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ATTENUATION OF THE RED BLOOD CELL STORAGE LESION TO ALLOW EXTENDED USE OF PREVIOUSLY CRYOPRESERVED (pRBC) UNITS IN AUSTERE ENVIRONMENTS

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14. ABSTRACT
Transfusion of blood is optimal treatment for blood loss. Resuscitation strategies emphasize transfusion of packed red blood cells (pRBCs) and fresh frozen plasma in equal ratios with minimal use of crystalloid fluids. The need for readily available pRBCs poses significant challenges for inventory/management/distribution. An additional issue is that pRBCs will degrade during storage. Our study examined strategies to attenuate progression of the red blood cell storage lesions in previously cryopreserved pRBCs. We hypothesized that post-thaw treatments of previously cryopreserved pRBCs would inhibit components of storage lesion. Methods: We examined three strategies to potentially attenuate the accelerated storage lesion after previous cryopreservation. First, experiments evaluated effect of post-thaw pH buffering on storage lesion development. Next, experiments treated post-thaw pRBCs with amitriptyline. The third set of experiments included testing AS-7 as an alternative to AS-3 after cryopreservation, thawing, and deglycerolization. Conclusion: The red blood cell storage lesion is more severe following cryopreservation. The storage lesion is exacerbated in an acidic environment. Post-deglycerolization storage in AS-3 with the addition of amitriptyline may attenuate aspects of the storage lesion. Storage of previously cryopreserved pRBCs in AS-7 may decrease some aspects of the storage lesion.

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1.0 BACKGROUND

Transfusion of blood products is the optimal treatment for blood loss from hemorrhage after trauma (1). Damage control resuscitation strategies emphasize the transfusion of packed red blood cells (pRBCs) and fresh frozen plasma (FFP) in equal ratios with the minimization of crystalloid containing fluids (2). The need for ready availability of pRBC units can pose significant inventory, management, and distribution challenges. An additional issue is that packed red blood cells begin to degrade after donation. This process, termed the "red blood cell storage lesion" is a series of biochemical and physiological changes that accumulate over time and lead loss of clinical effectiveness of transfused erythrocytes (3) as well as additional harmful consequences in the clinical setting (4-8).

One potential method to decrease harm from transfusion of stored pRBC units is through the use of cryopreserved blood. First described in the 1960s, cryopreservation allows storage of pRBC units for up to ten years prior to thawing, deglycerolization, and transfusion (9-11). Recent clinical studies confirm that the immediate use of previously cryopreserved pRBC units to ameliorate anemia after blood loss is safe and effective (12-14). Distribution and storage of cryopreserved pRBC units in theater with deglycerolization upon anticipated demand provides a potentially powerful tool to ensure ready availability of pRBC units.

Currently, cryopreserved pRBC units may be stored for up to 14 days after thawing and deglycerolization (15). This time point is based on recovery of intact cells following transfusion as thawed pRBC units have increased erythrocyte membrane fragility (16, 17). There is concern that these changes may limit safe use of cryopreserved pRBCs to the immediate period after thawing. Recent data from our laboratory indicates that the onset and rate of development of the red blood cell storage lesion are accelerated in previously cryopreserved pRBC units, with accelerated hemolysis, increased phosphatidylserine expression (a marker of red blood cell senescence), and rapid formation of harmful red blood cell microparticles (18). Thus, while previously cryopreserved pRBC units offer an advantage over traditional units in terms of very long term storage, this positive benefit may be eroded rapidly after thawing by the accelerated development of the red blood cell storage lesion.

The overall goal of the current project was to examine potential strategies to attenuate the progression of the red blood cell storage lesion in previously cryopreserved packed red blood cell units. We hypothesized that novel post-thaw treatments of previously cryopreserved pRBC units would inhibit components of the storage lesion over the ensuing 14-day post-thaw storage period. In order to achieve this goal, we proposed three specific aims:

> **Specific Aim 1:** Determine the effect of post-thaw potential of hydrogen (pH) buffering on development of the red blood cell storage lesion.
>
> **Specific Aim 2:** Determine the effect of inhibition of the acid sphingomyelinase on the red blood cell storage lesion.
>
> **Specific Aim 3:** Determine the effect of post-thaw storage solution on development of the red blood cell storage lesion.

The data resulting from these studies provide information to potentially improve the quality and stability of previously cryopreserved pRBC units after thawing and deglycerolization. They also highlight a potential way forward to improve the quality of previously cryopreserved pRBCs.

2.0 METHODS

Standard units of leukoreduced human pRBCs in additive solution-3 were obtained from the Hoxworth Blood Center (Cincinnati, Ohio). The pRBC units were collected in citrate phosphate double Dextrose (257.6 millimoles per litre (mmol/L) glucose, 105.0mmol/L citric acid, 18.5mmol/L monosodium Phosphate, pH 5.7). Units were stored at 4 degrees Celsius (^{O}C) for a period of 24 hours in additive solution-3 (Nutricel; 55.5mmol/L glucose, 70.1mmol/L Sodium chloride, 20mmol/L sodium phosphate, 12mmol/L citric acid, 2.2mmol/L adenine, pH 5.8). These units were split in half by volume on day 2, with half utilized as control units and stored under standard conditions. The other half of the red cell units were placed in glycerol (Glycerolyte 57 solution; Fenwal Inc, Lake Zurich, Ill) at cryopreserved at -80^{O}C using the ACP 215 Automated Cell Processor (Haemonetics Corp., Baintree, Mass). On day 3, pRBCs were thawed, deglycerolized, washed, and prepared for transfusion.

To determine the effect of post-thaw pH buffering on development of the red blood cell storage lesion, groups of human pRBC units were collected under standard conditions, then underwent cryopreservation. The previously frozen pRBCs were placed in different storage solutions after thawing and deglycerolization. The first group was placed in standard additive solution-3 (AS-3). The second was stored under more acidic storage conditions by titrating additive solution-3 to a pH of 4.5 with 1M hydrogen chloride (HCl). The third group was placed in more alkaline conditions by titrating additive solution-3 to a pH of 8.5 with 1M Sodium hydroxide (NaOH). The fourth group was placed in an enhanced buffering solution generated by replacing citric acid in additive solution-3 with 12.5 mmol sodium bicarbonate, resulting in no initial pH change but enhancing the buffering capacity.

Acid sphingomyelinase (ASM) is an enzyme that catalyzes the breakdown of sphingomyelin to ceramide and phosphoryl choline under conditions of cellular stress (19). Ceramide, in turn, serves as a harmful pro-inflammatory mediator and causes cellular damage. Our recent work has demonstrated that ASM activity increases during pRBC storage and that inhibition of the ASM activity results in decreased accumulation of red blood cell microparticles during storage and less lung inflammation upon transfusion of older pRBC units (20). To examine the effect of inhibition of acid sphingomyelinase in previously cryopreserved pRBC units on the severity of the red blood cell storage lesion, human pRBC units were purchased, cryopreserved, thawed, deglycerolized, and prepared as above. Packed red blood cell units were divided into three groups. Group 1 was placed in AS-3. Group 2 was treated with 125 μM amitriptyline, a well described specific ASM inhibitor (20). Group 3 was treated with a corresponding volume of vehicle.

As early as 1993, it was recognized that AS-3 may not be the optimal solution for storage of cryopreserved pRBCs after thawing and deglycerolization (21). Additive solution-7 (AS-7) is a new solution for pRBC storage that was recently approved by the FDA. The solution is designed to have increased buffering capacity as well as additional phosphate to optimize RBC metabolism. Previous studies indicate that AS-7 mitigates aspects of the red blood cell storage lesion under standard storage conditions (22), but the effect of AS-7 on the red blood storage lesion after previous cryopreservation is unknown. To examine the effect of storage in AS-7 on progression of the red blood cell storage lesion after previous cryopreservation, human pRBC units were purchased, cryopreserved, thawed, deglycerolized, and prepared as above. Packed red blood cell units were divided into two groups. Group 1 was placed in AS-3. Group 2 was placed in AS-7.

All units were stored under standard storage conditions for at least 14 days (see results). At day 4, 7, and 14 of storage, pRBC units were sterilely accessed and evaluated. Biochemical parameters, including lactate, glucose, hemoglobin, hematocrit, and pH were determined by portable analyzer. Free hemoglobin and advanced oxidation protein products (AOPP) were determined by ELISA. Microparticle concentrations, phosphatidylserine externalization, Band-3 expression, and cell complexity were determined by flow cytometry as we have previously described (23, 24). Osmotic fragility was determined by incubation of erythrocytes from each group in 0.8, 0.68, 0.56, 0.44, 0.32, and 0 % saline followed by assay for free hemoglobin.

Due to anticipated variability in the development of the red blood cell storage lesion between donors (3, 25), red blood cell unit results were normalized to the original collected donor unit where noted. The original collected unit is denoted as the standard unit (std) and the data from the cryopreserved units and test conditions is presented as fold change for each individual condition.

3.0 RESULTS

Aim 1: Experiments to determine the effect of post-thaw pH buffering on development of the red blood cell storage lesion

In this series of experiments, we evaluated the effect of alteration of post-thaw storage solution pH on the RBC storage lesion. Units of pRBCs underwent cryopreservation followed by thawing, deglycerolization, and then subsequent storage in standard storage solution (AS3, pH 5.9), or AS-3 with altered pH. We examined the effect of acidic (pH 4.5), basic (pH 8.5 after treatment storage solution with NaOH), and buffered pH (pH 6.35 after treatment of storage solution with Sodium bicarbonate ($NaHCO_3$) on several parameters of the red blood cell storage lesion. Initial analysis was carried out at days 4, 7, 10, and 14 after deglycerolization. For the purposes of clarity, the data from 14 days of storage is presented unless otherwise noted.

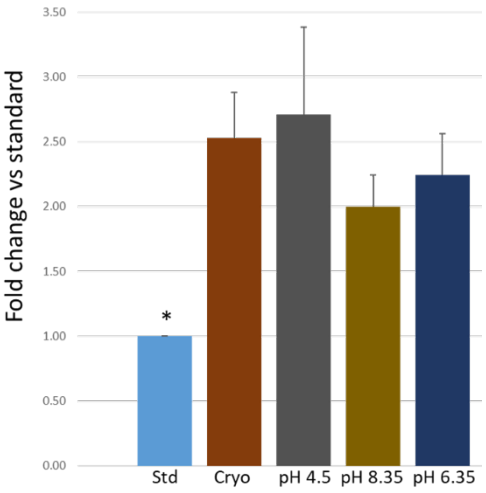

Figure 1. Free hemoglobin in pRBCs after storage for 14 days under standard (std) conditions, after cryopreservation (cryo), or after cryopreservation and storage in modified pH solutions as indicated. N=6 for each condition. *p<0.05 vs all other groups.

When units were evaluated for free hemoglobin release after 14 days of storage under standard conditions, we found that storage under buffered conditions (pH 6.35) was associated with decreased hemoglobin release as compared to storage in standard AS-3 (data not shown). This is similar to our previous findings (18). When we analyzed units stored under modified pH conditions after previous cryopreservation, we found that cryopreservation resulted in increased free hemoglobin levels under all conditions as compared to standard storage (**FIGURE 1**). Storage at pH of 8.35 appeared to result in decreased free hemoglobin release, but this trend did not reach statistical significance.

We next examined the stored units for accumulation of AOPP. These toxins develop during oxidative stress during storage. We found that previous cryopreservation was associated in increased accumulation of AOPP at 14 days of storage. There was no significant effect of pH on AOPP accumulation under these conditions (**FIGURE 2**).

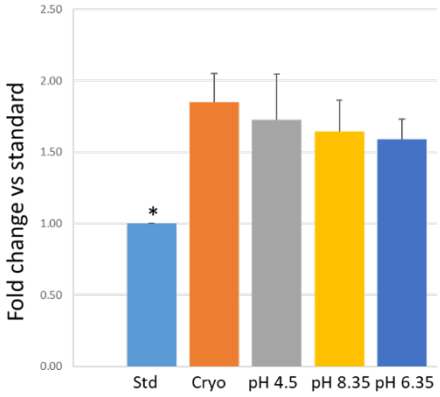

Figure 2. AOPP in pRBCs after storage for 14 days under standard (std) conditions, cryopreservation (cryo), or cryopreservation and storage in modified pH solutions as indicated. N=5 for each condition. *p<0.05 vs all other groups.

In addition to AOPP, we assayed the stored units for accumulation of hydrogen peroxide, a potentially harmful toxin. We found that previous cryopreservation resulted in increased hydrogen peroxide concentrations in units stored under standard or modified pH (**FIGURE 3**). Our data also demonstrated that storage of previously cryopreserved pRBCs in a buffered solution at pH 6.35 was associated with decreased hydrogen peroxide as compared to standard AS-3 after cryopreservation (**FIGURE 3**).

Microparticles are small extracellular vesicles that may contain bioactive mediators. We have previously demonstrated that stored pRBC units shed microparticles in increasing concentrations under standard storage conditions (26) and that these microparticle may mediate harmful events after resuscitation and transfusion, such as lung injury and thrombosis (26, 27). We have previously demonstrated that microparticles accumulate in previously cryopreserved pRBC units at a faster rate than during standard storage. In our next series of experiments the effect of pH modulation after cryopreservation on the microparticle accumulation for up to 14 days of storage. We found that previous cryopreservation was associated with increased microparticle accumulation at 14 days of storage (**FIGURE 4**). Our data indicate that storage in a more acidic pH (pH 4.5) exacerbated microparticle accumulation. Storage under higher pH conditions did not ameliorate the microparticle accumulation associated with cryopreservation (**FIGURE 4**).

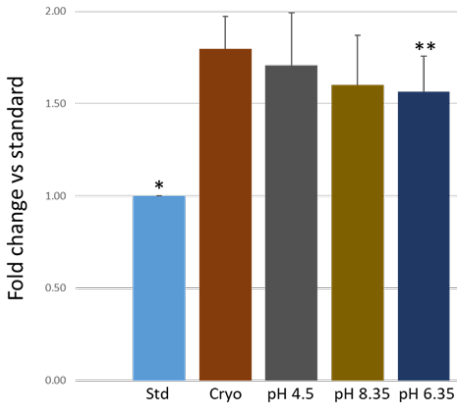

Figure 3. Hydrogen peroxide (H2O2) in pRBCs after storage for 14 days under standard (std) conditions, cryopreservation (cryo), or cryopreservation and storage in modified pH solutions as indicated. N=5 for each condition. *p<0.05 vs all other groups; **p<0.05 vs cryo group.

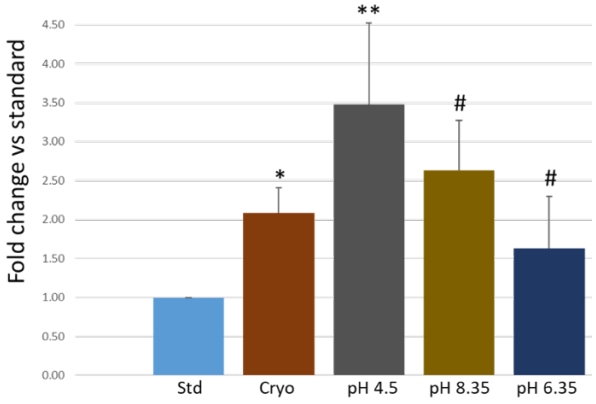

Figure 4. Microparticle accumulation in pRBCs after storage for 14 days under standard (std) conditions, cryopreservation (cryo), or cryopreservation and storage in modified pH solutions as indicated. N=6 for each condition. *p<0.05 vs standard, ** p<0.05 vs cryo, # p<0.05 vs standard.

Phosphatidylserine is an important component of cell membranes. Normal internalized, the exposure of phosphatidylserine on the outer leaflet of red blood cells is regarded as a sign of senescence and likely leads to the removal of the given erythrocyte from the circulation. Phosphatidylserine exposure is regarded as an important marker of the red blood cell storage lesion as it is an indicator of the likely survival of the erythrocytes after transfusion. We next determine the effect of previous cryopreservation on phosphatidylserine exposure. Our data demonstrated that previous cryopreservation, followed by storage under standard or alkaline conditions, is not associated with increased phosphatidylserine exposure (**FIGURE 5**). Storage in acidic (pH 4.5) conditions after previous cryopreservation accelerated phosphatidylserine exposure.

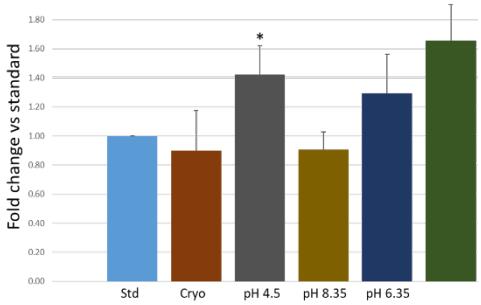

Figure 5. Phosphatidylserine exposure in pRBCs after storage for 14 days under standard (std) conditions, cryopreservation (cryo), or cryopreservation and storage in modified pH solutions as indicated. N=6 for each condition. *p<0.05 vs standard.

In the next series of experiments, we examined the effect of storage condition on pRBC unit hemoglobin, hematocrit, and potassium at day 4 and 14 after cryopreservation and deglycerolization. We did not find significant changes to these parameters with altered storage pH (data not shown).

Packed red blood cell storage is associated with decreased lactate production. When we evaluated pRBCs at 14 days after standard storage or cryopreservation, we found that previous cryopreservation is associated with significantly decreased lactate levels as compared to standard storage (**FIGURE 6**). Cryopreservation followed by placement in a storage solution with a pH of 4.5 exacerbated the decreased lactate production. Storage in a more basic or buffered pH did not improve lactate production (**FIGURE 6**).

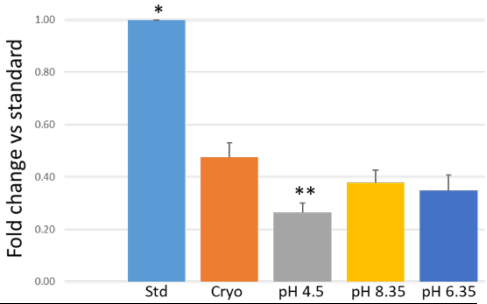

Figure 6. Lactate concentrations pRBCs after storage for 14 days under standard (std) conditions, cryopreservation (cryo), or cryopreservation and storage in modified pH solutions as indicated. N=6 for each condition. *p<0.05 vs other groups. ** p<0.05 vs cryo.

Currently, previously cryopreserved pRBCs are cleared for use for up to 14 days after thawing and deglycerolization. Thus, one limit on the potential use of cryopreserved pRBCs in the far-forward or remote setting is the relatively short shelf life after deglycerolization. In order to examine the effect of storage in altered pH solutions during prolonged storage, pRBC units underwent treatment and storage as described above, but extended the storage period for forty four days. When we examined the stored pRBCs for microparticle accumulation, we found that cryopreservation was associated with increased microparticle concentrations (**FIGURE 7**). There was a trend toward further increased microparticle accumulation during storage at pH of 4.5, but this did not reach statistical significance.

When we assayed these units for phosphatidylserine, we found that any alteration in the pH beyond the conditions found in standard storage solution resulted in increased phosphatidylserine exposure (**FIGURE 8**). Previously cryopreserved pRBCs demonstrated similar phosphatidylserine exposure as pRBC units stored under standard conditions.

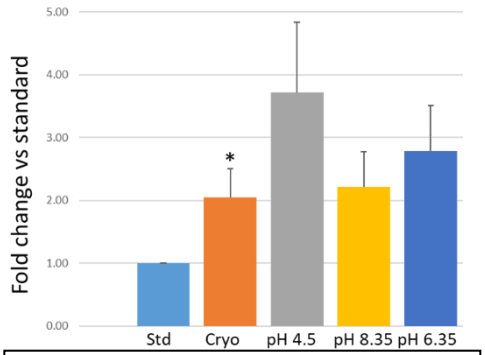

Figure 7. Microparticle concentrations in pRBCs after storage for 44 days under standard (std) conditions, cryopreservation (cryo), or cryopreservation and storage in modified pH solutions as indicated. N=4 for each condition. *p<0.05 vs standard conditions.

In the next series of experiments, we examined the effect of storage condition on pRBC unit hemoglobin, hematocrit, and potassium at day 30 and 34 after cryopreservation and deglycerolization. We did not find significant changes to these parameters with altered storage pH (data not shown).

We next assayed this extended storage units for lactate production. Our data indicate that cryopreservation was associated with suppressed lactate production (**FIGURE 9**). Storage in a more acidic pH was associated with further decreased lactate production.

When we evaluated these samples for release of free hemoglobin into the storage media, we found that cryopreservation was associated with increased free hemoglobin at storage day 44 (**FIGURE 10**). This increase was exacerbated by a lower pH.

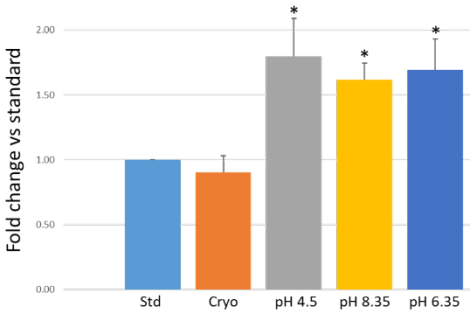

Figure 8. Phosphatidylserine exposure in pRBCs after storage for 44 days under standard (std) conditions, cryopreservation (cryo), or cryopreservation and storage in modified pH solutions as indicated. N=4 for each condition. *p<0.05 vs other groups.

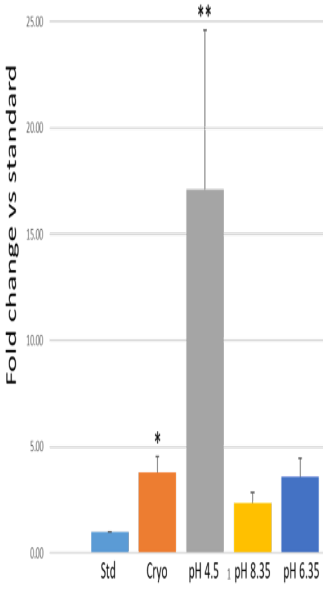

Figure 10. Free hemoglobin in pRBCs after storage for 44 days under standard (std) conditions, cryopreservation (cryo), or cryopreservation and storage in modified pH solutions as indicated. N=6 for each condition. *p<0.05 vs cryo. ** p<0.05 vs other

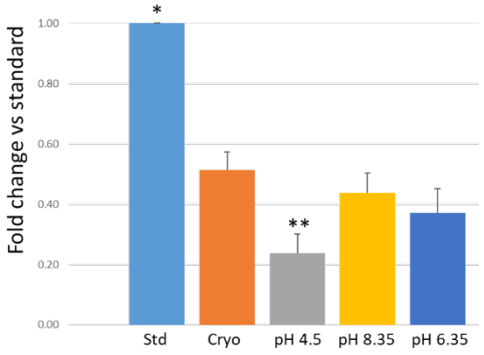

Figure 9. Lactate concentrations in pRBCs after storage for 44 days under standard (std) conditions, cryopreservation (cryo), or cryopreservation and storage in modified pH solutions as indicated. N=6 for each condition. *p<0.05 vs other groups. ** p<0.05 vs cryo.

Assays for AOPP, hydrogen peroxide, and partial pressure of oxygen indicated that each of these parameters were increased in pRBC units that were previously cryopreserved when compared to standard storage. We did not find that pH of the stored units altered these parameters (**FIGURES 11 and 12**).

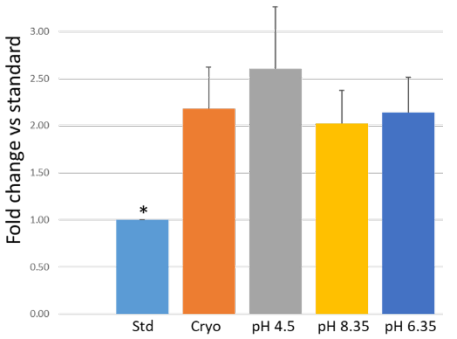

Figure 11. AOPP in pRBCs after storage for 44d under standard, cryopreservation (cryo), or cryopreservation and storage in modified pH solutions as indicated. N=6 for each condition. *p<0.05 vs other

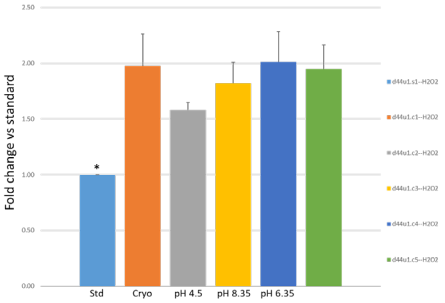

Figure 12. Hydrogen peroxide in pRBCs after storage for 44d under standard (std), cryopreservation (cryo), or cryopreservation and storage in modified pH solutions as indicated. N=6 for each condition. *p<0.05 vs other groups.

Aim 2: Experiments to evaluate the effect of inhibition of the Acid Sphingomyelinase on the red blood cell storage lesion.

Acid sphingomyelinase (ASM) is an enzyme that catalyzes the breakdown of sphingomyelin to ceramide and phosphoryl choline under conditions of cellular stress (19). Ceramide, in turn, serves as a harmful pro-inflammatory mediator and causes cellular damage. Our recent work has demonstrated that ASM activity increases during pRBC storage and that inhibition of the ASM activity results in decreased accumulation of red blood cell microparticles during storage and less lung inflammation upon transfusion of older pRBC units (20). Based on this data, we hypothesized that inhibition of acid sphingomyelinase in previously cryopreserved pRBC units will blunt onset and severity of the red blood cell storage lesion.

In order to test this hypothesis, human pRBC units were purchased, cryopreserved, thawed, deglycerolized, and prepared as above. Packed red blood cell units were divided into three groups. Group 1 was. Group 2 was treated with vehicle (PBS). Group 3 was treated with 125 μM amitriptyline, a well described specific ASM inhibitor (19). Units were then stored under standard storage conditions for up to 14 days and analyzed for development and severity of the red blood cell storage lesion. Standard storage pRBC units were treated in an identical fashion and served as additional controls.

After 14 days of storage, we noted differences between standard storage pRBCs and those undergoing cryopreservation in terms of several aspects of the red blood cell storage lesion. These findings were similar to our previous data (data not shown); (18). We found no differences between previously cryopreserved pRBCs stored in AS-3 as compared to those treated with vehicle or treated with amitriptyline with regards to pH, glucose metabolism, or lactate (data not shown).

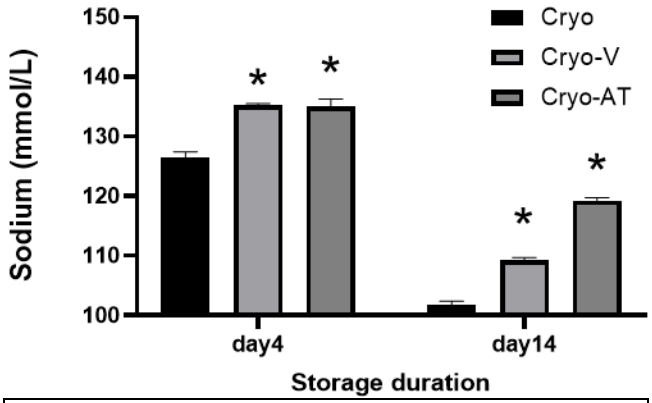

Figure 13. Sodium levels after cryopreservation, then storage in AS-3, AS-3 with vehicle (V), or AS-3 with amitriptyline (AT). N=4 for each condition. *p<0.05 vs other groups.

Cryopreservation followed by treatment with amitriptyline was associated increased sodium concentrations at days 4 and 14 of storage (**FIGURE 13**). We also found increased hemoglobin (**FIGURE 14 left**) and hematocrit (**FIGURE 14 right**) after storage with amitriptyline.

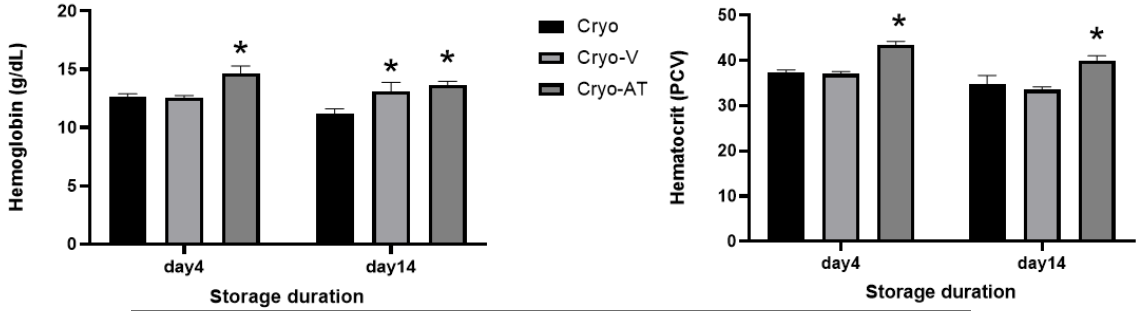

Figure 14. Hemoglobin (left) and hematocrit (right) after cryopreservation, then storage in AS-3, AS-3 with vehicle (V), or AS-3 with amitriptyline (AT). N=4 for each condition. *p<0.05 vs cryopreserved group.

We have previously demonstrated that amitriptyline treatment of pRBCs during storage leads to decreased microparticle accumulation. When we examined microparticle accumulation in pRBCs stored under standard conditions, we found that amitriptyline treatment was associated with decreased microparticle concentration (data not shown). In previously cryopreserved pRBCs, amitriptyline treatment resulted in increased microparticle concentrations at day 4 of storage, decreased concentrations at day 7, but no difference by day 14 (**FIGURE 15**).

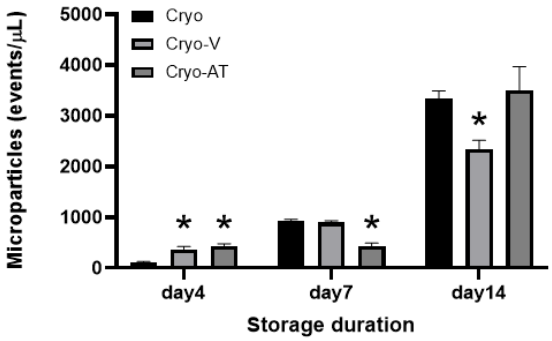

Figure 15. Microparticle concentrations after cryopreservation, then storage in AS-3, AS-3 with vehicle (V), or AS-3 with amitriptyline (AT). N=4 for each condition. *p<0.05 vs cryopreserved group.

In the next series of experiments, we determined the effect of amitriptyline treatment on free hemoglobin and susceptibility to osmotic stress. Treatment with amitriptyline after cryopreservation was associated with decreased free hemoglobin at 14 days of storage (**FIGURE 16**), but there was no difference with vehicle. Treatment with amitriptyline rendered previously cryopreserved pRBCs more susceptible to hemolysis in dilute saline solution at 4 and 7, but not 14 days of storage (data not shown).

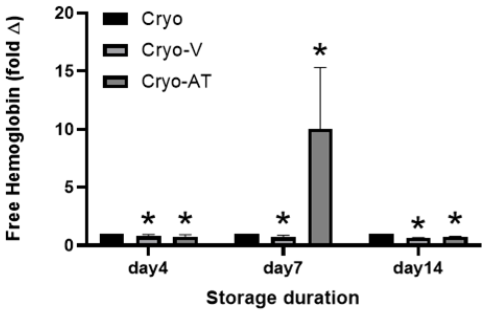

Figure 16. Free hemoglobin concentrations after cryopreservation, then storage in AS-3, AS-3 with vehicle (V), or AS-3 with amitriptyline (AT). N=4 for each condition. *p<0.05 vs cryopreserved group.

We then examined the effect of amitriptyline treatment on RBC morphology and band 3 expression. As determined by flow cytometry, there were no changes in morphology or erythrocyte volume that were noted (data not shown). Band 3 expression was increased at 4 and 14 days after deglycerolization and storage (**FIGURE 17**). In a similar fashion, phosphatidylserine was increased at day 4 and 7, but not day 14 of storage (**FIGURE 18**).

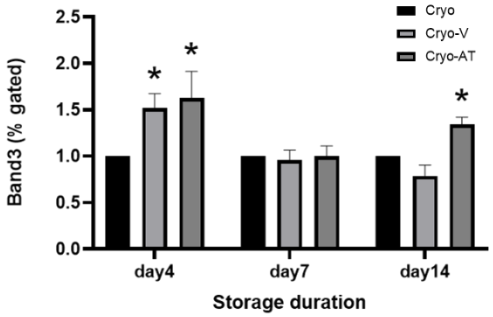

Figure 17. Band 3 expression after cryopreservation, then storage in AS-3, AS-3 with vehicle (V), or AS-3 with amitriptyline (AT). N=4 for each condition. *p<0.05 vs cryopreserved group.

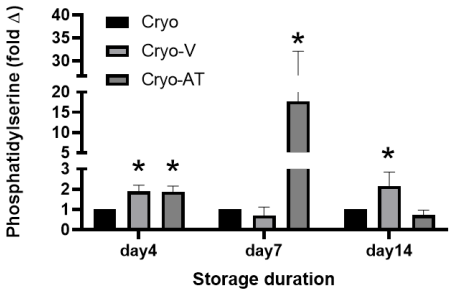

Figure 18. Phosphatidylserine expression after cryopreservation, then storage in AS-3, AS-3 with vehicle (V), or AS-3 with amitriptyline (AT). N=4 for each condition. *p<0.05 vs cryopreserved group.

<u>Aim 3: Experiments to determine the effect of post-thaw storage solution on characteristics of the red blood cell storage lesion.</u>

Current post-thaw processing of cryopreserved pRBC units involves removal of glycerol from the units with a series of washing steps. The erythrocytes are then suspended in Additive Solution-3 (AS-3, Nutricel) for storage and potential transfusion up to 14 days later. As early as 1993, it was recognized that AS-3 may not be the optimal solution for storage of cryopreserved pRBCs after thawing and deglycerolization (21). Additive solution-7 (AS-7) is a new solution for pRBC storage that was recently approved by the FDA (22). The solution is designed to have increased buffering capacity as well as additional phosphate to optimize RBC metabolism. Previous studies indicate that AS-7 mitigates aspects of the red blood cell storage lesion under standard storage conditions (22), but the effect of AS-7 on the red blood storage lesion after

previous cryopreservation is unknown. We hypothesized that post-thaw storage in AS-7 would result in decreased progression of the storage lesion after previous cryopreservation.

In order to test this hypothesis, human pRBC units were purchased, cryopreserved, thawed, deglycerolized, and prepared as above. Packed red blood cell units were divided into two groups. Group 1 was be placed in AS-3 and stored under standard conditions. Group 2 underwent cryopreservation, then deglycerolization and storage in AS-3. Group 3 was stored in AS-7 under standard storage conditions. Group 4 underwent cryopreservation, then deglycerolization and storage in AS-7. Units were then stored under standard storage conditions for up to 14 days with sample removal and assays performed as described below.

When we examined the effect of post-cryopreservation storage in AS-7, we found that this resulted in increased pH at day 4 after deglycerolization (**FIGURE 19**). This difference was not present by day 14.

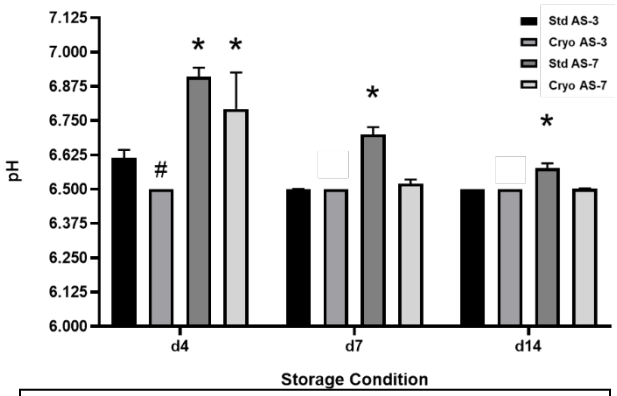

Figure 19. pH after cryopreservation (cryo), then storage in AS-3 or AS-7. N>4 for each condition. #p<0.05 vs others. *p<0.05 vs standard group (std).

Next, we determined lactate production and glucose utilization after cryopreservation and storage in either AS-3 or AS-7. We found no significant differences between previously cryopreserved pRBCs subsequently stored in AS-3 as compared to those stored in AS-7 (data not shown). Lactate production was the same at day 4 (**FIGURE 20**). At days 7 and 14, lactate production was less in previously cryopreserved pRBCs than those stored under standard conditions. At day 14, there was increased lactate production in pRBCs stored in AS-7 after cryopreservation as compared to those stored in AS-3.

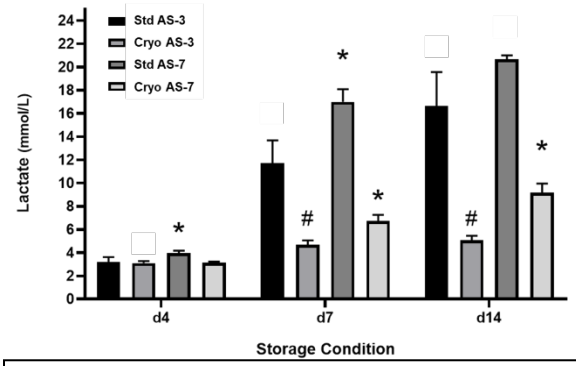

Figure 20. Lactate after cryopreservation (cryo), then storage in AS-3 or AS-7. N>4 for each condition. Day 4: *p<0.05 vs other groups; Day 7: #p<0.05 vs standard (std) conditions; *p<0.05 vs other groups.

In the next series of experiments, we assayed the stored samples for free hemoglobin. We found that cryopreservation followed by storage in AS-3 was associated with increased free hemoglobin as compared to non-cryopreserved units stored in AS-3 (**FIGURE 21**). Previous cryopreservation followed by storage in AS-7, as compared to AS-3, was associated with decreased free hemoglobin at day 7 of storage.

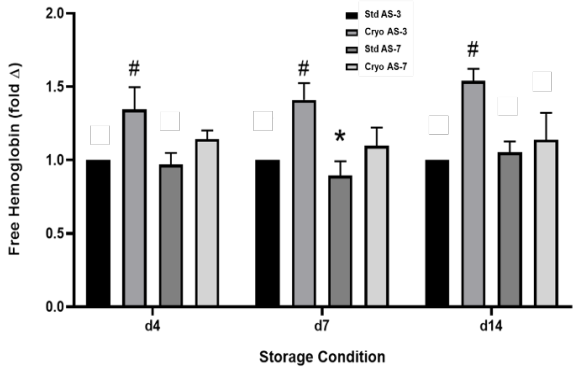

Figure 21. Free hemoglobin after cryopreservation (cryo), then storage in AS-3 or AS-7. N>4 for each condition. Day 4: #p<0.05 vs standard AS-3; Day 7: #p<0.05 vs cryo AS-7, *p<0.05 vs cryo AS-7; day 14 #p<0.05 vs std AS-3.

When we examined microparticle shedding under the same conditions, we found less microparticles in pRBCs previously cryopreserved, then stored in AS-7, as compared to those stored in AS-3, at day 4 and day 7 of storage (**FIGURE 22**). This difference was not present at day 14.

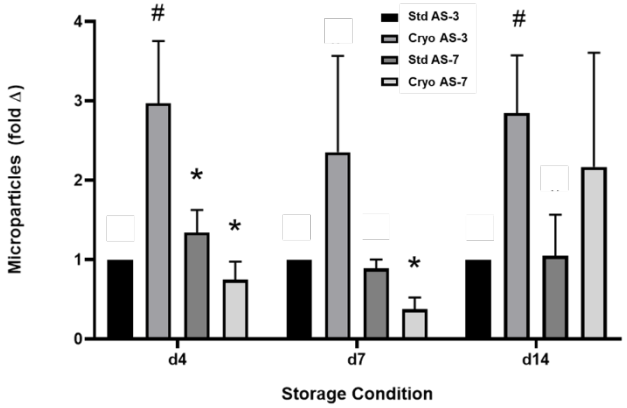

Figure 22. Microparticles after cryopreservation (cryo), then storage in AS-3 or AS-7. N>4 for each condition. Day 4: #p<0.05 vs standard AS-3, *p<0.05 vs cryo AS-3; Day 7 *p<0.05 vs cryo AS-3; day 14 #p<0.05 vs std AS-3.

We next evaluated the effect of storage in AS-3 or AS-7 after cryopreservation on advanced oxidative protein products. We found no differences between these groups at days 4, 7, or 14 of storage (data not shown). When we analyzed the units for phosphatidylserine externalization and Band 3 expression, we found no significant differences between these two groups at days 4, 7, or 14 of storage (data now shown).

In a final series of experiments, we examined the effect of cryopreservation followed by storage in AS-3 or AS-7 on osmotic fragility. At day 4, 7, and 14, we found that both cryopreservation groups demonstrated more hemolysis than the standard storage groups (**FIGURE 23**). At day 14, there was greater hemolysis in pRBCs stored in AS-3 after cryopreservation than those stored in AS-7.

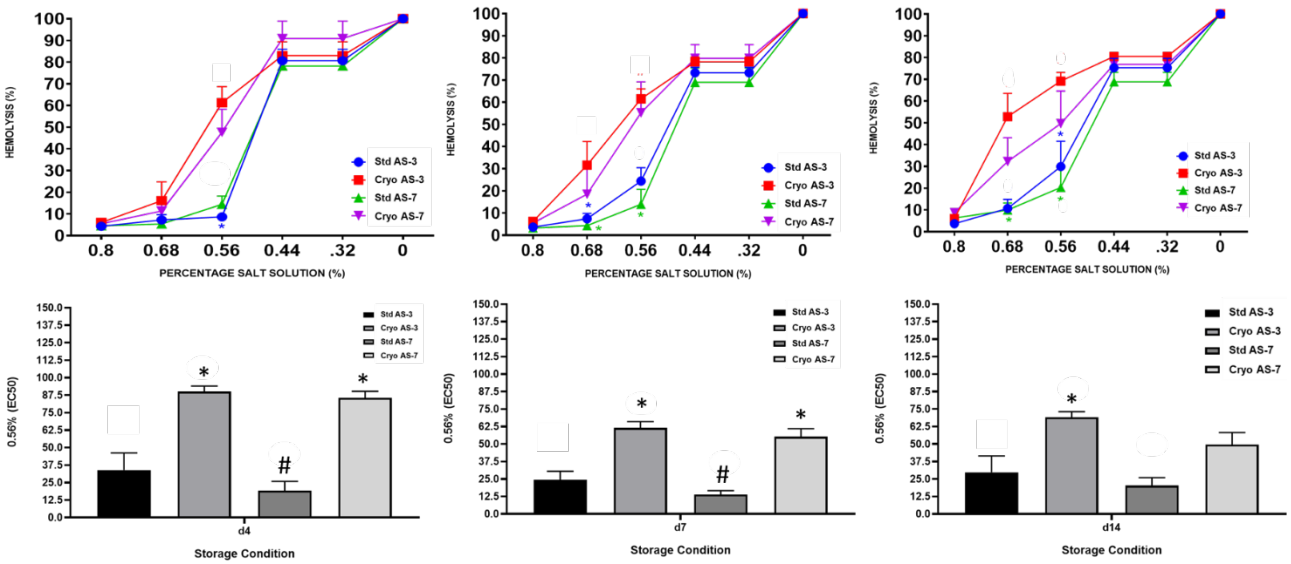

Figure 23. Osmotic fragility after cryopreservation (cryo), then storage in AS-3 or AS-7. N>4 for each condition. Day 4 and day 7: *p<0.05 vs other groups, # p<0.05 vs std AS-3; Day 14, p<0.05 vs other groups.

4.0 DISCUSSION

In the present studies, we examined three strategies to potentially attenuate the accelerated red blood cell storage lesion that is seen in pRBCs after previous cryopreservation. In each series of experiments, the initial units were split prior to cryopreservation and thus each pRBC unit was able to serve as its own control. This strategy helped to minimize inherent differences in the red blood cell storage lesion that are noted in individuals after donation.

In experiments for Specific Aim 1, we evaluated the effect of post-thaw pH buffering post-thaw pH buffering on development of the red blood cell storage lesion. We found that several aspects of the storage lesion, including microparticle accumulation, phosphatidylserine externalization, and decreased lactate production were exacerbated by storage in a more acidic environment. These findings were also generally similar during extended post-deglycerolization storage of pRBCs. Storage in a more alkaline environment did not result in attenuation of the storage lesion. Based on these experiments, we recommend that storage in an acidic environment be avoided.

Another finding from the extended storage experiments is that the storage lesion did not appear to become more proportionally severe in previously cryopreserved pRBCs over the duration of the storage period. Based on this, further studies on extended storage of pRBCs post-deglycerolization may be warranted in order to determine if this is a potential strategy for extending the utility of these pRBC units.

In experiments for Specific Aim 2, we treated post-thaw and post-deglycerolized pRBCs with amitriptyline, an inhibitor of the acid sphingomyelinase. We found that several aspects of the storage lesion, including decreased sodium, hematocrit, hemoglobin, and Band 3 as well as increased free hemoglobin and microparticle shedding, were attenuated in the pRBCs treated with amitriptyline after deglycerolization, albeit similar effects were often seen with vehicle as well. Based on these data, further studies of storage solution modification should be considered as this may lead to greater mitigation of the red blood cell storage lesion after thawing and deglycerolization.

In experiments for Specific Aim 3, we tested AS-7 as a potential alternative to AS-3 after cryopreservation, thawing, and deglycerolization. Several aspects of the storage lesion, including initial storage pH, lactate production, microparticle shedding, and susceptibility to osmotic stress were improved with AS-7 storage at some timepoints after deglycerolization. In our opinion, this data is not of sufficient significance to recommend changing the post-thaw storage solution to AS-7, but additional studies with AS-7 or modified storage solutions should be considered.

5.0 CONCLUSIONS

Based on our data, we have reached the following conclusions:

1. The red blood cell storage lesion is more severe and accelerated following cryopreservation.
2. After cryopreservation, storage in an acidic environment exacerbates the red blood cell storage lesion.
3. Storage of previously cryopreserved pRBCs in a more alkaline or buffered environment is no more advantageous than storage in AS-3.
4. Post-deglycerolization storage in AS-3 with the addition of amitriptyline may attenuate aspects of the red blood cell storage lesion.
5. Storage of previously cryopreserved pRBCs in AS-7 may decrease some aspects of the storage lesion.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

^{O}C	degrees Celsius
AOPP	advanced oxidation protein products
ASM	Acid sphingomyelinase
HCl	hydrogen chloride
pRBC	packed red blood cells
pH	potential of hydrogen
FFP	fresh frozen plasma
mmol/L	millimoles per litre
M	moles of solute per liter of solution
NaOH	Sodium hydroxide
Std	Standard
AS-3	Additive solution-3
AS-7	Additive solution-7