under investigation.

.

CHAPTER 4

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Introduction

The understanding of the storage lesion and its effect on transfusion related injury is just beginning to be understood. The results of the research presented in this thesis contribute to the understanding of how various components of the storage lesion could contribute to transfusion related injury as well as insights into treatments to attenuate injury. In this final chapter, a general discussion of our results is provided as well as potential avenues for future studies.

The Red Blood Cell as a Component to the Storage Lesion

Research into mechanisms that mediate increased morbidities and mortality associated with transfusion of older RBC have focused primarily on the storage media and what it contains. Several studies have shown that hemoglobin, free iron and microparticles contribute to injury associated with transfusion. However there is also another element that we believe could contribute to the storage lesion and that is the aged intact RBC. Cell free hemoglobin and microparticles are removed fairly quickly after transfusion. Hemoglobin is bound to haptoglobin and removed by monocytes within

minutes, provided that hemoglobin concentrations do exceed that of haptoglobin. Ninety percent of microparticles are removed by Kupffer cells of the liver within 30 minutes of transfusion [57]. However due to the nature of transfusion, 75% of the RBC are still within circulation 24 hours post transfusion. These older RBC could have a chronic effect on inflammation that could endure past the acute injury caused by cell free hemoglobin and microparticles.

As we have shown, aged RBC show an increase in NO-scavenging as compared to fresh RBC. Our data show a spectrum of NO-scavenging rates: at 42 days of storage, some donor RBC see little change in NO-scavenging rates as compared to day 0, whereas other donor NO-scavenging rates have increased to 40 times that seen at day 0.. This heterogeneity of NO-scavenging rates could correlate to more RBC degradation products and thus increase transfusion toxicity. A longitudinal study of NO and nitrite scavenging rates at different storage times compared to other components of the storage lesion such as hemolysis and microparticle formation could show NO-scavenging rates as a predicator of storage lesion. Another avenue of exploration concerning aged RBC is determining how long these cells remain in circulation. These old RBC "echinocytes" are denser as compared to discoid RBC. It is possible to separate echinocytes from other RBC through high speed centrifugation. Once separated, these older cells could be labelled and assessed for viability upon transfusion. It might be that these older RBC are removed quickly from circulation by the macrophage system. We also used a washing technique in our studies to improve transfusion; however we did not assess changes in the intact RBC during washing. We speculate that these older RBC would be fragile and more likely to lyse during the process but it would be important to investigate if this was

the case. This could be done by measuring RBC NO-scavenging rates pre-and post-washing to see if NO scavenging decrease which would indicate that older RBC were removed.

Endogenous Aging of the RBC

This idea of older RBC having an increase in NO-scavenging and nitrite scavenging has lead our lab to the idea that endogenous RBC could have differing levels of NO-scavenging and nitrite scavenging. RBC have a life span of approximately 120 within circulation. If a blood sample is taken from a healthy individual, one would see that the RBC contained therein would consist of a heterogeneous population of cell ages which are characterized by different sizes, densities, surface areas, volumes and antioxidant statuses. Using high speed centrifugation, RBC can be sorted according to densities. When separated in this manner, age corresponds to density, with younger RBC at the top having the lowest density and RBC age increasing with density [167].

Using this method of separation our lab has been able to compare the capabilities of the younger RBC to that of older RBC to assess NO scavenging and/or nitrite oxidation [58]. The results seen were an increase in NO-scavenging and nitrite oxidation associated with the older/denser RBC.

We hypothesized that the heterogeneous population could change in disease states, where there could be an increase or decrease in older/denser RBC. It has been shown that during a normal pregnancy there is increase in RBC production which results in younger *in vivo* RBC. In contrast, it has been shown that in preeclampsia, a disease

associated with hypertension and decreased NO-bioavailability, the patient does not experience this increase in younger RBC [63]. To investigate this idea of changes in RBC population we have started to collect some preliminary data comparing the NO scavenging rate of women experiencing preeclampsia to that of women with a normal pregnancy. Preliminary data we have collected suggests that there is a trend in pre-eclamptic women having increased NO-scavenging rates as compared to both healthy women and pregnant women (Figure 4-1). Additionally, as we look at NO-scavenging as a function of gestational age we see that the RBC of pre-eclamptic women exhibit a higher NO-scavenging rate earlier in their pregnancy compared to that of non-pre-eclamptic pregnant women. This increased NO-scavenging rate seen in pre-eclamptic women could be associated with the hypertension seen in this condition. We may see that pre-eclampsia does change RBC NO-scavenging rates as tests continue, but this phenomenon might be present in other diseases. Several diseases have noted dysfunction in NO-signaling including many vascular diseases and diabetes. It would be interesting to see if an increase in NO-scavenging is seen in RBC from these different disease backgrounds.

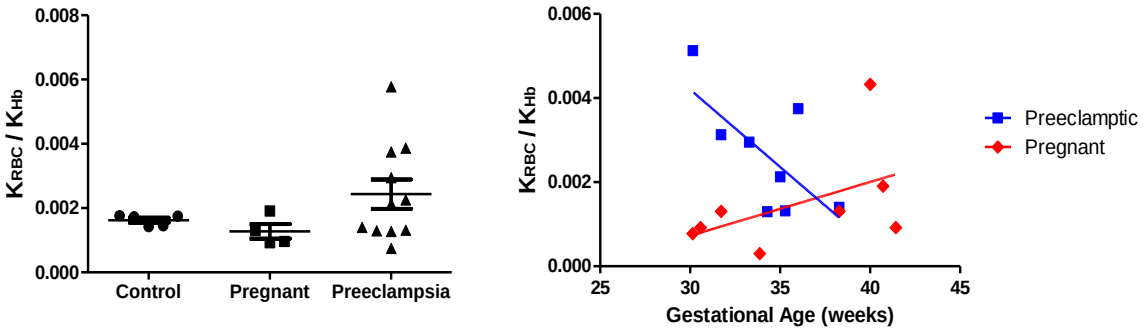

Figure 4-1 NO scavenging of pregnant, preeclamptic, and nonpregnant women
RBC are collected upon hospital visit and assayed within an hour of blood being taken. Preeclampsia patient are defined as having a blood pressure higher than 140/90 twice at least 4 hours apart and more than 300mg of protein in urine. Nitric oxide scavenging is assayed by a competition experiment as explained in chapter 2.

Heme contributes to the storage lesion

In chapter 3 we show that inhibition of the TLR4 receptor results in a decrease in lung injury and increase in survival when mice were transfused with stored blood. This inhibition of the TLR4 receptor could prevent its activation by cell free heme which we have shown to be present in the storage media. Further studies need to be performed to validate heme as the activator of the TLR4 receptor rather than endogenous ligands present due to tissue damage and inflammation. To do this we can use hemopexin treatment prior to transfusion. Hemopexin binds hemin, preventing its activation of the TLR4 receptor. Hemopexin has been shown to avert respiratory failure in sickle cell mice when mice display signs of acute chest syndrome [60].

Another area of interest is determining the contribution of cell free hemoglobin, hemin, microparticles and aged RBC to transfusion toxicity. Our mice model of trauma –

hemorrhage demonstrates a >90% lethality when transfused with 10day stored RBC. Using the data we collected to characterize murine blood banking, we can generate separate preparations of cell free hemoglobin, hemin, and microparticle concentrations as would be seen at 10 days of storage. Using these preparations we can administer each of these separately or in combination to assess lethality and lung injury. In this way we can identify what are the prime instigators of toxicity and from there develop further therapies for the storage lesion. Our studies indicate that activation of TLR4 to be the major cause for injury since an inhibition of TLR4 attenuated lung injury at 5 days storage and increased survival at 10 days of storage, whereas washing RBC only increased survival at 10 days. This suggests that heme may play a significant role in toxicity, however this may not be the case since TLR4 could be activated by endogenous ligands as a result from damage caused by elements other than heme. Whatever the results may indicate, this knowledge could inform us as to better means of therapy and prevention of transfusion injury.

Using blood bag segments, we have shown that heme concentrations increase with storage time. Segments do not reliably represent conditions within the blood bag and have been shown to overestimate hemolysis [154]. It would be important to assess heme levels in actual bags to see if heme levels are increasing. In our assessment of heme levels we noted heterogeneity in heme concentrations. A longitudinal study that looks at heme levels, as well as other factors in the blood storage lesion such as hemolysis and microparticle formation, over donors could reveal that early levels of heme could result in more severe RBC damage during storage. This knowledge could

alert blood banks about storage lesion toxicity potential in RBC prior to storage and could alter storage duration.

Heterogeneity of blood donors

Current FDA standards require 75% of RBC to remain in circulation 24 hours post transfusion. It has been noted that RBC viability over storage time differs among individuals. Recent studies have shown that although most stored blood maintain this standard when transfused at the maximum storage time, there are outliers on both sides of the expiration date with some feasibly able to be stored longer than the maximum storage time and others needing to be used before the posted expiration date [168]. We have seen this variance in the parameters we measure in the storage lesion including NO scavenging rates of RBC, cell free hemoglobin and heme concentrations. There are several possibilities for these differences seen in storage. One study of particular interest compared the hemolysis rate and mechanical fragility of stored blood by donor of similar background (age, non-smoking, blood type) to determine variability in donors [169]. Interestingly, this study showed that there was a two-fold variability in mechanical fragility amongst donors and an outlier of the group was reported to have hereditary hypertriglycermidemia. This concept that the condition of the donor prior to donation may have an effect on storage lesion has interested me and is something I wish to pursue. Some questions I hope to answer are: can conditions such as the donor's age and diet affect RBC viability during storage? Age and diet have been shown to affect the RBC. It has been shown that as a person ages the spectrum of endogenous RBC densities change.

Older individuals have an increase in less dense RBC as compared to younger individuals. These less dense RBC would indicate that they are younger RBC, however when assessed by an erythrophagocytosis assay, older individual's less dense RBC were more likely to be phagocytized compared to RBC from younger donors [62].

Diet can affect RBC morphology; it has been shown that cholesterol availability has an effect on RBC membrane composition and deformability [97, 98]. It would be interesting to do a longitudinal study assessing aspects of the storage lesion including RBC NO scavenging rate, hemolysis, microparticle formation, and heme levels of RBC donated from older compared to younger individuals, or comparing individuals with a low cholesterol diet to those with a high cholesterol diet. This concept could be taken to our murine model of trauma/hemorrhage. A comparison of differing ages and diets of mice prior to blood donation could be assessed for transfusion related toxicity. Understanding how age and diet affect the storage lesion could allow blood banks to prioritize when to use blood for transfusion.

Another aspect that could affect the variance is the preparation of RBC prior to storage. As noted in chapter 2, difference in centrifugation containers changed hemolysis rates. It was also noted by Gkoumassi and coworkers that an increase centrifugation time during blood processing resulted in increased hemolysis [125] . This idea that changes in the preparation could have effects on storage is worth investigating. A comparison of centrifugation containers, times and speed could be conducted and used to improve blood banking procedures.

APPENDIX/APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: December 17, 2012

TO: RAKESH PRAVINCHANDRA PATEL, Ph.D.
BMR2-532 2182
FAX: (205) 934-7447

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: RBC Age and Potentiation of Transfusion - Related Pathology in Trauma Patients
Sponsor: NIH
Animal Project Number: 121208998

As of December 17, 2012, the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Rabbits	B	50
Rats	B	100

Animal use must be renewed by December 16, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 121208998 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

RE: LiebertPub Website Customer Question

Ballen, Karen <KBallen@liebertpub.com> Tue, Apr 1, 2014 at 3:06 PM
To: "ryan.stapley@gmail.com" <ryan.stapley@gmail.com>

Dear Ryan:

Copyright permission is granted for you to r-use content from your article originally published in ANTIOXIDANTS & REDOX SIGNALING.

Kind regards,

Karen Ballen

Manager, Reprints, Permissions and Liebert Open Option

From: ryan.stapley@gmail.com [mailto:ryan.stapley@gmail.com]
Sent: Tuesday, April 01, 2014 11:55 AM
To: Ballen, Karen
Subject: LiebertPub Website Customer Question

Name - Ryan Stapley
Position - Graduate student
Department - Pathology
Institution/affiliation - UAB
Address Line1 - 113 Hickory Knolls
City - Birmingham
State - ALABAMA
Country - USA
Zip - 35226
Email - ryan.stapley@gmail.com
Phone - 8016414317

Copyright Permission

Editorial Office <editorial@portlandpress.com> Wed, Apr 2, 2014 at 6:01 AM
To: Ryan Stapley <ryan.stapley@gmail.com>

Dear Ryan

You do not need to apply for permission to re-use material of which you are an author. However, we ask that you add a credit line wherever re-published material occurs. Our preferred credit lines can be found on our website under the following link:

http://www.portlandpress.com/pp/journals/rights.htm

Please do not hesitate to contact us again if you have any further queries,

Best regards

Helene

Helene Pettersson

Editorial administrator

The Biochemical Society/ Portland Press Ltd

Charles Darwin House

12 Roger Street

London WC1N 2JU

T: 020 7685 2410 (International: +44 20 7685 2410)

F: 020 7685 2469 (International: +44 20 7685 2469)

E: Helene.Pettersson@portlandpress.com

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Apr 16, 2014

This is a License Agreement between Ryan Stapley ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3370820390278
License date	Apr 16, 2014
Licensed content publisher	John Wiley and Sons
Licensed content publication	Journal of Physiology
Licensed content title	Nitric oxide formation versus scavenging: the red blood cell balancing act
Licensed copyright line	© 2012 The Authors. The Journal of Physiology © 2012 The Physiological Society
Licensed content author	Benjamin Y. Owusu,Ryan Stapley,Rakesh P. Patel
Licensed content date	Jul 27, 2012
Start page	4993
End page	5000
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	Effects of Storage on the Red Blood Cell and Implications in Transfusion
Expected completion date	May 2014
Expected size (number of pages)	100
Total	0.00 USD
Terms and Conditions	

REFERENCES

1. Ellis, H., *James Blundell, pioneer of blood transfusion.* Br J Hosp Med (Lond), 2007. **68**(8): p. 447.
2. Landsteiner, K. and P. Levine, *On Individual Differences in Human Blood.* J Exp Med, 1928. **47**(5): p. 757-75.
3. CoE, S., *Guide to the preparation, use and quality assurance of blood components.* 13 edn, 2007.
4. Schiffer, C.A., et al., *A randomized trial of leukocyte-depleted platelet transfusion to modify alloimmunization in patients with leukemia.* Blood, 1983. **62**(4): p. 815-20.
5. Sniecinski, I., et al., *Prevention of refractoriness and HLA-alloimmunization using filtered blood products.* Blood, 1988. **71**(5): p. 1402-7.
6. Andreu, G., et al., *Prevention of HLA immunization with leukocyte-poor packed red cells and platelet concentrates obtained by filtration.* Blood, 1988. **72**(3): p. 964-9.
7. van Marwijk Kooy, M., et al., *Use of leukocyte-depleted platelet concentrates for the prevention of refractoriness and primary HLA alloimmunization: a prospective, randomized trial.* Blood, 1991. **77**(1): p. 201-5.
8. Yazer, M.H., et al., *The effect of prestorage WBC reduction on the rates of febrile nonhemolytic transfusion reactions to platelet concentrates and RBC.* Transfusion, 2004. **44**(1): p. 10-5.
9. *Leukocyte reduction and ultraviolet B irradiation of platelets to prevent alloimmunization and refractoriness to platelet transfusions. The Trial to Reduce Alloimmunization to Platelets Study Group.* N Engl J Med, 1997. **337**(26): p. 1861-9.
10. Ichinose, F., J.D. Roberts, Jr., and W.M. Zapol, *Inhaled nitric oxide: a selective pulmonary vasodilator: current uses and therapeutic potential.* Circulation, 2004. **109**(25): p. 3106-11.
11. van de Watering, L.M., et al., *Beneficial effects of leukocyte depletion of transfused blood on postoperative complications in patients undergoing cardiac surgery: a randomized clinical trial.* Circulation, 1998. **97**(6): p. 562-8.
12. Bilgin, Y.M., et al., *Double-blind, randomized controlled trial on the effect of leukocyte-depleted erythrocyte transfusions in cardiac valve surgery.* Circulation, 2004. **109**(22): p. 2755-60.
13. Koch, C., et al., *Transfusion and pulmonary morbidity after cardiac surgery.* Ann Thorac Surg, 2009. **88**(5): p. 1410-8.
14. Koch, C.G., et al., *Transfusion in coronary artery bypass grafting is associated with reduced long-term survival.* Ann Thorac Surg, 2006. **81**(5): p. 1650-7.

15. Habib, R.H., et al., *Role of hemodilutional anemia and transfusion during cardiopulmonary bypass in renal injury after coronary revascularization: implications on operative outcome.* Crit Care Med, 2005. **33**(8): p. 1749-56.
16. Ferraris, V.A., et al., *Intraoperative transfusion of small amounts of blood heralds worse postoperative outcome in patients having noncardiac thoracic operations.* Ann Thorac Surg, 2011. **91**(6): p. 1674-80; discussion 1680.
17. Commision, T.J., *Proceedings from the National Summit on Overuse.* Physician Consortium for Performance Improvement, 2012: p. 14-15.
18. Hebert, P.C., et al., *A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. Transfusion Requirements in Critical Care Investigators, Canadian Critical Care Trials Group.* N Engl J Med, 1999. **340**(6): p. 409-17.
19. Hajjar, L.A., et al., *Transfusion requirements after cardiac surgery: the TRACS randomized controlled trial.* JAMA, 2010. **304**(14): p. 1559-67.
20. Carson, J.L., et al., *Liberal or restrictive transfusion in high-risk patients after hip surgery.* N Engl J Med, 2011. **365**(26): p. 2453-62.
21. Benjamin, R.J. and B.I. Whitaker, *Boom or bust? Estimating blood demand and supply as the baby boomers age.* Transfusion, 2011. **51**(4): p. 670-3.
22. Greinacher, A., et al., *Implications of demographics on future blood supply: a population-based cross-sectional study.* Transfusion, 2011. **51**(4): p. 702-9.
23. Lau, E.H., et al., *Predicting future blood demand from thalassemia major patients in Hong Kong.* PLoS One, 2013. **8**(12): p. e81846.
24. Currie, C.J., et al., *Evaluation of the future supply and demand for blood products in the United Kingdom National Health Service.* Transfus Med, 2004. **14**(1): p. 19-24.
25. Purdy, F.R., M.G. Tweeddale, and P.M. Merrick, *Association of mortality with age of blood transfused in septic ICU patients.* Can J Anaesth, 1997. **44**(12): p. 1256-61.
26. Marik, P.E. and W.J. Sibbald, *Effect of stored-blood transfusion on oxygen delivery in patients with sepsis.* JAMA, 1993. **269**(23): p. 3024-9.
27. Pettila, V., et al., *Age of red blood cells and mortality in the critically ill.* Crit Care, 2011. **15**(2): p. R116.
28. Spinella, P.C., et al., *Duration of red blood cell storage is associated with increased incidence of deep vein thrombosis and in hospital mortality in patients with traumatic injuries.* Crit Care, 2009. **13**(5): p. R151.
29. Weinberg, J.A., et al., *Duration of red cell storage influences mortality after trauma.* J Trauma, 2010. **69**(6): p. 1427-31; discussion 1431-2.
30. Offner, P.J., et al., *Increased rate of infection associated with transfusion of old blood after severe injury.* Arch Surg, 2002. **137**(6): p. 711-6; discussion 716-7.
31. Koch, C.G., et al., *Duration of red-cell storage and complications after cardiac surgery.* N Engl J Med, 2008. **358**(12): p. 1229-39.
32. Leal-Noval, S.R., et al., *Influence of erythrocyte concentrate storage time on postsurgical morbidity in cardiac surgery patients.* Anesthesiology, 2003. **98**(4): p. 815-22.

33. Vamvakas, E.C. and J.H. Carven, *Transfusion and postoperative pneumonia in coronary artery bypass graft surgery: effect of the length of storage of transfused red cells.* Transfusion, 1999. **39**(7): p. 701-10.
34. Andreasen, J.J., et al., *Storage time of allogeneic red blood cells is associated with risk of severe postoperative infection after coronary artery bypass grafting.* Eur J Cardiothorac Surg, 2011. **39**(3): p. 329-34.
35. Gajic, O., et al., *Acute lung injury after blood transfusion in mechanically ventilated patients.* Transfusion, 2004. **44**(10): p. 1468-74.
36. Taylor, R.W., et al., *Impact of allogenic packed red blood cell transfusion on nosocomial infection rates in the critically ill patient.* Crit Care Med, 2002. **30**(10): p. 2249-54.
37. Katsios, C., et al., *Red blood cell transfusion and increased length of storage are not associated with deep vein thrombosis in medical and surgical critically ill patients: a prospective observational cohort study.* Crit Care, 2011. **15**(6): p. R263.
38. Gajic, O., et al., *Transfusion-related acute lung injury in the critically ill: prospective nested case-control study.* Am J Respir Crit Care Med, 2007. **176**(9): p. 886-91.
39. Cohen, B. and I. Matot, *Aged erythrocytes: a fine wine or sour grapes?* Br J Anaesth, 2013. **111 Suppl 1**: p. i62-70.
40. Tinmouth, A., et al., *Clinical consequences of red cell storage in the critically ill.* Transfusion, 2006. **46**(11): p. 2014-27.
41. Steiner, M.E., et al., *Addressing the question of the effect of RBC storage on clinical outcomes: the Red Cell Storage Duration Study (RECESS) (Section 7).* Transfus Apher Sci, 2010. **43**(1): p. 107-16.
42. Lacroix, J., et al., *The Age of Blood Evaluation (ABLE) randomized controlled trial: study design.* Transfus Med Rev, 2011. **25**(3): p. 197-205.
43. Bennett-Guerrero, E., et al., *Evolution of adverse changes in stored RBCs.* Proc Natl Acad Sci U S A, 2007. **104**(43): p. 17063-8.
44. Stan, A. and E. Zsigmond, *The restoration in vivo of 2,3-diphosphoglycerate (2,3-DPG) in stored red cells, after transfusion. The levels of red cells 2,3-DPG.* Rom J Intern Med, 2009. **47**(2): p. 173-7.
45. Raat, N.J., et al., *The effect of storage time of human red cells on intestinal microcirculatory oxygenation in a rat isovolemic exchange model.* Crit Care Med, 2005. **33**(1): p. 39-45; discussion 238-9.
46. Antonelou, M.H., et al., *Red blood cell aging markers during storage in citrate-phosphate-dextrose-saline-adenine-glucose-mannitol.* Transfusion, 2010. **50**(2): p. 376-89.
47. Rael, L.T., et al., *The effect of storage on the accumulation of oxidative biomarkers in donated packed red blood cells.* J Trauma, 2009. **66**(1): p. 76-81.
48. Kriebardis, A.G., et al., *RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components.* Transfusion, 2008. **48**(9): p. 1943-53.

49. Korgun, D.K., S. Bilmen, and A. Yesilkaya, *Alterations in the erythrocyte antioxidant system of blood stored in blood bags.* Res Commun Mol Pathol Pharmacol, 2001. **109**(5-6): p. 357-63.
50. Dumaswala, U.J., et al., *Protein and lipid oxidation of banked human erythrocytes: role of glutathione.* Free Radic Biol Med, 1999. **27**(9-10): p. 1041-9.
51. Alayash, A.I., R.P. Patel, and R.E. Cashon, *Redox reactions of hemoglobin and myoglobin: biological and toxicological implications.* Antioxid Redox Signal, 2001. **3**(2): p. 313-27.
52. Rogers, M.S., et al., *Pro-oxidant effects of cross-linked haemoglobins explored using liposome and cytochrome c oxidase vesicle model membranes.* Biochem J, 1995. **310 (Pt 3)**: p. 827-33.
53. Belizaire, R.M., et al., *Resuscitation with washed aged packed red blood cell units decreases the proinflammatory response in mice after hemorrhage.* J Trauma Acute Care Surg, 2012. **73**(2 Suppl 1): p. S128-33.
54. Hod, E.A., et al., *Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation.* Blood, 2010. **115**(21): p. 4284-92.
55. Cortes-Puch, I., et al., *Washing older blood units before transfusion reduces plasma iron and improves outcomes in experimental canine pneumonia.* Blood, 2014. **123**(9): p. 1403-11.
56. Baek, J.H., et al., *Hemoglobin-driven pathophysiology is an in vivo consequence of the red blood cell storage lesion that can be attenuated in guinea pigs by haptoglobin therapy.* J Clin Invest. **122**(4): p. 1444-58.
57. Willekens, F.L., et al., *Liver Kupffer cells rapidly remove red blood cell-derived vesicles from the circulation by scavenger receptors.* Blood, 2005. **105**(5): p. 2141-5.
58. Owusu, B.Y., et al., *Effects of erythrocyte aging on nitric oxide and nitrite metabolism.* Antioxid Redox Signal, 2013. **19**(11): p. 1198-208.
59. Piazza, M., et al., *Hemin and a metabolic derivative coprohemin modulate the TLR4 pathway differently through different molecular targets.* Innate Immun, 2011. **17**(3): p. 293-301.
60. Ghosh, S., et al., *Extracellular hemin crisis triggers acute chest syndrome in sickle mice.* J Clin Invest, 2013. **123**(11): p. 4809-20.
61. Zallen, G., et al., *Age of transfused blood is an independent risk factor for postinjury multiple organ failure.* Am J Surg, 1999. **178**(6): p. 570-2.
62. Sheiban, E. and H. Gershon, *Recognition and sequestration of young and old erythrocytes from young and elderly human donors: in vitro studies.* J Lab Clin Med, 1993. **121**(3): p. 493-501.
63. Lurie, S., *Density distribution of erythrocyte population in preeclampsia.* Gynecol Obstet Invest, 1992. **33**(2): p. 94-7.
64. Shiva, S., et al., *The detection of the nitrite reductase and NO-generating properties of haemoglobin by mitochondrial inhibition.* Cardiovasc Res, 2011. **89**(3): p. 566-73.
65. Laczko, J., C.J. Feo, and W. Phillips, *Discocyte--echinocyte reversibility in blood stored in CPD over a period of 56 days.* Transfusion, 1979. **19**(4): p. 379-88.

66. Lawrence, A.C., J.M. Bevington, and M. Young, *Storage of blood and the mean corpuscular volume.* J Clin Pathol, 1975. **28**(5): p. 345-9.
67. Card, R.T., et al., *Deformability of stored red blood cells. Relationship to degree of packing.* Transfusion, 1982. **22**(2): p. 96-101.
68. Card, R.T., N. Mohandas, and P.L. Mollison, *Relationship of post-transfusion viability to deformability of stored red cells.* Br J Haematol, 1983. **53**(2): p. 237-40.
69. Huang, Y.X., et al., *Human red blood cell aging: correlative changes in surface charge and cell properties.* J Cell Mol Med, 2011. **15**(12): p. 2634-42.
70. Donadee, C., et al., *Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion.* Circulation. **124**(4): p. 465-76.
71. Gyongyossy-Issa, M.I., et al., *Prestorage leukoreduction and low-temperature filtration reduce hemolysis of stored red cell concentrates.* Transfusion, 2005. **45**(1): p. 90-6.
72. Dezfulian, C., et al., *Role of the anion nitrite in ischemia-reperfusion cytoprotection and therapeutics.* Cardiovasc Res, 2007. **75**(2): p. 327-38.
73. Azarov, I., et al., *Mechanisms of slower nitric oxide uptake by red blood cells and other hemoglobin-containing vesicles.* J Biol Chem. **286**(38): p. 33567-79.
74. Hod, E.A., et al., *Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron.* Blood, 2011. **118**(25): p. 6675-82.
75. Weinberg, J.A., S.R. Barnum, and R.P. Patel, *Red blood cell age and potentiation of transfusion-related pathology in trauma patients.* Transfusion. **51**(4): p. 867-73.
76. Keszler, A., et al., *The reaction between nitrite and oxyhemoglobin: a mechanistic study.* J Biol Chem, 2008. **283**(15): p. 9615-22.
77. Wennmalm, A., et al., *Metabolism and excretion of nitric oxide in humans. An experimental and clinical study.* Circ Res, 1993. **73**(6): p. 1121-7.
78. Liu, X., et al., *Nitric oxide uptake by erythrocytes is primarily limited by extracellular diffusion not membrane resistance.* J Biol Chem, 2002. **277**(29): p. 26194-9.
79. Vaughn, M.W., et al., *Erythrocytes possess an intrinsic barrier to nitric oxide consumption.* J Biol Chem, 2000. **275**(4): p. 2342-8.
80. Lancaster, J.R., Jr., *Simulation of the diffusion and reaction of endogenously produced nitric oxide.* Proc Natl Acad Sci U S A, 1994. **91**(17): p. 8137-41.
81. Han, T.H., et al., *Erythrocyte nitric oxide transport reduced by a submembrane cytoskeletal barrier.* Biochim Biophys Acta, 2005. **1723**(1-3): p. 135-42.
82. Ignarro, L.J., *Nitric oxide as a unique signaling molecule in the vascular system: a historical overview.* J Physiol Pharmacol, 2002. **53**(4 Pt 1): p. 503-14.
83. Cantu-Medellin, N. and E.E. Kelley, *Xanthine oxidoreductase-catalyzed reduction of nitrite to nitric oxide: insights regarding where, when and how.* Nitric Oxide, 2013. **34**: p. 19-26.

84. Koch, C.G., et al., *Morbidity and mortality risk associated with red blood cell and blood-component transfusion in isolated coronary artery bypass grafting.* Crit Care Med, 2006. **34**(6): p. 1608-16.
85. Lundberg, J.O., E. Weitzberg, and M.T. Gladwin, *The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics.* Nat Rev Drug Discov, 2008. **7**(2): p. 156-67.
86. Kevil, C.G., et al., *Inorganic nitrite therapy: historical perspective and future directions.* Free Radic Biol Med. **51**(3): p. 576-93.
87. Pinder, A.G., et al., *Nitrite directly vasodilates hypoxic vasculature via nitric oxide-dependent and -independent pathways.* Br J Pharmacol, 2009. **157**(8): p. 1523-30.
88. Cosby, K., et al., *Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation.* Nat Med, 2003. **9**(12): p. 1498-505.
89. Huang, Z., et al., *Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control.* J Clin Invest, 2005. **115**(8): p. 2099-107.
90. Lundberg, J.O., et al., *Nitrate and nitrite in biology, nutrition and therapeutics.* Nat Chem Biol, 2009. **5**(12): p. 865-9.
91. Kevil, C.G. and R.P. Patel, *Preserving vessel function during ischemic disease: new possibilities of inorganic nitrite therapy.* Expert Rev Cardiovasc Ther, 2008. **6**(9): p. 1175-9.
92. Rodriguez, C., et al., *Sodium nitrite therapy attenuates the hypertensive effects of HBOC-201 via nitrite reduction.* Biochem J, 2009. **422**(3): p. 423-32.
93. Cholette, J.M., et al., *Washing red blood cells and platelets transfused in cardiac surgery reduces postoperative inflammation and number of transfusions: results of a prospective, randomized, controlled clinical trial.* Pediatr Crit Care Med, 2012. **13**(3): p. 290-9.
94. Weinberg, J.A., et al., *Transfusions in the less severely injured: does age of transfused blood affect outcomes?* J Trauma, 2008. **65**(4): p. 794-8.
95. Weinberg, J.A., et al., *Duration of red cell storage influences mortality after trauma.* J Trauma. **69**(6): p. 1427-31; discussion 1431-2.
96. Janz, D.R., et al., *Longer storage duration of red blood cells is associated with an increased risk of acute lung injury in patients with sepsis.* Ann Intensive Care, 2013. **3**(1): p. 33.
97. Croce, M.A., et al., *Transfusions result in pulmonary morbidity and death after a moderate degree of injury.* J Trauma, 2005. **59**(1): p. 19-23; discussion 23-4.
98. Zilberberg, M.D., et al., *Red blood cell transfusions and the risk of acute respiratory distress syndrome among the critically ill: a cohort study.* Crit Care, 2007. **11**(3): p. R63.
99. Silverboard, H., et al., *The role of acute blood transfusion in the development of acute respiratory distress syndrome in patients with severe trauma.* J Trauma, 2005. **59**(3): p. 717-23.
100. Doyle, M.P. and J.W. Hoekstra, *Oxidation of nitrogen oxides by bound dioxygen in hemoproteins.* J Inorg Biochem, 1981. **14**(4): p. 351-8.
101. Cherry, T., et al., *Transfusion-related acute lung injury: past, present, and future.* Am J Clin Pathol, 2008. **129**(2): p. 287-97.

102. Weinberg, J.A., et al., *Age of transfused blood: an independent predictor of mortality despite universal leukoreduction.* J Trauma, 2008. **65**(2): p. 279-82; discussion 282-4.
103. Zhu, H., et al., *Impaired adenosine-5'-triphosphate release from red blood cells promotes their adhesion to endothelial cells: a mechanism of hypoxemia after transfusion.* Crit Care Med, 2011. **39**(11): p. 2478-86.
104. Reynolds, J.D., et al., *S-nitrosohemoglobin deficiency: a mechanism for loss of physiological activity in banked blood.* Proc Natl Acad Sci U S A, 2007. **104**(43): p. 17058-62.
105. Azarov, I., et al., *Nitric oxide scavenging by red blood cells as a function of hematocrit and oxygenation.* J Biol Chem, 2005. **280**(47): p. 39024-32.
106. Doherty, D.H., et al., *Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin.* Nat Biotechnol, 1998. **16**(7): p. 672-6.
107. Kim-Shapiro, D.B., A.N. Schechter, and M.T. Gladwin, *Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics.* Arterioscler Thromb Vasc Biol, 2006. **26**(4): p. 697-705.
108. Liu, X., et al., *Diffusion-limited reaction of free nitric oxide with erythrocytes.* J Biol Chem, 1998. **273**(30): p. 18709-13.
109. Joshi, M.S., et al., *Nitric oxide is consumed, rather than conserved, by reaction with oxyhemoglobin under physiological conditions.* Proc Natl Acad Sci U S A, 2002. **99**(16): p. 10341-6.
110. Sakai, H., et al., *Encapsulation of concentrated hemoglobin solution in phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier.* J Biol Chem, 2008. **283**(3): p. 1508-17.
111. Gladwin, M.T., et al., *Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation.* Am J Physiol Heart Circ Physiol, 2006. **291**(5): p. H2026-35.
112. Huang, K.T., et al., *The reaction between nitrite and deoxyhemoglobin. Reassessment of reaction kinetics and stoichiometry.* J Biol Chem, 2005. **280**(35): p. 31126-31.
113. Cantu-Medellin, N., et al., *Effects of T- and R-state stabilization on deoxyhemoglobin-nitrite reactions and stimulation of nitric oxide signaling.* Nitric Oxide. **25**(2): p. 59-69.
114. Isbell, T.S., M.T. Gladwin, and R.P. Patel, *Hemoglobin oxygen fractional saturation regulates nitrite-dependent vasodilation of aortic ring bioassays.* Am J Physiol Heart Circ Physiol, 2007. **293**(4): p. H2565-72.
115. van Faassen, E.E., et al., *Nitrite as regulator of hypoxic signaling in mammalian physiology.* Med Res Rev, 2009. **29**(5): p. 683-741.
116. Gelderman, M.P., et al., *Serial oxygen equilibrium and kinetic measurements during RBC storage.* Transfus Med. **20**(5): p. 341-5.
117. Vaughn, M.W., et al., *Erythrocyte consumption of nitric oxide: competition experiment and model analysis.* Nitric Oxide, 2001. **5**(1): p. 18-31.
118. Vitturi, D.A., et al., *Regulation of nitrite transport in red blood cells by hemoglobin oxygen fractional saturation.* Am J Physiol Heart Circ Physiol, 2009. **296**(5): p. H1398-407.

119. Weinberg, J.A., et al., *Microvascular response to red blood cell transfusion in trauma patients.* Shock, 2012. **37**(3): p. 276-81.
120. Lang, J.D., Jr., et al., *Inhaled NO accelerates restoration of liver function in adults following orthotopic liver transplantation.* J Clin Invest, 2007. **117**(9): p. 2583-91.
121. Donadee, C., et al., *Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion.* Circulation, 2011. **124**(4): p. 465-76.
122. Gelderman, M.P., et al., *Serial oxygen equilibrium and kinetic measurements during RBC storage.* Transfus Med, 2010. **20**(5): p. 341-5.
123. Dalsgaard, T., U. Simonsen, and A. Fago, *Nitrite-dependent vasodilation is facilitated by hypoxia and is independent of known NO-generating nitrite reductase activities.* Am J Physiol Heart Circ Physiol, 2007. **292**(6): p. H3072-8.
124. Ormerod, J.O., et al., *The role of vascular myoglobin in nitrite-mediated blood vessel relaxation.* Cardiovasc Res. **89**(3): p. 560-5.
125. Gkoumassi, E., et al., *Hemolysis of red blood cells during processing and storage.* Transfusion, 2012. **52**(3): p. 489-92.
126. Kim-Shapiro, D.B., J. Lee, and M.T. Gladwin, *Storage lesion: role of red blood cell breakdown.* Transfusion. **51**(4): p. 844-51.
127. Reynolds, J.D., D.T. Hess, and J.S. Stamler, *The transfusion problem: role of aberrant S-nitrosylation.* Transfusion. **51**(4): p. 852-8.
128. Roback, J.D., et al., *Insufficient nitric oxide bioavailability: a hypothesis to explain adverse effects of red blood cell transfusion.* Transfusion. **51**(4): p. 859-66.
129. van de Watering, L., *Red cell storage and prognosis.* Vox Sang, 2011. **100**(1): p. 36-45.
130. Vandromme, M.J., G. McGwin, Jr., and J.A. Weinberg, *Blood transfusion in the critically ill: does storage age matter?* Scand J Trauma Resusc Emerg Med, 2009. **17**: p. 35.
131. Mangalmurti, N.S., et al., *Loss of red cell chemokine scavenging promotes transfusion-related lung inflammation.* Blood, 2009. **113**(5): p. 1158-66.
132. Baron, D.M., et al., *Pulmonary hypertension in lambs transfused with stored blood is prevented by breathing nitric oxide.* Anesthesiology. **116**(3): p. 637-47.
133. Yu, B., et al., *Diabetes augments and inhaled nitric oxide prevents the adverse hemodynamic effects of transfusing syngeneic stored blood in mice.* Transfusion. **52**(7): p. 1410-22.
134. Winslow, R.M. and M. Intaglietta, *Red cell age and loss of function: advance or SNO-job?* Transfusion, 2008. **48**(3): p. 411-4.
135. Alayash, A.I., *Haptoglobin: old protein with new functions.* Clin Chim Acta. **412**(7-8): p. 493-8.
136. Schaer, D.J., et al., *CD163 is the macrophage scavenger receptor for native and chemically modified hemoglobins in the absence of haptoglobin.* Blood, 2006. **107**(1): p. 373-80.
137. Sakai, H., et al., *NO and CO binding profiles of hemoglobin vesicles as artificial oxygen carriers.* Biochim Biophys Acta, 2008. **1784**(10): p. 1441-7.

138. Wang, D., et al., *Transfusion of older stored blood and risk of death: a meta-analysis.* Transfusion, 2012. **52**(6): p. 1184-95.
139. Weinberg, J.A., S.R. Barnum, and R.P. Patel, *Red blood cell age and potentiation of transfusion-related pathology in trauma patients.* Transfusion, 2011. **51**(4): p. 867-73.
140. Wagner, S.J., et al., *Research opportunities in optimizing storage of red blood cell products.* Transfusion, 2014. **54**(2): p. 483-94.
141. Baek, J.H., et al., *Hemoglobin-driven pathophysiology is an in vivo consequence of the red blood cell storage lesion that can be attenuated in guinea pigs by haptoglobin therapy.* The Journal of clinical investigation, 2012. **122**(4): p. 1444-58.
142. Francis, R.O., et al., *Glucose-6-phosphate dehydrogenase deficiency in transfusion medicine: the unknown risks.* Vox sanguinis, 2013.
143. Gladwin, M.T. and D.B. Kim-Shapiro, *Storage lesion in banked blood due to hemolysis-dependent disruption of nitric oxide homeostasis.* Curr Opin Hematol, 2009. **16**(6): p. 515-23.
144. Belizaire, R.M., et al., *Microparticles from stored red blood cells activate neutrophils and cause lung injury after hemorrhage and resuscitation.* J Am Coll Surg, 2012. **214**(4): p. 648-55; discussion 656-7.
145. Larsen, R., et al., *A central role for free heme in the pathogenesis of severe sepsis.* Sci Transl Med, 2010. **2**(51): p. 51ra71.
146. Janz, D.R., et al., *Association between haptoglobin, hemopexin and mortality in adults with sepsis.* Crit Care, 2013. **17**(6): p. R272.
147. Schaer, D.J., et al., *Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins.* Blood, 2013. **121**(8): p. 1276-84.
148. Stapley, R., et al., *Erythrocyte storage increases rates of NO and nitrite scavenging: implications for transfusion-related toxicity.* Biochem J, 2012. **446**(3): p. 499-508.
149. Liu, C., et al., *Mechanism of faster NO scavenging by older stored red blood cells.* Redox Biol, 2014. **2**: p. 211-9.
150. Alexander, J.T., et al., *Red blood cells stored for increasing periods produce progressive impairments in nitric oxide-mediated vasodilation.* Transfusion, 2013. **53**(11): p. 2619-28.
151. Zwart, A., et al., *Multicomponent analysis of hemoglobin derivatives with reversed-optics spectrophotometer.* Clin Chem, 1984. **30**(3): p. 373-9.
152. Piknova, B., et al., *The reaction of cell-free oxyhemoglobin with nitrite under physiologically relevant conditions: Implications for nitrite-based therapies.* Nitric Oxide, 2009. **20**(2): p. 88-94.
153. Zimring, J.C. and S.L. Spitalnik, *On the appropriate use and interpretation of animal models in transfusion medicine research.* Transfusion, 2013. **53**(10): p. 2334-9.
154. Kurach, J.D., et al., *Segments from red blood cell units should not be used for quality testing.* Transfusion, 2014. **54**(2): p. 451-5.

155. Hu, X., et al., *The level of complement activation fragments is higher in red blood cell units than segments.* Transfus Apher Sci, 2013. **49**(3): p. 692-3.
156. Weinberg, J.A., et al., *The deleterious effect of red blood cell storage on microvascular response to transfusion.* J Trauma Acute Care Surg, 2013. **75**(5): p. 807-12.
157. Nicholson, S.E., et al., *Transfusion-related acute lung injury in a rat model of trauma-hemorrhage.* J Trauma, 2011. **70**(2): p. 466-71.
158. Lei, C., et al., *Inhaled nitric oxide attenuates the adverse effects of transfusing stored syngeneic erythrocytes in mice with endothelial dysfunction after hemorrhagic shock.* Anesthesiology, 2012. **117**(6): p. 1190-202.
159. Bennett-Guerrero, E., et al., *Randomized study of washing 40- to 42-day-stored red blood cells.* Transfusion, 2014.
160. Cauwels, A., et al., *Nitrite protects against morbidity and mortality associated with TNF- or LPS-induced shock in a soluble guanylate cyclase-dependent manner.* J Exp Med, 2009. **206**(13): p. 2915-24.
161. Pickerodt, P.A., et al., *Sodium nitrite mitigates ventilator-induced lung injury in rats.* Anesthesiology, 2012. **117**(3): p. 592-601.
162. !!! INVALID CITATION !!!
163. Yu, M., et al., *HMGB1 signals through toll-like receptor (TLR) 4 and TLR2.* Shock, 2006. **26**(2): p. 174-9.
164. Brennan, T.V., et al., *Heparan sulfate, an endogenous TLR4 agonist, promotes acute GVHD after allogeneic stem cell transplantation.* Blood, 2012. **120**(14): p. 2899-908.
165. Okamura, Y., et al., *The extra domain A of fibronectin activates Toll-like receptor 4.* J Biol Chem, 2001. **276**(13): p. 10229-33.
166. Yazji, I., et al., *Endothelial TLR4 activation impairs intestinal microcirculatory perfusion in necrotizing enterocolitis via eNOS-NO-nitrite signaling.* Proc Natl Acad Sci U S A, 2013. **110**(23): p. 9451-6.
167. Piomelli, S. and C. Seaman, *Mechanism of red blood cell aging: relationship of cell density and cell age.* Am J Hematol, 1993. **42**(1): p. 46-52.
168. Dumont, L.J. and J.P. AuBuchon, *Evaluation of proposed FDA criteria for the evaluation of radiolabeled red cell recovery trials.* Transfusion, 2008. **48**(6): p. 1053-60.
169. Tarasev, M., et al., *Similar donors-similar blood?* Transfusion, 2014. **54**(3 Pt 2): p. 933-41.