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SAVE IT - DON'T WASTE IT! MAXIMIZING UTILIZATION OF ERYTHROCYTES FROM PREVIOUSLY STORED WHOLE BLOOD

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Abstract

Background: Recent military and civilian experience suggests that fresh whole blood may be the preferred for treatment of hemorrhagic shock, but its use is limited by its 21-day shelf life. The red blood cell storage lesion and coagulation status of packed red blood cells (pRBCs) salvaged from expired whole blood are unknown. We hypothesized that packed red blood cells can be salvaged from previously stored whole blood.

Methods: Cold stored, low-titer, O-positive, non-leukoreduced, whole blood units were obtained at 21 days of storage. Erythrocytes were separated by centrifugation, resuspended in AS-3, and stored for 21 additional days as salvaged pRBCs. The red blood cell storage lesion parameters of microvesicles, Band-3, free hemoglobin, annexin V, and erythrocyte osmotic fragility were measured and compared to pRBCs prepared at the time of donation and stored in AS-3 for 42 days (standard pRBCs). In additional experiments, murine pRBCs were prepared from expired whole blood units and compared to those stored under standard conditions. Mice underwent hemorrhage and resuscitation with standard and salvaged pRBC units and serum cytokines and free hemoglobin were determined.

Results: There were no significant differences in microvesicle formation or cell-free hemoglobin concentration between salvaged and standard pRBCs. There was decreased Band-3 and increased phosphatidylserine in the salvaged units as well as greater osmotic fragility. Salvaged pRBCs maintained consistent clot firmness. After hemorrhage and resuscitation in a murine model, salvaged pRBCs did not demonstrate increased serum cytokine levels.

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Conflict of interest statement

The authors declare that they have no conflicts of interest related to this study.

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Conclusions: Salvaged pRBCs from previously stored whole blood accumulate the red blood cell storage lesion in a similar fashion to standard pRBCs and maintain consistent coagulability when reconstituted with plasma. Salvaged pRBCs are not associated with an increased inflammatory response when used for resuscitation in a murine model. Salvaged pRBCs may be a viable product for utilization in the treatment of traumatic hemorrhagic shock.

Level of Evidence: Level II (prospective laboratory study, therapeutic)

Keywords

whole blood; red blood cell storage lesion; erythrocyte salvage; microvesicles

Background

There is no adequate substitute for the use of blood products for resuscitation after trauma and hemorrhage. Recent military and civilian trauma resuscitation experience has demonstrated the superiority of resuscitation with blood components in ratios approaching reconstituted whole blood (1–5). Potential additional benefits of whole blood include excellent hemostatic profile (6, 7) and reduced transfusion volumes (8) as compared to the use of blood component or crystalloid therapy. This experience has led to renewed interest in the use of whole blood for resuscitation after trauma in the military and civilian environments (9, 10).

Enthusiasm for, and the implementation of, whole blood in trauma resuscitation has been diminished by several concerns, including wastage of units in practice due to the relatively short shelf life (21 days when stored in CP2D as compared to 42 days for packed red blood cell units and 1 year for fresh frozen plasma), economic concerns, and the efficiency of focused provision of individual specific blood components to address deficits (10). Thus, whole blood for trauma resuscitation is available at relatively few civilian trauma centers in the United States (11).

One potential strategy to decrease potential wastage of stored whole blood is to prepare and store packed red blood cells units (pRBCs) as the whole blood units expire. While technically feasible, this is not common blood banking practice (11). In addition, pRBCs develop significant biochemical changes as they age, commonly termed the “red blood cell storage lesion” (12, 13). Previous studies from our and other laboratories have demonstrated that the red blood cell storage lesion is associated with significant alterations to erythrocytes and potential harm to pRBC recipients in the setting of massive transfusion (14–16). The red blood cell storage lesion appears to occur in erythrocytes in stored whole blood cell units as well (16). Recent work from our laboratory suggests that the red blood cell storage lesion in whole blood differs from the changes found in pRBCs, with possible implications for resuscitation after hemorrhage (17).

We undertook the present study in order to increase our understanding of the red blood cell storage lesion as it occurs in erythrocytes salvaged from previously stored whole blood. We hypothesized that erythrocytes can be salvaged from previously stored whole blood without a significantly accelerated red blood cell storage lesion. We further theorized that transfusion

of these salvaged packed red blood cells would not result in an increased inflammatory response to resuscitation in our murine model of hemorrhagic shock and resuscitation.

Methods

Human blood banking

Cold stored, low-titer, O-positive, non-leukoreduced, whole blood units were donated, screened, and then stored in citrate phosphate double dextrose (CP2D) under standard storage conditions for 21 days. Standard packed red blood cells units were donated under the standard protocol employed by our regional blood bank and stored under standard storage conditions for 42 days. Unused, de-identified whole blood units were acquired on the date of their expiration. The process of whole blood and packed red blood cell acquisition was reviewed by our institutional review board and approved as “non-human subjects research”. The platelets were removed by centrifugation for 300g for 7 mins with aspiration and discard of the supernatant. Subsequently, the blood underwent centrifugation at 1000g for 15 minutes. The supernatant and buffy coat were discarded, the remaining erythrocytes were re-suspended in AS-3 storage solution at a ratio of 2:9, and then stored for an additional 21 days under standard storage conditions prior to analysis.

Murine blood banking

Murine experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Cincinnati. Murine whole blood banking was performed using a modification of our previously characterized protocol (12). Male 8–10 week old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with intraperitoneal pentobarbital (0.1 mg/gram body weight) and underwent donation blood draw via cardiac puncture. Packed red blood cell units were prepared with density gradient centrifugation at 400g for 40 minutes with subsequent resuspension of the erythrocytes in AS-3 storage solution in a 2:9 ratio and storage for up to 14 days. We have previously determined this to be the equivalent of 42 days of pRBC storage in human units (12). Whole blood units were stored in CP2D, in a 1:7 ratio, and stored up to 7 days after previous experiments indicated that this was equivalent to 21 days of human whole blood storage. On day 7 of storage, density gradient centrifugation was utilized to isolate the erythrocytes from the whole blood units. The erythrocytes were then resuspended in AS-3 in a 2:9 ratio to generate salvaged packed red blood cells units. Salvaged pRBC units were stored for an additional 7 days prior to analysis or transfusion.

Determination of the red blood cell storage lesion

At intervals, human and mouse pRBC units were analyzed for components of the red blood cell storage lesion. Microvesicle accumulation, Band-3 membrane protein expression, and phosphatidylserine expression were determined using flow cytometry with protein specific antibodies for CD-235a and Ter-119 (for erythrocyte specificity in humans and mice, respectively), Eosin-5-maleimide (EMA; for Band-3 binding), and Annexin V (for phosphatidylserine binding), respectively, as we have described previously (18). Hemoglobin concentration was determined by colorimetric assay (Hemoglobin Colorimetric Assay Kit, Biovision, Milpitas, CA). Murine erythrocyte viability was determined via a flow

cytometric assay utilizing acetoxymethyl ester of calcein (Calcein-AM) marker (Millipore Sigma, St. Louis, MO). Susceptibility of red blood cells to osmotic stress was determined by suspending aliquots of erythrocytes in solutions containing increasing concentrations of sodium chloride (0, 0.32, 0.44, 0.56, 0.68, and 0.8% NaCl) for 30 minutes, followed by centrifugation at 10,000 x g for 10 minutes with analysis of the supernatant absorbance measured via a microplate spectrophotometer (BioTek Cytation 5, Winooski, VT). The hemolytic increment was calculated and EC50 determined by the hemolytic increment of each sample when suspended in the 0.56% NaCl solution. The coagulation potential of stored erythrocytes was determined by mixing aliquots of pRBC units in a 1:1 ratio with human or murine plasma, then analyzing viscoelastic coagulation parameters by rotational thromboelastometry (ROTEM, TEM Systems Inc, Durham, NC). The extrinsic pathway coagulation (EXTEM) contribution to clot was measured for each blood sample.

Murine Model of Hemorrhagic Shock

Hemorrhage and resuscitation were carried out as previously described (19). Briefly, male C57BL/6 mice were anesthetized with intraperitoneal pentobarbital (0.1 mg/gram body weight) followed by groin clipping and sterile preparation with povidone-iodine solution and alcohol. The skin was incised, femoral vessels exposed, and the femoral artery cannulated with a tapered polyethylene catheter. The catheter was connected to pressure transducers for continuous hemodynamic monitoring of the mice (AD Instruments Lab Chart). To avoid hypothermia, the cannulated mice were placed on a circulating water blanket maintained at 41°C. After 10 minutes of equilibration, hemorrhagic shock was obtained by withdrawing blood to achieve a mean arterial pressure (MAP) of 25 ± 5 mmHg and maintained for 60 minutes. (20) The volume (mL) of blood required to achieve the desired hemorrhagic shock MAP was recorded for each mouse. Following hemorrhagic shock, mice were resuscitated with standard or salvaged pRBCs to achieve a MAP greater than 70 mm Hg $\pm$ 5 mm Hg. The volume (mL) of fluid or blood required to achieve the appropriate resuscitation was recorded for each resuscitation group. The mice were monitored for 15 minutes following resuscitation, femoral artery decannulated, and euthanized at 1-hour post procedure end. Sham animals underwent femoral artery cannulation and hemodynamic monitoring for 90 minutes, without hemorrhage or resuscitation.

Cytokine Analysis

One hour after hemorrhage and resuscitation, mice were sacrificed, and blood obtained. Serum samples were analyzed for inflammatory chemokines and cytokines as described in the results utilizing a flow cytometry-based cytometric bead array assay (BD Biosciences, San Jose, CA).

Statistical analysis

A power analysis was performed for the hemorrhagic shock and resuscitation murine model in order to determine the appropriate sample size. The sample size was based on the ability to detect a 15% change in IL-6 levels with 90% power and an alpha of 0.05.

GraphPad Prism was utilized (San Diego, CA) to perform statistical analysis of data via ANOVA or t-test as noted in the results. $P < 0.05$ was deemed statistically significant. Data is presented as mean $\pm$ standard error of the mean.

Results

Development of red blood cell storage lesion in salvaged human pRBC units

In initial experiments, we determined the severity of the red blood cell storage lesion in pRBC units from previously expired whole blood as compared to pRBC units stored under standard storage conditions. We examined accumulation of red blood cell microvesicles, which have previously been linked to harm after resuscitation (21), as well as expression of Band-3 and phosphatidylserine, two key markers of erythrocyte membrane integrity. We found no significant differences between standard storage and salvaged pRBC units in terms of microvesicle accumulation (FIGURE 1A). However, salvaged pRBCs demonstrated increased phosphatidylserine (FIGURE 1B) and reduced Band-3 expression (FIGURE 1C).

Free hemoglobin accumulation in stored pRBC units is an important indicator of erythrocyte integrity and potential function after transfusion. We next determined free hemoglobin in standard storage pRBCs and in pRBC units salvaged from previously stored whole blood. We found no significant difference in supernatant hemoglobin between these two groups (FIGURE 1D).

In order to determine the susceptibility of stored red blood cells to osmotic stress, we subjected aliquots of stored erythrocytes to solutions containing increasing concentrations of sodium chloride, then determined the effect of these solutions on hemolysis. Our data indicate that pRBCs salvaged from previously stored whole blood are more susceptible to osmotic stress than those stored under standard conditions (FIGURE 2A and 2B).

The direct red cell effect (DRE) is an important component of clot formation after hemorrhage. In order to determine the coagulation potential of stored erythrocytes, we mixed aliquots of pRBC units in a 1:1 ratio with human plasma and then analyzed the viscoelastic coagulation parameters by rotational thromboelastometry. Salvaged pRBCs demonstrated an increase in clotting time over the storage period (FIGURE 2C) but maintained the same maximum clot firmness (FIGURE 2D).

Development of red blood cell storage lesion in salvaged murine pRBC units

We next examined the severity of the red blood cell storage lesion in pRBC units from previously expired murine whole blood as compared to pRBC units stored under standard storage conditions to investigate the translatable effect from human to murine experiments in which donors are homogeneous and controlled. Salvaged murine pRBCs demonstrated significantly reduced microvesicle accumulation by day 14 of storage as compared to the standard pRBCs (FIGURE 3A). In contrast to the human pRBCs, there was no significant difference in phosphatidylserine expression between standard-storage and salvaged pRBCs (FIGURE 3B) and Band-3 expression was somewhat greater in salvaged pRBCs (FIGURE 3C). Whereas cell free hemoglobin was unchanged in salvaged human pRBCs, the supernatant hemoglobin was reduced in murine salvaged pRBCs (FIGURE 3D). There was

no difference in the susceptibility of the pRBC units to osmotic stress (FIGURE 4A and B). Determination of erythrocyte viability demonstrated a greater proportion of viable erythrocytes in pRBCs salvaged from previously stored whole blood (FIGURE 4C). In order to determine the direct red blood cell contribution to clot formation, murine fresh-frozen plasma was combined with pRBCs in a 1:1 ratio and the extrinsic component of the clot formation (EXTEM) was analyzed via ROTEM. The salvaged pRBCs demonstrated an increase in clot formation time when compared to standard-storage pRBCs. There were no difference in clotting time, alpha angle, or maximum clot firmness (data not shown).

Salvaged pRBC units are effective for resuscitation after hemorrhage

In order to determine the utility of pRBC units salvaged from previously stored whole blood for resuscitation in a murine model of hemorrhage, mice were subjected to severe controlled hemorrhage, then resuscitated with pRBC units stored under standard conditions or pRBC units salvaged from previously stored whole blood. Sham mice underwent femoral vessel cutdown and cannulation, but not hemorrhage. There were no significant differences in volume of blood shed in order to reach target MAP or the volume of pRBCs needed to resuscitate the mice in each group (data not shown).

We next determined serum cytokine levels 1 hour after hemorrhage and resuscitation with either standard storage pRBCs or pRBCs salvaged from previously stored whole blood units. Hemorrhage, followed by resuscitation with pRBCs stored under standard conditions, resulted in increased serum levels of interleukin-6 and keratinocyte chemoattractant (KC; FIGURES 5A and 5B) compared to Sham. Resuscitation with pRBCs salvaged from previously stored whole blood units was associated with decreased levels of these pro-inflammatory cytokines as compared to resuscitation with standard storage pRBCs (FIGURES 5A, 5B). There were no differences between pRBC groups with respect to macrophage inflammatory protein 1- α (MIP-1 α), interleukin-10, TNF, IL-1 β , or monocyte chemoattractant protein -1 (MCP-1) (data not shown). The serum of the mice resuscitated with salvaged pRBCs after hemorrhage demonstrated reduced cell-free hemoglobin as compared to standard storage pRBCs (FIGURE 5C).

Discussion

In the present study, we investigated the red blood cell storage lesion in packed red blood cell units salvaged from expired whole blood. We evaluated these units by characterizing several aspects of the red blood cell storage lesion, including microvesicle formation, Band-3 and phosphatidylserine expression, supernatant hemoglobin, osmotic fragility, and the direct red blood cell effect in clot formation. Additional experiments compared standard storage pRBC units with salvaged pRBC units for resuscitation in a murine model of hemorrhage and resuscitation. Our data indicate that several parameters of the red blood cell storage lesion, most notably Band-3 and phosphatidylserine expression as well as susceptibility to osmotic stress, were more severe in human pRBC units salvage from previously stored whole blood, while other aspects of the storage lesion, including free hemoglobin, an important indicator of erythrocyte lysis, and microvesicle concentrations, potential mediators of harm after transfusion, were similar between the two groups.

Use of whole blood for trauma resuscitation was first described during World War I (22, 23) and was subsequently identified as the sole therapy required in trauma resuscitation in “cases in which blood volume and oxygen carrying elements are needed”(24). In the late 20th century, whole blood fell out of favor as the primary therapy in the management in hemorrhagic shock as concerns about safety and storage limitations surfaced. During this era, crystalloid and colloid solutions generally replaced blood for initial trauma resuscitation, at least partially due to the increased incidence of transfusion-transmitted infections from locally obtained fresh whole blood and the inability of the blood supply system to guarantee its safety(25), as well as increasing fractionation of donated blood into blood components in order to maximize storage time and shelf life. Recent experience in military and civilian trauma settings has highlighted the importance of replacing blood lost with similar fluids. This has sparked renewed interest in the use of whole blood for trauma resuscitation (9–11, 25).

Wastage of blood and blood products is an ongoing concern in the United States civilian and military trauma systems. Reasons for wastage include improper storage, failure to return to blood bank prior to storage, contamination, but most commonly, expiration. Out of the 12.4 million packed red blood cell and whole blood units distributed in 2013, 3.4% were wasted due to expiration. Approximately 12.0% of whole blood units distributed were outdated prior to use, an increase from previous years (26). This wastage is especially significant in rural, austere, or military environments with limited resource availability(27). Interventions focusing education on proper utilization of “in-date” units have been implemented to reduce blood wastage, but do not address wastage resulting from expiration(28–30). Specific strategies to mitigate the impact of whole blood unit wastage, including salvage of erythrocytes and creation of packed red blood cell units, would be an important approach to maximize the availability of blood and blood products.

One aspect of the red blood cell storage lesion that has been the subject of intensive study is the accumulation of microvesicles during the storage period. We previously demonstrated that pRBC-derived microvesicles induce neutrophil priming, cause endothelial cell activation, lead to a transient hypercoagulable state, and result in increased lung inflammation in transfusion recipients (14, 21, 31). We have also previously shown that decreased microvesicle formation and abrogation of interactions between pRBC-derived microvesicles and endothelial cells results in decreased endothelial cell activation and lung injury (15, 32). Our data from the present study indicates that microvesicle formation is not increased in salvaged human pRBCs and is decreased in salvaged murine pRBCs. Taken together, this data indicates that salvaging pRBCs from previously stored whole blood does not accelerate microvesicle formation.

Our data suggest that some aspects of the red blood cell storage lesion may be accelerated in salvaged pRBC units. Specifically, we found that Band-3 and phosphatidylserine expression worsened in salvaged human units. In addition, we found that susceptibility to osmotic stress and clotting time were also increased. The clinical implications of these findings are unclear and will be the subject of further investigation, including donor-specific variables such as age and gender. Of note, cell-free hemoglobin, an important indicator of erythrocyte lysis, was similar between the two groups. We also found that several aspects of the red blood cell

storage lesion were attenuated in salvaged murine units. We suspect that the whole blood environment may be protective against some aspects of the storage lesion, therefore improving the resistance of the erythrocytes to further aging. We have previously investigated similarities (and important differences) in the red blood cell storage lesion in human and murine models (20). Our present findings confirm important differences between the models and highlight the need to continue the evaluation of strategies to attenuate storage lesion formation in a variety of model systems.

The whole blood storage lesion remains relatively poorly understood and under investigated. In the present study, we did not examine the effect of previous whole blood storage on the possibility of subsequent salvage of plasma or platelets. A recent study demonstrates that whole blood maintains significant hemostatic potential, both in terms of plasma and platelet contributions to clot, for a longer duration during storage than was previously presumed (33). As our experience with whole blood storage increases, our understanding of the potential uses of previously stored whole blood must improve as well.

While our data study shows promise with regard to the ability feasibility and safety of salvaging red blood cells from expired whole blood, our data should be interpreted with caution. The method of salvaging red blood cells from expired whole blood is a process that would still require access to blood banking equipment (ie. centrifuge, leukoreduction filters, etc.). While these supplies are easily accessible in hospitals, they may not be readily available in military, rural, or austere environments. Thus, our findings may not be directly applicable to all military or rural settings.

Several of the markers of the red blood cell storage lesion, including osmotic fragility, were less severe in salvaged murine pRBCs but had differing results in the human studies. Inter-donor variability, while controllable in murine studies, is impossible to eliminate during the acquisition of human samples. Although donor variability in human packed red blood cell donation has been studied, these factors and their impact on patients are not fully understood (34, 35). Previous studies have suggested that several donor factors, including gender, may impact the quality of donated red blood cells and outcomes following blood transfusion (36–38). The blood products used in this study were de-identified prior to transfer. Therefore, we do not have donor-specific variables available for the present analysis. In the future, we plan to further examine the impact of donor-specific variables such as age and gender on the formation of the red blood cell storage lesion in standard-storage packed red blood cells. We will apply these findings to investigate storage lesion formation in salvaged packed red blood cells. Furthermore, we will further explore how to decrease the storage lesion and improve storage quality (and possibly the duration) of the storage of the salvaged packed red blood cells.

In conclusion, our data suggest that salvage of erythrocytes from previously stored whole blood is associated with increases in some aspects of the red blood cell storage lesion, but not with microvesicle formation or erythrocyte hemolysis. Use of salvaged pRBC units for resuscitation was not associated with increased inflammation in a murine model of hemorrhage and resuscitation. Our data indicate that salvaging pRBC units from whole blood is feasible and may be a strategy to decrease waste of this precious resource.

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Figure 1.

Standard storage and salvaged pRBCs were analyzed for aspects of the red blood cell storage lesion. Microvesicle counts (A), Band-3 expression (B), and phosphatidylserine expression (C) were determined by flow cytometry. Supernatant hemoglobin (D) was determined by colorimetric assay. n=6 per group. *p<0.05.

Figure 2.

Human pRBCs stored under standard conditions and those salvaged from previously stored whole blood were placed in decreasing concentrations of sodium chloride to determine susceptibility to osmotic stress (A, B). $n=4$ for each group, $*p<0.05$ vs standard group. Salvaged pRBCs demonstrated a prolongation of the clotting time and no change in maximum clot firmness as they aged (C, D). $n=5$ for each group, $p<0.05$ vs indicated group.

Figure 3.

Murine standard storage and salvaged pRBCs were analyzed for aspects of the red blood cell storage lesion. Microvesicle counts (A), Band-3 expression (B), and phosphatidylserine expression (C) were determined by flow cytometry. Supernatant hemoglobin (D) was determined by colorimetric assay. n=5 per group. *p<0.05.

Figure 4.

Murine pRBCs stored under standard conditions and those salvaged from previously stored whole blood were placed in decreasing concentrations of sodium chloride to determine susceptibility to osmotic stress (A, B). $n=5$ for each group. Salvaged pRBCs demonstrated increase erythrocyte viability (C). $n=5$ for each group, $p<0.05$ vs indicated group.

Figure 5.

The serum of mice that underwent hemorrhagic shock and resuscitation with either standard (STD) or salvaged pRBCs was analyzed for inflammatory marker and cell-free hemoglobin. Serum of mice treated with salvaged pRBCs demonstrated reduced IL-6 (A), KC (B), as well as a reduction in cell-free hemoglobin after hemorrhage and resuscitation. $n > 5$ in each group. * $p < 0.05$ vs indicated groups.