



Vascular Effects of the Red Blood Cell Storage Lesion

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Transfusion of RBCs is often clinically necessary—and life-saving—for anemic patients. RBCs can be stored for up to 42 days between the time of donation and the time of transfusion. For many years, investigators have studied the biochemical changes that occur in RBCs stored before transfusion (the RBC “storage lesion”). More recently, clinical studies have suggested that RBC units stored for long periods (often described as > 14-21 days) may mediate adverse effects in the recipient, leading to morbidity and mortality. Unfortunately, these effects are difficult to identify and study because there are no agreed-upon mechanisms for these adverse events and few good assays to study them in individual transfusion recipients. We have proposed the hypothesis of insufficient NO bioavailability (INOBA) to explain the adverse events associated with transfusion of older RBC units. INOBA postulates that the combination of impaired NO production and increased NO scavenging by stored RBCs, together with reduced NO synthesis by dysfunctional endothelial cells, collectively reduce NO levels below a critical threshold in vascular beds. In this situation, inappropriate vasoconstriction occurs, leading to reduced blood flow and insufficient O₂ delivery to end organs. If confirmed, the INOBA hypothesis may lead to improved methods for blood storage and collection, as well as new screening and matching tools for blood donors and transfusion recipients.

Introduction

Despite dramatic improvements in blood safety (particularly with respect to infectious disease transmission), posttransfusion complications still occur. Because their frequency, pathophysiology, and mechanism are unclear, some of the more difficult of these complications to address are the purported adverse events associated with transfusion of aged (“nonfresh”) RBC units compared with “fresh” units.¹ There are no effective clinical or laboratory assessments to determine whether these patients experience negative clinical consequences from stored blood. Nonetheless, there are epidemiologic data to suggest that such effects may be relatively common and are of major clinical significance.¹

RBC storage time and posttransfusion morbidity and mortality

A recent large retrospective study of 6002 cardiovascular patients who received 19 584 transfusions investigated adverse events related to the storage age of RBCs.¹ Patients who received older units (15-42 days of storage) had higher rates of in-hospital mortality (2.8% vs 1.7%, $P = .004$), as well as higher rates of extended intubation, renal failure, and sepsis compared with those who received fresher units (≤ 14 days of storage). One-year mortality was also significantly greater in patients who received older units compared with those receiving fresher units (11.0% vs 7.4%; $P < .001$). Similarly, in a retrospective review of 9 studies that included > 2800 cardiac surgery, trauma, and intensive care unit (ICU) patients, there was an increased rate of mortality, multiorgan failure, infections, and length of hospital stay in proportion to the age of the RBC units.²

Not all studies are in agreement, however. Edgren et al performed a cohort study of transfusion recipients in Sweden and Denmark (1995-2002). A total of 404 959 transfusion episodes were evaluable. There was no significant increase in the 7-day risk of death for

patients who received older versus fresher units (hazard ratio, 1.05; 95% confidence interval, 0.97-1.12). A small but statistically significant trend toward an increased 2-year risk of death in those who received older units (hazard ratio, 1.05; 95% confidence interval, 1.02-1.08) was found, but it was ascribed to weak confounding.³ Because the primary studies performed to date have been retrospective, there is the concern that their results have been affected by unintended biases.

A better estimate of the risks associated with the transfusion of older versus fresher stored units of blood likely awaits the results of prospective, randomized clinical trials (RCTs). The National Heart, Lung and Blood Institute–funded Red Cell Storage Duration Study (RECESS) is a multicenter RCT in which patients undergoing complex cardiac surgical procedures will be transfused with RBC units stored for 10 days or fewer versus RBCs stored for at least 21 days.⁴ Approximately 1434 patients will be enrolled, and outcomes including multiple organ dysfunction score, all-cause mortality, and other measures of organ dysfunction will be tracked. The “Age of Blood Evaluation” (ABLE) study, a double-blind multicenter RCT, will compare results from transfusion of RBCs stored for 7 days or less (“fresh”) against those stored an average of 15-20 days (“standard issue”) for adult ICU patients.⁵ The study will enroll 2510 patient with the objective of detecting a 5% absolute risk reduction by transfusion of fresh RBCs. Outcomes of interest include 90-day all-cause mortality, ICU mortality, organ failure, and nosocomial infections. The “Age of Red Blood Cells in Premature Infants” (ARIPI) RCT differs from RECESS and ABLE in that it will study transfusion outcomes in approximately 450 pediatric patients as a function of RBC storage interval (7 days or less versus standard practice).⁶ Outcomes of interest are mortality and disorders specific to neonatal patients, including necrotizing enterocolitis, retinopathy of prematurity, bronchopulmonary dysplasia, and intraventricular hemorrhage.

The endothelium controls vasodilation through the regulation of NO signaling

The microcirculation functions as an actively adjusting vascular circuit to match blood flow (and oxygen delivery) to local tissue oxygen needs.^{7,8} The endothelium, which releases mediators to regulate the contraction/relaxation of underlying smooth muscle and therefore vessel diameter, plays a very important role in microcirculatory function.^{9,10} These mediators include NO, thromboxane, endothelin, and endothelium-derived hyperpolarizing factor.⁷⁻¹⁰ For our studies, we have focused on the role of NO (both synthesized and released by RBCs and the endothelium) in the effects of stored RBCs.

NO production and signaling by the endothelium is fairly well characterized. Endothelial NO synthesis is controlled by nitric oxide synthase (NOS) activity. Of the 3 isoforms of NOS (neuronal, inducible, and endothelial), Endothelial NOS is thought to be the major source of NO for regulating vasoregulation.^{9,10} Endothelially produced NO diffuses to the underlying smooth muscle, where it activates guanylate cyclase and elicits muscle relaxation and vasodilation.^{9,10}

Reduced NO production is seen in patients with endothelial dysfunction, a common finding in many patients including those with cardiovascular disease (CVD).¹¹ Because a large number of transfusions are given to seriously ill patients, many transfusion recipients would be expected to have some degree of dysfunctional vascular NO signaling. Reduced NO production due to endothelial dysfunction can be studied using noninvasive ultrasound techniques. These individuals can also be identified through their biomarker profiles, particularly those affected by oxidative stress, which is involved in the pathophysiology of CVD. In patients with CVD, the total burden of risk factors, including oxidative stress markers, is correlated with endothelial dysfunction.¹²⁻¹⁴ These markers include hsCRP, LpPLA₂, and GSH/GSSG.¹⁵⁻¹⁸

RBCs can also control arterial tone and regulate local blood flow

There are also substantial data to suggest that RBCs can control local blood flow (hypoxic vasodilation) through the regulation of NO concentrations.¹⁹ The ability of RBCs to monitor local oxygen concentrations and regulate blood flow to ensure that oxygen is delivered to tissues in need is physiologically attractive. However, in contrast to the situation with endothelium, there is substantial disagreement regarding the mechanisms by which RBCs perform this function. Three different mechanisms have been put forward to explain how RBCs, upon sensing local hypoxia, could stimulate vasodilation and thereby increase blood flow: (1) release of ATP²⁰⁻²³, (2) release of NO from its storage form as S-nitrosylated hemoglobin (SNO-Hb)^{7,24}, and (3) reduction of circulating nitrite to NO during the R-to-T allosteric transition of Hb.²⁵⁻²⁷

In the first mechanism, ATP released by RBCs binds to purinergic receptors on endothelial cells, stimulating the production of NO and other substances that produce vasodilation.^{21,28} This model is supported by data showing that physiologically meaningful levels of ATP are released from RBCs under hypoxic conditions, that direct ATP application to vessels can stimulate vasodilation, and that ATP release is susceptible to physiologically attractive feedback mechanisms, including ADP and NO.^{21,28}

The SNO-Hb model postulates that NO equivalents are carried in RBCs as S-nitrosylated moieties on cysteine β -93 of Hb (SNO-

Hb).^{29,30} NO is then released from SNO-Hb during Hb deoxygenation to promote vasodilation when oxygen tension is low (hypoxic conditions).^{19,31} Problems with this model have been raised.³²⁻³⁴ For example, if SNO-Hb were an important precursor of vasoactive NO, then arteriovenous gradients in SNO-Hb should be identified, but this has not happened. Furthermore, transgenic mice expressing human Hb with an alanine substitution for cysteine β -93 preventing the synthesis of SNO-Hb demonstrate normal, RBC-dependent hypoxic vasodilation.³⁵

In the third model, Hb acts as an allosterically regulated nitrite reductase that catalyzes the formation of nitrite to NO, with SNO-Hb being formed as a side product.^{36,37} This has been reviewed in detail recently.³⁸ In addition, myoglobin can also reduce nitrite to NO, and the importance of this mechanism in cardiac function and response to ischemic events was shown with a myoglobin-knockout mouse.³⁹⁻⁴¹ Despite the elegance of the proposed allosteric mechanism³⁸ to regulate NO production based on local oxygen concentrations, this mechanism suffers from a problem also seen with the SNO-Hb model: how does the extremely short-lived NO molecule diffuse from Hb to the underlying smooth muscle?

NO scavenging is another mechanism by which RBCs (and hemoglobin) may regulate NO signaling

A fourth mechanism that may function in conjunction with (or instead of) those described above is the scavenging of NO by Hb. With this mechanism, Hb free in plasma (as a consequence of RBC hemolysis), Hb encapsulated in RBC-derived microparticles, or Hb in intact RBCs (less likely, particularly because RBCs circulate in the middle of the bloodstream away from the endothelium) consumes NO produced by endothelial cells, reducing NO levels and thus inhibiting the vasodilatory response.^{42,43} This mechanism is supported by studies in sickle cell disease and others showing that plasma free Hb can scavenge NO, reducing its bioavailability and causing clinical sequelae.^{44,45} Free Hb increases as RBCs break down during blood storage, suggesting that transfusion of long-storage-age RBCs may lead to a bolus infusion of NO-consuming Hb. Furthermore, RBC breakdown also leads to the formation of Hb-containing microparticles. Unlike intact RBCs, microparticles can flow close to the endothelium, bringing Hb close to the sites of NO synthesis, which may further accentuate NO scavenging after transfusion.⁴⁶

INOBA may underlie adverse effects associated with transfusion of older RBC units

Disruption of NO production/signaling mediated by stored RBCs could account for adverse effects ascribed to transfusions of stored RBCs. Interestingly, the INOBA hypothesis not only addresses this requirement, but also provides a role for recipient factors including endothelial function. This unifying hypothesis can be stated as follows: When the cumulative effects of RBC transfusions and recipient factors reduce local NO bioavailability to levels below a critical threshold, tissue perfusion is insufficient to meet metabolic demands, leading to morbidity and mortality in the transfusion recipient.

The INOBA hypothesis postulates that adverse events after transfusion are more likely to occur when the RBC-specific dysfunction that occurs with blood storage is combined with endothelial dysfunction, surpassing a threshold where NO bioavailability is insufficient to produce appropriate vasodilation, causing reduced

blood flow to vital organs and morbidity/mortality. As described below, the INOBA hypothesis can be tested in both reductionist in vitro systems and in patients and healthy volunteers receiving transfusions.

Effects of stored RBCs on vasoreactivity in vitro

In the first series of studies, we prepared leukoreduced AS-3 packed red blood cell (pRBC) units from volunteer donors and stored the units for up to 42 days under standard conditions. At selected times, we sampled the blood and performed aortic ring assays to determine the effects of blood storage on the ability of acetylcholine (ACh)-stimulated NO release from the endothelium to stimulate smooth muscle relaxation in rat aortic sections. These results showed that the presence of fresh pRBCs, even at low final hematocrits (~ 1%), could interfere with NO-mediated vasodilation in rat aortic rings. Compared with fresh RBCs, RBCs stored for 3-14 days produced a significant shift in the dose-response curve, with a reproducible 50% greater inhibition of relaxation. The inhibitory activity further increased with extended storage: RBCs stored for 28-42 days almost completely eliminated ACh-stimulated NO-mediated vasodilation. If the aortic rings were prerelaxed first, the subsequent addition of RBCs also caused contraction, indicating that the stored RBCs actively antagonize vasodilation.

One hypothesis to explain these effects is that stored blood hemolyzes, releasing free Hb that is known to scavenge NO. To address this possibility, we centrifuged the blood and removed the supernatant before adding the RBCs to the organ baths (mimicking a "volume reduction process" in the blood bank) or washed the cells 3 times with an excess of saline before testing the RBCs (mimicking blood component "washing"). The washing approach partially reversed the effects of stored RBCs. Washing RBCs that had been stored for 28-42 days before testing slightly abrogated their inhibitory effect on vasodilation, suggesting that some of the observed inhibition may be prevented by removing plasma free Hb, Hb-containing microparticles, or other factors from the plasma that are released during RBC storage. In addition, the membrane-permeable free radical scavenger Tempol (1mM) also partially blocked the inhibitory effects of stored RBCs, indicating that reactive oxygen species may be involved in this effect. Samples taken in parallel were tested for ATP and 2,3-diphosphoglycerate (2,3-DPG), and results were consistent with previously published reports.

Effects of stored RBCs on in vivo responsiveness to NO release

We sought to perform an in vivo equivalent of the aortic ring studies described above. These investigations are based on the hypothesis that whereas stored RBCs may have an effect on vascular physiology, this effect may be exacerbated if transfusion recipients have underlying vascular dysfunction (resulting, for example, from atherosclerotic or diabetic vascular disease). These studies were approved by our institutional review board. Volunteer hospitalized patients for whom a transfusion had been ordered were randomized to receive either a fresh (< 10 days old) or an aged (> 21 days old) blood transfusion. Endothelial function was assessed by flow-mediated dilatation (FMD) assays of the brachial artery prior to, during, 1 hour after, and the next day after transfusion. Twenty subjects have been studied to date. The mean age was 60 ± 20 years, 50% were male, 7 had a cancer diagnosis, and 6 required transfusion due to surgical blood loss, 3 due to chemotherapy, 2 due to gastrointestinal bleeding, and 3 due to other medical disease. The

mean FMD before transfusion was $5.0\% \pm 1.5\%$. During transfusion, FMD (corrected for shear rate) tended to increase ($P = .056$) and returned toward baseline after 1 hour of transfusion and the following day. The pattern of change in those who received fresh and aged blood was not significantly different. Whereas FMD appeared to return to baseline or even lower after transfusion in those who received aged blood, it appeared to trend higher in those receiving fresh blood. Therefore, there was a trend toward a difference in the endothelial functional response in those receiving fresh versus aged blood, although we plan to enroll a total of 30 subjects (based on power analysis) before the final statistical analysis. The subjects enrolled to date have been heterogeneous in terms of baseline endothelial dysfunction, pretransfusion hematocrits, medications received, time of day, and meal schedules (all factors that influence FMD).⁴⁷

Global metabolic screening for biochemical changes in RBCs with storage

With few exceptions, the biochemical changes that occur in RBCs during extended storage and that may underlie adverse effects of stored RBCs are not known. We undertook a comprehensive metabolic screening to identify biochemical metabolites that changed significantly in concentration during RBC storage as a first step to dissecting the mechanistic aspects of the RBC storage lesion. Six age-, race-, and sex-matched volunteers donated whole blood units (500 ± 50 mL) that were processed and stored as AS-3 pRBCs. At defined times during storage (0, 3, 7, 14, 28, and 42 days), aliquots were removed and snap-frozen. All 36 samples were simultaneously extracted and analyzed by GC/MS and LC/MS/MS. Biochemicals were identified by comparisons against a library of mass spectra derived from purified standards. Proprietary visualization and interpretation software was used to confirm the consistency of peak identification between samples. A general linear statistical model analysis was applied to log-transformed data, incorporating donor and time as the main effects.

A total of 185 identified biochemicals were quantitated in RBCs. As internal confirmation of the methods, 2,3-DPG declined to undetectable levels by storage day 14, whereas lactate increased progressively during storage, as previously documented. By 3 days of storage, 25 biochemicals had increased significantly ($P \leq .05$) in concentration compared with day 0, whereas 6 had decreased significantly. By 42 days, 56 markers increased and 47 decreased significantly. Using a general linear model, 90 biochemicals changed in association with storage time. Intriguingly, changes in 132 markers were associated with donor-specific factors. Profound metabolic changes were identified in Rapoport-Luebering shunt, glycolysis, glutathione synthesis, branched-chain amino acid metabolism, and adenine catabolism. Several biochemicals increased significantly only after 14 days of storage, possibly in response to 2,3 DPG depletion, and may represent useful markers of RBC aging during storage. Interestingly, although there was a time-dependent decrease in glutathione levels, other biochemical data indicate that redox homeostasis in the RBCs was generally maintained over the storage period.

Summary and future directions

Whereas RBC transfusion is a very commonly used clinical therapy, it can be associated with adverse events, including the possibility of worse outcomes in patients transfused with older RBC units. The INOBA hypothesis postulates that some of the adverse events are due to time- and storage condition-dependent changes in NO

production (and/or increased scavenging) by RBCs coupled with endothelial dysfunction in transfusion recipients, leading to reductions in blood flow, and thus oxygen delivery, to end organs. Our studies of this hypothesis have led to the following observations: (1) older RBC units can block NO-mediated vasodilation in rat aortic ring models; (2) the mechanisms may involve mediators released into the supernatant of the blood component and/or reactive oxygen species that are present at higher levels in older units; (3) there is a trend toward confirmatory findings in hospitalized patients treated by transfusion, although the studies are still ongoing; and (4) global metabolic profiling has suggested some new targets to investigate regarding the RBC storage lesion and potential mediators that could impair vascular function. These results may translate into improved preservative/storage solutions for RBC units and to better methods to match blood donors to recipients based on metabolic parameters.

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Conflict-of-interest disclosure: The author declares no competing financial interests. Off-label drug use: None disclosed.

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