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Storage Time and Interdonor Variability's Effect on Red Blood Cell Storage Lesion

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Storage Time and Interdonor Variability’s Effect on Red Blood Cell Storage Lesion

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Bioengineering by Shawn Raymond Mailo

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2014
The thesis of Shawn Raymond Mailo is approved, and it is acceptable in quality and form for publication on microfilm:

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University of California, San Diego

2014
For my parents, sister, and friends for always unconditionally supporting me and my dreams
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Abbreviations and Symbols

RBC – Red Blood Cell

WBCs – White blood cells

HIV – Human immunodeficiency virus

TRALI – Transfusion Related Acute Lung Injury

ATP – Adenosine triphosphate

2,3 DPG – 2,3-Disphosphoglycater

Hb – Hemoglobin

K+ – Potassium

P50 – Partial pressure of oxygen needed to 50% saturate red blood cells

β – Filter perfusion resistivity

Pl – Initial rise in filtration pressure for blood solution

Po – Initial rise in filtration pressure for saline

V – MCV/FPV

MCV – Mean corpuscular volume

FPV – Filter pore volume

h – Hematocrit

SD – Standard Deviation
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ABSTRACT OF THE THESIS

Storage Time and Interdonor Variability’s Effect on
Red Blood Cell Storage Lesion

by

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Master of Science in Bioengineering

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Professor Pedro Cabrales, Chair

Transfusion medicine is dependent upon the ability to hypothermically preserve red blood cell (RBC) function ex vivo. Since the 1950’s, expiration dates have been utilized to determine the viability of stored blood for transfusions. These expiration dates were based on the ability of stored RBCs to survive once inside a transfusion recipient. However, recent evidence suggests that the cell parameters that influence rheology, oxygen transport, and metabolism of hypothermically stored RBCs may be compromised, potentially leading to microvasculature occlusion and tissue hypoxia in transfused patients. Using storage time as a sole determinant of RBC quality ignores these physiological changes and the variability of these cell properties that may depend on other factors other than age. The main objectives of this thesis were to
observe physiological trends of RBCs during storage, the interdonor variability of these changes, and to evaluate correlations between various cell properties. This thesis demonstrates that hypothermic storage leads to RBC rheological impairments, affected by changes in deformability and aggregation, which can be detected using a filter perfusion technique. Significant changes were also found in oxygen transport and cell metabolism. In addition, this thesis has shown interdonor variability in all of the RBC parameters that were tested; however, variability in all biochemical analytes was minimal and may not be physiologically significant. Through these investigations, this thesis has contributed to the advancement of the fields of transfusion medicine and biopreservation science.
1. Introduction

According to the 2011 National Blood Collection and Utilization Report about 15 million units of blood are transfused each year in the United States. These 15 million bags of blood are transfused into 5.4 million patients; over 50,000 of which experience adverse side effects from the transfusion \[1\]. Cases of adverse effects from bad blood transfusions such as microcirculatory complications, TRALI, inflammation, organ failure, and even mortality show a direct link to red blood cell (RBC) storage lesions such as decreased ability to deliver oxygen to tissues and organs, as well as increased hemolysis (cell death) of the transfused blood \[2\]. Current blood transfusion standards set by the Food and Drug Administration limit blood storage to a maximum of 42 days, deeming older blood unsuitable for transfusions. However, this expiration criterion dates back several decades and derives from previous research studies involving a controversial method of analysis which assessed blood’s shelf life based on the average age of blood that preserved 75% of the transfused blood in the body 24 hours after injection. Some studies show little difference between the effectiveness of blood stored for 20 days compared to blood stored at 42+ days whereas other researchers cite cases of blood stored for significantly less than 42 days that were also unsuitable for transfusion \[3\]. Clearly there are several conflicting data points; the inconsistencies in relating RBC survival rate to the number of days stored demonstrates the method of predicting the quality of blood remains unresolved.

A better understanding of RBC storage lesions will be immensely beneficial in studying its clinical effects on patients and help contribute information to the topic of
recent debate. Current studies are beginning to use time-independent metrics to predict the survival rate of transfused blood based on findings that suggest inter-donor variability as a factor of the rate of storage lesion. Rheological changes in shape, deformability, and aggregability makeup a large portion of storage lesions RBCs experience during storage. One innovative study correlated RBCs deformability with its ability to perfuse (an aggregate metric of deformability, aggregation, and shape) through a micro pore filter. This correlation proved to be high throughput and less expensive than comparable standard deformability tests such as aspiration and ektacytometry [4]. Biochemical changes like ATP, Lactate, Hemoglobin, Potassium, and 2,3-DPG levels are believed to account for the remaining segments of storage lesions. Although studies have shown how these biochemicals change over time, it has not examined whether there are significant interdonor variabilities present.

Further investigation of RBC rheological and biochemical parameters has great potential to improve blood storage and lead to greater understanding of RBC storage lesion. This study aims to prove the recent emergence of the idea that higher quality red blood cell transfusions may lead to better patient treatments, thereby reducing both adverse effects and the overall cost of healthcare. Likewise, the foundation of higher quality blood rests on the promise of certain physiological qualities and these studies could help determine that each individual donor’s blood deteriorates at different rates over time.
Specific Aims

1. Observe physiological trends of RBCs during storage (Rheology & Biochemical)
2. Investigate interdonor variability of changes in RBC physiology during storage
3. Evaluate correlation between physiological changes in RBCs during storage

The culmination of these blood studies would be of utmost importance to blood banks and hospitals which are primarily interested in better understanding the complexity of the biochemical and biomechanical changes of a RBC through storage and how they affect the success and safety of blood transfusions. Based on biochemical and rheological changes through storage, inter-donor deterioration differences could debunk the current time dependent expiration date entirely, possibly proving the previous measurement method to be insufficient and costly in relation to bad blood transfusions. The data from this experiment will serve for substantial future development for new standards in blood quality and high throughput medical devices to test the characteristics of blood.
2. Background

2.1 Red Blood Cells: Structure and Components

On average each human contains around 4-6 liters of blood and is about 8% of the body’s total weight [5]. Human blood consists of three major components: Plasma, Red Blood Cells, and Platelets. In order of volume content 55% of blood is plasma, 40-44% is RBCs, and the remaining 0-5% consists of WBCs and platelets [5]. Blood is the body’s main transport system, helping maintain homeostasis in multiple ways. Blood picks up food from the digestive system, oxygen from the respiratory system, and delivers these through the body. As blood delivers nutrients and oxygen, it also picks up waste products and delivers it to the liver and kidneys which act as filters, cleaning the blood. Furthermore, blood is the main transport mechanism for hormones, enzymes, buffers, antibodies, and other biochemical substances. Lastly, blood is a great regulator of the body’s temperature due to its high specific heat and conductivity. Blood’s oxygen transport capabilities come from the Red Blood Cells. As RBCs travel the circulatory system these cells pick up $O_2$ from the lungs and release it to the body’s tissues when squeezing through capillaries. The secondary functions of RBCs include vasodilation and vasoconstriction through selective release of ATP, Nitric Oxide, S-nitrosothiols, and hydrogen sulfide [5]. Vasodilation and vasoconstriction is important because it aids the adjustment of blood flow through the body. Mature Red Blood Cells have an oval biconcave disk shape and are approximately 6-8um in diameter [5]. Unlike most cells in the body, mature RBCs do not contain a nucleus, ribosomes, mitochondria, and other organelles. RBCs red color comes from its primary component hemoglobin, a molecule
comprised of 4 globin protein chains, 4 red heme pigments, and 4 iron atoms [5]. 200-300 million hemoglobin molecules are contained in one RBC, making up 95% of a RBCs dry weight [5]. Oxygen gets transferred through the circulatory system by attaching to these hemoglobin molecules. The biconcave shape of the RBC provides a large surface area aiding in this transfer of oxygen. Other than attributing to the biconcave shape, the RBC membrane also plays a large role in regulating blood type, cell deformability, and adhesion. Special RBC membrane proteins have antigens attached to them which govern the blood type of the person - most widely known are the A, B, and Rh antigens. Membrane skeletal proteins, via tensegrity, allow RBCs to squeeze through capillaries that are < 2um wide (less than half of the diameter of an RBC) and form back to its original shape after leaving the compressive microvessels. These proteins work in conjunction with spectrin, stretchable cytoskeletal proteins that adhere to the inside of the RBC plasma membrane [5]. Being able to change shape is required for RBC survival due to repetitive shearing forces and bursting strains. Other membrane proteins control cell adhesion, often to other cells or to the endothelium. Both adhesion and deformability are important because they greatly influence microcirculation speeds.

2.2 Current State of Blood Storage

Through extensive research conducted since the early 1900s, packed red blood cells are now able to be stored up to 35 - 49 days at 4°C, depending on which additive solution is mixed with the blood [6]. The current process of blood donation and transfusion begins with screening donors for criteria such as: intercourse with an HIV-positive person, traveling outside the country, or personal history of infectious diseases.
Donors who pass the initial screening then donate blood. At this point all donated blood is tested for syphilis, hepatitis, HIV, and other possibly hosted diseases [7]. From the disease free units of blood, RBCs are then centrifuged and leukoreduced into units of packed RBCs. This is performed because leukocytes were found to cause adverse effects to patients post transfusion and increase oxidative hemolysis in RBCs during storage. The units of packed RBCs are then checked for blood group and mixed with preservatives and anticoagulant. The unit of packed RBCs is then labeled indicating blood component, blood group, additive solutions, and expiration date [8]. The expiration date is based on two requirements that were made in the 1950s. The first requirement states that at the end of the storage period, hemolysis can be no higher than 1% of stored RBCs. The second requirement states that 75% of the transfused RBCs must remain present in circulation after 24 hours of transfusion [3]. This is tested by radioactive labeling of the transfused RBCs and analyzing a blood sample from the transfusion patients after 24 hours of being infused. Before transfusion the stored blood is crossmatched with the patient’s blood to avoid hemolytic reaction due to mismatched blood groups [8].

2.3 Shortcomings of Blood Storage

Despite all the precautions and procedures taken to preserve RBC viability, the blood storage process still has flaws [9]. Although storing blood at 4°C is intended to slow down RBC metabolism and the accumulation of harmful waste products, blood storage does not stop the metabolic process completely. As RBC metabolism and waste production progresses in storage, an array of changes in the cell start to happen, often
known as storage lesions. These changes are a result of normal RBC aging and alterations in the processes that are in charge of normal RBC homeostasis. Storage lesions have been widely researched and documented in many papers in order to shed light on what happens to blood during storage [3, 10, 11, 12, 13, 14]. Typically storage lesions in RBCs are categorized as either biochemical or biomechanical changes. However, these categories can be very broad and are typically interrelated to each other.

### 2.4 Biochemical Changes

Although biochemical changes do not directly affect the normal functions of RBCs, it is the downstream cellular processes that are altered due to these biochemical changes. Adenosine Triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) are the two major chemicals that govern the functionality of the RBC that drastically change during storage [10]. ATP, often known as the “molecular currency of energy”, is a coenzyme used by cells to promote metabolism. During storage ATP begins depleting from RBCs. The decrease in ATP has been associated with less phosphorylation of cellular kinases and the breakdown of cationic ion pumps [11]. Furthermore it has been observed that low levels of ATP leads to alterations in both the cell membrane and cytoskeleton, ultimately affecting the cell’s morphology [12]. Rejuvenation solutions have been created to restore RBC ATP levels after storage. Although ATP levels can be restored, some storage lesions linked to ATP are irreversible and cannot be fixed [12]. Similarly, 2,3DPG, an allosteric effector of RBC oxygen affinity, is found to decrease in concentration during storage [13]. As intracellular 2,3DPG levels fall, RBCs affinity to oxygen increases, characterizing the cell’s ability to transport and release oxygen to the
body [10]. As stored RBCs are infused into the patient, concentrations of 2-3DPG naturally regenerate over a 24-48 hour period [9, 14, 15]. In transfusions where the patient is in critical condition and oxygen delivery is greatly needed, this restoration period or 2-3DPG may have a significant effect on the outcome of the patient. Other biochemical changes in RBCs over storage include increases in calcium (often associated with cell morphological changes), decreases in pH, as well as decreases in intracellular-potassium [5].

2.5 Biomechanical Changes in Stored RBCs

This study primarily focuses on examining the biomechanical changes that occur in RBCs during storage. Biomechanical storage lesions are localized to the cellular membrane and cytoskeleton and can be specifically defined as: membrane and cytoskeleton protein oxidation, membrane phospholipid loss, abnormal rearrangement of membrane phospholipids, and morphological changes [10]. Phospholipids of the membrane are lost through a process called microvesiculation, which was first defined by Rumsby et al. in 1977 [16]. Microvesiculation is the process where fragments of the plasma membrane bleb off of the RBC and is often used for intracellular communication and cell apoptosis. As these microvesicles (approx. 150 nm in diameter) depart from the cell, they take along phospholipids, transmembrane proteins, cytoskeletal proteins, and hemoglobin [17, 18, 19]. This ultimately rearranges membrane phospholipids and changes the morphological features of the cell.

As a major concern for storage lesion, a variety of studies have shown the effects of oxidative injury to RBCs during storage [20, 21, 22, 23]. Oxidative storage lesions affect
RBC hemoglobin, the cytoskeleton, and cellular membrane phospholipids [20, 22, 23, 24, 25]. The effects created by oxidation is not fully understood by researchers, however it past research shows that cytoskeletal protein oxidation along with membrane lipid oxidation can disrupt the plasma membrane and cytoskeleton interface [23]. This disruption has been proposed as a possible contributor to the increase in microvesiculation of RBCs during storage [23, 26].

Collectively, biochemical and the previously described biomechanical changes create the overall morphological changes the RBC goes through during storage. As the cellular membrane and cytoskeleton deform, the RBC losses its biconcave shape. Its progressive change in shape is characterized by the loss of membrane lipids and the development of echinocytic spincules [27, 28]. The three main stages of morphological progression in order are discocyte, echinocyte, and sphere-echinocyte. At the most morphologically altered state, sphere-echinocyte RBCs experience significant decrease in the normally large ratio between surface area and volume as well as increases in hemoglobin concentration [5].

2.6 Changes in Rheology

Studies characterize the effect of the biomechanical changes in stored RBCs by analyzing rheological properties. Blood rheology can be defined as the fluid mechanic science of the flow and deformation of blood. In vivo rheological properties of blood are primarily influenced by deformability, cell to cell aggregation, and adhesion to endothelial cells. It is of clinical interest to study the rheological properties of blood
because it has a direct effect on the RBC’s ability to perfuse through microvascular networks where they participate in gas exchange [29, 30, 31].

2.6.1 Deformability

RBC deformability is the ability of a RBC to change shape under mechanical stresses without hemolysing. This is an important characteristic because RBCs must squeeze through capillaries that are < 2μm wide (less than half of the diameter of an RBC) and form back to its original shape after leaving the compressive microvessels. How a RBC deforms can be affected by magnitude of applied stress, directionality of the applied stress, geometry of the RBC, and its material properties [26]. RBCs are known to have viscoelastic behavior, which is found commonly in mammals. Like rubber or other elastic bodies, shapes changes due to deforming stresses are resisted by RBCs. Through research, the viscoelastic cell behaviors are influenced by the following intrinsic properties: Cell Geometry - the biconcave shape gives the RBC added surface area which allows the cell to undergo shape changes while maintaining a constant surface area. This ability to change shape with constant surface area requires significantly smaller forces than a shape change that requires surface area expansion; Cytoplasmic viscosity - largely determined from the mean corpuscular hemoglobin concentration (MCHC); Material properties of the cell membrane - viscoelastic properties of the membrane impart from the complex skeletal protein networks [26].

RBC deformability is a crucial factor in blood viscosity and microcirculation velocity. In large vessels, blood with less deformable RBCs have a higher frictional resistance between fluid lamina under laminar flow conditions [32]. In microcirculatory
flow, less deformable RBCs slow down, sometimes even blocking, blood flow when squeezing through 3 micron capillaries or 1 micron endothelial slits in the spleen. Furthermore, low deformable cells traversing through microcirculatory vessels are often removed from circulation due to loss of surface area and increased spherical shape [32].

Deformability is key topic in shelf life of stored blood and blood transfusion patient recovery. From donation to the time that it is transfused into a patient, stored packed red blood cells experience changes in deformability. Although further research is needed, there have been implications that RBC deformability could be used as an indicator for the quality of stored blood to be used in a blood transfusion [33]. To mimic physiological conditions, artificial capillary networks have been used to measure RBC perfusivity, a metric that has been highly correlated to RBC deformability [34]. With more evidence, measuring RBC deformability may have a large impact on how we manage and transfuse blood.

2.6.2 Aggregation

Storage also affects the aggregation of RBCs which plays a crucial role in hemorheology and may affect bloods ability to flow in microcirculation. Aggregation occurs when RBCs stick together forming a single file line often referred to as a rouleaux. RBCs tend to aggregate when particle membrane proteins react with plasma proteins or polymers [35]. As the RBC membrane proteins change during storage it is postulated that RBC’s tendency to aggregate changes as well. Aggregation is also dependent on shear forces, forming at low shear rates and breaking up into individual cells at high shear rates [36]. In terms of rheology, the viscosity of blood during low shear flows is directly
correlated with the aggregation of RBCs. A slight change in *in vivo* blood viscosity may cause critical effects to the microcirculation of a patient. Therefore, it is necessary to understand how RBC aggregation is altered during storage [37].

### 2.7 Unit-to-Unit Variability

Focusing on the age of stored blood as the sole metric of storage lesion, RBC viability, and overall stored blood quality ignores the changes in RBCs that are not time dependent. Differences in blood between donors should also be considered when determining blood quality for transfusion. There have been previous studies showing that some donors have RBCs that store poorly, and that inter-donor differences in blood have been a major factor in variation of RBC recovery post transfusion [38, 39, 40]. Additionally, there have also been reports indicating inter-donor differences in ATP levels of donated blood [41]. Some studies believe the variability in between donors may stem from the differences in metabolic age of the RBCs when collected [42, 43]. However, there may be other donor-dependent factors involved in RBC variability such as gender, race, and overall donor health. Increasing the complexity of the matter at hand, storage lesion has also been reported to be greatly affected by the blood unit manufacturer. Specifically, unit production methods, type of additive solution, transport procedure, position of bag during storage, and storage temperature have all been found to alter blood storage lesion [44, 45, 46, 47]. Overall storage time is one out of many factors that define storage lesion of RBCs. By expanding our assessment of blood quality to incorporate variability between units, significant improvements can be achieved in both post transfusion patient recovery and stored blood management.
2.8 Determining Quality of Stored Blood using Rheology

Incorporating rheological tests on stored blood before transfusion will improve the blood quality assessment that is currently used in hospitals today. As previously mentioned blood quality is only determined by the percentage of transfused RBCs still present in the patient 24 hours after transfusion. Although this shows how blood survives in the recipient’s circulatory system, it does not convey that actual functionality of the blood. If RBCs are not traversing capillaries and transporting oxygen, patients can become ill and experience transfusion related injuries. Furthermore, it fails to address biochemical levels, oxygen affinity, and cellular mechanics all of which are considered key factors of RBC’s main function of oxygen transport. Considering this argument, new and better methods of determining RBC function must be implemented when examining the quality of stored blood before transfusion.

In terms of a diagnostic device standpoint, out of the main factors of RBC function (metabolic levels, rheological, oxygen transport), rheological properties are one of the easiest and quickest to obtain. Although important, current technology has not found an efficient way of testing oxygen transport in a timely fashion. The Hemox analyzer was created by TCS Scientific Corporation and is the only device that measures oxygen transport. The process of using the Hemox analyzer for oxygen transport is very complicated, takes a minimum of 2 hours, and is very sensitive to human error. In case of a critically ill patient, waiting two hours for blood can lead to further injury and even death. On the contrary, rheological properties have numerous measuring techniques and are partially representative of biochemicals levels due to their interrelationship. Current technologies for measuring rheological properties of blood include perfusion through
filters, rheoscopes, ektacytometers, micropipetting, and optical tweezers [48]. Although micropipetting and optical tweezers are time consuming, filter perfusion, rheoscopes, and ektacytometers all are high throughput devices and are suitable for real time diagnostics. Rheoscopes and ektacytometers are known to have better reproducibility than filter perfusion setups but are also more costly and fragile [4]. With the goal of providing hospitals around world with an economical process of testing the quality of stored blood, this thesis chose to analyze rheological properties and inter-donor variation of stored blood using a filter perfusion setup.
3. Methods

3.1 RBC Collection and Processing

Venous whole blood was drawn from 9 healthy donors using the standard blood bank phlebotomy procedures at the San Diego Blood Bank. Blood was drawn into sterilized bags which stayed sealed for the rest of the processing sequence. Units of blood were then placed in a refrigerated centrifuge (4°C) and spun to separate plasma from the RBCs. The plasma was then pressed into a satellite bag which was sealed off and removed from the original bag. Next, the red cell additive solution, AS-1 was drained into the red cells, remaining in the primary bag. The red cells and additive solution were mixed thoroughly, inverted and flowed by gravity through a leukoreduction filter and into a final product bag, already attached to the primary bag (the whole set, bags, filter, etc. were one unit, closed system). After filtration was completed, the empty bag and filter were sealed off and discarded. The filtered red cell product was then held in refrigerated storage (1-6°C) until 24 hours after blood draw to wait for the viral testing results. Once cleared, the units of blood were attached with special aliquot bags to ensure sterile storage and sampling for this study. The bags were then transported to UCSD’s Functional Cardiovascular Engineering Laboratory in coolers packed with ice and subsequently placed in a refrigerator for permanent storage at 4°C.
3.2 Contamination Prevention

Figure 1. Blood bag setup with branched aliquot bags for sterile sampling

Prevention of contamination was crucial for obtaining accurate data. In order to draw blood samples weekly while preventing the blood from coming in contact with the outside environment, an aliquot bag setup was used. As seen in figure 1, a normal unit of blood is hooked up to a branch of tubes that have individual aliquot bags at the end. To obtain one blood sample, all but one tube branch were clamped off. Blood was then allowed to flow into the single open aliquot bag. Once the aliquot bag was full, the open tube was irreversibility crimped closed using hermetic seal clips (Fenwal, Lake Zurich, Illinois). Next, the tubing in between the crimp and the aliquot bag was cut to allow usage of the blood sample from the aliquot bag. Unlike other methods used in storage lesion experiments, the aliquot bag setup prevents 100% of all blood contaminations from contact with the outside environment.
3.3 RBC Oxygen Transport and Biochemical Assays

In vitro RBC quality was assessed by conventional biochemical and oxygen transport assays including hematocrit, intracellular hemoglobin concentration, free hemoglobin, potassium (K+), ATP concentration, P50 (partial pressure of oxygen needed to saturate blood to 50% oxygen), 2,3 DPG, lactate, and pH. All tests were performed once a week for 6 weeks by the hematology lab of the UCSD Medical Center.

3.4 RBC Filter Perfusivity Preparation

Rheological properties were evaluated using a microfiltration technique. Samples of blood were first tested for hematocrit. This process involved placing a sample of blood in a capillary tube, centrifuging the blood using a Model C-MH30 microhematocrit centrifuge (Unico, Dayton, New Jersey) at 12,000rpm for 3 min, and then using a capillary micro-hematocrit reader to obtain the hematocrit.

Each blood sample was diluted with saline to get 10mls of 10% hematocrit blood. The diluted blood samples were then verified for 10% hematocrit using the previously-stated hematocrit testing procedure. After verification, the filtration apparatus was primed with the blood sample while insuring no bubbles stayed inside the apparatus.
3.5 Filtration Apparatus

The filtration apparatus included a syringe pump (Harvard Apparatus 22, Holliston, Massachusetts) that would pump blood samples out of a glass syringe (Becton Dickinson & Co, U.S.A) at constant flowrates. The blood would first flow through a straight tubular pressure sensor (Becton Dickinson & Co, U.S.A). After the sensor, blood was forced through a Teflon membrane filter with 5.0µm pores (Merck Millipore, Tullagreen, Carrigtwohill Co., IRL) and into a 1ml syringe (Becton Dickinson & Co, U.S.A) which served as a reservoir. The purpose of the reservoir was to inhibit dripping of the filtered blood which affected the pressure readings. Signals from the pressure sensor were processed through a BIOPAC MP150 amplifier and prefilter (BIOPAC Systems, Goleta, California) and then acquired by a BNC-2110 connector box (National Instruments, Austin, Texas). A DAC card digitized the signal which was further processed and stored by using a LabVIEW program (National Instruments, Austin, Texas).
3.6 Signal Processing

Figure 3. Pressure Vs Time data before and after being process by a LabVIEW butterworth lowpass filter

Signal Processing was used to suppress the inherent noise of the signal created from the pressure transducer. First, the BIOPAC amplified the pressure transducer signal with a gain of 5000 and then passed it through a low pass filter which had a cut off frequency of 300Hz. The signal was then sampled by the DAC board at a rate of 100Hz. Once all of the data was sampled, it was filtered once more using a 2nd stage low pass butterworth filter. A very low 1 Hz cut off frequency was used because the data was not expected to have a frequency. Figure 4 shows the effect of the 2nd stage butterworth filter on the signal acquired from the BIOPAC.
3.7 Experiment

The RBC suspensions were pumped through the micropore filters at a constant flowrate of 100µL/min while the pressure transducer measured the generated filtration pressure. Once the pressure of the pre-filtered blood plateaued, the pumped was turned off to allow the flowrate and pressure settle back to baseline. Once settled, this process was repeated for other flowrates (300, 500, 700, 900, 1100, 1300, 1500 µL/min). The delta pressure was calculated for each flow rate by subtracting the baseline pressure from the plateau pressure. The following graph represents this process.

Figure 4. Pressure Vs Time plot of stored red blood cells flowing through a micropore filter at different flow rates. ΔP (initial pressure rise) for the 300µL/min is shown.

For every flowrate, the filtration pressure generated by pumping saline solution alone was recorded before filtration of the RBC suspensions. These values were used to normalize pressure readings of the blood and to isolate the RBC’s effect on resistance from that of the saline. Theoretical modeling of RBC filtration curves perform by Skalak
et al. in 1983 [49] allowed for the calculation of the relative resistance in a pore bearing a red cell in passage to that in a pore with saline solution alone ($\beta$):

$$\beta = 1 + \left(\frac{P_1}{P_0} - 1\right) \frac{V}{h}$$

Where:

- $P_1$ = Initial rise in filtration pressure for blood solution
- $P_0$ = Initial rise in filtration pressure for saline
- $V$ = MCV/FPV
- MCV = Mean corpuscular volume
- FPV = Filter pore volume
- $h$ = Hematocrit

### 3.8 Statistical Analysis

Results were presented as both means and means ± standard deviation (SD). Physiological trends in rheological, oxygen transport, and biochemical data were represented by averaged values between all bags of blood for each individual time point (one per week). RBC interdonor variability measurements were shown by plotting red blood cell data for each individual bag against each other. The Two-Way ANOVA compared each individual bag of blood against each of the other bags of blood for every time point. Lastly, 3 correlation graphs were created to help define relationships between different parameters. Pearson’s $r$ coefficient and a linear trend line with 95% confidence interval bars were displayed with each correlation. All statistical calculations were performed using GraphPad Prism 4.01 (GraphPad Software, Inc., San Diego, CA) with statistical significance attributed to values of $P < 0.05$. 
4. Results

9 bags of packed red blood cells were used in this study to analyze storage lesion over a period of time. Each bag of blood came from unique randomized donors from the San Diego Blood Bank. The units of blood were leukoreduced and mixed with AS-1 additive solution to mimic common practice in the blood transfusion industry. During the storage period, several variables were measured weekly for each unit of blood for a total of 6 weeks.

4.1 Storage Lesion Trends and Interdonor Variability

4.1.1 Red Blood Cell Micropore Filter Per fusivity

Blood samples were tested for its rheological properties by flowing the samples through a micropore filter and measuring the pressure increase for different flow rates. As seen in figure 5 the pressure needed to flow the RBCs through the filter increased over time and for increasing flowrates. The greatest increase in pressure happened between

![Figure 5. Averaged data for all 9 units of blood: Pressure Vs. Storage Time Vs Flowrate (left); Pressure Vs Flowrate Vs Storage time](image-url)
weeks 3 and 4. The general increase in pressure over time for all flowrates were linear except at the lowest flowrate (100µL/min) which was more exponential.

The pressure increase was used to calculate the filter perfusion resistivity; which has commonly been used to define rheological properties such as aggregation and deformability. As can be seen in figure 6 the mean resistivity of the RBCs at a flowrate of 100µL/min increased from 1.5 in week 1 to 24.0 in week 6. Similar to what was found in the pressure data, the resistivity, tested at the lowest flowrate, increased exponentially in reference to storage time. At higher flowrates (500,700,900,1100,1500µL/min) resistivity increased as well, e.g. at the highest flowrate of 1500µL/min the resistivity increased from 1.7 in week 1 to 4.4 in week 6. However, tests at higher flowrate exhibited smaller increases in resistivity over time and increased in a more linear fashion.

Figure 6. Averaged data for all 9 units of blood: Resistivity Vs. Storage Time Vs Flowrate (left); Resistivity Vs Flowrate Vs Storage time
Figure 7. Progression trends in resistivity over time for individual bags. Each graph represents a different flowrate.
Another aspect of this study is to determine if there is a significant difference between donor’s blood and how it deteriorates during storage. Figure 7 shows each unit of blood’s change in resistivity over storage time for filtration test conducted at all flow rates. At a 100µL/min flowrate the units of blood all began with a resistivity of 5 or less and increased over time. The magnitude of which a unit of blood increased in resistivity varied greatly between units at this flowrate. It should also be noted that some units had a linear increase such as bag 4 while others had significant changes in slope as can be seen with bag1 and bag2. In comparison, the resistivity levels of all the blood units tested at the highest flowrate (1500µL/min) remained closer together throughout the storage period. The drastic changes of slope seen at the 100µL/min are attenuated in higher flow rates and form more linear changes.
Figure 8. Interdonor variability between bags of blood. Graphs are based off of results from 100 and 1500µL/min tests from week 1 and 6

For a different perspective, figure 8 show the resistivity values of each unit of blood compared to other bags for specific time points (week 1 & 6) and flowrates (100µL/min & 1500µL/min). At the earliest stages of storage, statistically significant differences in resistivity among the units of blood can already be seen. For all flowrates, Bag6 was found to be statistically different from all the other bags with a P value of P<0.0001. The effect of flowrate on resistivity measurements was very minimal for the first week of storage. However by week 6, majority of the units of blood had an order of magnitude difference between their resistivity level found at the 100µL/min and 1500µL/min flowrates. After analyzing interdonor variability of resistivity for week 6, it
was found that lower flowrates had less significant variance than their week 1 counterparts while the higher flows were found to have a slight increase in significant interdonor variability. Only Bag1 was statistically different from other bags with a P value of .0029 for the 100\(\mu\)L/min flowrate. At the 1500\(\mu\)L/min flowrate bags 1 and 9 were found statistically different from other bags with a P value of <0.0001.
4.1.2 Hematocrit

A minimal decrease was observed in the averaged hematocrit, starting at 63.2% in week 1 and ending at 61.8% in week 6. Significant variability in hematocrit was found between bags of blood. Furthermore, there was no one particular trend that the units of blood followed other than the small decline over the 6 weeks.

4.1.3 Hemoglobin Content

Similar decreasing trends were seen in Hb content over storage time. The average Hb concentration started at 19.6g/dL in week 1 and declined to 19.1g/dL by week 6. Interdonor variability was found in Hb content at all time points.
4.1.4 Free Hemoglobin

The average amount of free hemoglobin released from RBCs increased significantly from 1.3μMol to 42.8μMol over the course of 6 weeks in storage. This increase of free Hb followed a linear trend in respect to storage time. After week 1, free Hb had significant interdonor variability which increased with time.

4.1.5 Potassium

Average extracellular potassium increased linearly from 2.3mmol/L to 28.8mmol/L over the storage period, with the largest increase between week 4 and 5. Interdonor variability in potassium increased over time becoming statistically significant by week 3.
4.1.6 P50

The partial pressure of oxygen that is needed to saturate 50% of the RBCs (P50) was recorded to quantify oxygen transport capabilities. Over the storage period the P50 value dropped linearly from 27.2 mmHg to 9.9 mmHg, representing the RBC’s increase of oxygen affinity. Interdonor variability of the P50 was found every week of storage.

4.1.7 2,3 DPG

Average 2,3DPG concentrations decreased linearly from 12.6 µmol/gHb to 1.0 µmol/gHb over 6 weeks, reflecting its allosteric relationship with the P50 parameter. 2,3DPG had no significant difference during week 4 and declined in significant interdonor variability as storage time increased.
4.1.8 ATP

The average ATP concentration of RBCs followed a unique trend by first increasing from 4.4µmol/gHb to 6.8µmol/gHb during the first 4 weeks and then declining to 1.7µmol/gHb by week 6. Although every unit of blood followed this trend, interdonor variability was found at every time point of storage.

4.1.9 Lactate

Average RBC lactate concentration increased linearly from 1.4mmol/L to 29.2mmol/L over the 6 weeks of storage. Lactate had an increase interdonor variability in respect to time with significant differences starting in week 3.
4.1.10 pH

The average pH of the units of blood declined linearly from 7.1 to 6.5 from week 1 to week 6 of storage. Interdonor variability in pH was found in the units of blood at every time point. It should be noted that some donor’s blood pH declined in equal amounts between consecutive weeks while others, such as Bag 8, alternated between increasing and decreasing of pH levels.
4.2 Correlation Analyses

4.2.1 Free Hemoglobin and Potassium

![Correlation between free hemoglobin and potassium with linear regression trend line and 95% confidence interval](image)

Both Hb and Potassium are released in the plasma as RBC leakage and hemolysis increase. If one increases, it is expected to see the other increase as well. This is reflected by the high correlation coefficient $r = 0.99$ ($r^2 = 0.99$) between the two analytes and suggests a presence of RBC hemolysis or leakage.
2,3 DPG is an allosteric effector of oxygen transport. Therefore, we analyzed the correlation between changes in 2,3DPG concentrations and P50 levels. With a Pearson’s $r$ value of 0.97($r^2 = 0.95$), the allosteric relationship between the parameters is supported.
4.2.3 pH and Lactate

![pH vs Lactate](image)

On average, as lactate levels increased in the stored blood, the pH of the blood decreased. The Pearson’s $r$ value of $-0.97 (r^2 = 0.94)$ for the correlation between lactate and pH suggests that the analytes are inversely related. This has been observed in other studies which have found that high lactate levels in blood are associated with acidosis.
5. Discussion

RBC’s main function, oxygen delivery to tissues of the body, is largely dependent on its rheological and biochemical properties. Therefore, it is imperative to understand how these properties change over storage under standard blood banking conditions in order to ensure transfusion of functional RBCs to patients. This study aimed to address this lack of understanding by documenting trends in rheological and biochemical properties, the correlation between these properties, and how they vary between donors. More specifically this experiment monitored deformability and aggregation using a filter perfusion technique with various flowrates, as well as levels of hematocrit, hemoglobin, free hemoglobin, 2,3 DPG, P50, Lactate, Potassium, and pH.

This study demonstrated an increase in filter perfusion resistivity throughout the storage period which suggests decreases in deformability and increases in aggregation. Low flowrates exhibited much greater increases in filter resistivity between the beginning and end of storage in comparison to the highest flowrate (1500µl/min). Since aggregation plays a larger role in flow properties of blood at low flowrates while the effects of deformability are more prominent in higher flowrates, it is inferred that more significant changes in resistivity occurred due to aggregation than deformability.

Lactate increased as pH declined over time supporting previous reports associating high levels of lactate to acidosis. The average RBC P50 levels declined over storage which also represented the increase of RBC’s affinity to Oxygen. 2,3DPG, known to play a large roll in RBC oxygen affinity, decreased as well. Going against some studies that correlate ATP levels with deformability, the ATP content of the RBC units in this experiment had an increase of concentration up to week 4, followed up with a decrease.
until the end of the storage period. Up until week 6, ATP concentrations did not fall below a concentration of 2.7µmol/gHb, a minimum threshold at which RBCs have 90% chance of survival 24 hours after transfusion. With ATP levels lower than this threshold only for week 6, it is not likely that this results in the proposed adverse clinical outcomes. Plasma levels of potassium and free Hb rise during storage suggesting that hemolysis also increases as well since they are both intracellular molecules that are known to leave the cellular membrane after rupture. Significant differences between bags of blood were found in every parameter tested. Due to the high precision of the biochemical measurement methods, even small differences between donors were found to be statistically significant. However, it is hypothesized that the magnitude of these differences between donors are physiologically insignificant.

All of the measured data have been correlated with the duration of storage. However, none, except in cases of extreme hemolysis, have been shown to have critical quality thresholds that would ease the deciding process of whether to transfuse a patient with the blood at hand. All variables are continuous and most likely play some role in RBC storage lesion. The clinical significance of these variables is still unknown.

A better understanding of how RBCs change during storage is crucial to improving the outcomes of blood transfusions. Current studies are trying to evaluate the clinical effects of storage lesion: ABLE (effects due to the age of blood), ARIPi (effects due to the age of blood on infants), and RECESS (effects based on storage duration of RBCs) [50, 51, 52]. These studies, including other future research, will attempt to determine the appropriate management and utilization of stored blood based on stored blood attributes and individual patient’s needs. Although a substantial monetary burden
for blood banks, alternative forms of blood storage, such as RBC freezing and hypoxic storage, have already shown promise for longer storage life and function preservation of stored blood.

In this study, we were able to characterize and quantitate changes in rheology occurring throughout storage. However, other factors may have contributed to error in the data collected. During the filtration procedure, the temperature of the blood was not monitored. This could have affected viscosity of the blood solution and impaired the cell structures. Additionally the blood sample inside the syringe was not continually mixed during the experiment, leading to possible sedimentation of the RBCs. Sedimentation creates a nonhomogeneous solution which may have affected the flow resistance through the filter. Since new filters were used for each run of the experiment, variation between pore density, size, and volume should be taken into consideration. Mean corpuscular volume of the RBCs was used in the calculation of filter perfusion resistivity and was assumed to be the same for each unit of blood. In reality there are likely to be differences between donors and should be taken into account for more accurate data. Although there were many factors that may have contributed to error in the data, it is believed that general trends can be drawn from this study.
6. Future directions

To achieve a more robust conclusion this experiment should be repeated with a larger sample volume. With only 9 bags, the conclusions from this experiment may not accurately represent what happens in the whole population of stored blood. Since there were significant differences in RBC parameters between donors, it would be of interest to examine possible factors such as: age, gender, health, and athletic level. For example, a person who is athletic and young may have great differences in RBC function compared to a sedentary, obese, or elderly person. Being able to evaluate between good and bad donors has the potential to prevent not only high expenses due to processing already poorly functioning blood but also many adverse effects experienced by transfusion recipients. Lastly, although statistical differences were found between units of blood in respect to rheological, oxygen transport, and biochemical parameters, physiological relevance cannot be construed. A study which examined how the body reacts to various magnitudes of storage lesion, similar to what was seen in this experiment, will render what truly defines the quality of stored blood.
7. References


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