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Research Opportunities in Optimizing Storage of Red Blood Cell Products

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INTRODUCTION

Whether “older” red cells (RBCs) are as efficacious as “younger” RBCs is currently unresolved and awaiting the outcome of several large prospective clinical trials in the US and Canada. In an effort to proactively advance our understanding of the RBC storage lesion prior to the completion of these trials, NHLBI established a targeted research program in 2009 entitled, “Immunomodulatory, Inflammatory and Vasoregulatory Properties of Transfused RBC Units as a Function of Preparation and Storage.” The investigative teams funded by this initiative and their research topics have been previously reported.¹ These teams, as well as principle investigators from two of the large prospective clinical RBC storage trials, and other Transfusion Medicine experts gathered on August 28th and 29th, 2012 in Bethesda, MD for an NHLBI sponsored Working Group exploring “Strategies to Optimize Red Blood Cell (RBC) Products.” The first day consisted of progress reports from the eight investigative teams, a presentation about a canine sepsis model to investigate the effect of older blood on survival, and updates from two of the ongoing prospective RBC storage clinical trials. On the second day, researchers provided presentations on a planned study to identify genetic determinants that affect RBC storage, the influence of manufacturing method and irradiation on RBC in vitro quality during storage, development of a new additive solution, the effects of storage containers composition on RBC quality, post collection manipulations, and the possible impact of a reduced RBC shelf life on inventory. This article provides a summary of many of the presentations given during the two-day workshop.

REPORTS FROM RFA-HL-08-005: *IMMUNOMODULATORY, INFLAMMATORY AND VASOREGULATORY PROPERTIES OF TRANSFUSED RBC UNITS AS A FUNCTION OF PREPARATION AND STORAGE*

Microcirculatory perfusion: Role of Nitric Oxide, Nitrite, ATP and SNO-Hb

Scott R Barnum, Rakesh P Patel, and Jordan A Weinberg—The specific negative clinical manifestations associated with the transfusion of stored RBCs and the corresponding mechanisms responsible for such phenomena remain poorly defined. Our recent studies document that leukodepleted older RBC units potentiate transfusion-related toxicity in trauma patients.^{2–4} It is our hypothesis that the transfusion of relatively older blood may impede microvascular perfusion as a result of a loss of RBC-dependent control of nitric

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oxide (NO) mediated homeostasis concerning vasodilation, and immune cell and complement activation.⁵

To test this hypothesis, we conducted a prospective observational study that ties observation of the microcirculation during RBC transfusion to trauma patients to the *ex vivo* assessment of NO-mediated vascular homeostasis and inflammatory modulation. During transfusion, tissue oxygen saturation was assessed non-invasively by near-infrared spectroscopy and sublingual microcirculatory perfusion was concomitantly imaged using a hand-held microscope employing sidestream dark field illumination. Aliquots from both the RBC unit and the patient's pre- and post-transfusion serum were collected to assess the association between RBC storage age and mechanisms that modulate hypoxic microcirculatory vasodilation and complement-mediated activation of the inflammatory response.

We found that sublingual functional capillary density in trauma patients was negatively affected by transfusion of relatively older RBC units.⁶ We observed significant loss of whole-blood nitrite following transfusion of 1 RBC unit stored for >25 days, but not with younger RBCs.⁷ Using competition kinetic analyses and protocols that minimized contributions from hemolysis or microparticles, our data indicated that the consumption rates of NO increased ~40-fold and NO-dependent vasodilation was inhibited 2–4-fold comparing 42-day-old with 0-day-old RBCs.⁷ These results are probably due to the formation of smaller RBCs as a consequence of membrane loss during storage resulting in a greater surface to volume ratio for older RBCs. We also found that RBC storage did not affect deoxygenated RBC-dependent stimulation of nitrite-induced vasodilation. However, stored RBCs increased the rates of nitrite oxidation to nitrate in vitro. We are currently evaluating for changes in complement activation protein levels over RBC storage duration.

Mark T Gladwin and Daniel B Kim-Shapiro—We continue to explore the hypothesis that NO dysregulation underlies the RBC storage lesion, driven by increased hemolysis-mediated NO catabolism and loss of NO generating functionality.^{8, 9} NO is an important signaling molecule that regulates vascular tone, decreases platelet and endothelial cell activation, and thereby is anti-inflammatory. We have proposed that transfusion of older blood leads to a decrease in NO bioavailability which has deleterious downstream consequences.

Rapid NO scavenging by hemoglobin (Hb) is diminished by encapsulation in the RBC.¹⁰ Several mechanisms for reduced NO scavenging have been proposed: 1) rate limitations of NO diffusion to the RBC through the cell-free zone or unstirred layer surrounding RBCs;^{11–18} 2) diffusion of NO inside the RBCs¹⁹; 3) a finite permeability of the RBC membrane limiting the rate of NO entry.^{14, 20–23} All of these mechanisms break down upon hemolysis. We showed that storage-dependent hemolysis increases NO scavenging by cell-free Hb and Hb in RBC microparticles; the latter scavenges NO almost as fast as cell-free Hb.^{24, 25} Infusion of cell-free Hb and microparticle-containing supernatant from older stored blood increased mean arterial pressure in a rodent model whereas supernatant from fresher blood and cyanomethemoglobin did not.²⁴ These data provide inductive support for the hypothesis that transfusion of stored blood results in lower NO bioavailability, contributing to the storage lesion.

Normal NO bioavailability may also be affected by the action of a recently discovered RBC cell nitric oxide synthase (NOS).²⁶ During storage, RBC NOS function may decrease due to oxidative and membrane damage which could result in NOS uncoupling. Our studies suggest that there is a functional eNOS in RBC that regulates blood pressure (manuscript in review) but that it does not modulate in storage hemolysis.²⁷

One method to counter reduced NO bioavailability upon transfusion of stored blood is to neutralize NO scavenging of oxygenated free Hb by converting it to a non-NO scavenging form. Angeli's salt (AS) could do this since it releases HNO which reacts with oxygenated Hb to form methemoglobin. We have shown that AS preferentially reacts with cell-free Hb rather than RBC-encapsulated Hb. We have demonstrated that this preferential reactivity occurs in a canine model where AS was infused along with cell-free Hb.²⁸ The extent of methemoglobin was less than originally expected due to reaction of HNO with plasma thiols.²⁸ However, the intrinsic vasodilatory action of HNO was able to compensate for NO scavenging by Hb, neutralizing negative hemodynamic effects of the Hb.²⁸

Jonathan S Stamler and James D Reynolds—In 1996 we showed, for the first time, that RBCs dilate blood vessels and can represent a source of NO bioactivity independent of blood vessels.²⁹ As a result of these findings, the respiratory cycle of RBCs is now best-described as a three-gas system, comprised of NO in addition to O₂ and CO₂.^{29–31} The role of RBC-derived NO is to regulate microvascular blood flow, and efforts that only focus on blood O₂ content can fail to improve O₂ delivery resulting from insufficient blood flow.³² In particular, tissue perfusion is regulated by a response called hypoxic vasodilation, in which blood flow is increased by hypoxemia. Hb within RBCs is a principal transducer of this response, deploying NO bioactivity under hypoxia. NO bioactivity in blood exists principally in the form of S-nitrosoHb (SNO-Hb) whose formation and activity is regulated by oxygen tension (pO₂).^{33–35} In this model, transition from high to low pO₂ in arterioles (R to T transition in Hb) promotes the release of S-nitrosothiol (SNO)-based vasodilatory activity to maintain tissue perfusion consonant with metabolic demand. By extension, conditions that reduce SNO-Hb levels will reduce microvasculature blood flow.

Banked blood rapidly loses SNO-Hb and these losses impair vasodilation by RBCs.³⁶ The clinical relevance of SNO-Hb depletion is suggested by the failure of banked blood to improve tissue oxygenation following transfusion.³⁷ Major adverse events associated with transfusion (myocardial infarction, pulmonary edema, renal injury, multi-organ failure, and death)^{38–41} are also suggestive of the idea that administration of stored blood (deficient in SNO-Hb) may exacerbate rather than correct ischemia.³⁶ Because RBCs traffic through the microcirculation in line, impaired vasodilation by even a minor transfused fraction would be expected to adversely influence O₂ delivery. Banked RBCs would in effect plug the microcirculation. Since losses in SNO-Hb during storage are large, SNO-Hb levels are unlikely to normalize immediately post-transfusion.³⁶ Instead, transfused RBCs will act as sinks for NO and predispose the recipient to vasoconstriction and ischemic insult.

Identification of the SNO deficit of banked blood provides an opportunity for therapeutic intervention (renitrosylation therapy). Initial testing of an S-nitrosylating agent that restores SNO-Hb levels has yielded promising results: The vasodilatory activity of RBCs is normalized, blood flow and tissue oxygenation are improved, and organ function is preserved.^{36, 42–48} We are currently examining the utility of renitrosylation therapy in several pre-clinical and clinical studies.

John Roback—We explored whether young or stored RBCs elicited similar vasoreactivity. We first conducted *in vitro* studies. Aliquots from leukoreduced RBC units were added to rat aortic rings followed by vasodilatory stimuli. In the presence of day 0 blood, aortic rings significantly dilated in response to 10⁻⁵ M methacholine but not after incubation with RBCs stored for 42 days. Published data suggested the vasoinhibitory activity would be found in the storage-aged RBC (saRBC) supernatant, as degenerating saRBCs release Hb and arginase and reduce NO bioavailability.²⁴ However, washing saRBC samples did not reduce the inhibitory effect. We next quantified vasodilation in response to sodium nitroprusside (SNP), an NO donor. There was no inhibitory effect on

SNP-mediated vasodilation by either fresh or saRBCs indicating that the *in vitro* inhibitory effects of saRBCs did not involve NO scavenging and were due to intact RBCs.

We then studied vasoreactivity in healthy human recipients during transfusion of fresh or saRBCs. Autologous AS-1 RBCs were stored for 3–7 days and then transfused; the protocol was later repeated with another unit donated and stored for 35–42 days. Arterial acetylcholine (ACh) infusions were given to stimulate NO synthesis and blood flow responses were measured. Fresh transfusions had no effect but transfusion of saRBCs unexpectedly increased ACh-stimulated vasodilation 1 hour after transfusion. The effects were eliminated by the NOS inhibitor L-NMMA, suggesting an NO-related mechanism. These results provide evidence for acute vasoactive effects of saRBCs (but not fresh RBCs) in human transfusion recipients.

Finally, hospitalized patients for whom a transfusion had been ordered were randomized to receive a fresh (<10 days old) or saRBC (>21 days old). Non-invasive flow-mediated dilation (FMD) assays of the brachial artery were performed before, during, 1 hr after, and 24 hr after transfusion (n=41 subjects). Compared to pre-transfusion assessment, FMD increased acutely during transfusion (saRBC > fresh, not significant). By 24 hours post-transfusion, FMD results had decreased in both groups. However, this change was only significant for recipients of saRBCs ($p = 0.027$: during vs. 24; $p = 0.048$: pre vs. 24). These data suggest that saRBCs (but not fresh RBCs) in patients produce a vaso-inhibitory effect manifesting at 24 hrs post-transfusion.

Potential mediators of adverse effects: Iron, inflammation and microparticles

Steven L Spitalnik, Richard O Francis, and Eldad A Hod—We use murine, canine, and human models to focus on the downstream consequences resulting from rapid extravascular hemolysis of refrigerator storage-damaged RBCs. We continue to evaluate the “iron hypothesis,” which proposes that acute ingestion of a bolus of damaged RBCs by the monocyte-macrophage system, leads to catabolism of the cleared RBCs, releasing a large amount of iron from the ingested hemoglobin. This iron can acutely increase cytoplasmic iron levels and, by overwhelming the binding capacity of plasma transferrin, can acutely increase circulating levels of non-transferrin bound iron. These increases in non-protein bound iron can potentially lead to inflammation and an increased susceptibility to infection by particular pathogens. Indeed, transfusions of older, stored RBCs induced increased levels of non-transferrin bound iron in mice⁴⁹, dogs (manuscript submitted), and healthy human volunteers⁵⁰. Although these transfusions induced a pro-inflammatory cytokine response in mice and dogs, this was not seen with healthy human volunteers. Finally, these transfusions exacerbated infections *in vivo* in mice and *in vitro* using murine and human serum samples.

In addition to pursuing the studies described above, we became interested in factors that accelerate or ameliorate the RBC storage lesion. In particular, given that storage subjects RBCs to a significant oxidative stress, we propose that donors whose RBCs, for genetic and/or environmental reasons, are defective in handling oxidative stress may manifest the phenotype of a “poor storer.” In contrast, if their RBCs are very efficient at resisting oxidative stress, they may appear to be a “super storer.” Because RBCs contain a robust anti-oxidant system consisting of complex metabolic pathways with multiple enzymes and anti-oxidant proteins, any given donor may exhibit variability in multiple sections of these pathways. Therefore, to simplify investigation by using a well-defined model, we began studying human donors who are deficient in glucose-6-phosphate dehydrogenase (G6PD). G6PD-deficiency is the most common human enzymopathy and these RBCs, because they cannot generate sufficient amounts of NADPH and reduced glutathione, are highly susceptible to oxidant stress, resulting in lipid and protein oxidation, Heinz body production, phosphatidylserine exposure, and enhanced intravascular and extravascular hemolysis. Thus,

we hypothesize that RBC units from G6PD-deficient donors, which are present in the blood supply⁵¹, would exhibit an enhanced RBC storage lesion *in vitro* and decreased post-transfusion RBC recovery *in vivo*. Studies addressing this hypothesis are currently underway.

Charles Natanson—Older blood develops storage lesions and accumulates potentially injurious substances.⁵² Some studies report significantly increased mortality as blood ages.^{53, 54} We assessed the safety of older versus newer stored blood in transfused patients by conducting a meta-analysis of the published literature; and by conducting a study in a canine model of pneumonia to examine this question prospectively in a controlled setting.

In our meta-analysis, we searched PubMed, Scopus, and Embase for articles using title words *new* and *old* and *RBC* or *cells* and *storage* from 2001 to 2011 with mortality the variable of interest.⁵¹ The search yielded 99 studies of which 21 met inclusion criteria (18 observational studies and 3 small randomized controlled trials). The studies were predominantly in cardiac surgery (n = 6) and trauma (n = 6) patients, and included from 66 to 387,130 patients. A test for heterogeneity of study results ($I^2 = 3.7\%$, $P = 0.41$) showed the data could be combined using a random effects model. We found that older blood was associated with a statistically significant increase in the risk of death [odds ratio 1.16; 95% confidence interval (CI) (1.07–1.24)]. We estimated that 97 (63, 199; 95% CI) patients would need to be treated with only younger blood to save one life. Subgroup study analysis indicated the increased risk was not restricted to a particular type of patient, size of trial, or amount of blood transfused. The major limitation of this analysis was that it was based primarily on observational studies and an unknown unintentional bias could not be excluded.

In our blinded, randomized study using a validated canine model of sepsis,^{54, 55} and using modified standard blood banking and intensive care practices, we exchange transfused purpose-bred beagles infected with *S. aureus* pneumonia with either 7- or 42-day-old stored canine universal donor blood in 4 divided doses. Older blood significantly increased mortality, lung injury, and a shock injury score.⁵⁵ The older blood significantly increased pulmonary artery pressures during transfusion (4 to 16 h) and for 10 h afterward. Plasma-free Hb and NO consumption capability were significantly elevated and haptoglobin levels were decreased with older blood during and up to 32 hours after transfusion. Plasma non-transferrin-bound and labile iron were elevated only during transfusion (both $p = 0.03$). Low plasma haptoglobin levels and high NO consumption capability of plasma at 24 h were significantly associated with poor survival. With older blood, more severe lung injury, as evidenced by increased necrosis, hemorrhage, and thrombosis, was noted at the site of infection post-mortem.⁵⁵ It is unknown, however, if human blood transfused at the end of the storage period into critically ill patients would have similar effects as seen in this canine model.

Wenche Jy—We tested the hypothesis that microparticles shed in stored RBCs contribute to adverse transfusion outcomes and the risk of clinical complications in patients receiving transfusion for coronary artery bypass grafting (CABG) by affecting procoagulant activity, pro-inflammatory mediators, and the endothelium.

We have identified several factors affecting microparticle release and activities during storage. Non-leukoreduced RBCs produce platelet (PMPs), leukocyte (LMPs) and RBC (RMPs) microparticles. RMPs increased slightly before day 10 of storage, and then increased exponentially. PMPs rose steadily from day 0 peaking at day 20. LMPs increased after day 20, with levels rapidly increasing after day 30. PMP (days 0 to 20) and RMP (days 20 to 40) levels correlated with increasing MP-mediated procoagulant and inflammatory markers. Pre-storage leukoreduction decreased RMP generation by 20–40%, eliminated

PMP and LMP generation, and reduced total MP-mediated procoagulant and inflammatory markers by 40–60%. Levels of RMP released were proportional to platelet counts. Storage of leukoreduced RBC under anaerobic conditions resulted in 40–50% MP reduction. Mixing PMPs with RMPs in a ratio of 1:10 increased RMP procoagulant and proinflammatory activities by 50–60%.

Finally, a clinical study is ongoing in patients undergoing CABG who are randomized to receive either washed or unwashed RBCs. The 2 groups will be compared with respect to subclinical physiologic host responses including endothelial disturbances, inflammatory markers, and procoagulant responses. Transfusion complications and short-term (30 days) surgical complications will also be assessed. Non-transfused patients are serving as controls.

Neil Blumberg, Richard P Phipps, Sherry L Spinelli, and Jill M Cholette—We are interested in the mechanisms by which transfusion of allogeneic RBCs (and platelets) are associated with adverse recipient outcomes including fever and rigors, post-operative infection, lung injury, thrombosis, multi-organ failure and, mortality.⁵⁶ We hypothesized that both stored RBCs and accompanying supernatant mediate some of these effects through interactions with the transfusion recipient's platelets. A growing body of data demonstrates that platelets are involved in host defense, lung injury, and, of course, thrombosis.^{57–59} Previous work demonstrates that transfused soluble CD40L (CD154) secreted from activated platelets is a possible mediator of fever, rigors⁶⁰ and lung injury.⁶² Other candidate mediators in the supernatant of transfused RBCs include prostanoids that are recognized as mediators and markers of oxidative stress. This complex and diverse family of non-enzymatically generated mediators are present in transfused blood and substantially affect normal platelet function *in vitro* (unpublished data).

In addition to the supernatant, storage altered RBCs likely affect recipient immunity and host defenses, lung and vascular function, and hemostasis. We presented unpublished preliminary data from a retrospective observational study suggesting that in newborns and infants undergoing cardiac surgery, RBC storage age is strongly associated with post-operative infection.⁶² This association was not explained by total dose of RBCs transfused or patient co-morbidities, corticosteroids, operative variables, etc. In previous small randomized clinical trials, washed and leukoreduced transfusions were associated with improved survival in younger adults with acute leukemia⁶³ and reduced post-operative inflammation in pediatric cardiac surgery.⁶⁴ However in our preliminary data, washing did not appear to abrogate the association of storage duration with post-operative infection in pediatric cardiac surgery. We conclude that both stored RBCs and supernatant mediators affect post-transfusion morbidity and mortality.

Philip J Norris Rosemary Sparrow and Philip C Spinella—We have focused on understanding how transfusion-related immunomodulation (TRIM) is affected by the storage age of the RBCs. The first data measured in participants of the Age of Blood Evaluation (ABLE) study were presented. Microparticles, D-dimer and fibrinogen levels were measured. Fluctuations over the first 30 days post-transfusion were noted but the effects of storage (if any) will only be able to be assessed once the patient groups (fresh vs. standard issue blood) are unblinded when ABLE ends.

In addition to studying immune and coagulation parameters in transfused patients, we evaluated the RBC unit-derived microparticles effect on host immune responses. Microparticles from leukoreduced RBC units potentiated T cell survival and proliferation. This effect was mediated through activation of monocytes but was independent of blood storage time.

A paired study of AS-1 and SAGM stored RBCs found significant differences in numbers of released microparticles, including potentially pro-coagulant phosphatidylserine-bearing microparticles, level of hemolysis, and profile of adherence of stored RBCs to endothelial cells under continuous flow perfusion. Whether these *in vitro* differences between AS-1-RBCs and SAGM-RBCs translate into differences in behaviour *in vivo* following transfusion is not known. Nevertheless, these findings are intriguing from the perspective of the “continental divide” coined by van de Wating⁶⁵, who observed that the concern about the ‘age of blood’ stems more from North American studies than from European studies.

In other work, *in vitro* models of transfusion were developed to study the interaction of vascular endothelial cells with leukocytes. Stored RBCs appeared to modulate the interaction of neutrophils with endothelial cells. Further research will enable exploration of a “two-insult” TRIM model concept, help better understand the mechanisms of cell-cell interactions, and identify potential remedies.

REPORTS FROM STRATEGIES TO OPTIMIZE RED BLOOD CELL (RBC) PRODUCTS WORKING GROUP

Donor characteristics for optimal storage

Michael P Busch—While some elements of the RBC storage lesion itself are well known, including free Hb release, increasing levels of RBC-derived microparticles, and loss of RBC deformability and enzymatic functionality, there appears to be a large variation between blood donors in these properties. Little is known about the molecular mechanisms of hemolysis and how the propensity of erythrocytes to hemolyze in the setting of blood bank storage is modulated. There are >4,000 identified polymorphisms in hemoglobins, RBC membrane proteins, and enzymes that are known to affect life-span and stability, yet none have been evaluated for their effects on RBC storage parameters, post-transfusion recovery or half-life. These known genetic variants, most of which are Mendelian inherited somatic point mutations, likely represent only a few of many undiscovered variants that are likely to affect hemolysis during storage.

The Recipient Epidemiology and Donor Evaluation Study (REDS)-III RBC-Omics study will pursue the overarching hypothesis that genetic variation in donors underlies the variable propensity of erythrocytes to hemolyze during storage. To address our hypothesis, we propose to evaluate 14,000 distinct donors and their RBC donations at REDS-III blood centers. Samples from leukoreduced-RBC components will be analyzed for key storage parameters as a function of donor characteristics. Genetic and metabolomic studies will be conducted to understand the etiology of storage-related hemolysis, capacity to donate, Restless Leg Syndrome, and PICA. Finally, a shareable biorepository of phenotypically and genotypically characterized biospecimens derived from the stored RBC components will be established, allowing for further studies into the mechanisms underlying, and approaches for prevention of, the RBC storage lesion.

Processing and storage solutions

Jason P Acker—There are many differences in how RBC products are manufactured internationally. These methods include RBC production from whole blood (WB), apheresis collection, buffy coat (overnight hold) or PRP processing, anticoagulant solution, and whether units are leukoreduced.

We found that WB-derived RBCs had statistically greater volume (313 ± 30 mL) and Hb per unit (59 ± 8 g/unit) compared to those of apheresis RBCs (296 ± 16 mL, 55 ± 3 g/unit). Hematocrit ($61 \pm 2\%$ vs $63 \pm 3\%$), hemolysis ($0.11 \pm 0.04\%$ vs $0.22 \pm 0.05\%$), supernatant

K^+ (9.51 ± 4.55 mmol/L vs 15.31 ± 3.01 mmol/L) and supernatant Na^+ (120 ± 52 mmol/L vs. 149 ± 10 mmol/L) were less in WB derived RBCs than in apheresis RBCs. Within WB-derived RBC products, RBCs produced after an overnight hold had significantly less hemolysis and supernatant K^+ , but reduced oxygen dissociation parameters than those of PRP or WB-filtered RBC products. Differences were also observed between RBCs preserved with AS-1, AS-3 and SAGM. Significant differences in the number and composition of microparticles as a function of the manufacturing method were also observed.

Thus, not all RBCs are equivalent, and in-vitro quality differences should be considered when designing transfusion outcome studies.

Dana Devine—Irradiation of blood is a common post-production manipulation that may be conducted by hospital blood banks or by blood component manufacturers. There are regional differences in guidelines with respect to the allowable maximum age of blood at irradiation and the amount of storage time allowed after irradiation. North American guidelines are the most liberal, with blood of any age being considered acceptable for irradiation, and storage after irradiation allowable for up to 28 days or the normal 42 day post-collection outdate, whichever comes first. The storage time restriction after irradiation reflects the recognition that RBCs are damaged by 25 Gy. If hemolysis affects adverse reactions to stored blood products, then irradiation may increase hemolysis during storage.⁶⁶

Using a ‘pool and split’ model to avoid donor variability on study results, units of leukoreduced RBC stored in SAGM additive solution were irradiated on Day 1, and tested on days 1, 7, 14, 21 and 28 for a standard panel of physiological measures. Hemolysis increased more rapidly during storage, with higher levels even on Day 1. Unlike untreated RBCs which show a linear relationship between potassium leakage and storage time, irradiated RBCs showed an initial rapid increase ≈ 3 -times those of untreated cells by Day 7. Other parameters which were significantly worse in the irradiated arm included MCV, MCHC, lactate, and microvesicle production.

The RBC membrane ‘ghosts’ and the microvesicles in irradiated and non-irradiated study products were analyzed by proteomic techniques. Significant alterations in protein distribution among vesicles and ghosts were seen for band 3 and hsp70, with the relative enrichment of band 3 in the microvesicle fraction of the irradiated arm. The expression of somatin, a lipid-raft associated protein, was greater in microvesicles from irradiated products. These preliminary data suggest that irradiation causes aberrant distribution of cytoskeletal and ion transport proteins.

In summation, there is substantial decline in in-vitro quality parameters caused by irradiation that are apparent well before 28 days of storage.

John R Hess—Our group has improved RBC storage through licensure studies of a new additive solution, EAS-81, which increases buffering capacity to improve RBC metabolism during storage. Previous RBC additive solutions (ASs) provide nutrients, such as glucose, phosphate, and adenine, and membrane protectants, such as mannitol or citrate, with little regard to pH. The nutrients allow the RBCs to make ATP and survive as long as ATP lasts. However, pH is important as it defines the metabolic space in which the RBC glycolytic system can work.⁵² Above pH 7.2, glycolytic intermediates are shunted through the Rapoport-Leubring pathway, making 2,3-di-phosphoglycerate at the expense of ATP.⁶⁷ Below pH 6.5, phosphofructokinase is inhibited and useful glycolysis stops.⁶⁸ Blood in donors typically have pHs of 7.35, which are lowered to 7.1 by mixing with the acidic—and strongly buffering—CPD.⁶⁹ Addition of a conventional AS at pH 5.5 further lowers the pH

to 7.05. EAS-81 uses an alkaline pH of 8.4 and the buffer capacity of phosphate and bicarbonate (HCO_3^-) to raise the pH of the anti-coagulated suspension to 7.2. This maximizes the pH range available for titrating the metabolic waste products of glycolysis (mostly lactic acid). At the same time, the phosphate and HCO_3^- increase buffering capacity. The net result is that more glucose is consumed, ATP made, and the RBCs store better and longer.

In the licensure trial RBC recoveries averaged 88% at 6 weeks for units processed within 8 hours of collection, 86% at 6 weeks for units processed after a 24 hour warm hold, and 83% at 8 weeks for units processed within 8 hours. These values were better than those of AS-1 controls processed within 8 hours (82% at 6 weeks). Hemolysis and microvesicle production were also reduced.

Even better results appear possible by extending the methods developed for EAS-81. The general consensus is that better storage should be used to provide better RBC products to patients without extending the current 6-week storage period.

Plasticizers and storage systems

Naomi LC Luban—The care of critically ill neonates and children results in exposure to disposable plastic devices which are ubiquitous in the critical care environment. Many of these devices, including intravenous, nasogastric, gastrostomy and endotracheal tubes, oxygen masks, and umbilical catheters, are made with PVC and contain the leachable plasticizer, di-2-ethylhexyl phthalate (DEHP), which provides functionality and flexibility. Based on their small surface area and duration of exposure, infants with cardiorespiratory, hematological and renal disorders, especially those with exposure to ECMO and CBP circuits or undergoing exchange transfusion and dialysis, are at highest risk for DEHP exposure.

DEHP and its metabolites, in particular the monoester MEHP and the oxidative metabolites MEHHP and MEOH, are reproductive and developmental toxicants in animal models including primates, and classified as “endocrine disruptors”.^{70, 71} These metabolites have been measured in neonates and observed levels correlate with the criticality of care.^{72–75}

Bisphenol A (BPA), a component of polycarbonate plastics, is also an endocrine disrupter and protoestrogen whose adverse effects have similarly been documented in animals and humans.⁷⁶ BPA has been quantified in sick infants and associated with abnormal infant brain development and behavior.^{77, 78}

Endocrine disruptor toxicities of DEHP, its metabolites, and BPA include Sertoli cell damage, an increase in DNA breaks in sperm, delayed ovulation and polycystic ovaries, reduced kidney and hepatic function and hepatic and genitourinary malignancies.^{79, 80} While exposure and adverse effects can occur prenatally, post-natal exposure occurs through multiple routes including ingestion, inhalation, dermal and IV exposure. The unique physiology of the sick neonate, including hepatic glucuronidation and antioxidant pathways, quantity of intestinal lipase, minute ventilation and kinetics of plasma distribution, all contribute to higher risks for infants to DEHP, its metabolites and BPA.

The multiple source exposure of medical devices which likely occur in the critically ill infant and child is of potential public health concern.^{81, 82} Substitution of these devices with others that do not contain DEHP or BPA is not always feasible; costs associated with major manufacturing modifications may not be sustainable.

To that end, the American Academy of Pediatrics has recommended long-term studies to evaluate the effect of medical exposure to devices and plasticizers.⁸³ Additional studies could aid in investigating the mechanisms of how DEHP/MEHP, its metabolites, and BPA produce their reported adverse effects and contribute to a further understanding of what modifications to blood bags, administration sets and devices could best reduce exposure.

Larry J Dumont—Storage systems for RBC are intended to preserve the functional (i.e., efficacy) and safety properties of RBCs until transfusion. The systems must also be easy to use and provide rapid access in hospitals, especially during emergencies. Systems can also be designed to restore RBC properties which deteriorate during storage and include washing and biochemical interventions for rejuvenation.⁸⁴ These steps add complexity, cost, and decrease availability, but could be improved if these steps were shown to be clinically beneficial. Storage systems in the future may also contribute to prevention strategies by providing a bactericidal environment or removal of potentially harmful elements such as cytokines, chemokines, antibodies, free Hb, and RBC microvesicles.

Current and future RBC preservation include new additive solutions,⁸⁵ freezing, anaerobic storage⁸⁶ or addition of free radical scavengers. The use of DEHP plasticizer has serendipitously reduced hemolysis.^{87, 88} In rodents, DEHP and its metabolites have organ and reproductive toxicity effects, effects on the endocrine system, and possible carcinogenicity. The carcinogenicity effect in rodents is not generalizable to humans. The generalizability to humans of the other effects has not been conclusively proved. However, both the European Union⁸⁹ and the FDA⁹⁰ have stated through rules or opinion papers that the use of DEHP containing medical devices should be minimized, especially in high risk populations such as children and pregnant or nursing women. Given that neonates are one of the highest risk groups considering development, metabolism, potential dose, and potential life span, randomized trials are indicated to address these questions.

Several candidate DEHP or PVC replacements have been evaluated,⁹¹ but thus far the only successful replacement has been with BTHC plasticized PVC.⁹² DINCH, as another possible DEHP replacement, is currently being evaluated.⁹³ None of these substitutes have been found to be as effective as DEHP for the prevention of hemolysis during storage. Interestingly, mixing of the alternatively plasticized RBC bag during storage protects against hemolysis.

Summary

James P AuBuchon—Potential modifications of a unit after collection include new additive systems, anaerobic storage, leukoreduction, new storage bags, addition or removal of additive or rejuvenation solution, nitrosylation, washing and gamma or UV irradiation. The selection of the most beneficial additive system would need to be made immediately after collection as well as would the alteration of the RBC biochemistry through reduction of oxygen tension. The benefits of leukoreduction are understood to be maximized through removal of white cells before prolonged storage. Undesired changes in RBCs biochemistry could be effected through replenishment of additive solution and removal of supernate to diminish metabolic waste products. The impact of alternative containers in which to store RBCs and/or deliver them to the patient would also need to be considered.

Preparation of the unit for transfusion could attempt to return the RBCs to their “original” state (such as through replenishment of gases removed or lost during storage) or even go beyond that through rejuvenation of biochemical pathways that could not only allow return of the cell to a previous state but create a cell with desirable supranormal characteristics or capacities. Additionally, some steps that may currently be taken in order to avoid certain

types of reactions, e.g., washing, might be applied for additional purposes, but these may also have untoward impacts.

The primary quandary in consideration of options such as these with our current state of knowledge is summarized in our inability to answer the question: What's really important to patient outcome? If RBC storage is shown to produce detrimental clinical outcomes, we are, at present, uncertain which of the storage lesion's changes need to be prevented, reversed or treated in the recipient to reverse this. The interest of each research group in its particular view of the storage lesion is understandable, but more definitive and possibly a multisystem explanation of the pathophysiology of the negative impact of "old blood" will be required to choose which intervention is most beneficial. Absent that, we are left with delineating all the potential steps that *might* be taken to optimize a transfusion experience for the recipient, a listing which strains not only logistic and biochemical bounds but financial constraints!

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Appendix

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AREAS FOR FUTURE RESEARCH

The NHLBI Working Group on Strategies to Optimize RBC Products reached a consensus that significant advances in storage solutions or other methods to improve 24 hour RBC recovery and survival within the current 42 day storage period would be beneficial. While participants acknowledged that it may be difficult to definitively link a particular RBC storage-related alteration with clinical outcome, the working group recognized that well-designed prospective clinical trials are ongoing which should provide basic information on whether the transfusion of “older” RBCs is associated with significantly more morbidity or mortality than patients receiving “younger” RBCs.

After considering all of the information presented during the workshop and the opinions expressed by participants, the Working Group on Strategies to Optimize RBC Products suggested that further investigation in the following areas was indicated. These scientific priorities are not listed in any particular priority order although there was a strong consensus that the field was most hampered by a lack of robust methods to measure the effectiveness of RBC transfusions.

- Development of improved RBC storage/rejuvenation solutions
- Studies to improve storage bags such as those that investigate the use of plasticizers other than DEHP
- Studies to characterize the exposure of pediatric recipients to DEHP and investigate methods/practices to limit that exposure
- Improvement of methods or development of new methods to measure oxygen delivery to tissues
- Identification and development of surrogate markers related to oxygen delivery
- Methods to identify the sources and potentially reduce some variability of donor RBC in vitro alterations, post-transfusion viability and clinical effectiveness during storage
- Identification of markers that are correlated with good or poor RBC survival
- Clinical studies that link RBC in vitro characteristics with recipient outcome