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Previous cryopreservation alters the natural history of the red blood cell storage lesion

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Abstract

Background—During storage, packed red blood cells (pRBCs) undergo a number of biochemical, metabolic and morphologic changes, collectively known as the “storage lesion”. We aimed to determine the effect of cryopreservation on the red blood cell storage lesion compared to traditional 4°C storage.

Methods—Previously cryopreserved human packed red blood cells were compared to age matched never frozen packed red blood cells obtained from the local blood bank. The development of the red cell storage lesion was evaluated after 7, 14, 21, 28, and 42 days of storage at 4°C in AS-3 storage medium. We measured physiological parameters including cell counts, lactic acid and potassium concentrations as well as signs of eryptosis including loss of phosphatidylserine (PS) asymmetry, microparticle production and osmotic fragility in hypotonic saline.

Results—Compared to controls, previously cryopreserved pRBC at 7 days of storage in AS-3 showed lower red cell counts (3.7 vs 5.3×10^6 cells/uL, $p < 0.01$), hemoglobin (12.0 vs 16.5 g/dL, $p < 0.01$), hematocrit (33.0 vs 46.5% , $p < 0.01$), and pH (6.27 vs 6.72 , $p < 0.01$). Over 28 days of storage, storage cryopreserved pRBC developed increased cell free hemoglobin (0.7 vs 0.3 g/dL, $p < 0.01$), greater PS exposure (10.1 vs 3.3% , $p < 0.01$), and microparticle production ($30,836$ vs $1,802$ MP/uL, $p < 0.01$). Previously cryopreserved cells were also less resistant to osmotic stress.

Conclusion—The red blood cell storage lesion is accelerated in previously cryopreserved pRBC after thawing. Biochemical deterioration of thawed and deglycerolized red cells suggests that storage time prior to transfusion should be limited in order to achieve similar risk profiles as never frozen standard liquid storage pRBC units.

Introduction

The advent of anticoagulant and storage solutions in the early 20th century allowed longer term storage of blood products, enabling the use of allogeneic blood product transfusion to become a vital and widespread therapy in modern medicine. The 2011 National Blood

Collection and Utilization Survey estimated that 13.7 million allogeneic whole blood and packed red cell units were transfused in the United States alone during that year, with an additional 65,000 autologous units transfused.¹ Nearly half of all intensive care unit patients receive red blood cell transfusions.

In the early era of blood banking, availability of red cells was complicated by a limited donor supply and short shelf life of whole blood stored at 4° Celsius. The partitioning of blood components into erythrocyte, platelet concentrates and plasma spurred the development of additive solutions to supplement the volume of packed red blood cell units. Currently, F.D.A. approved additive solutions are composed of saline solutions containing citrate and phosphate buffers as well as glucose and adenine to supplement erythrocyte metabolism. These techniques increased the shelf life of erythrocytes to 42 days, significantly increasing the efficiency of blood banking.

The transfusion of stored red cell concentrates have been implicated in lung injury since this correlation was first appreciated in the military area in the 1960's.^{2,3} Since then, red cell and other blood component therapy has been associated with a multitude of adverse clinical outcomes.^{4,5} Furthermore, the duration of storage prior to transfusion has been implicated in worse post-operative outcomes. In a large study of cardiac surgery patients, transfusion of red cells stored greater than 2 weeks was associated with increased post-operative complications and increased mortality. The cause of this perceived inferiority of older blood is an area of much debate and study. Current hypotheses include (1) the depletion of intracellular metabolites, (2) erythrocyte membrane degradation, (3) inflammatory mediators, (4) and hemolysis. These changes taken together are known as the red blood cell storage lesion. Much frequently reviewed work regarding the mechanism leading to the so-called storage lesion already exists, however its clinical implication is still a matter of debate.²

In parallel to the development of red cell storage in anticoagulant and additive systems at 4° Celsius, techniques to preserve erythrocytes for an extended duration at -80° Celsius in high concentrations of glycerol have been developed. This storage technique has the advantage of allowing the red cells to be maintained in the frozen state for 10 years. The technique developed by Valeri and his colleagues has been an important adjunct to standard blood banking practices, particularly in military and expeditionary medicine as well as repositories of rare blood types, due to limited donor availability.⁶⁻⁸ Cryopreservation also provides a novel opportunity to minimize exposure of red cell transfusion recipients to potentially harmful metabolic byproducts found in aged packed red blood cells.^{9,10} Current FDA guidelines limit the use of thawed and deglycerolized erythrocytes to 14 days due to concerns related to red blood cell viability. Little is known of the effect of previous cryopreservation on aspects of the red blood cell storage lesion either in the near (up to 14 days) or long (up to 42 days) term. We undertook these experiments to develop a more sophisticated understanding of effect of previous cryopreservation on development of the red blood cell storage lesion during short- and long-term storage. We hypothesized that previous cryopreservation would alter the natural history of the red blood cell storage lesion.

Materials and Methods

Donor packed red blood cells

Donated human packed red blood cell units (n=5) were collected in citrate phosphate double dextrose (CP2D) (257.6 mmol/L glucose, 105.0 mmol/L citric acid, 18.5 mmol/L monosodium phosphate, pH 5.7). Units were stored at 4 degrees Celsius for a period of less than 6 days in additive solution-3 (Nutricel) (55.5 mmol/L glucose, 70.1 mmol/L sodium chloride, 20 mmol/L sodium phosphate, 12 mmol/L citric acid, 2.2 mmol/L adenine, pH 5.8). The red cells were preserved in glycerol using Glycerolyte 57 solution (Fenwal Inc., Lake Zurich, IL) using the ACP 215 Automated Cell Processor (Haemonetics Corp., Baintree, MA). After at least 1 year of storage at -80 degrees Celsius, the units were deglycerolized using the ACP 215 with the manufacturer's recommended protocol (Haemonetics Corp., Baintree, MA). Standard units of leukoreduced human packed red blood cells in additive solution-3 were obtained from the Hoxworth Blood Center (Cincinnati, Ohio) and used as controls. Storage age matching was based on the time after thawing and deglycerolization as compared to donation (for standard storage pRBC units). Comparisons to previously cryopreserved units of blood were made after identical storage durations in liquid phase at 4° Celsius. All units were cultured at the end of 42 days of storage to evaluate for bacterial contamination.

The use of human biological materials was approved by the University of Cincinnati Institutional Review Board. All donor health information was protected in accordance to institutional policy.

Biochemical Measurements

For biochemical measurements, samples of previously cryopreserved and control units were drawn from storage bags using 18 gauge needles through sterile sampling ports (Fenwal Inc., Lake Zurich, IL) and aliquoted into sterile polypropylene tubes for future testing to minimize exposure to repeated sampling of blood bags. Individual aliquots were stored at 1-6 degrees Celsius and tested at intervals during storage.

The packed red blood cell unit pH was determined using an Accumet AB electrochemical bench top meter (Fisher Scientific International Inc., Hampton NH) at 22 degrees C. Lactic acid (LA) and cell free potassium (K^+) were measured using point of care clinical blood analyzers (iSTAT, Abbott Laboratories, North Chicago, IL). Red blood cell concentration (RBC), hemoglobin (Hgb), hematocrit (Hct), and Mean Corpuscular Volume (MCV) were measured using a Coulter AcT diff Analyzer (Beckman Coulter Corp., Brea, CA). For determination of cell free hemoglobin, aliquots of packed red cells were centrifuged at 2,000 RCF $\times$ 10 minutes at 4 degrees C and the supernatant were sampled for cell free hemoglobin using centrifugation and absorbance spectroscopy on the cell free component. Oxygen dissociation curves and determination of oxygen p50 values was performed by ARUP laboratories (Salt Lake city, UT) and oxygen p50 measurements were obtained.

Osmotic Fragility

Previous methods reported by Mazeron and his colleagues were modified to produce erythrocyte osmotic fragility curves in hypotonic solutions.¹¹ Osmotic Fragility was determined by incubation of packed red cells in 10 volumes serially diluted saline solutions for 1 hour at room temperature. Cells were pelleted at $2,000 \text{ RCF} \times 10 \text{ minutes}$ at 4 degrees C and supernatant was sampled for free hemoglobin. Results were normalized to total hemoglobin and plotted against saline concentration. EC50 for 50% lysis was determined using SigmaPlot software.

Red Cell Membrane Symmetry and Microparticle Formation

Extracellular membrane phosphatidyl serine (PS) expression was determined by flow cytometry analysis using fluorescein conjugated Annexin V antibody (Abcam p.l.c., Cambridge, United Kingdom). Cells were co-incubated with phycoerythrin conjugated anti-CD235a antibody (BD Biosciences, San Diego, CA). Cells were analyzed on an Attune flow cytometer (Life Technologies, Carlsbad, CA). Erythrocyte and erythrocyte derived microparticles were determined by size gating using 1 and 6 μm polystyrene beads and glycophorin A positivity.

Statistical Analysis

All results are reported as mean $\pm$ standard deviation unless otherwise specified. All statistical tests were performed using unpaired student's t-tests with a significance set at 0.05. All figures are generated by SigmaPlot (Systat Software Inc., Chicago, IL).

Results

Post-processing packed red blood cell characteristics

During initial experiments, we determined characteristics of packed red blood cell units during the initial period after thawing and deglycerolization (**Figure 1**). Previously cryopreserved units contained lower concentrations of red cells as compared to standard storage pRBCs (3.7×10^6 vs 5.5×10^6 per μl , $p < 0.01$; **Figure 1A**). The hematocrit of previously cryopreserved packed red blood cell units was also lower than standard storage pRBCs units ($33.2 \pm 4.3\%$ vs $48.3 \pm 6.2\%$, $p < 0.01$; **Figure 1B**). In a similar fashion, the average hemoglobin concentration was lower in our previously cryopreserved units as compared to standard storage pRBCs units ($11.2 \pm 1.1 \text{ mg/d}$ vs $16.8 \pm 1.9 \text{ mg/dL}$, $p < 0.01$; **Figure 1C**). The mean corpuscular volume of previously frozen pRBC units was not significantly different than standard storage pRBCs units (**Figure 1D**).

Supernatant content of pRBC units during subsequent storage

Previous work from our laboratory showed that the pH of pRBCs decreases during storage.¹² We next compared the pH of previously cryopreserved pRBCs with that of standard storage pRBC units over time. The pH of cryopreserved pRBC units immediately after deglycerolization was 6.26 ± 0.07 as compared to 6.68 ± 0.04 in standard storage pRBC units ($p < 0.01$; **Figure 2A**). In each group, the extracellular pH decreased during subsequent storage. The extracellular pH of previously cryopreserved pRBC units decreased to

6.13±0.05 at 14 days of storage, then again to 5.61±0.07 at day 42 of storage (**Figure 2A**). A similar relative extracellular acidosis occurred in standard storage pRBC units with a pH of 6.59±0.05 at day 14. The pH appeared to stabilize over time in standard storage pRBC units (**Figure 2A**).

An additional well known aspect of the red blood cell storage lesion is the accumulation of extracellular potassium.¹³ We determined extracellular potassium levels in the supernatant of units stored after cryopreservation and deglycerolization or standard storage (**Figure 2B**). Initial cell free potassium levels in previously cryopreserved blood were lower than that of age matched controls, although this difference did not reach significance (9.28±1.02 vs 11.17±2.06, p=0.07). Both previously cryopreserved packed red blood cells and post-thaw storage-age matched standard storage pRBC units showed a similar rise in cell free potassium during 42 days of 4° C storage (**Figure 2B**).

In order to determine the effect of previous cryopreservation on hemolysis, we determined the effect of storage on accumulation of cell free hemoglobin. Cell free hemoglobin in previously cryopreserved pRBC units was 0.27±0.05 mg/dL as compared to 0.37±0.05 mg/dL in post-thaw storage-age matched standard storage pRBC units (p<0.01; **Figure 2C**). At 14 days of storage, free hemoglobin in previously cryopreserved and post-thaw storage-age matched standard storage pRBC units was similar. By day 42 of storage, cell free hemoglobin in previously cryopreserved units had surpassed that of post-thaw storage-age matched standard storage pRBC units (1.40±0.28 vs 0.78± 0.16, p<0.01; **Figure 2C**).

Extracellular lactic acid concentration in previously cryopreserved blood was 6.39±0.69 meq/L compared to 12.49 ±0.52 in post-thaw storage-age matched standard storage pRBC units (p<0.01; **Figure 2D**). The rate of lactic acid accumulation was significantly lower in previously cryopreserved blood as compared to post-thaw storage-age matched standard storage pRBC units (**Figure 2D**). After correcting for the concentration of red cells, the daily lactic acid production in previously frozen packed red blood cell units was lower than that of post-thaw storage-age matched standard storage pRBC units (0.21 vs 0.35 meq/L/million cells, p<0.01).

Membrane fragility, PS externalization and microvesiculation

Previously cryopreserved cells exhibited less resistance to hypo-osmotic lysis than post-thaw storage-age matched standard storage pRBC units. The sodium chloride concentration at which 50% of maximal lysis occurred (EC50) was 0.55±0.01% in previously cryopreserved units compared to 0.49±0.01% in post-thaw storage-age matched standard storage pRBC units at day 28 (p<0.01; **Figure 3**). This difference was also seen at 42 days (data not shown).

Loss of erythrocyte membrane asymmetry was also noted to be more pronounced in previously cryopreserved red blood cells. By flow cytometry, a subset of erythrocytes were demonstrated to have phosphatidyl serine exposure on the extracellular membrane (**Figure 4A**). The percent of erythrocytes showing this sign of red cell deterioration was 9.55±1.22% in cryopreserved blood by 28 days of storage compared to only 3.15±0.50% in standard storage erythrocytes (p<0.01; **Figure 4B**). This difference was less pronounced at day 42 of

storage with cryopreserved erythrocyte phosphatidyl serine exposure on $23.8 \pm 3.4\%$ of cells compared as compared to $22.0 \pm 5.1\%$ in standard storage pRBC units ($p=0.77$, data not shown).

An additional important measure of loss of red blood cell membrane integrity and aging is the production of microparticles.¹⁴ Red cell derived microparticles were demonstrated by dual fluorescent labelling of sub micrometer sized particles with erythrocyte lineage marker CD235 and phosphatidyl serine (**Figure 4C**). Red cell derived microparticles were higher by day 28 in previously cryopreserved units as compared to post-thaw storage-age matched standard storage pRBC units ($4.8 \pm 1.0 \times 10^4$ /uL vs $0.3 \pm 0.1 \times 10^4$ /uL, $p<0.01$; **Figure 4D**). This difference was also present at day 42 of storage (1.9×10^4 / uL vs $0.3 \pm 0.04 \times 10^4$ /uL, $p<0.01$, data not shown).

The process of deglycerolization and preparation for transfusion involves potentially violating the sterility of pRBC units and raises concerns for possible contamination. In order to assess for contamination, blood samples collected at the end of 42 days of storage were plated on trypsin soy agar plates and allowed to grow for 72 hours at 37 degrees C under standard cell culture conditions. No colonies were observed on any of the plates from previously cryopreserved or control packed red blood cells (data not shown). Romanowsky stain also did not show any significant contamination or abnormalities in red cell morphology in the erythrocyte concentrates (data not shown).

Oxygen dissociation curves were generated for three samples of cryopreserved blood as well as three control samples at 28 days of storage. The oxygen dissociation p50 for cryopreserved units compared to controls was not significantly different (data not shown).

Discussion

In the present study, we evaluated the effect of previous cryopreservation on aspects of the red blood cell storage lesion. Cryopreservation represents a potentially powerful method to store pRBC units, manage inventory and distribution, and mitigate potential harm from the red blood cell storage lesion. Previous clinical studies as well as our current data indicate that previously cryopreserved pRBC units, as prepared for transfusion, are a safe and effective means of ameliorating anemia.^{9,15} Previous preclinical studies have examined the effects of the duration of storage prior to cryopreservation and the duration of storage at -80 degrees Celsius.^{16,17} We undertook the present study to increase our knowledge about the effect of subsequent storage of previously cryopreserved pRBC units. Our data indicate that previous cryopreservation alters the onset and development of the red blood cell storage lesion and suggest that there is the potential to intervene in this process and prolong the effective shelf life of these pRBC units.

The current FDA approved method of glycerolization and deglycerolization with the Haemonetics ACP 215 requires removal of plasma and resuspension of red cells in 40% v/v glycerol and storage at less than -65 degrees Celsius. To date, there is no evidence to suggest that any appreciable deterioration of red cells occurs during this storage process. The FDA limit for storage in the frozen state is 10 years. The 14 day post thaw storage limit,

however, causes many logistical issues. It also raises the question of what changes in these previously frozen units compared to concentrates stored in similar storage systems at 4 degrees Celsius.

Our study obtained previously frozen blood from a single blood bank that was thawed and deglycerolized using the Haemonetics ACP 215 using the manufacturers recommended protocol. A review of the literature will reveal inter-institutional variability of red blood cell recovery.^{18,19} The current American Association of Blood Banks standard mandates a post thaw recovery 80% of the original red cells present prior to glycerolization. Although post thaw hematocrit in our study was lower than controls, this is not a clear marker for decreased recovery of cells as the reconstituted volume is not strictly controlled.

The significantly decreased pH found in previously frozen cells may be in part due to the high relative volume of AS-3, which has a pH of 5.8. During storage, both thawed units and controls units exhibited a steady decline in pH. Previous work has shown that dilution of red cells and buffering of the extracellular environment plays a beneficial role in the in vivo recovery. The experimental additive solutions proposed by Hess and colleagues²⁰ to prolong storage at 4° C may also be beneficial to previously frozen red cells. The clinical implications of this less than physiologic pH at the time of infusion are likely not significant given the relatively acidotic nature of intravenous normal saline, however this may play a larger role if applied in massive transfusion settings.

Despite the considerably lower concentration of red cells, extracellular potassium levels increased no less rapidly. A shift of potassium to the extracellular space in response to environmental acidosis occurs, although in vivo hyperkalemia is seen more commonly with non-anion gap acidosis. While this may suggest that membrane leak and cell lysis occurs more rapidly in thawed units, it is also likely a depletion of cellular energy impairs potassium equilibrium maintained by ATP dependent potassium pumps.²¹

The lactic acid accumulation occurs more rapidly in the controls than in the previously cryopreserved units, especially during the first 21 days of storage. Even when corrected for decreased red cell concentration, lactic acid generation was significantly decreased in previously frozen cells. It is apparent that even at 4°C glycolysis plays a key role in erythrocyte metabolism and maintenance of ATP dependent functions. Moreover, erythrocyte ATP release may play an important role in physiologic regulation of oxygen delivery.²² Flux through glycolytic enzymes may also be partially down regulated in response to the progressive acidosis which occurs sooner in frozen cells than in controls.

Membrane loss and the generation of hemoglobin containing microparticles play a significant role in the red blood cell storage lesion.²³ Similar to previous studies by Acker et. al. in 2008, we saw no statistically significant increases in microparticle formation immediately after thaw (data not shown).¹⁶ This may be partially attributed to the minimal pre-freeze storage duration of our cryopreserved red blood cells. While between day 28 and day 42 of storage, there appears to be a decrease in microparticle concentration, logically this is unlikely to be the case in a closed storage environment. Much more likely is that this is an effect of pre-analytical and analytical error inherent in measuring microparticles by

flow cytometry. Despite its many pitfalls including limited resolution of sub 400 nanometer particles and low inter-experimental comparability, flow cytometry remains the gold standard to count plasma microparticles and has been applied widely to particles derived from many cell types.²⁴ We also found that post thaw storage duration resulted in more rapid exposure of extracellular erythrocyte membrane phosphatidyl serine, which has been implicated in eryptosis and microparticle formation.²⁵

Our results suggest that the cryo-injury alluded to in previous studies may be intrinsic to the red cells themselves and is not removed by washing during the deglycerolization process. Based on these observations, aspects of the red blood cell storage lesion in previously frozen pRBCs reaches levels similar to nearly expired standard pRBCs at approximately 21-28 days after thaw. Previous work by Moore and colleagues also suggest storage of up to 3 week post thaw would be practical in AS-3.^{26,27} One practical consideration regarding the use of previously cryopreserved pRBC units is related to the composition of these units when recently prepared as compared to pRBC units stored under standard conditions for the duration of their shelf life. In order to evaluate this, we re-analyzed data presented in Figures 2 and 4 in order to directly compare these two groups. These data are presented in **Figure 5**. We found that several characteristics of the red blood cell storage lesion, including pH, lactate, potassium, free hemoglobin, erythrocyte phosphatidylserine expression, and microparticle counts were more favorable in recently thawed pRBC units than in pRBC units that were stored under standard conditions for up to 42 days. These data suggest that even though storage lesion progression is altered in previously cryopreserved units, recently thawed and deglycerolized units may be of higher quality than pRBCs stored under standard conditions for the full FDA shelf life. As storage solutions continue to improve, this comparison may become less pronounced. However the promise of novel storage solutions and improved post thaw reconstitution may have similar, if not greater, benefits on previously cryopreserved packed red blood cells.

Our findings are not without limitation and should be interpreted with caution. One potential limitation of our works is that ABO blood groups and donor characteristics were not controlled for between the groups. Previous studies have suggested that ABO group alters has been found to alter responses in certain settings of aged pRBCs²⁸, other data indicates that ABO group does not alter membrane fragility during storage²⁹ but the effect on the red blood cell storage lesion after cryopreservation is unknown and thus our findings should be interpreted with caution. In addition, lower erythrocyte concentrations in cryopreserved units may affect the development of the storage lesion. The changes we observed would favor lower erythrocyte concentrations in most situations, including the development of free hemoglobin and microparticle generation. Improvements to the deglycerolization process may have profound effects on its aging characteristics. Finally, all packed red blood cell units in our study were stored in polypropylene tubes after extraction from standard blood bags. Our previous data indicates that aliquots stored in polypropylene tubes exhibit similar aging characteristics to those stored in standard blood bags.^{12,14}

Conclusion

Cryopreservation of red blood cell units in at -80°C for a prolonged period is a promising adjunct to standard blood banking techniques. The post thaw characteristics are markedly different than fresh packed red blood cells and the development of the red blood cell storage lesion is accelerated in the post thaw period. Previously cryopreserved packed red blood cells may be favorable in situations where new donors are not readily available and storage duration at 4°C Celsius must be prolonged. Future studies focusing on decreasing the storage lesion in previously cryopreserved packed red blood cells may allow longer post-thaw storage time and provide additional longevity to these red cell products in vitro and in vivo.

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Figure 1.

(A) Erythrocyte cell counts, (B) hematocrit, (C) hemoglobin concentration and (D) mean corpuscular volume in standard storage (Control) or previously cryopreserved (Frozen) packed red blood cell units at 7 days of storage. * signifies $p < 0.01$ vs standard storage (Control) conditions.

Figure 2.

Comparison of pH (A), extracellular potassium concentration (B), free hemoglobin concentration (C), and lactic acid concentration (D) between standard storage (Control) or previously cryopreserved (Frozen) packed red blood cell units during storage in AS-3. * signifies $p < 0.05$ vs post-thaw storage-age matched standard storage (Control) pRBC units at each time point.

Figure 3.

Osmotic fragility of standard storage pRBCs (A) and previously cryopreserved pRBCs (B) in increasing concentrations of sodium chloride. Effective hypotonic lysis concentration of cryopreserved (Frozen) packed red blood cells at 28 days of storage was less than that of post-thaw storage-age matched standard storage (Control) pRBC units (C). * signifies $p < 0.01$ vs post-thaw storage-age matched standard storage (Control) pRBC units.

Figure 4.

Flow cytometry analysis and comparison of previously frozen and post-thaw storage-age matched standard storage erythrocytes at 28 days of storage. (A) Scatter plot of previously cryopreserved pRBCs stained for phosphatidyl serine. (B) Quantification phosphatidyl serine exposure on pRBC membranes in standard storage (Control) as compared to previously cryopreserved (Frozen) pRBC units. (C) Scatter plot of flow cytometry analysis of red blood cell derived microparticles in previously cryopreserved pRBC. (D) Quantification of pRBC derived microparticles in standard storage (Control) as compared to previously cryopreserved (Frozen) pRBC units at 28 days of storage. * signifies $p < 0.01$ vs post-thaw storage-age matched standard storage (Control) pRBC units.

Figure 5.

Comparison of (A) free hemoglobin, (B) lactic acid, (C) pH and (D) extracellular potassium concentration in previously cryopreserved pRBCs at 7 days of storage as compared to pRBCs stored under standard conditions for 42 days. (E) Phosphatidyl serine exposure and (F) pRBC derived microparticle concentration in previously cryopreserved pRBCs at 7 days of storage as compared to pRBCs stored under standard conditions for 28 days. * signifies $p < 0.05$ vs standard storage pRBC units.