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Effect of Packed Red Blood Cell Cryopreservation on Development of the Storage Lesion and Inflammation

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14. ABSTRACT

Recent clinical studies in trauma patients indicate that treatment of anemia by transfusion with previously cryopreserved packed red blood cells is superior to that of aged units stored under standard conditions, but the Food and Drug Administration currently restricts their use to 14 days after thawing. The effect of longer term liquid storage on development of the biochemical, metabolic, and morphologic changes collectively known as the red blood cell storage lesion is unknown. In the present study, we attempted to compare and quantify the development of the red blood cell storage lesion of previously cryopreserved red cell units to conventional blood storage. Our findings indicate that, in addition to significantly altered characteristics post thaw, the common indicators of red blood cell storage lesion developed more rapidly in previously cryopreserved red cells. To achieve a satisfactory safety profile for transfusion of previously cryopreserved red blood cells, these factors must be considered in addition to red cell viability and post-transfusion recovery. This investigation provides insight into the durability of these cells after storage at -80°C and may guide potential expansion of red blood cell cryopreservation in civilian, trauma, and military settings. Cryopreservation of red blood cell units 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. Post-thaw storage duration may be further studied to provide additional longevity to these red cell products in vitro and in vivo.

15. SUBJECT TERMS
Blood, cryopreserved blood, cryopreserved packed red blood cells, pRBCs, red blood cell storage lesion, blood storage

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1.0 SUMMARY

Recent clinical studies in trauma patients indicate that treatment of anemia by transfusion with previously cryopreserved packed red blood cells is superior to that of aged units stored under standard conditions, but the Food and Drug Administration currently restricts their use to 14 days after thawing. The effect of longer term liquid storage on development of the biochemical, metabolic, and morphologic changes collectively known as the red blood cell storage lesion is unknown. In the present study, we attempted to compare and quantify the development of the red blood cell storage lesion of previously cryopreserved red cell units to conventional blood storage. Our findings indicate that, in addition to significantly altered characteristics post thaw, the common indicators of red blood cell storage lesion developed more rapidly in previously cryopreserved red cells. To achieve a satisfactory safety profile for transfusion of previously cryopreserved red blood cells, these factors must be considered in addition to red cell viability and post-transfusion recovery. This investigation provides insight into the durability of these cells after storage at -80°C and may guide potential expansion of red blood cell cryopreservation in civilian, trauma, and military settings. Cryopreservation of red blood cell units 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. Post-thaw storage duration may be further studied to provide additional longevity to these red cell products in vitro and in vivo.

2.0 INTRODUCTION

The advent of anticoagulant and storage solutions in the early 20th century allowed for more long-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. Nearly half of all intensive care unit patients receive red blood cell transfusions [1].

In the early era of blood banking, the large demand for red cells was logistically complicated by a limited donor supply and short shelf life of whole blood at 4°C. 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 (pRBC) units and favorably dilute the product. Currently, Food and Drug Administration (FDA)-approved additive solutions are composed of saline solutions containing citrate and phosphate buffers as well as glucose and adenine to supplement erythrocyte metabolism [2]. These techniques increase the shelf life of erythrocytes to 42 days, significantly increasing the efficiency of blood banking.

The transfusion of stored red cell concentrates has been implicated in lung injury since this correlation was first appreciated in the military area in the 1960s [3,4]. Since then, red cell and other blood component therapies have been associated with a multitude of adverse clinical outcomes [5,6]. 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 longer 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. 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 unclear [3]. Current hypotheses include (1) the depletion of intracellular metabolites, (2) erythrocyte membrane degradation, (3) inflammatory mediators, and (4) hemolysis (Figure 1).

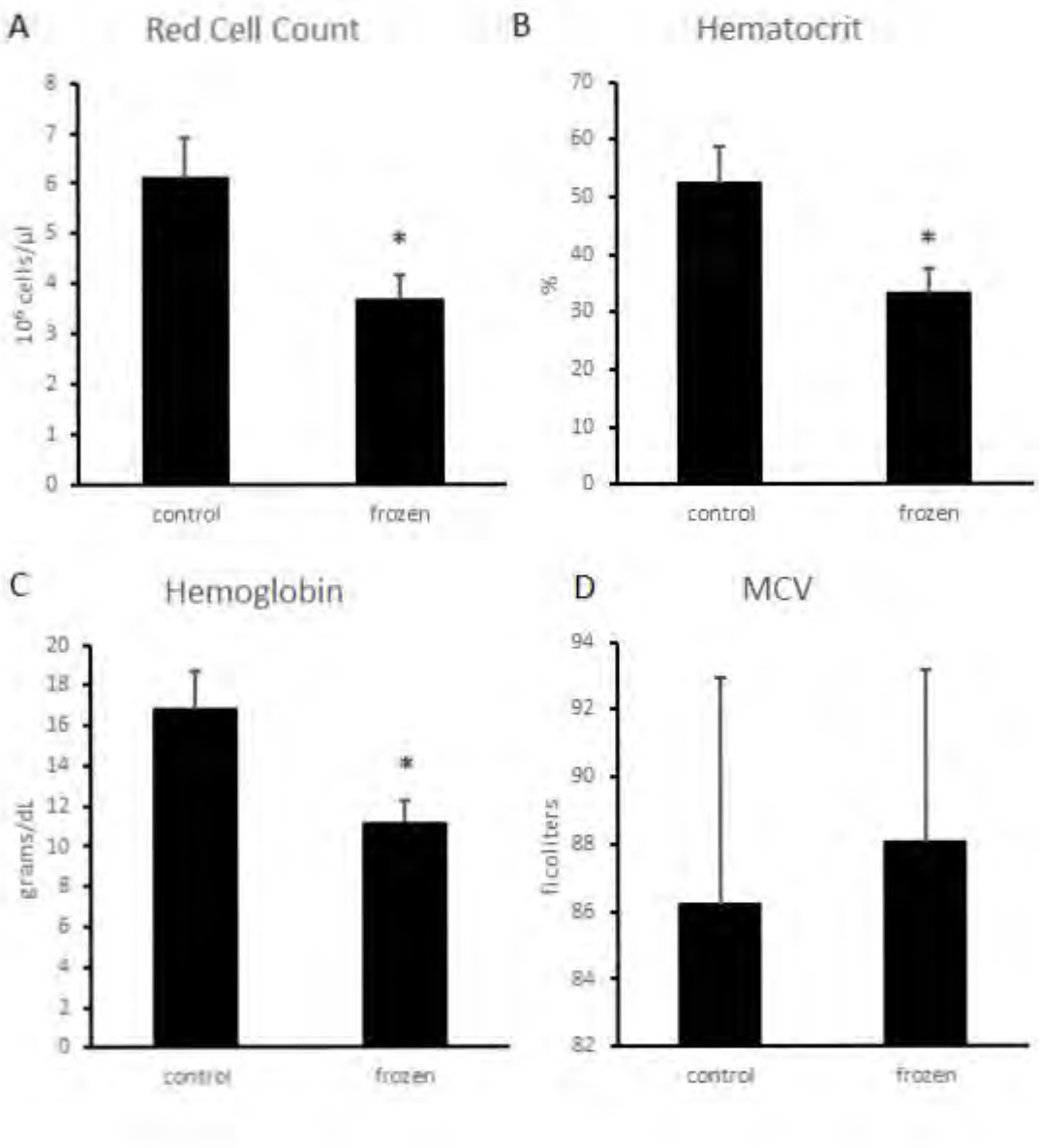

Figure 1. Storage lesion data.

Techniques to preserve erythrocytes for an extended duration at -80°C in high concentrations of glycerol may provide a solution to the red blood cell storage lesion. While these cells can be maintained in the frozen state for an essentially indefinite period of time, their use after thawing and removal of glycerol is currently limited to 14 days due in part to concerns about bacterial contamination [7,8]. The technique developed by Valeri and colleagues has been a useful adjunct to standard blood banking practices, particularly in military and expeditionary medicine, as well as repositories of rare blood types where donor availability is limited [9-11]. Cryopreservation also provides a novel opportunity to minimize exposure of red cell transfusion recipients to potentially harmful metabolic byproducts found in aged pRBCs [12,13]. Understanding the post-thaw characteristics of cryopreserved red cells will provide a more sophisticated understanding of the red blood cell storage lesion and the clinical risk and benefits of cryopreserved red blood cell transfusion. In this study, we aim to show that cryopreservation accelerates the post-thaw development of the red blood cell storage lesion.

3.0 MATERIALS AND METHODS

3.1 Donor Packed Red Blood Cells

Donated human pRBCs were collected in citrate phosphate double dextrose (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°C for a period of less than 6 days in additive solution-3 (AS-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 the membrane permeable cryoprotectant glycerol using Glycerolyte 57 solution (Fenwal Inc., Lake Zurich, IL) and the ACP 215 Automated Cell Processor (Haemonetics Corp., Baintree, MA). After at least 1 year of storage at -80°C, the units were deglycerolized using the ACP 215 in the FDA-approved manufacturer's recommended protocol (Haemonetics Corp., Baintree, MA). Standard units of leukoreduced human pRBCs were obtained from the local blood bank to be used as controls. All units were cultured at the end of 42 days of storage to ensure no bacterial contamination had occurred.

3.2 Biochemical Measurements

Samples from previously cryopreserved and control pRBC units were drawn from blood bags using 18-gauge needles through sterile sampling ports (Fenwal Inc., Lake Zurich, IL) and aliquoted for future testing to minimize exposure to repeated sampling of blood bags. Individual aliquots were stored at 1-6°C and tested at various time intervals during storage.

The packed cell unit pH was determined using the Accumet AB electrochemical benchtop meter (Fisher Scientific International Inc., Hampton, NH) at 22°C. Lactic acid (LA) and cell free potassium (K^+) were measured using point of care clinical blood analyzers (Abbott Laboratories, North Chicago, IL). Red blood cell concentration, hemoglobin (Hgb), hematocrit (Hct), and mean corpuscular volume were measured using a Coulter Ac·T diff Analyzer (Beckman Coulter Corp., Brea, CA). Aliquots of packed red cells were centrifuged at 2,000 relative centrifugal force x 10 minutes at 4°C and supernatant was sampled for cell free Hgb. Oxygen dissociation curves were performed by ARUP Laboratories (Salt Lake City, UT) and oxygen p50 measurements were obtained.

3.3 Osmotic Fragility

Osmotic fragility was determined by incubation of packed red cells in 10 volumes of serially diluted saline solutions for 1 hour at room temperature. Cells were pelleted at 2,000 relative centrifugal force x 10 minutes at 4°C and supernatant was sampled for free Hgb. Results were normalized to total Hgb and plotted against saline concentration. The sodium chloride concentration at which 50% of maximal lysis occurred (EC50) was determined using SigmaPlot software (Systat Software Inc., Chicago, IL).

3.4 Red Cell Membrane and Microparticle Formation

At 28 days of storage, red cells were removed from storage and extracellular membrane phosphatidylserine (PS) was quantified using fluorescein conjugated Annexin V (Abcam p.l.c., Cambridge, UK). Cells were co-incubated with phycoerythrin conjugated anti-CD235a (glycophorin A) 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. Cell morphology was also examined using Diff-Quik Romanowsky stains under light microscopy.

3.5 Statistical Analysis

All experiments were performed with at least n=5 to ensure reproducibility of results. Results are reported as average ± standard deviation unless otherwise specified. All statistical tests were performed using non-parametric Wilcoxon methods with a significance set at 0.05. All figures are generated by SigmaPlot and Microsoft Excel 2003 (Redmond, WA).

4.0 RESULTS

4.1 Initial Red Cell Concentrate Characteristics

Previous cryopreserved units contained lower concentrations of red cells (3.68×10^6 red cells per μL) compared to controls (3.68×10^6 vs. 6.10×10^6 per μL). This corresponded to an average Hct of 33.2±4.3% in frozen units while controls had an average Hct of 52.4±6.2%. Similarly, the average Hgb concentration was lower at 11.2±1.1 mg/dL in our cryopreserved units compared to 16.8±1.9 mg/dL in controls. The mean corpuscular volume of previously frozen units was not significantly different than controls (Figure 2).

4.2 Supernatant Content

The pH of cryopreserved units after thawing was 6.43±0.04 compared to 6.64±0.05 in controls. The extracellular pH had dropped to 5.83±0.2 and 5.61±0.07 by days 28 and 42 of storage, respectively. A similar relative extracellular acidosis occurred in controls with a pH of 6.17±0.18 by day 28. However, progressive decline in pH was not observed in controls, with pH of 6.25±.12 at day 42 (Figure 3).

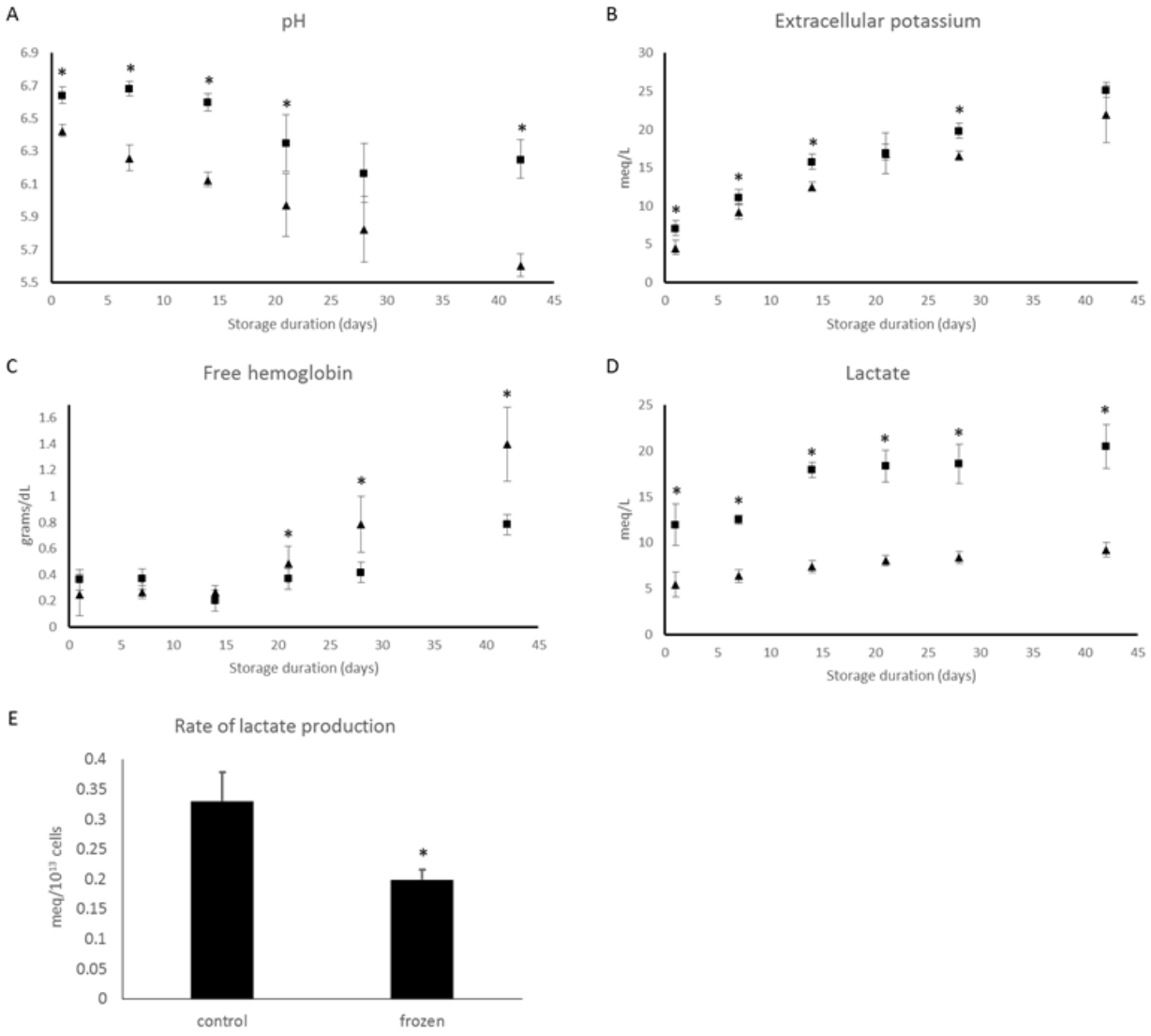

Figure 2. Corpuscular volume.

Cell free K^+ levels in previously cryopreserved blood were initially lower at 4.58±1.0 meq/L while age-matched controls had initial K^+ levels at 7.12±1.60 meq/L. Increases in K^+ levels in previously cryopreserved units were similar to controls, resulting in 16.17 meq/L and 23.6 meq/L at 28 and 42 days post thaw, respectively (Figure 3).

Initially, extracellular LA concentration in previously cryopreserved blood was 5.41±1.36 meq/L compared to 11.92±2.26 in age-matched controls. However, the rate of LA accumulation was profoundly lower in previously cryopreserved blood compared to controls with LA levels of 8.38±0.67 meq/L compared to 18.59±2.11 meq/L in controls by day 28 (Figure 4). While lactate generation appeared linear in previously frozen units throughout our experiment, the rate of rise in age-matched controls more closely followed a logarithmic or 2nd order polynomial function.

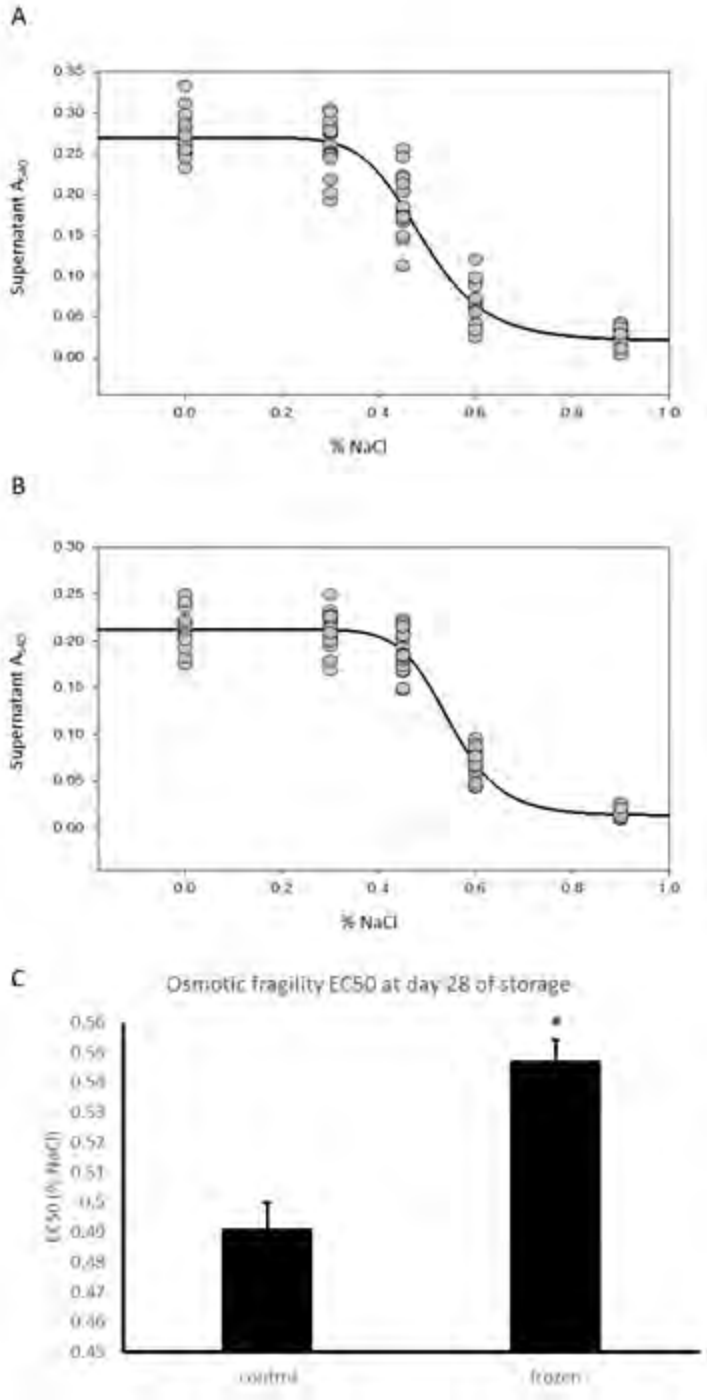

Figure 3. Progressive decline in pH.

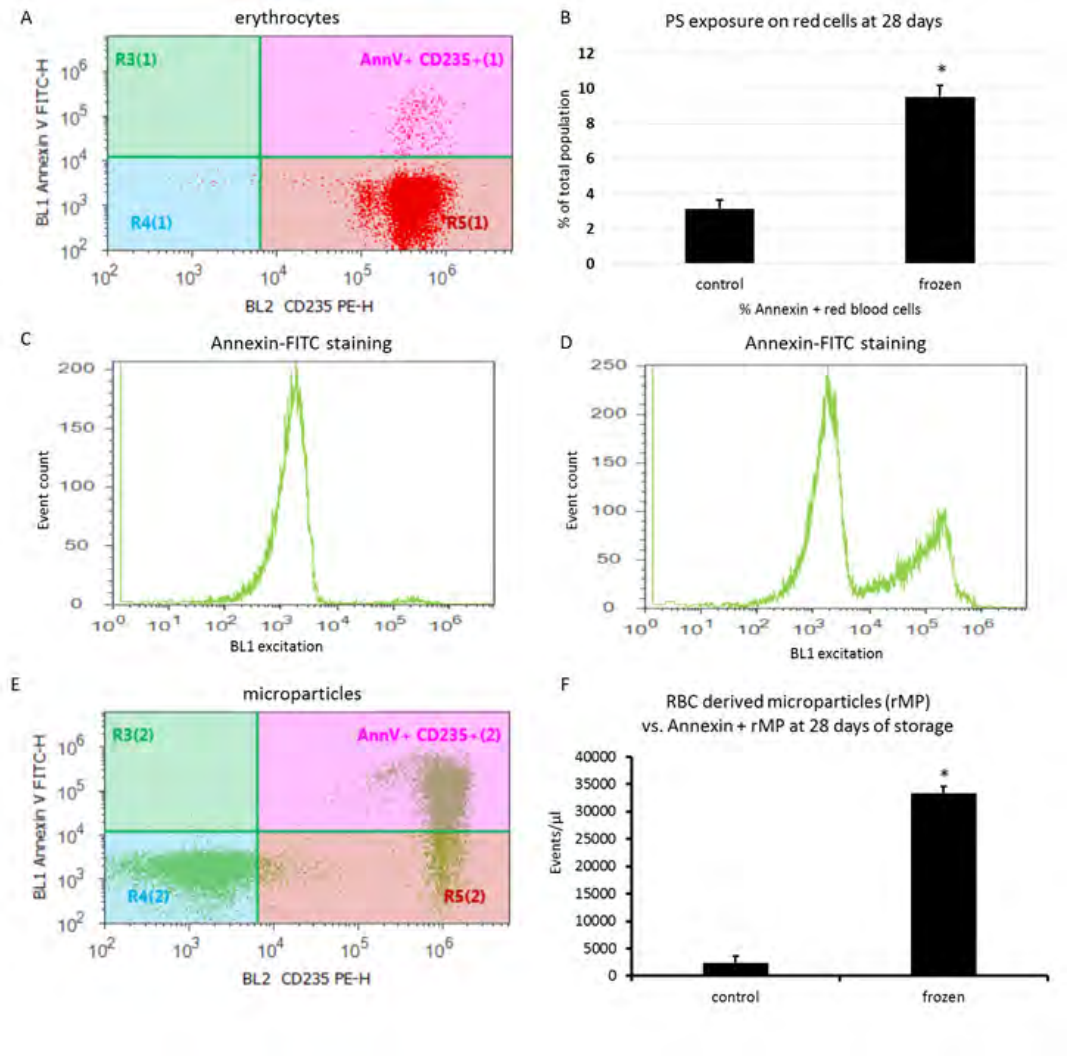

Figure 4. 28-day results.

Cell free Hgb in previously cryopreserved units was 0.56±0.10 mg/dL compared to 0.18±0.07 mg/dL in age-matched controls. By 28 days after thaw, previously frozen units had significant supernatant Hgb of 0.78±0.21 mg/dL. Controls had a significantly less supernatant Hgb of 0.41±0.08 mg/dL at day 28, but at day 42 controls were also visibly contaminated with 0.78±0.16 mg/dL Hgb.

4.3 Membrane Fragility, PS Externalization, and Microvesiculation

Previously cryopreserved cells exhibited less resistance to osmotic stress than controls. The EC50 was 0.55±0.01% in previously cryopreserved units compared to 0.49±0.01% in controls at day 28. This difference was stable through 42 days of storage compared to age-matched controls.

Loss of erythrocyte membrane asymmetry was also noted to be more pronounced in previously cryopreserved cells. By flow cytometry, PS exposure on the extracellular membrane was detected on 9.55±1.22% of cryopreserved red blood by 28 days of storage compared to only 3.15±0.50% of control erythrocytes. This difference was much less pronounced at day 42 of storage with cryopreserved erythrocyte PS exposure on 23.8±3.4% of cells compared to 22.0±5.1% in controls. Red cell derived microparticles were also significantly higher by day 28 in previously cryopreserved units (4.8 ±1.0 $x10^4/\mu L$ vs. 0.3±0.1 $x10^4/\mu L$), which persisted even at day 42 of storage (1.9 $x10^4/\mu L$ vs. 0.3±0.04 $x10^4/\mu L$).

5.0 DISCUSSION

In the present study, we attempted to compare and quantify the development of the red blood cell storage lesion of previously cryopreserved red cell units to conventional blood storage. Our findings indicate that, in addition to significantly altered characteristics post thaw, the common indicators of red blood cell storage lesion developed more rapidly in previously cryopreserved red cells. To achieve a satisfactory safety profile for transfusion of previously cryopreserved red blood cells, these factors must be considered in addition to red cell viability and post-transfusion recovery. This investigation provides insight into the durability of these cells after storage at -80°C and may guide potential expansion of red blood cell cryopreservation in civilian, trauma, and military settings.

The current FDA-approved method of glycerolization and deglycerolization with the Haemonetics ACP 215 requires removal of plasma, resuspension of red cells in 40% v/v glycerol, and storage at less than -65°C. To date, there is no evidence to suggest that any appreciable deterioration of red cells occurs at -65°C. The FDA limit for storage in the frozen state is 10 years [7]. 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°C.

Our study obtained previously frozen blood from a single blood bank thawed using the Haemonetics ACP 215 using the manufacturer's recommended protocol. A review of the literature reveals inter-institutional variability of red blood cell recovery [14,15]. The current American Association of Blood Banks standard mandates a post-thaw recovery of 80% of the original red cells present prior to glycerolization. Although post-thaw Hct 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. Neither total red cell mass nor ACP 215 wash effluents were evaluated in this study.

The significantly decreased pH found in previously frozen cells may be in part due to the high relative volume of AS-3, with a pH of 5.8. However, 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 play a beneficial role in the in vivo recovery. The experimental additive solutions proposed by Hess and colleagues [16] to prolong storage at 4°C may also be beneficial to previously frozen red cells. The clinical implications of this below-physiologic pH at time of infusion are likely not prohibitive given the relative acidosis nature of intravenous normal saline; however, this may play a larger role if applied in massive transfusion settings.

Other in vitro parameters associated with the red cell storage lesion are considered a cumulative effect. Presumably, with a lower concentration of red cells we would expect to see slower accumulation levels of these values. Instead, free Hgb in thawed units surpasses that seen in outdated standard packed cell supernatants by day 21 after thaw. Recent evidence shows that the transfusion of cell free Hgb and membrane bound extracellular Hgb induces dysregulation of nitric oxide mediated regulation of blood flow. As such, the expiration date of red blood cell products should consider the cell free Hgb burden of transfusion as well as percent hemolysis and post-transfusion recovery.

Despite the considerably lower concentration of red cells, extracellular potassium levels increased at a similar rate. A shift of potassium to the extracellular space may be occurring in response to environmental acidosis, although in vivo hyperkalemia is seen more commonly with non-anion gap acidosis. While this may suggest that membrane leak and cell lysis occur more rapidly in thawed units, it is also likely that a depletion of cellular energy impairs potassium equilibrium maintained by adenosine triphosphate (ATP) dependent potassium pumps [17].

Lactic acid accumulation was more pronounced in the first 21 days in controls than in cryopreserved units. Even when corrected for decreased red cell concentration, LA 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 physiological regulation of oxygen delivery [18]. Depletion of 2,3 diphosphoglycerate during storage may also play a role in post-transfusion Hgb-oxygen affinity. 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 Hgb containing microparticles plays a significant role in the red blood cell storage lesion [19]. Similar to previous studies by Holovati et al. in 2008, we saw no statistically significant increases in microparticle formation immediately after thaw (data not shown) [20]. 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. It is much more likely 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 [21]. We also found that post-thaw storage duration resulted in a more rapid exposure of extracellular erythrocyte membrane PS, which has been implicated in eryptosis and microparticle formation [22].

Our results suggest that the cryo-injury alluded to in many publications may be intrinsic to the red cells themselves and is not removed by washing during the deglycerolization process. Based on these observations, the red blood cell storage lesion in previously frozen blood has reached levels similar to outdated standard pRBCs at approximately 21-28 days after thaw. Our findings are consistent with previous suggestions that a storage of at least 3 weeks post thaw is possible in AS-3 [23,24]. Although in vivo survival of red cells after transfusion is the best measurement of erythrocyte quality and viability, these experiments were beyond the scope of our work.

6.0 CONCLUSION

Cryopreservation of red blood cell units 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 pRBCs, and the development of the red blood cell storage lesion is accelerated in the post-thaw period. We recommend that effects of the post-thaw storage period be further studied to generate potential strategies to increase quality and longevity of these red cell products.

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LIST OF ABBREVIATIONS AND ACRONYMS

AS-3	additive solution-3
ATP	adenosine triphosphate
EC50	sodium chloride concentration at which 50% of maximal lysis occurred
FDA	Food and Drug Administration
Hct	hematocrit
Hgb	hemoglobin
K^+	cell free potassium
LA	lactic acid
pRBC	packed red blood cell
PS	phosphatidylserine