

Evolution of adverse changes in stored RBCs

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Recent studies have underscored questions about the balance of risk and benefit of RBC transfusion. A better understanding of the nature and timing of molecular and functional changes in stored RBCs may provide strategies to improve the balance of benefit and risk of RBC transfusion. We analyzed changes occurring during RBC storage focusing on RBC deformability, RBC-dependent vasoregulatory function, and S-nitrosohemoglobin (SNO-Hb), through which hemoglobin (Hb) O₂ desaturation is coupled to regional increases in blood flow *in vivo* (hypoxic vasodilation). Five hundred ml of blood from each of 15 healthy volunteers was processed into leukofiltered, additive solution 3-exposed RBCs and stored at 1–6°C according to AABB standards. Blood was subjected to 26 assays at 0, 3, 8, 24 and 96 h, and at 1, 2, 3, 4, and 6 weeks. RBC SNO-Hb decreased rapidly (1.2×10^{-4} at 3 h vs. 6.5×10^{-4} (fresh) mol S-nitrosothiol (SNO)/mol Hb tetramer ($P = 0.032$, mercuric-displaced photolysis-chemiluminescence assay), and remained low over the 42-day period. The decline was corroborated by using the carbon monoxide-saturated copper-cysteine assay [3.0×10^{-5} at 3 h vs. 9.0×10^{-5} (fresh) mol SNO/mol Hb]. In parallel, vasodilation by stored RBCs was significantly depressed. RBC deformability assayed at a physiological shear stress decreased gradually over the 42-day period ($P < 0.001$). Time courses vary for several storage-induced defects that might account for recent observations linking blood transfusion with adverse outcomes. Of clinical concern is that SNO levels, and their physiological correlate, RBC-dependent vasodilation, become depressed soon after collection, suggesting that even “fresh” blood may have developed adverse biological characteristics.

adenosine triphosphate | hemoglobin | nitric oxide | S-nitrosothiols | transfusion

Every year in the US, ≈ 14 million units of blood are collected, and ≈ 13.9 million units of RBCs are administered to 4.8 million patients (1). Despite this widespread use, human blood products are licensed only on the basis of the procedures used for collection, processing, and storage. Mandated, specific testing ensures safety from infectious diseases and compatibility between blood product and recipient, but there is no regulatory or clinical efficacy standard specifying the clinical outcomes constituting an effective transfusion. In addition, RBC transfusion has not been subjected to the formal risk/benefit analysis that would be routine for new biological therapeutics.

RBCs may be stored for up to 42 days under controlled conditions before transfusion. However, numerous changes occur in RBCs during storage (collectively referred to as the “storage lesion”) that may alter their biological function, including delivery of oxygen to cells (2). Retrospective cohort studies have found a correlation between RBC storage duration and morbidity and mortality rates after transfusion (3–5), suggesting progressive storage lesions may be responsible for adverse outcomes. Despite these observational data, no large controlled clinical trials have been conducted to evaluate the relationship between the age of stored RBCs and clinical outcomes. RBC-transfused patients had worse outcomes than nontransfused patients matched for clinical variables in several studies (6–10). Moreover, in randomized clinical trials, a more liberal RBC transfusion strategy failed to benefit pediatric or adult patients with anemia and critical illness (11, 12),

raising the broader concern that RBC storage is problematic (2, 13) and could be improved. This is particularly relevant because, to date, the development of and outcomes with blood substitutes have been disappointing (14–16).

Although storage-induced changes in certain RBC molecular constituents have been studied, less is known about changes in RBC function with storage (2, 17, 18). One of the RBC’s principal functions is O₂ delivery, a product of changes in O₂ content and blood flow. Increases in O₂ affinity in stored RBCs, reflecting progressive decreases in 2,3-diphosphoglycerate (2,3-DPG) over the weeks of storage, are well documented (17, 18), and O₂ delivery by stored RBCs is deficient even early after processing and before significant decline in 2,3-DPG (19). However, less is known of how storage influences the role of the RBC in the O₂-dependent regulation of blood flow (“hypoxic vasodilation”), in part because this RBC function was only recently appreciated (20, 21). The O₂ sensor role of hemoglobin (Hb) subserves this RBC activity by dispensing vasodilator S-nitrosothiol (SNO) equivalents in proportion to the degree of hypoxia in the tissues it perfuses (20, 21). In concert with the oxygenation-induced allosteric transition, S-nitrosohemoglobin (SNO-Hb) forms in human blood when a nitric oxide (NO) equivalent binds to the conserved $\beta 93$ Cys thiol residue of Hb (≈ 1 in 1,000–10,000 Hb tetramers bind NO there). Conversely, RBCs perfusing tissues release limited fluxes of vasodilator SNO equivalents in proportion to Hb O₂ desaturation (22), matching regional blood flow with metabolic demand.

Previous studies have looked at particular aspects of storage alone or have not collected, processed, and stored RBCs consistent with standards of the AABB. Therefore, we conducted this study to simultaneously quantify multiple biochemical components of the RBC storage lesion and related storage-induced changes in RBC physiologic functions critical to O₂ delivery, particularly deformability and RBC-dependent vasoactivity. We reasoned that an impairment in RBC-dependent hypoxic vasodilation might under-

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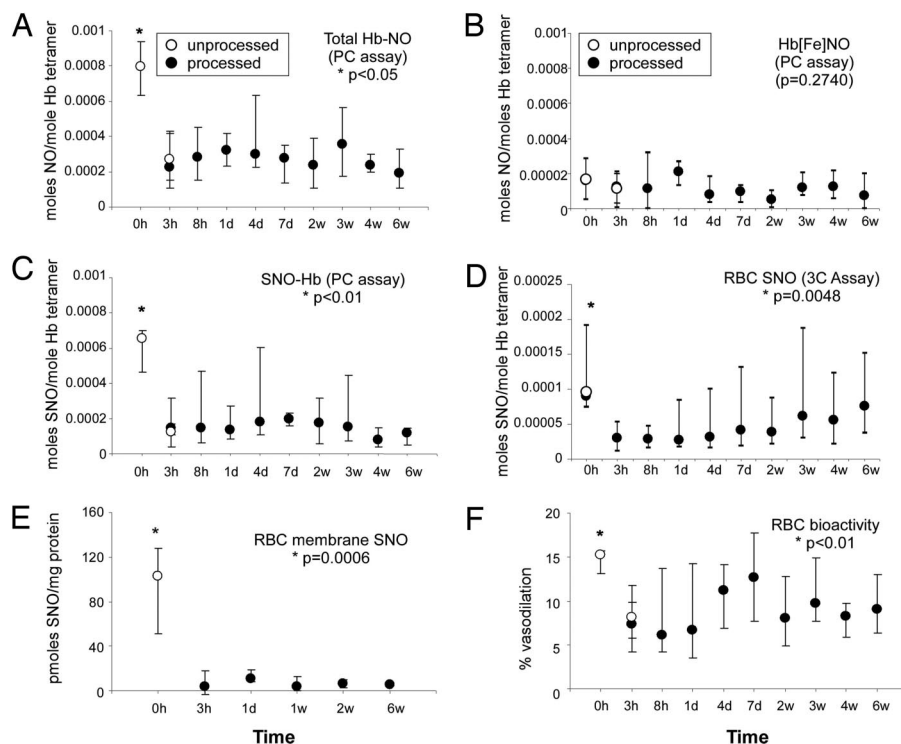
Abbreviations: AABB, organization formerly known as the American Association of Blood Banks; NO, nitric oxide; PS, phosphatidyl serine; SNOs, S-nitrosothiols; Hb, hemoglobin; (M)PC, (mercuric-coupled) photolysis-chemiluminescence; 3C, CO (carbon monoxide)-saturated cuprous chloride/cysteine assay; 2,3-DPG, 2,3-diphosphoglycerate; CP2D, citrate-phosphate-dextrose-dextrose anticoagulant solution; AS-3, additive solution-3.

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We used two complementary assays to measure bioactive forms of RBC NO (Fig. 2; individual data are shown in [SI Fig. 6](#)). Total Hb-bound NO and SNO-Hb decreased markedly from 0 h (fresh RBCs) to 3 h in unprocessed samples (i.e., samples unexposed to AS-3 and not leukofiltered, Fig. 2). Specifically, RBC SNO-Hb was 1.2×10^{-4} mol of SNO per mole of Hb tetramer (1 SNO per 7,915 Hb tetramers) at 3 h vs. 6.5×10^{-4} mol of SNO per mole of Hb (1 SNO per 1,527 Hb tetramers) in fresh RBCs. Total Hb-NO and



SNO-Hb were similarly depressed in processed samples at 3 h (the earliest postprocessing point), and remained markedly depressed for the 6 weeks of the study. Hb[Fe]NO (iron-nitrosyl hemoglobin, in which NO binds to Hb's heme iron) levels did not change significantly over time (Fig. 2). Total RBC SNO content, measured by the 3C assay, was also depressed in stored RBCs (3.01×10^{-5} mol of SNO per mole of Hb, or 1 SNO per 33,223 Hb tetramers) at 3 h relative to that in both fresh RBC controls (9.0×10^{-5} mol of SNO per mole of Hb, or 1 SNO per 11,106 Hb tetramers) and published normal values (Fig. 2) (22). There were nonsignificant trends toward later increases in total Hb-NO (Fig. 2A) and SNO(Hb) (Fig. 2 C and D) values.

The vasoactivity of fresh, air-exposed venous RBCs was similar in magnitude to that reported previously by us and others (21, 28–30). RBC vasoactivity was depressed by 3 h (Fig. 2; individual data are shown in [SI Fig. 6](#)). Similar depression of RBC vasoactivity was seen in time-control samples held for 3 h (Fig. 2). There was a nonsignificant trend toward recovery of RBC vasoactivity, peaking at a time (≈ 1 week) similar to the nonsignificant resurgence in SNO-Hb and total Hb-bound NO levels (Fig. 2). In separate experiments to probe the possible role of RBC-derived ATP in these responses, we investigated the influence of a nitric oxide synthase (NOS) inhibitor (L-NAME) on RBC-dependent hypoxic vasodilator responses (Fig. 3). The results show that L-NAME abolished responses to ATP, but not responses to RBCs in hypoxia. Nitrite elicited no significant vasodilation (in hypoxia) at a concentration of $1 \mu\text{M}$ (Fig. 3, n.s.), which is supraphysiological, consistent with recent findings (29–32); moreover, there was no unmasking of a response to nitrite by prior exposure of vessels to RBCs (Fig. 3).

RBC deformability decreased steadily over the 42 days of storage (Fig. 4; individual data are shown in SI Fig. 7). Deformability deteriorated over time at all shear stresses tested (data not shown).

Blood cultures on all units during storage showed no evidence of bacterial contamination. Consistent with this finding and with cytokine assays in units of uncontaminated, leukodepleted RBCs (33), levels of the proinflammatory cytokines IL-6, IL-8 (SI Fig. 8), IL-1 β , and tumor necrosis factor (TNF)- α (data not shown) in all samples were extremely low and did not rise during storage. Accordingly, only the first 76 were analyzed for cytokine content.

Discussion

The findings of this study are that, in blood that has been collected, processed, and stored by using blood-banking industry standard

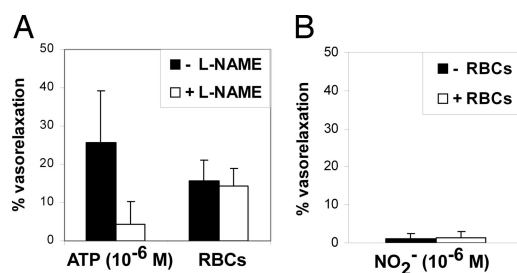


Fig. 3. Alternative mediators of RBC-dependent hypoxic vasodilation. (A) RBC-dependent hypoxic vasodilator responses are NOS-independent. Fresh washed human RBCs [0.4% hematocrit (Hct) or ATP (10^{-6} M)] were added to precontracted rabbit aortic rings at 1% O₂ [PO₂ 7 mmHg (mmHg = 133 Pa)] in the absence or presence of the NOS inhibitor L-NAME in tissue baths as described, and the percentage of vasorelaxation was measured. *, $P < 0.05$. (B) Minimal nitrite-induced vasodilation in the absence or presence of RBCs. Nitrite (1 μ M) was added to precontracted rabbit aortic rings at 1% O₂ (PO₂ 7 mmHg) in the absence or presence of RBCs (0.4% Hct). Data are mean $\pm$ SD from four experiments each.

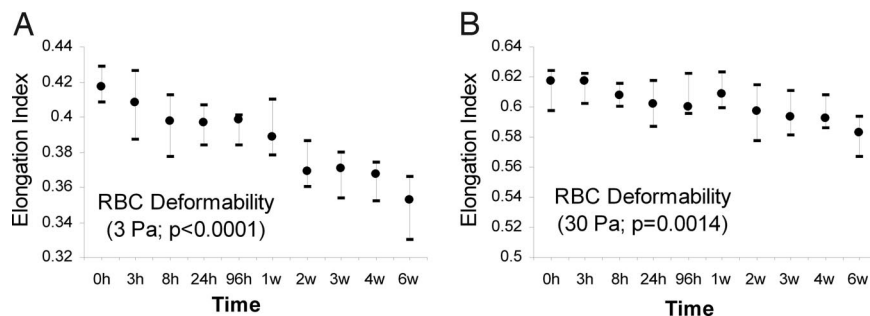


Fig. 4. RBC deformability as elongation index for two representative shear stress levels as a function of storage time. Values at 0 h are from unprocessed RBCs. Data are median with 25th and 75th percentiles. *P* values represent significance for change over time.

operating procedures, RBC SNO-Hb levels and RBC-dependent vasodilation are profoundly depressed immediately, whereas RBC deformability deteriorates more gradually over time. Thus, the time courses of individual functional changes differ markedly from one another. Recent studies suggest that these changes may be clinically relevant, and further study is needed to determine how these changes relate to the excess morbidity and mortality associated with transfusion (7–9, 12). In addition, we confirm numerous biochemical and functional changes previously reported during RBC storage, including some changes that may be related to our findings.

Our results bear directly on several key questions regarding the safety and efficacy of RBC transfusion. Although RBC transfusion is widely regarded as life-saving under some circumstances, growing evidence links transfusion of RBCs with increased mortality in certain high-risk patients. The emphasis over the last several decades has been on lesions that develop over the storage period. Observational studies in trauma, critical illness, and cardiac surgery, linking the administration of older blood to increased mortality (3–5), support the concern that transfusion of older blood may be detrimental. Evidence also exists that transfusion is detrimental in some settings regardless of RBC storage duration. Several studies have assessed the relationship between RBC transfusion and outcome, not accounting for the duration of storage of the blood. For example, an association was found between perioperative RBC transfusion and long-term mortality after coronary artery bypass graft surgery (7, 8). Consistent with these studies, among 24,112 patients with acute coronary syndrome, those who received a RBC transfusion had a significantly higher unadjusted rate of 30-day death (8.00% vs. 3.08%; $P < 0.001$) (9). This association persisted in an analysis that took into account other known risk factors. A clinical trial in critically ill adult patients demonstrated diminished survival (approaching statistical significance, $P = 0.1$) and significantly higher in-hospital mortality in patients randomized to “liberal” administration of RBC transfusions (12). In addition, mortality was greater in those subgroups of liberally transfused patients who were either less severely ill or under 55 years of age (12). These and other studies argue that any RBC transfusion may in fact be deleterious rather than beneficial in some patient populations (6). Recognition of the limited benefit or potential harm from RBC transfusion is suggested by the adoption of more restrictive, scenario-specific RBC-transfusion thresholds in some physician groups (34). The failure of RBC transfusion to provide a clinical benefit in other groups of pediatric or adult patients with anemia and critical illness raises the broader concern that RBC storage is problematic and could be improved (2, 6, 11, 12).

In support of the possibility that even fresh, processed RBCs are dysfunctional in some respects, we show that RBC SNOs are depressed by a factor of ≈ 4 at 3 h after collection. In earlier work, we showed that in venous RBCs in PBS (pH 7.4) held *ex vivo* for 30 min before assay (25°C, native PO_2), SNO-Hb levels declined markedly (30); similarly, under conditions mimicking blood bank-

ing (pH < 7.0), RBC SNO-Hb and RBC vasoactivity also decline early [see companion article (35)]. Together, these two observations motivated the current study. Our finding of early and marked RBC SNO-Hb depletion is strengthened by the use of two mechanistically distinct techniques [MPC and 3C (CO-saturated cuprous chloride-cysteine) assays]; indeed, the extent of SNO decline in RBCs (time point 0 h vs. postprocessing, AS-3-exposed RBCs, at 3 h) was similar by both methods (77% and 67% declines by MPC and 3C, respectively). The observation that RBC SNO-Hb and total Hb-NO (measured by the MPC assay) and RBC bioactivity were likewise depressed at 3 h in unprocessed samples suggests that time alone, rather than AS-3 exposure and/or leukofiltration, is sufficient to produce the deficiencies. RBC SNO-Hb (by MPC assay) and total RBC SNO content (measured by the 3C assay, and representing the sum of Hb-bound SNO, other protein-bound SNO, and S-nitrosoglutathione) are different but overlapping pools of SNO species. RBC SNO-Hb represents the major RBC SNO (21, 36), and one might expect the results to be quantitatively similar, despite the distinct mechanisms of the two assays. Indeed, recoveries of SNO standards in the presence and absence of high background Hb concentrations (as in RBC lysates) by 3C and MPC are essentially complete and therefore equivalent between the two assays. But herein and previously, basal levels of total RBC SNO content measured by 3C are several-fold lower than basal RBC SNO-Hb measured by MPC. The exact reason for the difference in basal values of RBC SNO(Hb) using the two techniques is unknown, but likely reflects the known differences in sample preparation. In addition, the ability of 3C to measure membrane vs. cytosolic SNO-Hb, as well as different subpopulations of SNO-Hb species (37), is currently unknown.

Taken together, our findings that total RBC SNO, RBC SNO-Hb, and total Hb-bound NO fall markedly and early in stored RBCs are consistent with the loss of SNOs. Hb[FeII]NO, which serves as a precursor to SNO-Hb (21) but is biologically inactive itself (30), did not change during processing and storage. (Total Hb-bound NO is equivalent to the sum of these two species). The SNO loss from RBCs may be either through export (e.g., to thiol SNO acceptors, such as glutathione and albumin, which were present in plasma until the red cells were removed) or degradation to an inactive NO metabolite and cannot be accounted for by accumulation of Hb[Fe]NO, which did not change. A functionally active endothelial NO synthase (eNOS)-type NOS has been reported in human RBCs and to produce NO continuously (38). Indeed, RBC eNOS-derived NO could serve as a substrate in formation of RBC SNO-Hb, thus providing RBC-eNOS-derived NO equivalents with a pathway for export and activity. However, the loss of RBC SNO(Hb) with storage is not a direct result of limitation of RBC eNOS, because in fresh RBCs, SNOs were abundant despite the absence of supplemental L-arginine or extracellular calcium (both of which are required for the reported RBC eNOS activity) and despite the presence of the calcium-chelator DTPA. The mechanistic basis for

the loss of RBC SNOs and their fate with RBC storage are currently under study by our group.

In contrast to some other RBC disorders involving SNOs, in which the loss of SNO-Hb was accounted for by a gain in inactive Hb[Fe]NO, the RBC storage lesion entails depression of SNO-Hb with no change in Hb[Fe]NO levels, so that total Hb-bound NO levels (the sum of Hb[Fe]NO and SNO-Hb) were also depressed. In addition, total RBC SNO (measured by the 3C assay, the sum of Hb-SNO, other protein-bound SNO, low-mass SNO) and levels of membrane SNOs, which are essential for RBC export of SNO bioactivity, were substantially depressed compared with levels in fresh RBCs and published values (22, 28, 39).

The initial vascular response to RBCs at low pO_2 is relaxation, and takes place within seconds, consistent with hypoxic vasodilation *in vivo* in humans. By contrast, the secondary vasoconstrictor phase requires minutes and is therefore of little or no biological significance because RBCs typically traverse capillaries within seconds (21). In our study, the timing of the changes in RBC vasoactivity and in RBC (Hb-bound) SNO were similar, whereas decreases in ATP were more gradual and less profound, as in previous studies (40). Further evidence that the loss of RBC-derived ATP during storage does not account for the weakening of hypoxic vasodilation to stored RBCs came from experiments in which a NOS inhibitor abolished responses to ATP, but not responses to RBCs in hypoxia. The weak vasodilator nitrite cannot account for these responses because, even in the presence of RBCs, the levels required for vasoactivity (micromolar) by nitrite exceed the values present in blood [Fig. 3 and Crawford *et al.* (29)]. By contrast, a SNO synthase function of RBC Hb can convert nitrite to bioactive SNO-Hb (41, 42), and our data do not exclude a role for this activity in maintaining the low, residual SNO-Hb levels seen during RBC storage or in the later, nonsignificant trend toward small increases in SNO(Hb) (Fig. 2 and SI Fig. 5).

RBC SNO-Hb levels correlate with the ability of RBCs to relax blood vessels in hypoxia (“hypoxic vasodilation”), a functional “bioassay” for the recently described role of the RBC in blood flow regulation *in vivo* (20–22). Derangements in RBC SNO-Hb mirror derangements in RBC vasodilator activity in several diseases studied to date, with the specific lesion varying from one disorder to another (20, 22, 28, 30, 36, 43). In diabetes mellitus, for example, Hb nitrosylation is increased because glycosylation of Hb favors the R structure, but RBC vasoactivity is depressed because the R-state tendency disfavors SNO release, consistent with the vasculopathy of diabetes (20). In sickle cell disease, RBC SNO-Hb and vasoactivity are depressed, and the degree of depression correlates with disease severity (28); in this case, SNO-Hb formation is suppressed because of differences in heme redox potential between sickle and normal human Hb. Hypoxic vasodilation to sickle RBCs is impaired because of both the SNO-Hb deficiency and abnormal transfer of SNO from Hb to thiols in the membrane protein anion exchanger 1 (AE1), an essential step in RBC-SNO-dependent vasorelaxation (28, 39). In patients with pulmonary arterial hypertension and hypoxemia, RBC SNO-Hb formation was deficient, and both the depressed RBC bioactivity and pulmonary vascular resistance were improved when RBC SNO-Hb was replenished *in vivo* (30). Thus, the *in vitro* RBC bioassay model recapitulates *in vivo* O_2 -sensitive function of the RBC in blood flow regulation in humans in health (21, 37), disease (44), and in corrective therapy.

We showed that, whereas changes in RBC-dependent vasodilation and SNO-Hb take place early after storage (within 3 h), changes in RBC deformability take place more gradually (days to weeks). We measured RBC deformability using the laser-assisted optical rotational cell analyzer (LORCA) assay, in which RBCs are subjected to increasing shear stress over several minutes (45, 46). Our prespecified major shear stress of interest was 3 Pa, a level that may be encountered in the microcirculation of humans (47–49). Deformability also decreased significantly over the 42-day storage period at 30 Pa, suggesting that the membrane defect induced by

storage is pronounced enough to moderately resist the ability to deform even at a very high level of shear stress. Less deformable RBCs could exacerbate organ ischemia in surgical and other critically ill patients. Normal RBCs are 7–8 μm in diameter, slightly wider than capillaries, so they must deform to traverse the microcirculation. RBCs that are less deformable can either block and obstruct capillaries or, more commonly, traverse the microcirculation at a significantly increased transit time, resulting in overall diminished O_2 delivery to organs (50, 51). Impaired RBC deformability was shown in patients with sepsis, a condition of microcirculatory pathology and organ injury (45, 52, 53), and the magnitude of changes in RBC deformability from septic patients is similar to those we observed in stored RBCs from healthy volunteers. The clinical relevance of this and other storage lesions requires further study.

The lack of RBC adhesiveness to endothelial cells or RBC exposure of phosphatidylserine (PS) during storage observed in this study suggests that stored, leukodepleted RBCs do not contribute to diminished O_2 delivery to tissues by either adhesion, as seen in sickle cell disease, or by direct activation of coagulation pathways through exposure of PS, as is postulated to occur in other RBC disorders.

The storage-related deficiency of SNO-Hb and impairments in RBC vasoactivity and deformability (38, 54) may disrupt normal O_2 delivery (a function of both blood flow and O_2 offloading) by RBCs in the microcirculation, predisposing to the excess morbidity and mortality associated with RBC transfusion in some patient groups (7–9, 12). Strategies to replenish SNOs in RBCs have been shown in other diseases to be effective and partially reverse the related impairment in RBC-dependent vasodilation and correlated physiologic derangements (28, 30). Restoring SNOs might also improve RBC deformability, which is sensitive to NO and its derivatives (54); further study is needed to determine whether strategies that replenish SNOs in stored RBCs, or prevent their loss, improve storage-induced rheological lesions. It is rational to test, in future clinical trials, whether replenishing SNOs in stored RBCs, or preventing their loss, will improve patient outcomes with RBC transfusion.

Materials and Methods

After written informed consent, healthy volunteers meeting eligibility criteria were enrolled at Duke University Medical Center (DUMC), whose Institutional Review Board approved the study.

Blood Donation and Processing. Blood was collected and processed by American Red Cross-certified technicians using techniques consistent with the Technical Manual of the AABB (55). Venous blood was collected in CP2D anticoagulant, leukofiltered, and stored in a monitored refrigerator. RBC aliquots were removed at the indicated intervals. For certain assays, blood was also collected into 10-ml Vacutainers (containing CP2D anticoagulant) and immediately analyzed (0 h, see SI Methods), avoiding the ≈ 3 -h delay inherent in the large-volume blood collection, leukofiltration, and processing required to prepare additive-solution RBCs. For some parameters, (2,3-DPG, RBC ATP, extraerythrocytic (free) Hb, 3C SNO, and cytokines), aliquots were taken and stored at $-80^\circ C$ for later batch analysis. To keep all personnel performing assays blinded to the duration of sample storage, all samples were assigned a unique identification code based on a computer-generated randomization list. See SI Methods for more details.

Assays. RBC-dependent vasoactivity was determined as changes in isometric tension in phenylephrine-precontracted rabbit aortic rings at low pO_2 , as described in SI Methods and previously (20, 21). **RBC SNO-Hb, Hb[Fe]NO, and membrane SNO assay.** Photolysis-chemiluminescence (PC) was described previously (21) and is detailed in SI Methods. Washed RBCs were lysed and Hb desalted by using a G-25 fine Sephadex column. Hb-bound NO was mea-

sured by PC assay in the presence (Hb[Fe]NO) and absence (total Hb-bound NO) of HgCl₂, which selectively cleaves SNO bonds. SNO-Hb is the difference between total Hb-NO and Hb[Fe]NO. The specificity of this technique has been demonstrated previously; of note, this assay is insensitive to nitrite and nitrate (whether or not additional thiols are present).

Total RBC SNO: Reduction in carbon monoxide (CO)-saturated copper/cysteine (3C). SNOs are selectively reduced to NO in a cuprous chloride- and CO-saturated cysteine solution that is replaced before the injection of each sample; released NO is detected as the chemiluminescent product of its reaction with ozone (22).

RBC deformability. RBC deformability was measured by using a laser-assisted optical rotational cell analyzer (LORCA) as described in *SI Methods* and previously (45, 46). *A priori*, we defined two main levels of shear stress, 3 and 30 Pa (49), with 3 Pa representing a clinically relevant level of shear stress in the microcirculation (47, 48).

Cytokine assays. Cytokines were determined in the storage medium as described in *SI Methods*.

RBC adhesion. RBC adhesion was determined as described (56), by using a variable-height flow chamber and TNF α -treated primary human umbilical vein endothelial cells as the adhesive substrate.

RBC ATP and 2,3-DPG. RBC ATP and 2,3-DPG content were determined after deproteinization by using standard techniques described in *SI Methods*.

Extraerythrocytic (free) Hb assay. A published technique (57) was used in modified form (*SI Methods*).

PS exposure. PS exposure on the RBC membrane was measured as described in *SI Methods*.

Blood gases. Blood gases were performed by using a CCX 8 blood gas analyzer (Nova Biomedical, Waltham, MA).

Blood culture. Blood culture was performed at the DUMC clinical microbiology laboratory by using standard methods.

Statistical methods and data integrity. Statistical methods and data integrity are described in *SI Methods*.

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