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Frozen, Old, or New? Comparing Biochemical Markers and Tissue Oxygenation in Transfused Blood.

A Baccalaureate Honors Thesis by Connor Wiles

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Abstract

The transfusion of red blood cells is a necessary therapy used to treat anemia that often results from traumatic hemorrhage. The traditional method of storing red blood cells prior to transfusion in the United States has been as a liquid in a refrigerated fashion at a temperature between two and eight degrees Celsius. Throughout the storage duration, the red cells undergo a series of structural, functional, and biochemical changes commonly known as the storage lesion. Increased length of storage has been associated with increased rates of infection, as well as higher incidents of mortality. We hypothesized that this prospective, randomized, double blinded study would demonstrate that a cryogenic freezing method of storing blood would result in higher tissue oxygenation for the patient, an increased biochemical profile of the blood, and decreased rates of negative outcomes compared to the liquid preservation method.

Adult trauma patients with an injury severity score of greater than 4 and an anticipated need for transfusion of at least one unit of blood were randomized to receive either CPRBCs or LPRBCs. 57 patients were randomized and received blood transfusions as part of either of the two groups. 22 patients received CPRBCs, and 35 patients received LPRBCs. Tissue oxygenation as well as 2,3-DPG concentration, ($p < 0.05$) was elevated in the patients whom received CPRBCs over the patients that received LPRBCs. The serum concentrations of haptoglobin, serum amyloid p, and C-reactive protein were all decreased in CPRBC units compared to the LPRBC units. Pro-inflammatory cytokines IL-8 and TNF- α were elevated in the LPRBC group compared to the CPRBC group ($p < 0.05$). Finally the D-dimer, which is an indicator of fibrinolysis, was higher in the LPRBC group ($p < 0.05$).

In conclusion, CPRBCs maintain a superior biochemical profile over LPRBCs, as well as provide potential for higher tissue oxygenation in patients, and also present a viable option to combat the severe blood shortage that constantly exists in the United States, by extending the shelf life of RBC units to ten years. This extension of storage time may also result in the prevention of an \$80 million loss annually.

Introduction

Anemia is defined as a decrease in the amount of red blood cells, or a less than normal concentration of hemoglobin in the blood. In trauma patients, this condition is usually onset by hemorrhage and the resulting coagulopathy from the injuries. Due to the methods of resuscitation, the often-urgent need for surgical intervention, the requirement for serial blood draws, and the abnormality of red blood cell (RBC) production in these patients, they are not often able to keep up with the body's requirement for oxygen. In

order to combat this issue, critically ill patients in the intensive care units receive an average of five units of packed red blood cells (PRBC).¹ Patients often receive transfusions of PRBC's in order to restore and maintain intravascular blood volume as well as increase blood pressure. Patients may also receive transfusions in order to increase oxygen perfusion in systemic tissues and organs, with the ultimate goal of preventing organ failure and death.²⁻⁴

The efficacy of the PRBC units is dependent on the RBC to traverse a network of micro-capillary blood vessels, reach the tissues, and offload the oxygen molecules it is carrying^{5,6}. Unfortunately, while awaiting transfusion, these units go through a series of structural and biochemical changes and develop a well documented storage lesion.⁷ These changes adversely affect the cells life span, as well as its shape and plasticity, which in turn negatively affect the cells ability to navigate the capillary networks and deliver oxygen to the tissues that need it. Changes in the nitric oxide (NO) metabolism, increases in the concentration of free hemoglobin, decreases in the concentration of 2,3-diphosphoglycerate (2-3 DPG), increased hemolysis, and residual leukocytes as well as platelets are additional factors that contribute to the storage lesion. NO depletion is an especially concerning issue as it is a crucial contributor to vasodilation which enables blood to reach end tissue easier, and can decrease blood pressure.

Likely as a result of this storage lesion, the transfusion of liquid preserved red blood cells (LPRBCs) has been linked to a longer hospital length of stay, prolonged coagulation times, an increase in the number of infections, increased occurrence of trauma related acute lung injury (TRALI), multiple organ dysfunction syndrome, and death^{8,9-15}. Longer storage times for LPRBC's have been associated with increases in these as well as other complications¹⁶. By nature of the donation process, donated blood is a mix of RBCs at varying time points along their life cycle. At this point it is impractical for banked blood to be selected for based on the cells age. However, the method used to store the blood can either have a neutral or negative effect, which will ideally leave them arrested in the state in which they were donated or further degrade the donated cells.

Currently, the Food and Drug Administration (FDA) approve LPRBCs, for storage at a temperature between two and eight degrees Celsius for up to 42 days. The RBCs are separated from the plasma and leukocytes, a solution of citrate-phosphate-dextrose-adenine is added, and the units are brought down to the desired temperature. Although erythrocyte metabolism is decreased at these temperatures, cells still undergo the morphologic changes that were described above.⁷ After observing these changes in conjunction with the many complications and negative outcomes associated with blood transfusions thought to be associated with storage methods, a method of freezing blood for prolonged safer storage was identified.

In 1950, Smith¹⁷ discovered that a small amount of blood mixed with glycerol could be cooled to a temperature of -70 degrees Celsius. Not long after, this method was further investigated and developed in order to freeze entire units of PRBCs while retaining normal function¹⁸. Many cell-specific cryopreservation protocols have been developed since that time. The methods vary in their processing techniques, as well as red cell concentrations, types of protective solutions, rates of thawing and freezing, storage temperature, and glycerol concentration. In the United states we use a high glycerol concentration in conjunction with a slow freezing and cooling process¹⁹. The process of

thawing and washing the cells in order to remove the glycerol from the units must be carefully controlled in order to prevent the lysis and degradation of the cells. In order to prevent the storage lesion from developing before the freezing process, the American Association of Blood Banks require RBCs to be frozen within 6 days of collection²⁰. The FDA approved a storage period of up to ten years for glycerol frozen RBCs at a temperature of -80 degrees Celsius.

In addition to the physiologic implications of transfusing LPRBCs, there is also the issue of blood shortages that are not able to be adequately addressed using this method, as well as the huge financial loss that our as well as other countries, suffer as a result of relying solely on this method. Due to the 42-day shelf life of LPRBCs, the units that reach this time point must be discarded, and has amounted to a loss of approximately \$80 million annually. Based on the shelf life, there are also seasonal and cyclical shortages that effect patient management. America's blood center's STOPLIGHT, which is the blood monitoring program in the United States, has reported that 30% of national sites had only a two day supply of blood on hand at any given time²¹. Although blood donation always increases during times of national disasters, it often takes up to two days for the blood to reach the locations and people that need it. Many lives are lost unnecessarily due to this issue that could be easily solved with a supplemental blood-banking program. Enter cryopreservation of banked blood.

During the conflict in South East Asia in the 1960s the FDA had approved storage for LPRBCs to a shortened 21-day period. This truncation of the storage period resulted in the destruction of almost 50% of the banked blood that Americans had overseas. Fortunately a significant number of Cryopreserved red blood cell (CPRBC) units had been sent to Da Nang, Vietnam around the same time. The CPRBCs virtually eliminated the deficit, and in addition, the patients whom had received the units experienced little or no transfusion reactions compared to the group that had received the LPRBCs²². Following the conflict in Vietnam, a number of institutions in the United States developed and implemented a cryopreserved blood storage program. Among them were Massachusetts General Hospital, Cook County Hospital, and 30 other institutions²³. The institutions that developed CPRBC storage programs used them as a supplement to their LPRBC programs, and they were very successful. However, due to the FDA eventually approving a 42-day shelf life for the LPRBCs, as well as advancements in the liquid storage techniques, the CPRBC programs were virtually abandoned. Although there are still CPRBC programs in the United States, we see them only in military settings such as the conflict in the Middle East, as well as small programs that help preserve units of rare blood types.

Historically the process of cryogenically freezing blood units has been timely, costly, and not as safe as LPRBC storage. The process was performed in an open system that was labor intensive and required technicians to manually inject the units with the glycerol substrate, and then wash them through various filters after they were thawed. This process was not only time consuming, but also introduced unnecessary risk for contamination. Furthermore, the units were only approved for a 24 hour storage period after the units had been thawed²⁴. The Haemonetics Corporation (Braintree, MA) has developed an automated system that has virtually removed all possibility for contamination and greatly increased the efficiency of the entire process. This has become the standard of practice for our institution's CPRBC program. Currently, we are using the

Automated Cell Processor (ACP) 215, which simultaneously thaws and deglycerolizes the blood units in a closed system. The machine is easy to use, and can simultaneously process two units at a time. When this machine is used for the deglycerolization process, the FDA has approved a 14-day shelf life at 4 degrees Celsius in standard storage solutions for each unit post thaw²⁵.

Recently, evidence from multiple studies has shown that the duration of storage for blood products greatly affects the efficacy and safety to the patient. In an ideal world, a fresh product that was transferred directly from donor to recipient would be supplied when the need for transfusion arises. Sadly, this will likely never be possible, and to shorten the allowed length of time for storage, would greatly and negatively affect our nation's blood supply. With the addition of a cryopreserved blood program used as a supplement to the already robust liquid storage program, we may be able to greatly increase the outcomes of our patients as well as save money for our institutions. Although a frozen blood storage program has been utilized in the military and academic setting, a widespread application in the civilian setting has yet to be explored²⁶⁻²⁸. Based on the body of evidence that supports negative results due to the storage lesion that develops after long term refrigerated storage, and the possible benefits of a frozen blood storage program, we have performed a pilot study to assess the theoretical advantages of this program with regard to tissue oxygenation and biochemical changes compared to the liquid preservation technique. Clinical outcomes, tissue oxygenation, and biochemical changes were assessed recognizing that the study was not adequately powered to make strong conclusions, but may provide evidence as to whether or not cryopreservation might be a valid supplemental method for the storage of blood products.

RESEARCH DESIGN AND METHODS

This was a prospective, randomized, double-blinded study. Approval from the university's Institutional Review Board was obtained. Patients were considered eligible if admitted to the Oregon Health and Science University trauma service with an Injury Severity Score (ISS) of greater than 4 and an anticipated need for a blood transfusion. Exclusion criteria included inability to obtain consent, massive transfusion within the previous three months, blood transfusion within the previous 24 hours, requirement for emergent transfusion, bilateral hand injuries, patients whom were pregnant, and children that were less than 15 years old. Patients requiring emergent transfusions were excluded on the basis of the frozen blood's requirement of a two-hour time period during which the units were thawed and deglycerolized. Patients with bilateral hand injuries were excluded on the basis of our requirement for placing the tissue oxygenation (StO₂) monitor on the patient's thenar eminence. Consent was obtained from the patient or legally authorized representative prior to transfusion of the first unit of blood.

Patients were initially randomized to one of three groups, new blood, old blood, or cryogenically frozen blood. Patients randomized to the new blood group would receive a transfusion of LPRBCs that had been stored for a period of no longer than 14 days. Patients randomized to the old blood group would receive LPRBCs that were stored for a period of time between 14 and 42 days. Patients randomized to the frozen blood group would receive CPRBCs that had been stored cryogenically for varying amounts of time.

Due to the lack of a statistically significant difference between the two LPRBC groups, they were combined to create one group of patients that received blood between the ages of 1 and 42 days old, stored in the traditional refrigerated fashion.

Once patients were randomized to one of the groups, the blood bank was immediately notified of their enrollment in the study. Blood bank staff would randomize the patients to one of the three groups using a random numbers generating program which related the number selected, to a number on a list corresponding to a specific group. Upon request by the treatment team for blood products for an enrolled patient, the blood bank would begin the thawing and deglycerolizing process if the patient were randomized to the frozen blood group. If the patient had been randomized to one of the other two groups, they would simply hold the refrigerated products for a time period of two hours. This was to maintain the blindness for the research staff, as well as the treatment team.

Following notification of the blood bank of a patient's enrollment, the research staff would collect basic demographic information on the enrolled subject. The information collected included sex, age, race, and ISS. Information such as outcomes, complications, and hospital length of stay, organ failure, transfusion reactions, surgeries, thrombotic events as well as mortality were also collected. A patient's participation in the study in no way affected their chances of receiving a transfusion, and all decisions to transfuse a patient were made at the discretion of the primary treatment team. In our facility patients will likely receive a transfusion if their hemoglobin level is less than 7 g/dL for normal patients, and less than 8 g/dL if the patient had incurred a head injury.

Multiple methods of cross matching were used based on the results of the patient's initial antibody screen. A serologic test, Antiglobulin, was performed using the Gel microtube method if a patient's serum showed no sign of antibodies. In this case, if there were also no discrepancies found in the ABO, and RH positive or negative type, then an electronic cross match was performed. If no antibodies were present in the serum, and discrepancies in the ABO, RH type was however found, then an immediate spin serologic test was performed.

Once the primary treatment team had placed an order for blood products for an enrolled patient, the research coordinator on call would receive a page notifying them of the order. The coordinator would then retrieve a near-infrared spectroscopy device (Hutchinson Technology, Hutchinson, MN) and place it on the patient's thenar eminence prior to transfusion of the products, and this would remain there for twelve hours post transfusion. The instrument works non-invasively by penetrating the tissue with near-infrared light that is able to measure the concentration of hemoglobin loaded with oxygen as well as the hemoglobin that is not carrying oxygen. The instrument calculates a ratio of oxygenated to deoxygenated hemoglobin and returns the value as a percentage of tissue oxygen saturation.²⁹

After placing the StO₂ Monitor, the research staff would then collect a 10cc baseline blood sample before the transfusion began. Blood samples were also collected from the patient post transfusion of the first unit, and following the transfusion of a second unit if one were administered. A 12-hour post transfusion time point blood sample was also obtained for all subjects. For each of these time points a thromboelastogram (TEG), as well as a complete blood count (CBC) was ordered. Standard coagulation tests as well as a D-dimer (Diagnostica Stago Inc., Parsippany, NJ), was also performed on the human samples. After the blood had been hung, 300cc of the unit was allowed to

transfuse into the patient, and at that point a 12cc blood sample was taken from the blood unit itself. A sample of the same volume was taken from each additional unit that the patient received. All human samples as well as samples taken from the individual units were assayed for biochemical markers and changes. The Bio-Plex Pro Human Acute Phase 4-Plex Panel (Bio-Rad Laboratories Inc., Hercules, CA) was used to assess Haptoglobin (Hg), serum amyloid P (SAP) and C-reactive proteins (CRP). Hg was used as an indicator that assessed the degree of hemolysis of the red blood cells in each sample, as it is a free hemoglobin scavenger. SAP and CRP were assessed because of their known anti-coagulation effects. Combined with Heparin, SAP can cause a hypocoagulable state^{30,31}. A sievers Nitric Oxide analyzer 280i (GE Analytical Instruments, Boulder, CO) was used to measure NO metabolites. A commercially available kit (Roche Diagnostics, Indianapolis, IN) was used to analyze the levels of 2,3-DPG in each sample. The Human Cytokine 8-Plex Assay (Bio-Rad Laboratories Inc., Hercules, CA) was used to measure cytokines IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, TNF- α , and IFN- γ .

If a patient suffered from organ failure, the data was assessed in order to look for correlations between the complication and the transfusion. Adult respiratory distress syndrome (ARDS) was defined using the American-European consensus conference³². The Denver Multiorgan Failure score was used to grade multiple organ failure³³. RIFLE classification as outlined by the Acute Dialysis Qualitative Initiative Workgroup was used to assess acute renal failure³⁴. Mean differences in maximum and minimum StO₂ data compared to baseline values were used to determine changes in oxygenation. An area under the curve analysis was used in order to identify trends as well as correct for minute-to-minute fluctuations in oxygenation that is typical of StO₂ measurements. Measurements taken at baseline, during transfusion, as well as post transfusion of the blood products were compared within each group and between each group. The data was found to be normally distributed, and was evaluated using student t-tests.

SPSS version 19 (IBM, Armonk, NY) was used to analyze all data. Continuous, normally distributed data were analyzed using Analysis of Variance (ANOVA) and paired student t-tests. Non-parametric data were analyzed using a Mann-Whitney U and Wilcoxon Rank Sum tests. Significance was set at the 95% confidence interval with a p value of less than 0.05.

RESULTS

A total of 57 patients participated in this study. Thirty-five patients received LPRBCs while the remaining twenty-two received CPRBCs. 11 patients whom received LPRBCs received two consecutive units, while 9 patients whom received CPRBCs were transfused two units (p=0.57). Median time for transfusions was 135 minutes (75-209 minutes). Both groups were similar in age, sex, mechanism of injury, ISS, and APACHE II score (Table 1). The length of storage for the CPRBCs was significantly longer with a mean age of 588 days while the LPRBCs mean storage age was 14 days (Table 2).

(n total=57)	LPRBC Group (n=35)	CPRBC Group (n=22)	p value
Age, median (IQR)	44 (30-54)	50 (26-66)	0.56
Gender (Male/Female)	23/12	16/6	0.58
Mechanism of Injury (blunt/penetrating)	34/1	19/3	0.29
ISS, median (IQR)	26 (13-33)	18 (10-27)	0.18
APACHE II Score, mean (SD)	11 (5)	11 (5)	0.88

Table 1 Patient demographics.

(n total=57)	LPRBC Group (n=35)	CPRBC Group (n=22)
Storage duration (days), median (IQR)	14 (9.8, 27.3)	588 (392, 756)
Haptoglobin (ng/mL), median (IQR)	73 (31, 105)	10 (4, 32)
C-reactive Protein (ng/mL), median (IQR)	5 (2, 10)	0.1 (0.0, 1.7)
Serum amyloid p (ng/mL), median (IQR)	34 (22, 54)	1.8 (0.6, 13.5)

Table 2 Stored blood characteristics for LPRBCs and CPRBCs (p<0.01)

The CPRBCs were found to have decreased serum concentrations of Hg, SAP, and CRP compared to the LPRBCs (Table 2). Pro-inflammatory cytokines IL-8 and TNF- α were elevated compared to baseline values in the LPRBC group, and this elevation was not observed in the CPRBC group (Figures 1,2). There was no difference observed in pro-inflammatory cytokines IL-2, IL-6, INF- γ , or GMCSF in either of the two groups at any time point (Figures 3-6). Nor was there a difference observed in anti-inflammatory cytokines IL-4, or IL-10 in either group at any of the time points (Figures 7,8). No difference in NO metabolites was observed in either group (Figure 9). The D-dimer was elevated in the LPRBC group at the 12 hour post transfusion time point (Figure 10) A difference in the concentration of 2,3-DPG between the two groups became statistically significant following transfusion of the second unit of blood and persisted through the 12 hour time point (Figure 11).

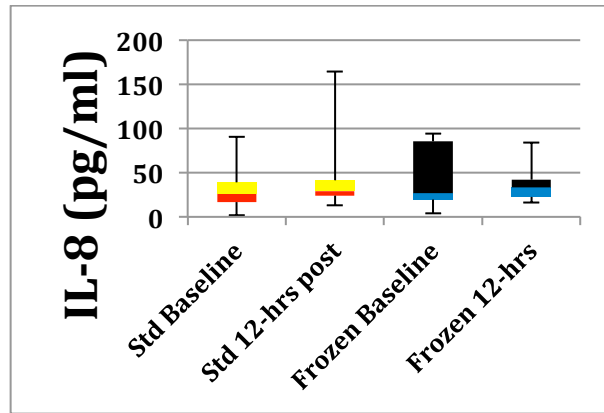


Figure 1 IL-8 concentration at baseline and 12-Hr time point for LPRBCs and CPRBCS

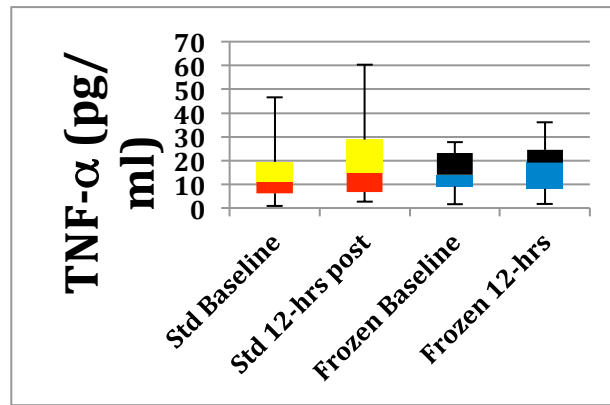


Figure 2 TNF-α concentration at baseline and 12-Hr time point for LPRBCs and CPRBCS

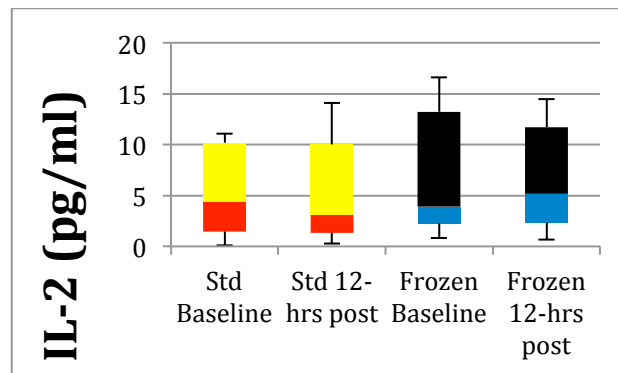


Figure 3 IL-2 concentration at baseline and 12-Hr time point for LPRBCs and CPRBCS

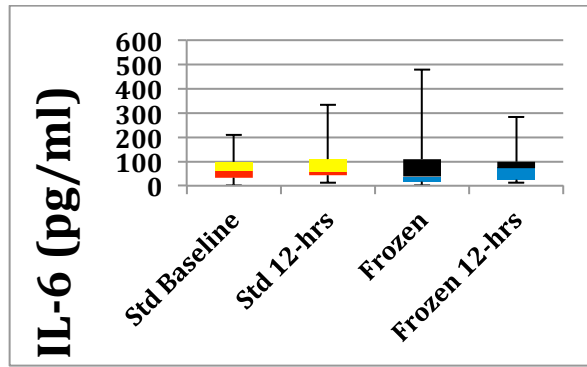


Figure 4 IL-6 concentration at baseline and 12-Hr time point for LPRBCs and CPRBCS

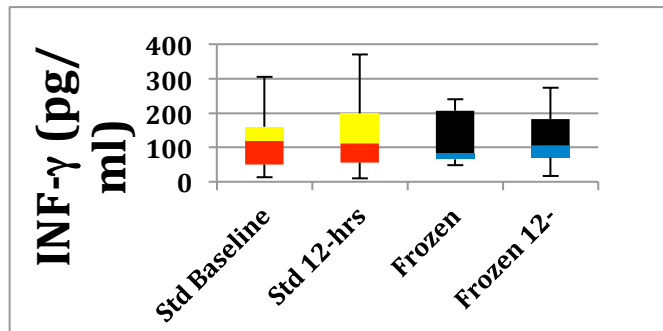


Figure 5 INF- γ concentration at baseline and 12-Hr time point for LPRBCs and CPRBCS

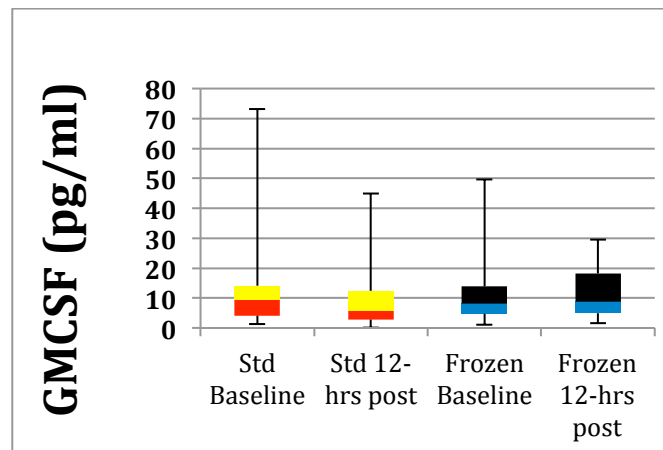


Figure 6 GMCSF concentration at baseline and 12-Hr time point for LPRBCs and CPRBCS

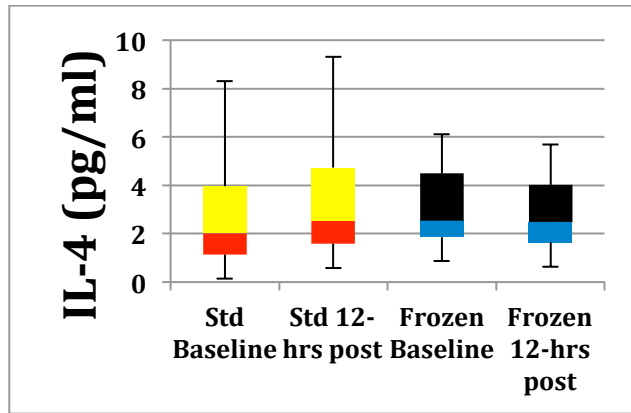


Figure 7 IL-4 concentration at baseline and 12-Hr time point for LPRBCs and CPRBCS

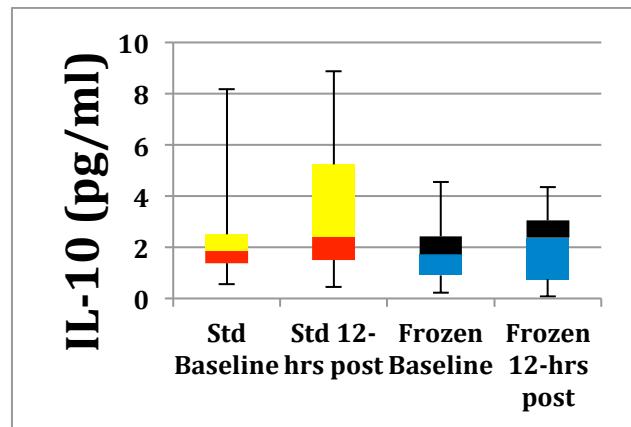


Figure 8 IL-10 concentration at baseline and 12-Hr time point for LPRBCs and CPRBCS

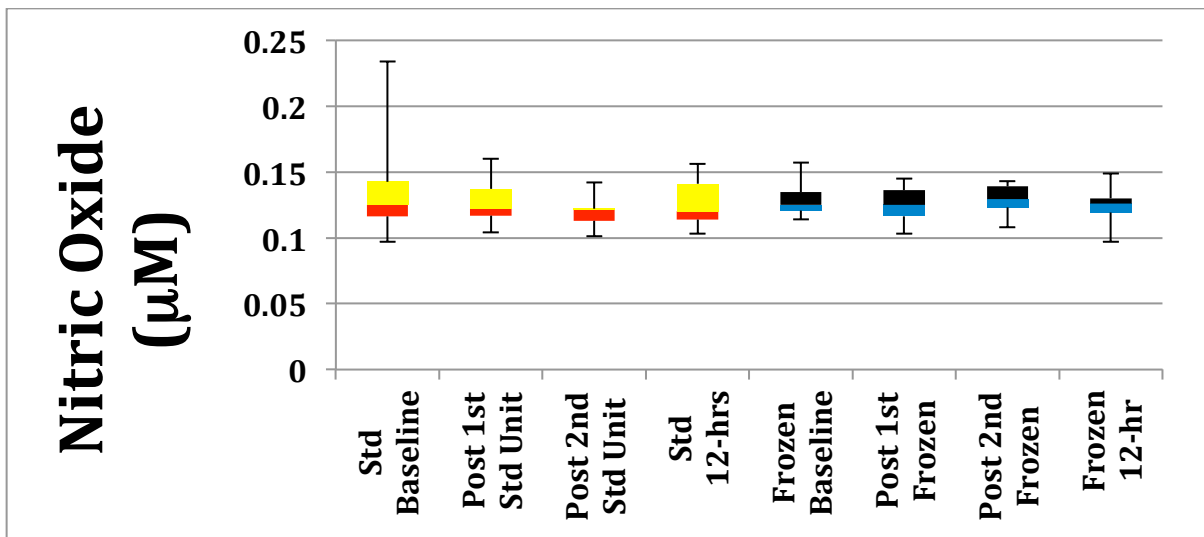


Figure 9 Concentration of NO Metabolites from baseline through 12-Hr time point

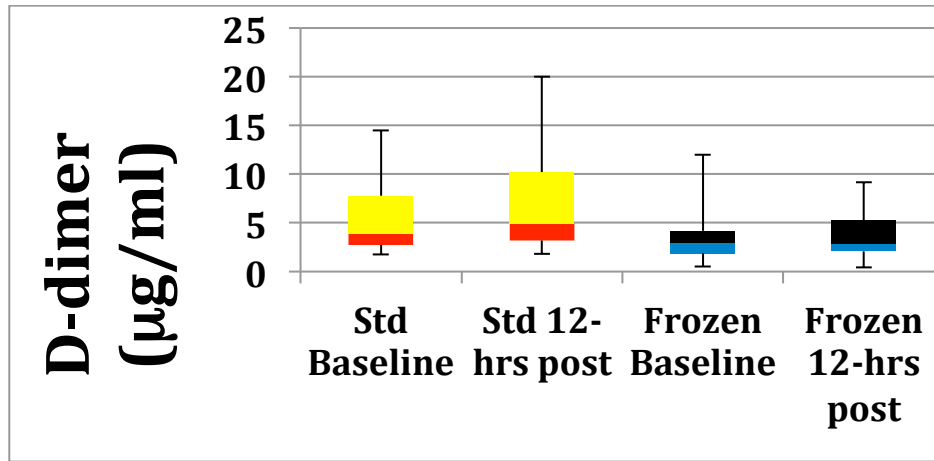


Figure 10 D-Dimer concentration at baseline and 12-Hr time points (p<0.05)

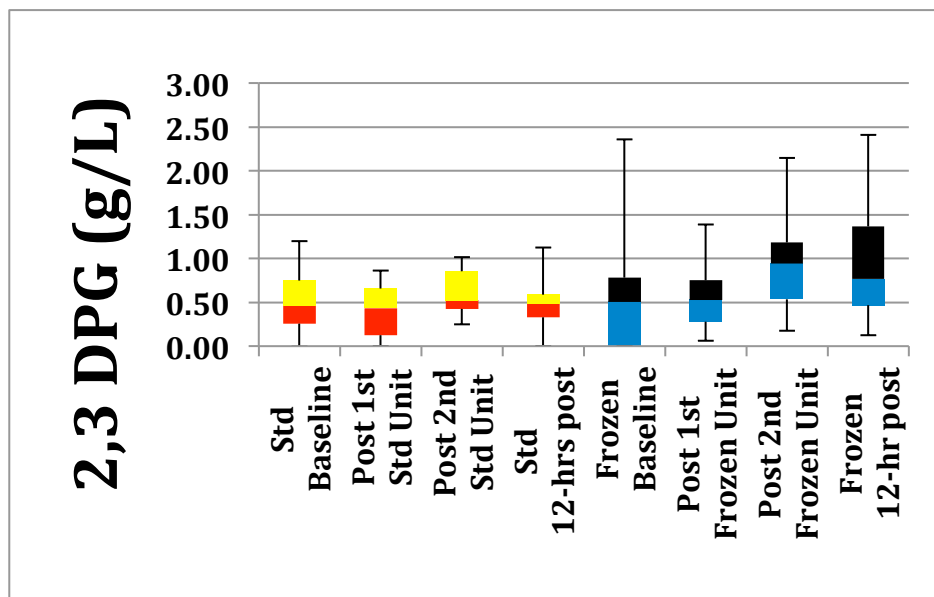


Figure 11 Concentration of 2,3-DPG at each time point in the LPRBC and CPRBC groups (p<0.05)

The mean values for StO₂ data throughout the study were compared between the two groups of patients. Only data that was continuously collected for patients through the twelve-hour time point was considered valid. After removing patients that had discontinuous data, there were 20 patients in the CPRBC group that were compared with 30 Patients in the LPRBC group. At baseline, there was no statistically significant difference in the tissue oxygenation between the two groups. However, from the time of transfusion initiation up through three hours post transfusion, the patients whom received CPRBCs demonstrated an increase in tissue oxygenation over the patients whom received LPRBCs. An elevated StO₂ tracing was noticed in the CPRBC group following the 180 minute time point, but was no longer statistically significant after that time.

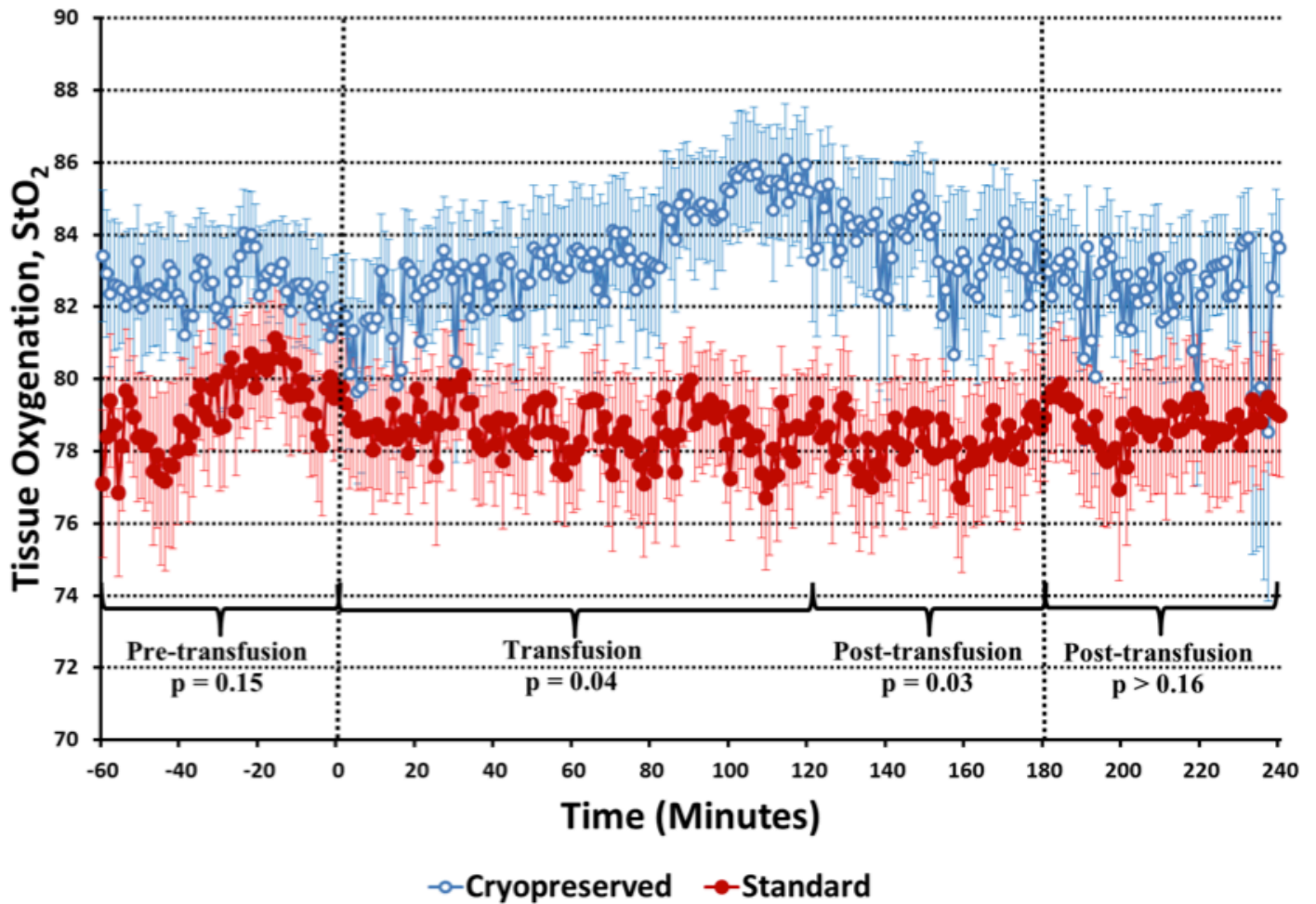


Figure 12 StO₂ tracing from baseline through the 12-Hr time point for LPRBC and CPRBC groups

While this study was not powered to demonstrate statistically significant differences in clinical outcomes, additional data were also assessed to screen for any major safety differences. No gross differences were noted between the two groups when comparing rates of respiratory failure, acute renal failure, organ failure, post transfusion fever, transfusion reactions, deep venous thrombosis, mortality, or median hospital length of stay (Table 3). There were no differences observed in TEG parameters (Table 4), with the exception of a slightly greater α -angle in the cryopreserved group following transfusion of the second RBC unit. Hematocrit (HCT) was evaluated before transfusion, immediately after transfusion, 12 hours after transfusion, and just before discharge. No differences were noted in HCT change following transfusion between the groups, and this remained true through the point of discharge (Fig. 13).

(n total=57)	LPRBC Group (n=35)	CPRBC Group (n=22)	p value
Respiratory Failure	32%	32%	0.97
Acute Renal failure	9%	5%	0.54
multiple-organ failure	12%	9%	0.75
Post transfusion fever	3%	0%	0.42
Transfusion reaction	0%	0%	NA
Deep venous thrombosis	15%	32%	0.13
Mortality	0%	0%	NA
Hospital length of stay, median (IQR)	11 (7-16)	16 (9-27)	0.1

Table 3 Outcomes for patients randomized to either of the two groups.

	LPRBC Group	CPRBC Group	p value
Baseline (R)	6.0 ± 1.7	5.9 ± 1.8	0.85
Post transfusion unit 1 (R)	6.6 ± 1.6	6.0 ± 1.2	0.32
Post transfusion unit 2 (R)	6.4 ± 1.4	5.9 ± 2.1	0.5
12-h post transfusion (R)	6.3 ± 1.9	6.9 ± 2.4	0.44
Baseline (MA)	70.1 ± 8.6	73.1 ± 8.2	0.27
Post transfusion unit 1 (MA)	70.8 ± 7.0	69.8 ± 8.9	0.71
Post transfusion unit 2 (MA)	66.4 ± 7.6	70.5 ± 7.3	0.19
12-h post transfusion (MA)	71.3 ± 7.9	72.8 ± 7.4	0.57
Baseline (α angle)	70.6 (67.3-73.2)	73.1 (67.7-75.9)	0.19
Post transfusion unit 1 (α angle)	68.1 (64.3-72.2)	70.7 (65.5-75.4)	0.24
Post transfusion unit 2 (α angle)	66.7 (62.0-69.2)	71.7 (68.3-75.6)	0.01
12 hours post transfusion (α angle)	68.3 (63.5-72.7)	68.3 (63.8-74.9)	0.89

Table 4 TEG Values for LPRBC and CPRBC groups; median, (IQR). R is the time to clot formation, MA is maximum clot strength, and α -angle is the fibrin cross-linking.

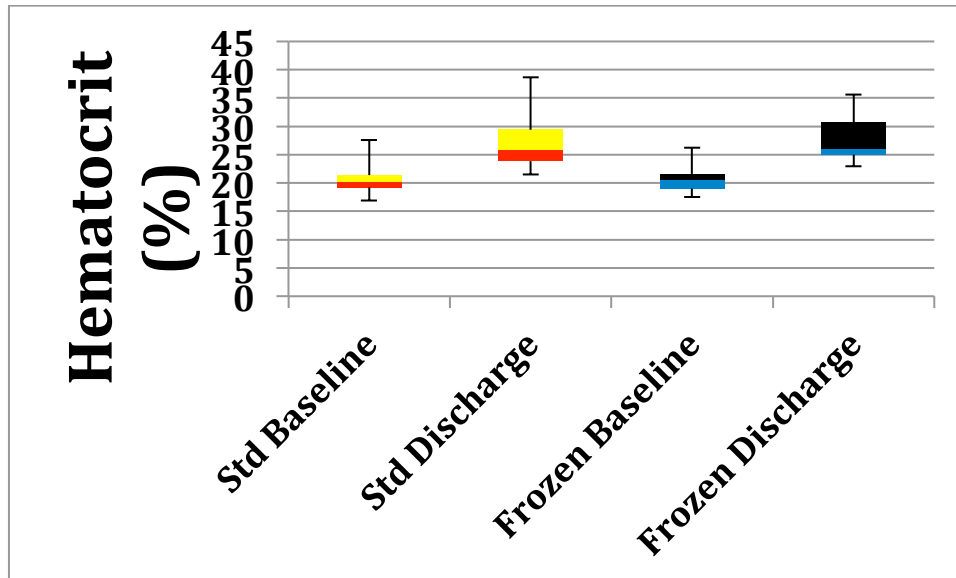


Figure 13 Hematocrit levels for LPRBC and CPRBC groups at baseline and at the time of discharge

DISCUSSION

The goals of any blood transfusion are to restore intravascular volume to an ideal range, increase and maintain blood pressure, increase peripheral tissue oxygenation, and ultimately to prevent organ failure and death. Blood storage techniques have developed as an ongoing process that has come a long way from where they originated. Today the LPRBC storage techniques are very safe from contamination, and have removed almost all risk of disease transmittance from donor to recipient. However the ideal concentrations and types of preservative solutions, storage length, and temperatures have yet to be determined in order to optimize the benefit to the patient.

Due to the increasingly large body of research that supports the existence of a storage lesion, and the fact that the storage lesion's detrimental effects are exacerbated as the length of storage time increases, storage methods have been identified as a potential target for improvement. The lesion has been linked to hypoxia³⁵, increased mortality, longer hospital lengths of stay, organ failure⁵², inflammatory protein related hypocoagulation^{30,31,36} hyperkalemia associated cardiac arrhythmias⁸, and increased levels of free hemoglobin which has been linked to patients becoming hypertensive and developing acute renal tubular injuries^{8,37,38} as well as many others, but the risks of transfusions still do not outweigh the benefits to the patient in most cases. Our responsibility to patients is to maximize the benefit while decreasing the risks as much as possible.

Although not adequately powered to make these conclusions, this study has shown no significant differences in clinical outcomes between the groups whom received CPRBCs and LPRBCs. These conclusions will be more closely examined at, and assessed for clinical significance with the multi-center study that is currently underway, but they have provided insight about the gross trends we are looking for. The main differences that our study identified, and was powered to look at were found in the difference in tissue oxygenation between the two blood groups as well as the difference

in the concentration of 2,3-DPG and the elevated levels of pro-inflammatory cytokines that were observed in the LPRBC group. The CPRBC group showed an elevated level of tissue oxygenation from the time of transfusion initiation through the three-hour time point, and was statistically significant throughout this period of time. The trend continued through the twelve-hour time point, but was no longer statistically significant after 180 minutes. It is likely that the increased tissue oxygenation results from the superior biochemical profile that is associated with the CPRBC units.

Our institution published a study that demonstrated that LPRBCs stored for a period of 20 days or less had no effect on the tissue oxygenation of patients, and LPRBCs that had been stored for a period of time greater than 21 days decreased the patient's tissue oxygenation³⁵. Based on our data, and seeing these trends now in multiple studies, it is theorized that the decrease in oxygenation may be linked to a lacking biochemical profile that usually aids in the offloading of oxygen. 2,3-DPG, which promotes the offloading of the oxygen molecules has been shown here to be decreased with the liquid storage method, and this decrease can be due to the acidic environment created by the red cells metabolizing glucose and generating protons during liquid storage that can degrade the molecule^{8,20}. Data has shown that cryopreservation at these temperatures arrests the biochemical pathways that are associated with this increased acidity, and seems to explain the superior biochemical profile associated with the CPRBC group.

O'Brien and Watkins³⁹ has shown that after thawing the CPRBC units, the levels of 2,3-DPG found in the individual units were similar to the levels found when they were frozen, and that their concentration was only affected by the amount of time between donation and freezing.

As the storage time increases and the storage lesion evolves, a major consequence is the devolution of the RBCs from deformable biconcave discs to non-deformable spherocytocytes⁷. This structural change in the red cells promotes adherence to the vascular endothelium, and prevents them from being able to navigate the small capillary networks in peripheral tissue, which further prevents oxygen from reaching the hypoxic tissues that need them⁴⁰.

During the thawing and deglycerolizing process that occurs before CPRBCs are transfused, they undergo a washing process as well. This may have an additional beneficial effect on the units by removing unwanted lysed cells, free hemoglobin, and other harmful proteins. A study is currently being designed that will look at the washing process independent of the CPRBC units.

Up to 30% of RBCs in blood units that are greater than 42 days old are considered non-viable, and when transfused, the body immediately removes them from circulation. Not only do the cells become non-viable, but they also dump their contents into the blood stream as they lyse. This increases the concentration of free hemoglobin, which is a known NO scavenger⁴¹. NO is a potent and crucial vasodilator that is a necessary component in order to widen vessels and allow the blood cells to gain access to the tissues that need them.

StO₂ changes are commonly used as predictors of mortality in trauma as well as neurological patients²⁹. The near infrared monitoring method we used to obtain the StO₂ data for these patients has been widely used to assess tissue perfusion in applications such as cerebral monitoring and monitoring for compartment syndrome^{42,43}. The fact that tissue oxygenation which is crucial to patients survival and rapid healing is decreasing

based on the length and method of storage, is an indicator that something must be changed in order to preserve the potential of PRBCs.

With the decreased oxygenation, the inferior biochemical profile, the inability to mobilize blood units for seasonal demands and natural disasters, and the more than \$80 million loss annually that results from our nation maintaining only a liquid preservation method of storage for PRBCs, it is clear there are advantages to having a supplemental cryogenic blood storage program. This study has begun to demonstrate that the Method of cryogenically freezing red blood cells is safe, maintains a superior biochemical profile, achieves increased levels of tissue oxygenation over liquid storage and provides the possibility of huge cost savings and better patient care. Currently we are in the middle of a multi-center study using similar methods that is better powered to make the conclusions we have outlined, and will look closer at the outcomes and transfusion reactions associated with patients receiving red blood cells.

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