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MURINE BLOOD BANKING: CHARACTERIZATION AND COMPARISONS TO HUMAN BLOOD

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Abstract

Blood transfusion remains an essential treatment of acute anemia. Current storage processes allow the efficient administration of blood products. Erythrocytes undergo morphological and biochemical changes during storage that may affect outcomes after transfusion. A reliable small-animal model would be ideal to examine the effects of stored blood products after transfusion. The objective of this study was to characterize the storage of murine erythrocytes for future application to animal models of acute anemia. Blood samples were collected from male mice and human volunteers, separated into components, and stored. At intervals, morphological and biochemical analysis was performed. Lactate, potassium, hemoglobin, and hemolysis were determined, and cell morphology was evaluated with light microscopy. Murine packed red blood cells (pRBCs) aged more rapidly than human samples. Murine pRBCs exhibited higher lactate levels (34.9 ± 1.3 mmol/L vs. 18.1 ± 1.0 mmol/L, mouse vs. human) and more severe acidosis as indicated by pH (6.56 ± 0.02 vs. 6.79 ± 0.04 , mouse vs. human). Murine pRBCs hemolyzed earlier (11.2 ± 3.7 g vs. 0.7 ± 0.3 g, mouse vs. human after 21 days of storage) and more rapidly than human pRBCs. Corpuscular changes consistent with red cell storage lesions appeared earlier in murine samples compared with human stored pRBCs. Compared with human pRBCs, murine pRBCs exhibit similar but more accelerated aging processes under standard storage conditions. Characterization of the murine red cell storage lesion will allow the application of stored blood components to future investigations into the treatment of acute anemia in experimental murine models.

Keywords

Red cell storage lesion; erythrocyte; blood preservation

INTRODUCTION

Allogenic blood transfusion remains an integral component of therapy for patients with acute anemia, with more than 50% of patients admitted to intensive care units receiving blood transfusions (1). During the most recent military conflicts, liberal blood component therapy has correlated with increased survival (2). However, transfusion of blood products is not without consequences. Recent studies have suggested that the transfusion of stored blood

is associated with increased mortality, septic complications, and organ failure after surgery and severe traumatic injury (3–6). Both the volume and the age of transfused blood products have been identified as independent risk factors for multisystem organ failure (3–6). In addition to causing transfusion-related complications, blood component therapy remains an expensive and limited medical resource.

Controversy exists in current transfusion practices, as the balance between the risks and benefits to blood component therapy has not been fully delineated. Recent efforts have increased our understanding of the “red cell storage lesion,” the constellation of changes erythrocytes undergo during the storage process. The clinical implications of the storage lesion are unknown but may play a role in complications after transfusion.

To better understand the consequences of transfusing stored blood products, a small-animal model for blood banking techniques is needed. A murine model of stored blood would provide substantial opportunity to study the effects of aged packed red blood cells (pRBCs) and plasma in the setting of hemorrhage, trauma, and immunosuppression. Although significant data exist regarding the storage lesion of human pRBCs (7), little is known about the aging process of murine erythrocytes (8). Once characterized relative to the storage process for human erythrocytes, a simple and reproducible model for storing murine blood products can be used to examine a broad spectrum of disease states seen in our patient populations.

MATERIALS AND METHODS

Animal model

Male C57/BL6 mice weighing 22 to 29 g were purchased from Harlan Laboratories (Indianapolis, Ind) were fed standard laboratory diet and water *ad libitum* and acclimated for 1 week in a climate-controlled room with a 12-h light-dark cycle. Experiments were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati. These experiments were performed in adherence to the National Institutes of Health guidelines on the use of laboratory animals.

Preparation of storage solutions

Citrate phosphate double dextrose (CP2D) (257.6 mmol/L of glucose, 105.0 mmol/L of citrate-citric acid, 18.5 mmol/L of monosodium phosphate, with a pH of 5.7) was prepared daily and used for anticoagulation (9). AS-3 (Nutricel) (55.5 mmol/L of glucose, 70.1 mmol/L of sodium chloride, 20 mmol/L of sodium phosphate, 12 mmol/L of citric acid, and 2.2 mmol/L of adenine, with a pH of 5.8) was used as medium for the storage of pRBCs (10).

Blood collection

Mice were anesthetized with intraperitoneal pentobarbital (0.1 mg/g body weight), and blood was collected via cardiac puncture into syringes pretreated with CP2D anticoagulant. Immediately after harvest, CP2D was added to fresh whole blood in a ratio of 1:7. The solution was gently mixed to avoid cell lysis.

Component separation

Anticoagulated whole blood was centrifuged within 30 min of collection at 1,000g for 15 min at 4°C. More than 90% of the plasma layer was removed from each sample. AS-3 was added to the remaining red cell volume in a ratio of 2:9 calculated from the original volume of whole blood collected. The two solutions were gently mixed to avoid cell lysis, incorporating the buffy coat into solution. This component was stored as pRBCs at 4°C on a nutator to allow for continual mixing.

Collection of human blood

Using a protocol approved by the University of Cincinnati Institutional Review Board, 50 mL of human whole blood was harvested from each of five healthy male volunteers after informed consent was obtained. The CP2D anticoagulant was added to each sample of fresh whole blood in a ratio of 1:7. Each sample was aliquoted and processed as previously described for murine pRBCs.

Sample analysis

Samples of pRBCs from humans and mice were analyzed at intervals from the day of collection (day 0) to 45 days after collection (day 45) to determine the effects of storage on the biochemical and hematologic parameters of pRBCs. Each sample was mixed gently before analysis. The following parameters were assayed on each sample: pH, lactate, potassium (I-STAT 1 Analyzer; Heska, Loveland, Colo), hemoglobin, and hematocrit (Coulter AcT10 cell counter; Beckman Coulter, Fullerton, Calif). Each sample was centrifuged at 6,000g for 10 min at 4°C. The supernatant was removed and analyzed for hemoglobin content as a measure of hemolysis. To document changes in RBC morphology over time, peripheral blood smears were performed at intervals after storage. After staining for erythrocytes, slides were evaluated with light microscopy and representative photomicrographs were shown (Protocol Hema 3 manual staining system; Fisher Scientific, Pittsburgh, Pa).

Statistics

Results are reported as the mean $\pm$ SEM. Two-tailed Student *t* test or ANOVA with Holm-Sidak testing for pairwise comparison was used where appropriate to determine significance. Statistical calculations were performed using SigmaPlot 10 software (Systat Software, Chicago, Ill) with significance set at values of $P \leq 0.05$. Sample sizes were $n = 5$ for each group at all time points analyzed.

RESULTS

In these experiments, we examined pH and lactate in mouse and human pRBCs as biochemical markers of red cell storage lesions. The pH of murine pRBCs was significantly lower from human pRBCs at every time point except on day 4 (Fig. 1). In addition, murine pRBCs, as compared with human samples, reached a lower pH at an accelerated rate. After 9 days of storage, murine pRBCs fell to a pH of 6.5 compared with human pRBCs that did not fall to a pH of 6.5 until day 21 (Fig. 1). The pH of murine pRBCs fell below the lower

limit of detection and was not able to be measured after 9 days; human pRBCs reached this level after 21 days.

In stored pRBCs, lactate levels increased more rapidly in murine than in human samples (Fig. 2). During early storage, lactate levels in murine pRBCs increased on average 2.72 mmol/day compared with 1.47 mmol/day in human pRBCs. An inflection point occurred in both murine and human pRBCs, after which both species demonstrated a plateau in lactate levels (Fig. 2). After 14 days in human and after 21 days in murine pRBCs, lactate levels no longer significantly increased with longer storage periods. At all time points, murine lactate levels were significantly higher than human lactate levels in stored pRBCs (Fig. 2).

Potassium levels in stored blood products increase over time, leading to levels of hyperkalemia that may limit the useful shelf life of banked pRBCs (11). At all time points after collection and storage, murine pRBCs exhibited higher levels of potassium compared with human pRBCs (Fig. 3). Murine pRBCs developed a rapid increase in potassium levels during the first 10 days of storage. After 10 days of storage, murine pRBCs were increased 14.7-fold compared with a 6.3-fold increase in potassium levels in human pRBCs. After 21 days of storage, the degree of hyperkalemia in murine pRBCs no longer increased. Potassium levels in stored human pRBCs increased during the first 28 days of storage but did not subsequently change with prolonged storage periods (Fig. 3).

Hemoglobin concentration was measured as another indicator of stability of pRBCs during storage. The hemoglobin levels of murine pRBCs were similar to human pRBCs during the entire length of storage time, with the exception of 28 days of storage (Fig. 4). Over time, human pRBCs exhibited no significant increase or decrease in hemoglobin levels. Hemoglobin concentration in murine pRBCs did not change over time. Only after 28 days of storage did hemoglobin levels differ significantly from any earlier time point (Fig. 4).

The degree of hemolysis in stored samples was determined by measuring supernatant hemoglobin concentrations after centrifugation of murine and human pRBCs. Hemolysis of murine pRBCs increased compared with human pRBCs at all time points except on the day of collection (day 0) and days 2, 4, and 7 (Fig. 5). After prolonged storage, from days 14 to 35, the hemolysis of murine pRBCs increased at an average rate of $0.71 \text{ g} \pm 0.01 \text{ g}$ of hemoglobin per day, compared with the slower rate of $0.07 \text{ g} \pm 0.03 \text{ g}$ of hemoglobin per day seen in human pRBCs (Fig. 6). No significant hemolysis occurred until after 14 days of storage in either murine or human pRBCs.

Over time, pRBCs undergo typical morphological changes characteristic of the red cell storage lesion. At intervals, we examined mouse and human RBC morphology using light microscopy. Changes in murine RBCs consistent with storage are seen as early as 2 days after collection (Fig. 7B). Characteristic findings included the crenation of erythrocytes with the formation of spicules, as well as a reduction in mean corpuscular hemoglobin concentration, seen by progressive loss of the central clearing found in normal RBCs. Over time, murine erythrocytes underwent lysis, evident in smears taken from day 21, 28, and 35 samples (Fig. 7, E–G). Human erythrocytes do not show evidence of loss of central clearing until after 21 days of storage (Fig. 7, L–N). The morphological changes seen in murine

erythrocytes are present to a lesser degree in human erythrocytes and appear after longer storage times (Fig. 7, H–N).

DISCUSSION

In the present study, we collected, processed, and stored murine and human pRBCs. We characterized the biochemical, hematological, and morphological changes seen in murine pRBCs after prolonged storage relative to changes in human pRBCs. In doing so, we have characterized a reproducible small-animal model of blood banking, applying the identical anticoagulant and red cell storage media used in our institution's blood collection center. These results indicate the presence of the red cell storage lesion in a mouse model, with potential application to various injury models requiring transfusion.

Transfusion of stored blood products continues to be an important component of the treatment of disease states across a broad spectrum of medical specialties. Conflicting clinical data have emerged regarding the risks and benefits of blood transfusion. In the intensive care setting, pRBC transfusion has been associated with increased mortality and more severe end-organ damage (12). The Transfusion Requirements in Critical Care study provided evidence supporting a more conservative transfusion practice (13). Several retrospective and prospective studies have found similar results when examining critically ill patients, leading to more restrictive patterns of transfusion during the past decade (14–18).

Current data from the military and civilian trauma databases show improved clinical outcomes with more liberal transfusion practices termed *damage control resuscitation* strategies. In the setting of severe hemorrhagic shock, transfusing a high ratio of fresh frozen plasma to pRBCs may lead to decreased morbidity and mortality (2, 19). Controversy exists in current transfusion practices in both cardiac and transplant patient populations as well. Blood transfusion has been associated with an increased incidence of heart failure after coronary artery bypass grafting and well as improved renal allograft survival after transplantation (20, 21).

Given conflicting data regarding the benefits of blood transfusion, recent research efforts have focused on the effect of storage on pRBCs. Several clinical studies have demonstrated increased mortality, multisystem organ failure, and infection rate when pRBCs stored for longer than 14 days are used for resuscitation after traumatic injury (4–6). Similarly, the transfusion of pRBCs stored for longer than 14 days was associated with worse clinical outcomes after cardiac surgery (3). A reproducible small animal model of blood banking will provide a valuable vehicle for understanding the effects of transfusing stored blood components in each of these clinical settings.

Current U.S. Food and Drug Administration guidelines require a post-transfusion RBC viability of 75% or greater after 24 h (22). Recent research has documented the *in vivo* survival of stored murine erythrocytes after transfusion (23). In contrast to the rapid deterioration of rat erythrocytes over time (8), murine erythrocytes displayed a modest decrease in RBC viability, analogous to the changes seen in human RBCs during storage (23). After 14 days of storage in CPDA-1, murine RBCs fell below the current requirement

for 75% post-transfusion survival (23). In our study, murine RBCs anticoagulated in CP2D and stored in AS-3 documented a much earlier increase in other significant parameters but did not exhibit any significant evidence of hemolysis until after 14 days of storage.

In addition to post-transfusion viability, other relevant changes in pRBCs collected from mice occur relative to human samples. Samples of stored murine pRBCs demonstrated more severe acidosis, as evidenced by a lower pH and increased lactate levels. Although a low pH is initially beneficial to maintain adequate levels of ATP synthesis, prior studies suggest that if pRBCs are stored in additive solutions with higher pHs, increased 2,3-diphosphoglycerate synthesis occurs at the expense of adenosine triphosphate (ATP), resulting in decreased RBC viability (24). A low basal level of ATP generation during storage is required to maintain erythrocyte viability and is sustained until the pH falls below 6.5 (25). In murine pRBCs, the pH falls below this critical pH after 9 days compared with 21 days in human pRBCs. This low pH may subsequently cause increased RBC lysis, as ATP stores are depleted and further synthesis is impaired, accelerating hemolysis. Acidosis, one of the three components of the “lethal triad” of traumatic injury, leads to increased mortality after injury (26). The transfusion of stored blood products with significant acid-base imbalances may be partly responsible for these clinical consequences. In addition, the transfusion of a high lactic acid burden may be unsafe when given to unstable trauma patients with tissue ischemia and may affect complications seen with the transfusing aged blood products in this population (5). Resuscitation strategies using fresher blood products may attenuate the acidosis that follows severe injury and may be safer in the setting of tissue trauma.

The transfusion of a high potassium load in blood stored for prolonged periods has documented clinical consequences including neonatal deaths after cardiac surgery (27). With progressive damage of the RBC membrane, increased permeability leads to potassium leakage. This, compounded by the impaired sodium-potassium pump at low storage temperatures, exacerbates the loss of intracellular potassium (22). Hyperkalemia itself may not affect clinical outcomes unless transfused rapidly or in a large volume, as in massive transfusion protocols. Current research is attempting to address hyperkalemia in stored blood products by warming, washing techniques, and new additive solutions to avoid these complications, all of which could be studied using small-animal models (28).

As expected, hemoglobin levels in murine and human pRBCs stored for 35 and 45 days, respectively, were not significantly different, with the exception of a single time point. The rate of hemolysis, however, as determined by measuring free hemoglobin concentration after centrifugation, was much faster in murine pRBCs. Both populations of RBCs initially remained stable, then hemolyzed over time, reflecting an exhaustion of ATP stores and inevitable RBC death (29). The life span of circulating murine erythrocytes is known to be shorter than human RBCs, approximately 50 days compared with 120 days (30), and we expect a more rapid rate of murine RBC death during storage. For clinical comparison, the hemolysis of murine pRBCs after 12 days correlates with the degree of hemolysis found in a unit of human pRBCs stored for 35 days. The average length of storage of blood products sent overseas and used for military resuscitation and treatment is 33 days (31). The translation of this information into a clinical model may have significant implications in studying the treatment of hemorrhagic shock and trauma.

The morphological changes observed in our RBC samples are consistent with known storage lesions. During storage, human erythrocytes lose their typical biconcave disc shapes, undergoing crenation, and forming echinocytes (9). Over time, stored RBCs shed spicules from the cell membrane, leading to decreased surface areas, increased membrane fragility, and loss of deformability, all of which may have implications after transfusion (29). Compared with murine pRBCs, human pRBCs remain relatively stable initially. After 35 days of storage, however, the central clearing has disappeared, and RBCs have transitioned to spherocytocytes. In murine blood cells, the appearance of echinocytes occurs much earlier and progresses steadily starting at 2 days of storage. By day 21, nearly 50% of the murine erythrocytes appear lysed, consistent with the degree of hemolysis seen at this time point. At day 35, no normal murine erythrocytes remain, with 98% of RBCs hemolyzed.

Our goal was to develop a reproducible model of murine blood storage, with indicators that could correlate with human blood storage. The ability to combine these data with known preclinical models provides the opportunity to apply this method of blood storage to injury models requiring blood transfusion. The results obtained from this investigation allow us to draw parallels between the aging process of human and mouse stored RBCs, at time points throughout the storage process. Although murine erythrocytes undergo an accelerated aging process compared with human RBC, the ability to relate the two processes temporally is critical to reproducing human diseases in a rodent model. In doing so, we can potentially examine stored murine RBCs in models of hemorrhagic shock, traumatic injury, cardiac and transplant surgery, and other causes of acute anemia for which blood transfusion remains a crucial component of medical therapy.

In conclusion, the transfusion of stored blood components, although currently administered under more restrictive guidelines, remains an integral part of the treatment of acute anemia. The need to more closely evaluate the ramifications of blood transfusion has generated significant interest in examining the effects of storage on blood components. A known collection of changes develops over time in human stored pRBCs, and these same changes are found in murine stored pRBCs. This study, after characterizing the murine red cell storage lesion, will permit the addition of stored components to the future study of transfusion-related research in murine models of anemia.

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Fig. 1. pH in samples of human and murine pRBCs stored in AS-3 at 4°C

* $P < 0.05$ vs. human, # $P < 0.001$ vs. identical marks, ## $P < 0.05$ vs. identical marks. $n = 5$ for each group at all time points.

Fig. 2. Lactate levels in human and murine pRBCs during storage

Murine and human samples were followed for 35 days and 45 days, respectively and were significantly different from each other at all time points. * $P < 0.001$ vs. human, # $P < 0.05$ vs. identical marks, ## $P < 0.01$ vs. identical marks. $n = 5$ for each group at all time points.

Fig. 3. Time-related changes in potassium in human and murine pRBCs after storage
 Murine and human pRBCs were stored for 35 days and 45 days, respectively. * $P < 0.05$ vs. human, # $P < 0.05$ vs. identical marks, ## $P < 0.05$ vs. identical marks. $n = 5$ for each group at all time points.

Fig. 4. Time-related changes in hemoglobin levels in human and murine pRBCs stored in AS-3 at 4°C

Murine and human samples were followed for 35 days and 45 days, respectively. * $P < 0.03$ vs. human, # $P < 0.05$ vs. identical marks. $n = 5$ for each group at all time points.

Fig. 5. Time related changes in hemolysis expressed as supernatant hemoglobin level in human and murine pRBCs during storage

Murine and human pRBCs were followed for 35 days and 45 days, respectively. * $P < 0.05$ vs. human, # $P < 0.05$ vs. identical marks, ## $P < 0.05$ vs. identical marks. $n = 5$ for each group at all time points.

Fig. 6. Rate of hemolysis in human and murine pRBCs during storage, expressed as change in free hemoglobin concentration of supernatants over time, after 14 days of storage
* $P < 0.001$ vs. human.

Fig. 7. Changes in RBC morphology over time

Photomicrographs A through G represent murine erythrocytes at storage intervals from day of collection to 35 days of storage. Photomicrographs H through N represent human erythrocytes stored in AS-3 at 4°C after identical lengths of storage time.