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Molecular mechanisms of erythrocyte aging

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Abstract

Anemia and hemorrhagic shock are leading causes of morbidity and mortality worldwide, and transfusion of human blood products is the ideal treatment for these conditions. As human erythrocytes age during storage in blood banks they undergo many biochemical and structural changes, termed the red blood cell ‘storage lesion’. Specifically, ATP and pH levels decrease as metabolic end products, oxidative stress, cytokines, and cell-free hemoglobin increase. Also, membrane proteins and lipids undergo conformational and organizational changes that result in membrane loss, viscoelastic changes and microparticle formation. As a result, transfusion of aged blood is associated with a host of adverse consequences such as decreased tissue perfusion, increased risk of infection, and increased mortality. This review summarizes current research detailing the known parts of the erythrocyte storage lesion and their physiologic consequences.

Keywords

blood banking; review; storage lesion

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Introduction

Hemorrhagic shock is the second-leading overall cause of early death as well as the most common cause of potentially preventable death in traumatically injured patients (Kauvar et al., 2006). In order to ameliorate this shock state, patients must undergo resuscitation in order to restore circulatory volume, reverse metabolic acidosis, and attenuate cellular hypoxia. Although the ideal resuscitation strategy remains controversial, this process commonly involves infusion of crystalloid salt solutions as well as the transfusion of human blood products (Holcomb et al., 2013). The failure to achieve specific endpoints of resuscitation is associated with an increased incidence of multiple organ failure and death (Brakenridge et al., 2011).

Several studies now suggest that transfusion of human blood products, including the use of stored red blood cell units (pRBCs), is the ideal treatment of hemorrhagic shock in the acute setting (Holcomb et al., 2007; Makley et al., 2010a; Holcomb and Pati, 2013; Schorn and Phillippi, 2014). Unfortunately, this use of blood products may result in later harm to the patient. Liberal transfusion strategies for pRBC have been associated with poor clinical outcomes and increased mortality in critically ill patients (Hébert et al., 1999; Vincent et al., 2002; Corwin et al., 2004; Gong et al., 2005; Holst et al., 2014; Robertson et al., 2014). The etiology of this harm is unclear, but it is thought that a combination of transfusion of larger volumes of blood (Sauaia et al., 1994), as well as use of older pRBCs (Weinberg et al., 2008, 2010; Mangalmurti et al., 2009), drive this effect. Due to these concerns, understanding the harm caused by transfusion of pRBC units has become an area of intense study in the care of trauma patients. Unfortunately, most of the literature regarding the adverse effects of transfusing aged pRBCs is retrospective and observational, and thus our understanding of the true effect of the age of pRBCs at the time of transfusion will remain limited until prospective trials are completed.

Standard blood banking inventory management relies on a first in, first out system whereby the oldest viable pRBC units are often used first (Koch et al., 2013). Thus, the average age of transfused pRBCs ranges from 20 to 30 days (Shapiro et al., 2003; Weinberg et al., 2008; Brown et al., 2014). As the life span of erythrocytes is 120 days, each pRBC transfused is a homogenous collection of cells in various phases of the aging process. This means that patients, when transfused, may be exposed to erythrocytes present in pRBCs that range in age from 42 to 162 days in chronological age.

As pRBCs age, they develop changes in biochemical and molecular parameters. Collectively, these changes are known as the red blood cell or erythrocyte ‘storage lesion’ (Koch et al., 2013). Compared to fresh units, transfusion of aged pRBCs has been associated with increased rates of pneumonia, sepsis, multi-organ failure, and mortality (Purdy et al., 1997; Zallen et al., 1999; Offner et al., 2002; Koch et al., 2008; Weinberg et al., 2008). In this review we summarize storage methods of pRBCs, the various molecular components of the red cell storage lesion that result from that storage, as well as the clinical effects that result from transfusion of aged blood.

Blood collection and storage

Human blood is collected from donors as whole blood directly into a solution anticoagulant citrate and nutrient phosphate and dextrose, or by apheresis into a solution of acid citrate dextrose (Hess, 2010a). Blood collected by apheresis is filtered free of most leukocytes and platelets. Whole blood collections are centrifuged to separate the erythrocytes from the anticoagulant solution, plasma, and buffy coat containing leukocytes and platelets. One method, the buffy coat method, involves draining the erythrocytes from the bottom of the collection bag and leaving the buffy coat and plasma behind. The other method requires a gentler centrifugation, which leaves the platelets suspended in the plasma. The platelet-rich plasma is removed and the remaining cells are passed through a leukocyte reducing filter that removes most leukocytes and platelets. The importance of this process, called leukoreduction, is a matter of debate in the transfusion community, and will be discussed in more detail later.

The concentrated erythrocytes are then mixed with a storage solution and stored at 1–6°C for up to 42 days (Wehrli, 2012). The first pRBC additive solution, developed in the late 1970s, contained saline-adenine-glucose (SAG) (Sparrow, 2012). Multiple variations of this formula have been developed in the years since. These additive solutions (AS) all contain membrane protectant sugars, volume for dilution of metabolic waste products, and nutrients, especially adenine (Cancelas et al., 2014). Three of these solutions have been approved for use in the United States (AS-1, AS-3, AS-5), while several others are in use around the world (SAGM, MAP, PAGGSM) (Sparrow, 2012). These storage solutions aid in the preservation of erythrocytes during storage, but aging and degradation still occurs in a predictable fashion, which is the focus of the current review.

Erythrocyte storage lesion

Biochemical parameters

The classic biochemical characteristics of the pRBC storage lesion include decreased ATP, 2,3-diphosphoglycerate, and pH, along with increased potassium, free hemoglobin, and lactate (Bennett-Guerrero et al., 2007; Hess, 2010b). Potassium and other ion imbalances result from the inactivation of membrane pumps during cold storage (Flatt et al., 2014). Erythrocytes lack mitochondria, and thus depend on glycolysis for energy. As erythrocytes age in storage, they metabolize glucose and generate lactate and protons as end products. These initial changes are seen rapidly, often in the first 2 weeks of cold storage (Karon et al., 2009). Excessive 2,3-diphosphoglycerate interacts with membrane proteins, causing structural changes, and also impacts the erythrocyte’s ability to deliver oxygen to the tissues (Tsai et al., 2004; Flatt et al., 2014). While these levels are normalized after transfusion, it takes up to 1 week for a full recovery (Solheim et al., 2004). Stored pRBCs also generate proinflammatory cytokines that have been implicated in subsequent tissue injury after transfusion (Seghatchian, 2006; Belizaire et al., 2012a; Matot et al., 2013).

Complement anaphylatoxins C3a and C5a, as well as the membrane attack complex (MAC), are also found in stored pRBCs (Solheim et al., 2003; Seghatchian, 2006). MAC levels increase as a function of time and have been suggested as the primary complement-mediated

contributor to the storage lesion (Hu et al., 2014). The bolus of complement complexes received by a patient during transfusion of aged pRBCs likely leads to the activation of a number of host cell types, including endothelial cells, B cells, and circulating erythrocytes (Stowell et al., 2012), resulting in substantial complement-mediated inflammatory processes (Weinberg et al., 2011).

Morphology

During storage, the erythrocyte structural proteins, lipids, and carbohydrates undergo oxidative injury (Hess, 2010b). There is a time-dependent reduction in glutathione and glutathione-peroxidase activity (Dumaswala et al., 1999), resulting in a sensitivity to oxidation that increases with time of storage (Şekeroğlu et al., 2012). Oxidation of fatty acids results in lipid peroxidation and increased levels of malondialdehyde, which has known cytotoxic effects and can cross-link erythrocyte membrane phospholipids and proteins (Şekeroğlu et al., 2012). Formation of the spectrin-actin-protein 4.1 complex decreases during storage (Wolfe et al., 1986). Band 3, usually freely mobile, aggregates into large oligomers that may result in phospholipid loss (Kriebardis et al., 2007; Karon et al., 2009). This is the result of both protein loss and oxidative cross-linking.

The cumulative result of these changes is that aging erythrocytes lose membrane domain as well as their classic biconcave disc shape, subsequently changing to echinocytes and spherocytes with a loss of their normal deformability (Figure 1) (Izzo et al., 1999; Bennett-Guerrero et al., 2007; Relevy et al., 2008; Karon et al., 2009; Hess, 2010b). Phosphatidylserine, normally on the intracellular side of the plasma membrane, becomes externalized (Hess, 2010b). These membrane changes and increased fragility contribute to increased acidosis and hemolysis observed during the storage of pRBCs, as well as decreased erythrocyte survival following transfusion (Haradin et al., 1969; Berezina et al., 2002; Bennett-Guerrero et al., 2007). A summary of common alterations to erythrocytes during storage can be found in Table 1.

Microparticles

During storage, pRBCs develop erythrocyte-derived microparticles (MPs) (Xiong et al., 2011; Gao et al., 2013). MPs are sub-cellular particles that range in size from 0.1 to 1.0μm (Lacroix et al., 2010). Although the process of MP formation is incompletely understood, it has been linked to spectrin oxidation (Wagner et al., 1987) as well as ATP depletion (Lutz et al., 1977). Erythrocyte-derived MPs contain hemoglobin, lipids, band 3, glycophorins, actin, lipid raft proteins, caspases, Fas, and extracellular phosphatidylserine, but lack spectrin and ankyrin (Greenwalt et al., 1984; Kriebardis et al., 2008; Koshiar et al., 2014). Stomatin has been implicated in the formation of storage-associated MPs (Salzer et al., 2008; Bosman et al., 2012), suggesting a raft-mediated mechanism of MP formation. The band 3 in MPs shows age-related aggregation and degradation (Bosman et al., 2012). There is also an age-related recruitment of proteins from the plasma, ubiquitin-proteasome system, and small G proteins to MPs (Bosman et al., 2012).

Experimental findings support the hypothesis that MP formation is part of a self-protective mechanism whereby damaged proteins or membrane patches are selected for removal in

order to preserve the health of the remaining erythrocyte (Willekens et al., 2008; Bosman et al., 2012). This concept is further supported by the finding that red cell exchange, a procedure for treating patients with complicated sickle cell disease, results in a specific decrease in the number of erythrocyte-derived MPs in the circulation (Mahfoudhi et al., 2012). MP formation from circulating erythrocytes is increased in patients with hemoglobinopathies (Westerman et al., 2008), and by substituting normal erythrocytes for genetically abnormal erythrocytes, MP formation is reduced.

Evaluation of pRBC MPs via flow cytometry, the most common technique utilized in the laboratory setting, is limited because of the small size of these particles (Lacroix et al., 2010). Using techniques such as nanoparticle tracking analysis and dynamic light scattering, the average size of circulating MPs has been determined to range from 100 to 300 nm (Lawrie et al., 2009; Aatonen et al., 2014). However, the lower limit of detection by flow cytometry is 488 nm (Yuana et al., 2011). As such, much of the existing literature characterizing these MPs can only be interpreted as a description of MPs >500 nm. By combining flow cytometry and other available assays it is possible to build a more complete picture regarding these particles, but we are still quite limited by the available technology when it comes to understanding this increasingly important field of study.

Sphingolipids

Sphingolipids are essential eukaryotic cell membrane components and play an important role in cell signaling (Aguilera-Romero et al., 2014). Sphingolipid metabolism appears to play an important role in many of the erythrocyte structural changes that may occur during aging, as shown by Dinkla et al. (2012). By incubating erythrocytes with varying doses of sphingomyelinase, they were able to induce formation of ceramide-enriched platforms, phosphatidylserine exposure, band 3 changes, and CD59 clustering. These changes resulted in decreased cell size, increased cell fragility, and MP formation. Further, because inflammation can increase sphingomyelinase secretion, they suggest this as a contributing mechanism for the anemia of chronic illness. Sphingolipids have also been implicated in membrane changes that occur in sickle cell disease (Awojoodu et al., 2014) and Gaucher disease (Adar et al., 2008). Ceramides have also been suggested to form pores in erythrocyte membranes (Hatakeyama et al., 1999). Finally, mass spectrometry studies have shown phospholipid interactions to be involved in the membrane budding process that drives MP formation (Bicalho et al., 2013). Lipids, specifically sphingolipids, are clearly important components of erythrocyte membrane dynamics involved in the aging process.

Physiologic effects and clinical consequences

Morphology

Under normal flow conditions, erythrocytes, usually 6–9 μm in diameter, are able to flex their discoid shape in order to squeeze through capillary vessels that are only 3–6 μm wide (Stadler and Linderkamp, 1989). The decreased deformability of aged erythrocytes leads to reduced capillary flow and decreased oxygen delivery to tissues (Hess, 2010b). These changes also negatively impact the survival of transfused erythrocytes (Card, 1988). As these damaged cells attempt to pass through capillary beds, they aggregate and break down,

resulting in hemolysis, microthrombosis, and occlusion of these tiny vessels. Aged erythrocytes have also been shown to have increased adhesion to endothelial cells (Anniss and Sparrow, 2006; Relevy et al., 2008), likely due to the increased phosphatidylserine on the external erythrocyte membrane (Hess, 2010b). This mechanism has been implicated in the microangiopathy and anemia associated with diabetes, sickle cell disease, and cancer (Klug et al., 1974; Cohen, 1981; Tsukada et al., 2001).

Free hemoglobin

Storage of pRBC units results in increased extracellular hemoglobin. This ‘cell-free hemoglobin’ is actually a combination of MP-bound and free floating hemoglobin and may have significant effects when transfused (Greenwalt et al., 1991). Under normal physiological conditions, nitric oxide (NO) is generated by endothelial nitric oxide synthase and plays a role in controlling blood flow by inducing relaxation of blood vessel smooth muscle (Palmer et al., 1987; Walford and Loscalzo, 2003). When present, cell-free hemoglobin scavenges nitric oxide (NO) at a significantly higher rate than erythrocyte-encapsulated hemoglobin (Vaughn et al., 2000; Azarov et al., 2005). Erythrocyte-derived MPs also contain large concentrations of hemoglobin and play a role in NO consumption (Donadee et al., 2011; Liu et al., 2013). NO scavenging by stored blood supernatant increases with length of storage, and is directly proportional to heme concentration (Donadee et al., 2011). Cell-free hemoglobin plays a role in ROS formation (Donadee et al., 2011), and NO uptake is also affected by the oxygenation status of hemoglobin (Azarov et al., 2005).

The cumulative impact of free hemoglobin in the pRBC unit is that transfusion may result in significant NO scavenging and reduction in the recipient. This reduction in NO is thought to impede endothelial-dependent vasodilation and subsequently affect end-organ perfusion (Bennett-Guerrero et al., 2007; Reynolds et al., 2007; Rigamonti et al., 2008; Neuman et al., 2014). The resultant impaired vasodilation may have a significant clinical impact. Decreased cerebral perfusion may be a reason that cardiac surgery patients receiving older pRBCs are at an increased risk for post-operative delirium (Brown et al., 2014). In a rat model of hemorrhage shock, transfusion of aged pRBCs compared to fresh pRBCs has negative effects on liver perfusion and necrosis (Matot et al., 2013). Baek and coworkers showed that transfusion of aged pRBCs with haptoglobin reduces intravascular hemolysis, acute hypertension, vascular injury, and kidney dysfunction associated with transfusion of cell-free hemoglobin (Baek et al., 2012). Similarly, Baron et al. demonstrated an increase in pulmonary artery pressure following transfusion of old blood, which they were able to mitigate with inhaled NO (Baron et al., 2012). By either scavenging the free hemoglobin or replacing NO it may be possible to ameliorate the negative effects of transfusing aged pRBCs.

One benchmark for pRBC storage is the survival of 75% of erythrocytes up to 24 h after transfusion (Dumont and AuBuchon, 2008). Given that one pRBC contains 220–250 mg of iron, transfusion recipients must rapidly clear greater than 50 mg of iron per pRBC transfused (Ozment and Turi, 2009). Hod et al. have described the effects of non-transferrin-bound iron in both animal models and human trials (Hod et al., 2011, 2010). Following

transfusion, iron deposition is visibly evident in the liver, spleen, and kidney. This free iron leads to increased systemic inflammation and has been linked to increased infection susceptibility, specifically *Escherichia coli* proliferation. This mechanism is likely similar to the increased infection susceptibility seen in hemochromatosis patients (Khan et al., 2007). Clinical studies have correlated age of pRBCs transfused, as well as number of units transfused, with infectious complications (Mynster and Nielsen, 2000; Horvath et al., 2013). Indeed, patients receiving multiple transfusions are also getting a bolus of cell-free hemoglobin, and this appears to have multiple negative consequences.

Microparticles

Transfusion of aged pRBCs has been clinically associated with increased incidence of deep vein thrombosis (Spinella et al., 2009). Elevated levels of erythrocyte-derived MPs in patients have been associated with increased thrombin formation and complement activation (Jy et al., 2013; Koshiar et al., 2014; Zecher et al., 2014). In animal models, transfusion of aged pRBCs causes increased coagulopathy compared to fresh pRBCs (Vlaar et al., 2010). MPs from aged pRBCs have been shown *in vitro* to induce thrombin generation, potentially due to increased phosphatidylserine expression, possibly in a factor XII-dependent manner (Sweeney et al., 2009; Van Der Meijden et al., 2012; Gao et al., 2013). Coagulopathies associated with microparticles, including those from erythrocytes, are well described and cause significant morbidity.

Transfusion of stored blood in cardiac surgery patients is associated with increased rates of pneumonia (Horvath et al., 2013), and MPs in stored blood may play a role. Aged pRBCs cause increased lung microvascular permeability and neutrophil migration compared to fresh pRBCs (Mangalmurti et al., 2009). This finding is either the result of MP accumulation in aged blood (Belizaire et al., 2012a,b) or other factors in the pRBC supernatant (Silliman et al., 1998). In mouse and rat models, washing aged pRBCs prior to transfusion resulted in diminishing the lung injury (Vlaar et al., 2010; Belizaire et al., 2012a). Initially, the injurious substance was thought to be cytokines or other cell-signaling proteins. However, recent evidence has indicated that MPs are a significant cause of lung inflammation (Belizaire et al., 2012b).

Leukoreduction

The issue of leukoreduction is a hotly debated topic. Blood banks in Canada and most of Europe routinely leukoreduce pRBCs prior to storage, whereas practice patterns in the United States vary. Basic science evidence suggests that leukoreduction improves viscoelastic properties of stored pRBCs as well as reduces immunoglobulins, complement proteins, and cytokines that are generate during storage (Solheim et al., 2003; Seghatchian, 2006; Nagura et al., 2013; Sowemimo-Coker, 2014). From a theoretical standpoint, leukocytes in stored blood are more biologically active than erythrocytes and are responsible for the deleterious production of inflammatory proteins that drive the storage lesion. Thus, by banking blood that has been cleansed of leukocytes, the storage lesion will be greatly improved.

The Society of Thoracic Surgeons and the Society of Cardiovascular Anesthesiologists clinical practice guidelines in 2011 recognizes level IIa evidence supporting the use of leukoreduced blood, specifically for patients undergoing cardiac procedures (Ferraris et al., 2011). In clinical studies, leukoreduction has been shown to reduce infectious complications of pRBC transfusion (Fergusson et al., 2004; Blumberg et al., 2007; Friese et al., 2008). Leukoreduction has also been associated with improved mortality (van de Watering et al., 1998; Hébert et al., 2003), decreased length of stay (Fung et al., 2006), fewer febrile transfusion reactions (Hébert et al., 2003; King et al., 2004), and reduced rates of acute respiratory distress syndrome (Plurad et al., 2008) in transfusion recipients. However, there are several clinical studies that show no difference in outcomes with leukoreduction (Sharma et al., 2002; Wallis et al., 2002; Llewelyn et al., 2004; Nathens et al., 2006; Capraro et al., 2007; Phelan et al., 2007; Frietsch et al., 2008; Watkins et al., 2008). As no data suggest that leukoreduction is more harmful than not performing leukoreduction, implementation of practice may revolve cost-effectiveness (Cleemput et al., 2006), as the cost of leukoreducing one unit of pRBCs is between $20 and $40 (van Hulst et al., 2005). While historic opinion has been to purify pRBCs as much as possible to avoid patient harm, further research is necessary to determine when a specific benefit exists for leukoreducing blood for storage. Additional work is needed in this area of controversy as stronger basic and clinical evidence would support universal leukoreduction. If universal leukoreduction is not necessary, the specific population of transfusion recipients who would benefit from leukoreduction needs to be defined.

Conclusion

The ability to acquire, store, and transfuse human blood products is absolutely necessary in modern medicine, especially for the treatment of hemorrhage and anemia. Much progress has been made in understanding the erythrocyte storage lesion and its clinical implications. As erythrocytes age, in storage as well as in circulation, they undergo a variety of biochemical and structural changes. In the body, aging cells are targeted for elimination before becoming harmful. However, upon transfusion, millions of aged and senescent erythrocytes are introduced into a patient who is already under physiologic stress. Subsequently, the burden of acidic, hemolytic, and inefficient erythrocytes can create problems for the new host, such as increased susceptibility to infection and impaired end-organ perfusion. pRBC aging and storage will continue to be a fervent area of research until blood banks around the world are able to adequately meet the needs of their patient populations.

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Biography

From left to right: Richard Hoehn, Peter Jernigan, and Alex Chang are surgical residents at the University of Cincinnati College of Medicine, currently participating in a research fellowship. Their areas of research interest include blood banking, trauma, and sphingolipids. Timothy Pritts is a trauma surgeon at the University of Cincinnati, with research interests including blood banking, shock and resuscitation, lung injury, and traumatic brain injury. Michael Edwards is the chairman of surgery at the University of Cincinnati, with specific research interests including sphingolipids and translational medicine, and is also an avid car racing enthusiast.

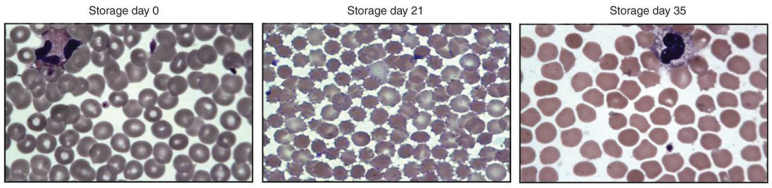

Figure 1.

Light microscopy images demonstrating changes in human erythrocyte morphology during storage. pRBCs were stored in AS-3 at 4°C. Over time, erythrocytes develop loss of biconcave disc shape, subsequently changing to echinocytes and spherocytes. Images adopted from Makley et al. (2010b).

Table 1

Characteristics of the erythrocyte storage lesion.

Biochemical	Structural
↓TP	↓membrane domain
↓2,3 diphosphoglycerate	↓deformability
↓pH	↑microparticles
↓nitric oxide	↑spherocytosis
↑potassium	↑hemolysis
↑free hemoglobin	↑band 3 oligomers
↑lactate	↑ceramide rafts
↑cytokines	↑external phospatidylserine
↑reactive oxygen species	↑aggregability
↑soluble lipids	↑osmotic fragility
↑complement proteins	↑protein cross-linking