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Transfused Older Stored Red Blood Cells Improve the Clinical Course and Outcome in a Canine Lethal Hemorrhage/Reperfusion Model

Steven B. Solomon^{1,*}, Irene Cortés-Puch^{1,*}, Junfeng Sun¹, Kenneth E. Remy¹, Dong Wang¹, Jing Feng¹, Sameena S. Khan^{1,2}, Derek Sinchar³, Daniel B. Kim-Shapiro⁴, Harvey G. Klein⁵, and Charles Natanson¹

¹Critical Care Medicine Department, Clinical Center, NIH, Bethesda, Maryland, USA ²University College Dublin, Belfield, Dublin, Ireland ³Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, PA, 15213 ⁴Department of Physics and the Translational Science Center, Wake Forest University, Winston-Salem, NC 27109 ⁵Department of Transfusion Medicine, Clinical Center, NIH, Bethesda, Maryland, USA

Abstract

BACKGROUND—In canine models, transfused older stored red blood cells (RBCs) hemolyze *in vivo* resulting in significantly increased intravascular cell free hemoglobin (CFH) and non-transferrin bound iron (NTBI). During canine bacterial pneumonia with septic shock, but not in controls, older stored RBCs were associated with significantly increased lung injury and mortality. It is unknown if in shock without infection transfusion of older RBCs will result in similar adverse effects.

STUDY DESIGN AND METHODS—Two-year-old purpose-bred beagles (n=12) were transfused similar quantities of either older (42-day) or fresher (7-day) stored universal donor canine RBCs 2.5 hours after undergoing controlled hemorrhage (55ml/kg).

RESULTS—With older transfused RBCs, CFH (p<0.0001) and NTBI (p=0.004) levels increased, but lung injury (p=0.01) and C-reactive protein levels (p=0.002) declined and there was a trend toward lower mortality (18% *versus* 50%). All 3 deaths after transfused fresher red cells resulted from hepatic fractures. Lowered exogenous norepinephrine requirements (p<0.05) and cardiac outputs (p<0.05) after older transfused RBCs were associated with increased CFH levels that have known vasoconstrictive nitric oxide scavenging capability.

CONCLUSIONS—In hemorrhagic shock, older RBCs altered resuscitation physiology but did not worsen clinical outcomes. Elevated CFH may lower norepinephrine requirements and cardiac outputs ameliorating reperfusion injuries. With hemorrhagic shock, NTBI levels persist in contrast

Address correspondence to Steven B. Solomon, Ph.D., Critical Care Medicine Department, NIH Clinical Center, 10 Center Drive, Room 2C145, NIH, Bethesda, Maryland 20892. Tel: 301-435-2287; Fax: 301-480-5493; SSolomon@cc.nih.gov.

*S.B.S and I.C.P share first authorship and contributed equally to the manuscript.

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to the increased clearance, lung injury, and mortality in the previously reported infection model. These preclinical data suggest that whereas iron derived from older RBCs promotes bacterial growth, worsening septic shock mortality during infection, release of CFH and NTBI during hemorrhagic shock is not necessarily harmful.

Keywords

Blood storage lesion; red blood cells; hemoglobin; iron; transfusion medicine; vascular biology; animal models of vascular pathology; hemorrhagic shock; hemorrhage-reperfusion

INTRODUCTION

Transfusion of stored red blood cells (RBCs) is one of the most commonly used medical therapies. Each year, approximately 15 million units are collected in the United States. RBCs units are licensed for up to 42-days of refrigerated storage prior to transfusion.^{1,2} The customary practice instituted for efficient inventory control assigns patients needing a transfusion the oldest compatible unit available (“first in, first out”). However clinical and laboratory studies have raised concerns that the changes that develop as refrigerated RBCs age, known as the “storage lesion,” result in blood that is less safe than blood stored for short durations.^{3–5} Neither the clinical importance of these changes nor the “critical age” of stored RBCs has been determined. Multiple randomized controlled trials comparing transfused stored blood of different ages in various patient populations have been completed^{6–8} or are ongoing^{9–11}. Since the mean age for a RBC unit transfused in the United States is 17.9 days,² and intentionally “aging” blood beyond this usual practice is unacceptable for a human trial, even moderately sized clinical trials may fail to detect significant adverse effects of the oldest RBCs, particularly in subgroups of patients. Clinically relevant animal studies allow examination of the age extremes of stored RBCs as well as control of such factors as volume of transfused RBCs, severity of illness, and comorbidities.

In a canine model of *S. aureus* pneumonia, transfusion of 42-day old stored RBCs markedly increased lung injury and mortality rates.⁴ These increased risks after transfusion of older blood were associated with ongoing *in vivo* hemolysis, resulting in high levels of cell free hemoglobin (CFH), and non-transferrin bound iron (NTBI). These data were consistent with the hypothesis that high levels of CFH increase vasoconstriction by scavenging nitric oxide (NO) at sites of infection contributing to vascular endothelial injury and worsening the severity of the pneumonia. The increased levels of NTBI afforded a potential source of iron for bacterial proliferation that may have exacerbated the pulmonary infection and thereby increased mortality.¹² The relative importance of CFH compared with NTBI as a mechanism for exacerbating the injury observed with older blood transfusion remains unclear. Transfusion of older blood in the absence of infection, in otherwise normal (control) canines, resulted in comparable increases in hemolysis and cell free hemoglobin and greater elevations in NTBI levels, but no lung damage or mortality.¹² These findings suggest that infection and/or critical illness would be necessary for older blood to augment risks.

We hypothesized that the adverse effects observed after massive transfusion of older blood required the presence of established infection and that transfusion during a lethal inflammatory illness without infection would not be associated with increased risks. To evaluate this hypothesis, we developed an infection-free canine model of acute hemorrhagic shock and reperfusion injury. We replaced withdrawn blood after 2.5 hours with an equivalent amount of either fresh or older stored RBCs. The volume transfused was comparable to the amount of blood received by animals in our exchange-transfusion and infection model. We observed organ injury and evidence of increased inflammation during hemorrhagic shock with delayed reperfusion. However, without infection present, massive amounts of older stored transfused blood did not worsen outcome.

MATERIALS AND METHODS

Study Synopsis

Twelve purpose-bred beagles (12 to 28 months old, 9 to 12.5 kg) were randomized to be transfused either older (6 week, n=6) or fresher (1 week, n=6) CPDA-1 stored, commercially available, leukocyte-reduced, universal donor canine blood (DEA1.1, ABRINT, Dixon, CA), following acute hemorrhage. Blood was collected, prepared, transported, and stored under conditions comparable to those required for human blood transfusion. Except for the age of stored blood transfused, all animals were treated identically throughout the 96-hour study duration. On day 0, animals were phlebotomized 55ml/kg whole blood from a femoral arterial line over 30 minutes. Two and a half hours following acute hemorrhage, animals were transfused 55 ml/kg over 30 minutes with a combination of either older or fresher packed red blood cells (RBCs), and thawed fresh frozen plasma (ratio one to one, volume of packed RBCs to fresh frozen plasma (Animal Blood Resources Inc., Dixon, CA). Investigators and caregivers were blinded to treatment assignments during the entire experiment. The statistician (JS) was aware of treatment groups and monitored survival weekly but did not have contact with study animals or their caregivers.

Each study week (three consecutive weeks), four animals were transfused with older (n=2) or fresher (n=2) stored blood 2.5 hours after hemorrhage and received standard intensive care ancillary therapies similar to those used in non-experimental clinical veterinarian small animal intensive care units. These measures included sedation, mechanical ventilation, fluids, and vasopressors given according to standardized protocols based on physiological endpoints, as well as standard prophylactic measures (see below). Animals were continuously monitored and cared for by a clinician or trained technician throughout the experiment. After 96 hours, animals still alive were considered survivors and were euthanized. The study protocol was reviewed and approved by the National Institutes of Health Clinical Center Institutional Animal Care and Use Committee. Please see also the SUPPLEMENTAL MATERIALS AND METHODS *Detailed Study Design, and Statistical Methods* as well as previous descriptions of the canine model¹³ for details.

Canine Blood Banking and Transfusion Practices

The methods of obtaining and transfusing canine universal donor blood and fresh frozen plasma and the characteristics of the canine blood employed from 1 to 6 weeks of storage in

comparison to human stored blood have been previously described.⁴ The stored RBC and plasma bags were cultured after opening to detect contamination. None of the cultures showed bacterial growth.

Laboratory measurements

Canine levels of CFH ($\mu\text{mol/L}$) were determined by Drabkin's method, as previously described.⁴ Canine NTBI and PLI plasma levels were determined in a commercial laboratory (aFerrix, Tel Aviv, Israel) using a proprietary assay.⁴ CRP was determined using a commercially available kit (eBioscience, San Diego, CA).

Histology

Immediately following death or euthanasia (survivors), complete necropsies were performed on all animals. Selected tissue samples from the heart (each ventricle), lungs (cephalic and caudal, left and right), liver, kidneys (medulla and cortex, left and right) and spleen were collected for potential analysis.

RESULTS

Survival

Survival rates or times did not differ significantly during experimental massive acute hemorrhage followed by transfusion of an equivalent amount of either older or fresher RBC (42 vs. 7 day old RBC, $p=\text{NS}$; Figure 2A). Transfusion with fresher stored RBCs after massive hemorrhage was associated with 50% mortality (3/6) compared to 18% mortality (1/6) in animals transfused with older stored RBCs. Despite a trend that unexpectedly favored animals transfused with older RBCs, the study was halted to evaluate secondary data in order to investigate the unexpected result and minimize animal use.

To investigate the causes of mortality, necropsies were performed on all animals directly following death or at 96 h, after euthanasia, in survivors. All three animals transfused with fresher blood that died before 96 h were observed to have fractures and vascular necrosis in the right medial lobe of the liver (Figure 2B), as well as 300 to 600 ml of free blood in the peritoneal space. Massive hemorrhage associated with liver fractures was the sole cause of death. In the three animals receiving 7-day old RBCs surviving to 96 h, no liver abnormalities or peritoneal hemorrhage were observed. The sole death prior to 96 h in animals transfused with older blood resulted from exsanguination into the subcutaneous tissue occurring over days through a ruptured femoral artery at the site of catheter placement. Abnormalities of the femoral artery or liver were not observed in the five animals that received older stored RBCs and survived to 96h. There were no significant lesions in any of the 12 animals on gross pathology of the lung, heart, kidneys, pancreas or gastrointestinal tract.

Lung Injury

Transfused fresher RBCs significantly increased lung injury compared to older RBCs as measured by the lung injury score (LIS)⁴ at 20 h, and the LIS score was higher without reaching statistical significance from 8 to 16 h after acute hemorrhage (Figure 3). The

evidence of impaired lung function was consistent with the trend toward decreased survival in the animals receiving fresher stored blood. The component parameters of the LIS score are provided in Supplemental figure 1.

Inflammatory Response

C-reactive protein (CRP) was measured as an acute phase protein associated with inflammatory injury. CRP levels increased significantly in response to transfusion from 10 to 48h in animals transfused with either older or fresher blood ($p=0.0002$; all values, compared to baseline)(Figure 4). By 48 h, CRP levels were declining in animals transfused with older blood, while levels in those transfused with fresher blood continued to rise ($p=0.002$; old vs. fresh blood levels at 48h). The continued increase in CRP levels in response to receiving fresher blood is consistent with the severe liver damage observed after 48h during necropsy in these animals.

Cardiovascular function

To investigate the cardiovascular response to reperfusion with older *versus* fresher blood after massive acute hemorrhage, we examined serial changes from baseline in MAP and vasopressor requirements. During transfusion, there was a similar mean increase in MAP with fresh and older blood ($p = \text{ns}$). However, at 8 h (4 h after transfusion), animals receiving older blood had a significantly higher MAP in comparison to those transfused with fresher blood ($p = 0.007$, Figure 5A). The MAP in some animals receiving older blood exceeded the upper limit of the desired range per protocol (80–100 mmHg for sedated and ventilated canines). Further, from 8 to 24 h, animals receiving older units of blood consistently required significantly lower doses of norepinephrine support to maintain MAP in the normal range in comparison to those receiving fresher blood ($p < 0.05$ at 8 h, 12 h, 16 h, 20 h after hemorrhage, Figure 5B). Transfusion with older blood was associated with little or no requirement for blood pressure support with norepinephrine (<0.1 mcg/kg/min) throughout the early reperfusion period (8 and 12 h). We calculated a shock reversal score that reflects the vascular response to shock by taking into account the level of vasopressor support (norepinephrine) needed to maintain the MAP within a normal range.⁴ Older blood transfusion significantly improved the shock reversal score starting at 8 h (4 h after the end of the transfusion) until 16 h later (T24 h), in comparison to fresher blood transfusion (p -values ranging from 0.01 to <0.0001 , Figure 5C). Animals receiving older RBCs also had a statistically significant increase in the systemic vascular resistance index (SVRI) at 8, 12 and 20 h after hemorrhage (Figure 5D).

We further examined serial changes in the cardiac index in response to reperfusion. Following transfusion, there was a significant decrease in cardiac index (CI) with older compared to fresher stored blood transfusion (p -values ranging from 0.05 to 0.004 between 8 h and 16 h after hemorrhage, Figure 5E). The lack of a similar decrease in cardiac index in animals receiving fresh blood probably reflects their greater doses of norepinephrine, a vasopressor with beta cardiac stimulating effects in addition to alpha vascular peripheral vasoconstrictor effects.

To insure that the observed cardiovascular effects were not due to differences in fluid resuscitation, we serially measured mean CVP (Figure 6A), PAOP (Figure 6B), and hematocrit (HCT, Figure 6C). We found no significant differences between the two groups in these serial measures either during transfusion or after through 96 h (data not shown from 72 h to 96 h).

Serial mean plasma levels of Cell free hemoglobin (CFH), Non-transferrin bound iron (NTBI) and Plasma labile iron (PLI)

Transfusion of older blood in our model of canine pneumonia⁴ was associated with hemolysis and increased levels of CFH, NTBI and PLI. In the hemorrhage reperfusion injury model, CFH was significantly increased in animals transfused with older compared to fresher RBC at 4h (30 minutes after transfusion) ($p = 0.05$) and levels continued to increase and remained significantly elevated at 24 h ($p < 0.0001$) and 48 h ($p < 0.0001$) (Figure 7A). NTBI levels were abnormal ($>0.2 \mu\text{M}$) in animals transfused with older RBC and significantly greater than after fresher RBC transfusions at 10 h ($p = 0.01$) and at 24 h ($p = 0.004$) (Figure 7B). PLI also reached abnormal levels in response to transfusion with older blood by 10 h but was not significantly different at that time point when compared to transfusion with fresher stored blood, and returned to baseline levels by 24 h (Figure 7C).

Renal and Liver function

We examined serial mean levels of electrolytes, blood urea nitrogen (BUN), creatinine (Cr), aspartate aminotransferase (AST), alanine transaminase (ALT), Creatinine phosphokinase (CPK), and glucose (Glu) (Supplemental Table 1). There were no significant differences or abnormalities in electrolytes, Glu, renal (BUN, Cr,) or liver (ALT) function between animals transfused with older compared to fresher stored RBC. A significant increase in AST and CPK at 24 h was observed in animals receiving older compared to fresher stored RBC (both, $p < 0.05$) likely reflecting the increased rate of hemolysis or muscle ischemia. None of the other measurements outlined in the methods and Figure 1 showed marked changes as a result of age of transfused blood nor were there any other significant differences in their levels from baseline levels.

DISCUSSION

Transfusion of fresher stored RBCs following hemorrhagic shock was associated with 50% mortality (3/6) compared to 18% mortality (1/6) in animals transfused with older RBCs. The sole death in the older stored blood group was associated with iatrogenic hemorrhage from an arterial puncture site, nevertheless analyzed under intention to treat. These results were unexpected and opposite to previous *S. aureus* pneumonia studies that showed significantly improved survival rates when transfusing fresher stored RBCs.⁴ Also during hemorrhagic shock, in contrast to the infection model, animals receiving fresher stored RBCs had significantly more impairment in lung function and required more vasopressors to maintain blood pressure near normal.⁴ In this model, all three non-survivors transfused with fresher RBCs developed liver fractures with vascular necrosis, causing intraperitoneal hemorrhage. All animals exhibited marked increases in CRP levels after the prolonged shock and reperfusion as would be expected with a systemic inflammatory response. However, fresher

RBCs produced significantly more profound CRP elevations. By this measure, as with survival and liver fractures and the measures of cardiac and pulmonary function, animals transfused with fresher blood appear to fare worse in a hemorrhage/ reperfusion injury model, a significantly different and opposite effect of fresher transfused stored blood in an infection-transfusion model.

From these studies it is unclear whether transfused older RBCs were protective or fresher RBCs were harmful during reperfusion injury. Older transfused RBCs were associated with a different hemodynamic profile than fresher RBCs. Following transfusion, animals receiving older stored RBCs were able to maintain MAP in the normal range with only minimal doses of exogenous norepinephrine. We attribute these effects to the augmented release of CFH from older transfused RBCs, scavenging of NO and vasoconstriction leading to elevation of MAP.¹⁴ The significantly increased need with fresher blood to maintain MAP with norepinephrine, a vasopressor with beta one cardiac stimulatory agonist activity,^{15,16} may explain why the cardiac index was significantly higher and systemic vascular resistance index was significantly lower compared to animals receiving older blood. Higher cardiac output after transfusion of fresher blood reflects a higher flow resuscitation, potentially increasing organ perfusion and congestion.¹⁷ In contrast, after resuscitation with older RBC, the MAP was maintained in the normal range mainly by the effect of CFH scavenging NO. This pure vasoconstrictive effect, in contrast to norepinephrine, had no additional cardiac stimulatory activity. Therefore transfusion with older RBCs resulted in a lower CI and more gradual resuscitation overall than with fresher RBCs. Reducing the speed^{18–20} and/or the flow²¹ of resuscitation is believed to attenuate reperfusion injury. Lee et al¹⁸ found that in a rat model of hemorrhage-transfusion that gradual, step-wise resuscitation decreased mortality, liver injury, cytokine and radical oxygen species generation in comparison to rapid resuscitation. Similar findings have been reported in animal models of hemorrhage-reperfusion using fluid resuscitation instead of blood¹⁹ and in ischemia-reperfusion models.²⁰ Rapid resuscitation with high pulmonary artery flow rates has also resulted in more severe injury after lung transplantation in an animal model.²¹ Consistent with these findings, in our model, older blood resulted in a slower, gradual reperfusion with improved outcomes.

CFH scavenging of NO may have other benefits in the setting of hemorrhage and reperfusion injury aside from the direct hemodynamic effect. The role of NO during ischemia-reperfusion is complex.^{22–28} NO can be protective in ischemic reperfusion injury,²⁹ by improving microcirculation, reducing activation of cell-death pathways, enhancing mitochondrial function, and reducing the inflammatory response.^{22,23} Some have suggested that Release of NO from RBCs may improve tissue oxygenation through vasodilation.³⁰ However, excess release of NO may paradoxically result in cell and tissue damage by combining with superoxide radicals to form the highly toxic radical species, peroxynitrite, which can exert cytotoxic effects.³¹ During ischemia-reperfusion injury²⁵ as well as during hemorrhagic shock²⁶ excessive NO production has been linked to worse outcomes and inhibition of inducible nitric oxide synthase found to be beneficial in both canine and rodent experimental models.^{26,27}

We hypothesized, based on our previous bacterial pneumonia⁴ and normal control studies,¹² that infection was necessary to produce harmful effects when older blood is transfused. This study confirms that lethal inflammatory reperfusion injury without infection fails to produce similar results. To better define the mechanism(s) of the infection-older blood injury, we compared across our three models (hemorrhage-reperfusion, infection, and normal controls) the association of the levels of NTBI and CFH with the degree of lung injury and survival^{4,12} (Figure 8). The volume of stored older and fresher blood transfused was similar in all three models.^{4,12} Transfusion of older *versus* fresher blood led to a significantly greater increase in NTBI levels in the hemorrhage model than in the infection model (Figure 8A and B). However unlike the infection model, but similar to the hemorrhage model, marked increases in NTBI levels were seen when normal control animals were transfused with older in comparison to fresher RBCs (Figure 8C).^{4,12} In contrast, transfusion of older compared to fresher blood produced similar rises in CFH levels in all three models (Figure 8D–F).^{4,12} Transfusion with older compared to fresher blood produced significantly greater increases in lung injury and mortality rates in the infection model when compared to the controls and/or the hemorrhage model (Figure 8G–L).^{4,12} Elevation of CFH alone, and NO scavenging alone, cannot easily explain the marked increase in lung injury and mortality with older blood during infection because CFH and NO scavenging capability levels were similarly increased across the three models. In contrast, after older blood transfusion, the marked elevations in NTBI levels seen in normal controls and during reperfusion injury were not seen during infection. Disappearance of NTBI after massive older blood transfusion was associated with significantly increased lung injury and mortality rates (Figure 8E and G), compared to reperfusion injury and normal controls.^{4,12} The steep decline over time in the elevated NTBI levels during infection, but not during non-infectious inflammatory injury, is consistent with the notion that bacteria utilized the available plasma iron to promote growth and worsen outcomes.^{5,12,32,33} These findings are consistent with the findings in a murine model of transfusion with infection and in studies of normal volunteers.^{5,34,35} In contrast, released NTBI from older RBCs did not increase morbidity and/or mortality in the absence of infection.

In multiple clinical trials, chemically altered CFH, known collectively as Hemoglobin Based Oxygen Carriers (HBOCs) and used to replace stored RBCs, proved toxic.³⁶ This was particularly evident in trauma and patients with coronary artery disease whose impaired vasculature may be particularly at risk following NO scavenging. However the different characteristics of the molecules and differing features of administration likely explain the seemingly contradictory findings in clinical trials of HBOCs in trauma with hemorrhage compared to our experimental model of hemorrhage/reperfusion injury which demonstrated elevated levels of CFH. In the trauma studies HBOCs were given in gram quantities³⁷ and used as a replacement for blood for resuscitation.³⁷ Further, with HBOCs the CFH is modified to produce larger and/or more stable molecules by cross-linking the alpha and beta hemoglobin chains, polymerizing the hemoglobin molecules, or linking to polyethylene glycol, among the various methods to modify oxygen affinity, reduce NO scavenging and to keep the oxygen carrier in the intravascular space.³⁸ Our hemorrhage model differs from these clinical trials by resuscitating with blood which releases native CFH. Much lower

concentrations of CFH are generated by transfusions of old RBCs and the protein is released slowly over time. Native CFH is rapidly cleared from the circulation.

Interpretation of our findings is subject to several limitations. The number of animals is small and survival differences were not statistically significant. However the numerous positive physiology changes with older stored blood support the conclusion that such transfusions are not detrimental and may be beneficial in this model. All three non-survivors transfused with fresh blood showed the same very specific damage to the right middle lobe of the liver as the cause of death, an uncommon finding in clinical hemorrhagic shock. However this finding is consistent with an ischemia-reperfusion injury found in animal models and is not plausible as occurring by chance. The suggestive beneficial effects of older blood in this canine model of hemorrhage/reperfusion injury may not be applicable to other types of non-infectious inflammatory injuries. The volumes transfused are large and the differences in age of stored blood are extreme. This is a unique animal model and others have shown increased vascular damage,³⁹ histologic renal³⁹ or liver injury,⁴⁰ and increased levels of markers of renal, liver and muscle injury^{41,42} with older stored transfused blood in other experimental hemorrhage models; in some of these models, NO reversed this damage. However, Abadi et al,⁴³ in a rodent model of hemorrhagic shock and transfusion also failed to observe worsened organ injury or survival with older compared to fresher blood transfusion. The reasons for these differences are unknown, but may be related to the model studied. Canine RBCs may experience proportionally more damage by 42 days of storage than do human cells; however increased RBC fragility would not explain the improved physiology observed with the transfusion of older RBCs. Finally, this study does not address the effects of transfusing different mixtures of old and fresh RBCs. Others have reported that transfusion of fresh murine cells reverses the adverse effects of older stored RBCs.⁴⁴

In conclusion, transfusion with older stored RBCs does not appear to have detrimental effects during controlled hemorrhage in a canine model of severe inflammation without infection and in normal canine controls. Limited data suggest that in some situations such as resuscitation for hemorrhagic shock, transfusions with older RBCs may even prove to be beneficial. Further animal studies varying the timing and degree of hemorrhage as well as the vasopressors and/or resuscitation fluids are clearly warranted. Our findings support the hypothesis that iron availability is the major factor explaining the harmful effects of older transfused blood during infection. Taken together, these findings indicate that the risk/benefit profile of older blood may vary depending on the subpopulation transfused. Transfusion of older stored RBCs can appear safe in clinical trials of medical and surgical patients, but in selected subgroups of these patients, release of iron and CFH may affect the clinical course and alter the outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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D.K.-S. is listed as a coauthor on a patent application on methods of treating hemolysis and on patent applications related to development of blood substitutes using recombinant human hemoglobin.

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B. Pathology, right middle lobe, 7-day old blood non-survivor

Figure 2. Survival and liver infarct of right middle lobe

In the top panel (A) are Kaplan-Meier plots throughout comparing survival with 42-day-old (solid line) and 7-day-old (dotted line) blood following acute hemorrhage. In the bottom panel (B) is shown an example of the gross morphologic changes in liver found in all three non-surviving animals that were hemorrhaged and then transfused with 7-day old blood. The image to the left shows the median liver lobe with hemorrhagic infarct changes in situ. The image to the right shows the same liver infarct once separated from the rest of the liver parenchyma. A ruler is provided for reference.

Figure 3. Serial mean ($\pm$ SE) Lung Injury Scores (LIS)

LIS data from a previously published scoring system that increases the sensitivity to detect pulmonary damages.⁴ Components include mean pulmonary artery pressure, alveolar-arterial oxygen gradient, plateau pressure, O₂ saturation, and respiratory rate. The LIS is plotted over time (x-axis) for animals receiving 42-day-old blood or 7-day-old blood. A P-value indicates a significant difference between older and fresher blood exists at a time point.

Figure 4. Serial Mean levels of C-reactive protein

Serial mean ($\pm$ SE) plasma levels of C-reactive protein (mg/dL), a measure of inflammatory injury, are plotted over time (x-axis) for animals receiving 42-day-old (solid line) or 7-day-old (dotted line) blood. A p-value indicates a significant difference between older and fresher blood at a time point.

Figure 5. Serial mean cardiovascular response

Serial mean ($\pm$ SE) values of mean arterial pressure (panel A), norepinephrine levels (panel B), shock score* (panel C), systemic vascular resistance index (panel D) and cardiac index (panel E) are plotted over time (x-axis) for animals receiving 42-day-old (solid line) or 7-day-old (dotted line) blood (mean $\pm$ SE). A p-value indicates a significant difference between older and fresher blood exists at a time point. *The shock score accounts for the level of vasopressor support (norepinephrine) needed to maintain the mean arterial pressure in a normal range for sedated and ventilated canines (80–100 mm Hg).⁴

Figure 6. Serial mean ($\pm$ SE) measures of intravascular volume

This figure uses the same format as Figure 4, except that central venous pressure (panel A), pulmonary artery occlusion pressure (panel B) and hematocrit (panel C), are plotted on the y-axis. Similarly, p-values indicate that a significant difference between older and fresher blood exists at a time point.

Transfused Stored Blood

Figure 7. Serial mean ($\pm$ SE) plasma levels of cell-free hemoglobin and excess iron

This figure uses the same format as Figure 4, except that plasma levels of cell-free hemoglobin (panel A), non-transferrin bound iron (panel B) and plasma labile iron (panel C), are plotted on the y-axis. Similarly, p-values indicate that a significant difference between older and fresher blood exists at a time point.

Figure 8. Comparison of the effect of older versus fresher blood transfusion on the levels of excess iron and cell-free hemoglobin plasma and the degree of lung injury and survival in three experimental canine models: *S. aureus* pneumonia, hemorrhage-reperfusion injury and normal controls

This figure compares the effect of older (grey bars) *versus* fresher (open bars) blood transfusion on non-transferrin bound iron levels (panels A, B and C), cell-free hemoglobin levels (D, E, F) and LIS (G, H, I) 24 hours after transfusion in an experimental model of bacterial pneumonia (panels A, D, G, J), hemorrhage-reperfusion injury (panels B, E, H, K) and normal controls (C, F, I, L). Data are expressed in mean $\pm$ SE. In panels J, K and L,

survival proportion (%) at the end of the experiment (96 hours) is compared for the three models in a similar fashion. Data regarding the bacterial pneumonia model and normal controls have been previously published. A p-value indicates that a significantly different effect of older *versus* newer blood transfusion exists comparing the two models. The volume of blood transfused was similar in all three models.