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Renitrosylation of banked human red blood cells improves deformability and reduces adhesivity

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Abstract

Background—Transfusion of red blood cells (RBCs) is a frequent healthcare practice. However, unfavorable consequences may occur from transfusions of stored RBCs and are associated with RBC changes during storage. Loss of S-nitrosohemoglobin (SNO-Hb) and other S-nitrosothiols (SNOs) during storage is implicated as a detriment to transfusion efficacy. It was hypothesized that restoring SNOs within banked RBCs would improve RBC functions relevant to successful transfusion outcomes, namely increased deformability and decreased adhesivity.

Study Design and Methods—Stored human RBCs were incubated with nitric oxide (NO) donors PROLI/NO and DEA/NO (disodium 1-[2-(carboxylato)-pyrrolidin-1-yl]diazene-1-ium-1,2-diolate and diethylammonium (Z)-1-(N,N-diethylamino)diazene-1-ium-1,2-diolate) under different experimental conditions (e.g., aerobic/anaerobic incubation, NO donor to RBC ratio). SNO restoration was evaluated in vitro and in vivo as a means to improve RBC function after storage.

Results—Incubation of RBCs with the NO donors resulted in tenfold greater levels of SNO-Hb versus untreated control or sham RBCs, with significantly higher Hb-bound NO yields from an NO dose delivered by DEA/NO. RBC incubation with DEA/NO at a stoichiometry of 1:62.5 NO:Hb significantly increased RBC deformability and reduced adhesion to cultured endothelial cells. RBC incubation with DEA/NO also increased S-nitrosylation of RBC cytoskeletal and membrane proteins, including the beta spectrin chain. Renitrosylation attenuated both RBC sequestration in the lung and the mild blood oxygen saturation impairments seen with banked RBCs in a mouse model of transfusion.

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Author contributions

D.A.R. conceived the study, designed and performed experiments, analyzed data and wrote the manuscript; H.Z. performed experiments and analyzed data; M.W.F. designed experiments, analyzed data and edited the manuscript; and B.H. performed experiments and analyzed data; C.L.H. and G.M.P. produced the deconvolution program and analyzed data. T.J.M. conceived the study, designed experiments, analyzed data and wrote the manuscript.

Conclusions—RBC renitrosylation using NO donors has promise for correcting deficient properties (e.g., adhesivity, rigidity, and SNO loss) of banked RBCs and in turn improving transfusion outcomes.

Keywords

Nitric oxide; S-nitrosohemoglobin; storage lesion; erythrocyte; S-nitrosothiol

Introduction

In 2011, nearly 13.8 million units of whole blood and red blood cell (RBC) units were transfused in the United States.¹ Blood transfusion is a ubiquitous, expensive, and essential healthcare practice. Unfortunately, a possible negative link between banked RBC age and outcome in some patients has drawn concern. RCTs indicating no benefit of liberal RBC-transfusion strategies in varying anemic patient populations have also renewed questions about adverse changes present early in RBC storage.^{2–4}

Functional alterations of RBCs correlated with longer storage include reduced deformability and increased RBC adhesivity to endothelial cells (ECs),^{5–8} these alterations increase the resistance to blood flow.⁹ Together with RBC-potentiated hypoxic vasodilation,¹⁰ RBC deformability and adhesivity can influence tissue perfusion and oxygen delivery. Vessel injury, or even occlusion with organ morbidity are potential consequences of transfusing older RBCs dysfunctional in these properties.⁸

Progressive changes such as decreased pH, increased hemolysis, loss of 2,3-diphosphoglycerate and concomitant increased O₂ affinity are well documented.^{5,11–13} Within three hours of storage, levels of nitric oxide (NO) bound to hemoglobin (Hb) in the form of an S-nitrosothiol (SNO) group at the β 93 cysteine thiol are significantly depressed.^{5,14} As storage proceeds, this SNO deficiency persists.⁵ Thus, functional and biochemical changes occur over varying time windows during RBC banking. Concern has grown about the effectiveness of RBC units stored for longer periods (e.g., 3–6 weeks), but there is evidence that the early changes (hours to days) may also be clinically significant. Specifically, in hospitalized, anemic patients, more frequent RBC transfusion may predispose to harm, even though anemia itself is an independent risk factor for poor outcomes in these patients (e.g., cardiac surgery, trauma, sepsis).¹⁵

The importance of NO/SNO interactions with RBCs is well established,^{10,16–18} although debate and investigation of the mechanisms continue.^{19–21} Delivery of vasodilatory NO/SNO derivatives from the reactive thiol groups of S-nitrosohemoglobin (SNO-Hb), simultaneous with delivery of O₂ from the heme groups, allows the RBC to control blood flow by matching O₂ delivery to regional metabolic demand.¹⁰ NO from activated leukocytes or NO donor molecules can augment RBC deformability concentration-dependently in situ,^{22,23} and NO synthase (NOS) inhibition can decrease deformability.²⁴ NO's well-documented anti-adhesive properties may deter injurious RBC-EC interaction, a recognized consequence of transfusing older RBCs.^{25,26} Indeed, the effects of exogenous NO or inhibiting NOS have underscored a role for NO in modulating RBC adhesion to

endothelium.^{27–30} The impact of NO on these determinants of RBC function suggests a beneficial role for NO-based therapy in transfusion medicine.³¹

Replenishing the lost NO/SNO content (i.e., renitrosylation) of stored RBCs was efficacious in improving stored RBC-induced in vitro vasodilation,¹⁴ coronary blood flow,¹⁴ and tissue oxygenation in animal models of transfusion.³² But some RBC functions, including adhesivity to endothelium and RBC deformability may be worsened, rather than ameliorated, by NO.³³ We hypothesized that controlled incubation of older (i.e., 35–42 days) stored human RBCs with NO donors would augment levels of bioactive NO species, such as SNOs, in RBCs. We further hypothesized that renitrosylation could improve deformability, mitigate endothelial adhesion, and lead to physiological benefits in transfused mice. RBC renitrosylation can be achieved by incubation with aqueous NO solution, SNOs (i.e., *S*-nitrosocysteine), or exposure to nitroating gas.^{14,32} *N*-Diazoniumdiolates (NONOates) release NO at physiological pH. The NO-release kinetics of NONOates vary by chemical structure, allowing controlled SNO restoration.

Here, we studied the effects of the NONOates PROLI/NO and DEA/NO, well-characterized NO donors with disparate NO-release kinetics that release ample NO within practical timeframes (i.e., minutes).³⁴ Hb is by far the most abundant protein in the RBC. Given the very high affinity of heme groups for NO, it is likely that Hb(Fe)NO is a major initial reaction product of Hb and NO under anaerobic conditions.³⁵ With subsequent reoxygenation of the deoxygenated RBCs, SNO-Hb forms. We therefore assayed HbFeNO and SNO-Hb to measure renitrosylation efficacy. The NO/SNO content of aged RBCs was studied at varying NO:Hb molar incubation ratios, donor NO-release kinetics, and coordination of incubation time and allosteric modulation of Hb. To test whether restoration of RBC NO/SNO adducts effectively rectifies “RBC storage lesions” and improves subsequent post-transfusion outcomes, we assessed NO/SNO content and distribution, RBC deformability, and RBC adhesivity to endothelial cells after renitrosylation in vitro, and examined the physiological effects of NO/SNO restoration in transfused mice.

Materials and Methods

Materials

Human RBCs from the segmented tubing (“segments”) of units stored in Additive Solution-3, 35–42 days old, were from Duke Transfusion Services with IRB approval. Disodium 1-[2-(carboxylato)-pyrrolidin-1-yl]diazene-1-ium-1,2-diolate (PROLI/NO) and diethylammonium (Z)-1-(*N,N*-diethylamino)diazene-1-ium-1,2-diolate (DEA/NO) were from Cayman (Ann Arbor, MI). All other reagents were analytical grade and used as received unless noted otherwise.

RBC exposure to NO donors as a function of incubation conditions

See SI (Supporting Information) for details. Briefly, RBCs (35–42 d) were washed 3x in excess phosphate-buffered saline (PBS, pH 7.4) with 100 μ M diethylenetriaminepentaacetic acid (DTPA). Washed RBCs were adjusted to 35% hematocrit and then deoxygenated (35 min). RBC suspensions were incubated with NONOate at the indicated ratios for 15 min

under varying conditions: aerobic, anaerobic, or anaerobic for 5 min and aerobic for the remaining 10 min. RBCs were centrifuged at 2700g to remove non-decomposed NONOate at 15 min. The RBCs were resuspended and aerated with mixing to promote reoxygenation. After 5 min of reoxygenation, the RBCs were washed twice in PBS/DTPA. Control stored RBCs underwent no treatment after initial washing. Sham-treated stored RBCs underwent deoxygenation for 50 min, reoxygenation as described, and washes.

NO/SNO content on Hb and RBC membrane

See SI for details. Briefly, RBCs were lysed in H₂O with DTPA (1:4 packed RBC:H₂O). Hemolysate was desalted, partially purified, and analyzed by photolysis-chemiluminescence (MPC).¹⁰ Methemoglobin was determined in a subset of NO-loaded RBC lysates using spectral deconvolution as previously described³⁶. Membranes were prepared via RBC lysis in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer with DTPA and centrifugation at 18,500g for 15 min. Residual Hb was removed from membranes by serial washes in MOPS. Membrane pellets were solubilized, and assayed SNO was normalized to membrane protein.

Deformability of RBCs was studied in RBCs suspended in isotonic polyvinylpyrrolidone (PVP); final hematocrit was 0.21%. The elongation index (EI), was measured with an ektacytometer (LORCA; Mechatronics, Netherlands) at varying shear stresses; higher EI indicates greater deformability.³⁷

Resin-assisted capture for detection of nitrosylated RBC membrane proteins (SNO-RAC)

The SNO-RAC procedure was described previously.³⁸ See SI.

Adhesion of RBCs to nonactivated, cultured endothelial cells (ECs)

RBC-EC adhesion was measured as described.³⁹ Individual experimental curves were constructed for adhesion vs. shear stress, and for group comparisons, values at 1, 2, and 5 dyne cm⁻² were interpolated from the individual shear vs. adhesion curves, then averaged. See SI for additional details.

Nude mouse transfusion model included pulse oximetry and post-mortem analysis of RBC sequestration within lungs.²⁶ RBCs labeled with the fluorescent dye PKH26 (Sigma) were exposed (or not) to DEA/NO (1:62.5 ratio to Hb tetramer). Harvested lungs were counterstained with DAPI (4,6-diamidino-2-phenylindole) and assessed for tissue fluorescence using filters for PKH26 (red) and DAPI (blue). For tissue fluorescence imaging quantification, exposure times were held constant between the Control RBC and DEA/NO-RBC transfused pairs. Twenty lung fields were chosen randomly and the fluorescence intensity for each field was quantified using a Texas red filter and Adobe Photoshop CS5 software. The values were averaged for the 20 fields to obtain mean fluorescence intensity values. The group average of the mean (and SEM) values from each mouse was then calculated and expressed graphically. DEA/NO exposure did not alter fluorescent signal from PKH26, or its stability (see SI, Fig. S6).

Statistical analysis

Results are presented as mean and SEM except where otherwise indicated. Paired comparisons were by Student's t-test, and multiple-group data were analyzed by ANOVA with post-hoc testing using Tukey's or Dunnett's tests, as appropriate.

Results

Selection and characterization of NONOates

NO generation from NONOates varies based on pH and temperature;³⁴ we therefore characterized the kinetics of NO-release under fixed pH (7.4) at 25 deg. C. Complete decomposition of PROLI/NO produced 2 molar equivalents of NO within minutes (Figure S1A), and DEA/NO released 1.5 mol of NO/mol with a half-life of ~16 min. Accordingly, a 2.67-fold higher DEA/NO concentration generated the same molar NO as PROLI/NO during a 15-min incubation, given the markedly slower release by DEA/NO (Figure S1B).

SNO-Hb restoration in banked RBCs: Coordinate optimization of aeration status and timing

We aimed to determine experimental conditions favorable for reinitrosylation (i.e., enhanced NO/SNO content) of banked RBCs nearing their expiration. RBC exposure to NO under anoxic or hypoxic conditions, followed by aeration of the NO-loaded RBCs, favors transfer of NO from the heme to Cys β 93, and subsequently to the RBC membrane.^{10,16,40} To determine whether this pathway promoted post-storage reinitrosylation by NONOates, we incubated RBCs with NONOates (final NO:Hb ratio of 1:250) for 15 min under anaerobic conditions, aerobic conditions, or a combination thereof. After incubation and reoxygenation, SNO-Hb was formed from either of the NONOates under all conditions (Figure 1A). Notably, the sham procedure itself (deoxygenation followed by reoxygenation) did not alter stored-RBC levels of total Hb-bound NO, SNO-Hb, or Hb[Fe]NO in RBCs stored for 35–42 days (Figure 1). Moreover, basal Hb-bound NO levels (SNO-Hb and Hb[Fe]NO) in these segment-derived stored RBCs were similar to those we previously documented in RBC unit bags.^{5,41–43} Significantly higher heme-bound NO (Hb[Fe]NO) was quantified after DEA/NO treatment under anaerobic versus aerobic conditions (Fig. 1B). Similarly, highest levels of total NO bound to Hb (total Hb-NO) after RBC exposure to PROLI/NO were formed under deoxygenated conditions (Figure 1C). Collectively, these data show that anaerobic conditions promote superior NO loading of RBCs with NONOates; therefore this condition was selected for subsequent studies. Notably, anaerobic conditions also deter the unfavorable oxyhemoglobin reaction with NO that yields nitrate and methemoglobin (metHb). We measured metHb in a subset of RBCs exposed to the NO donors and in none, even at 1:25 NO:Hb ratios, did the metHb fraction exceed 2.81%. Mean (and SEM) metHb was 0.44 ($\pm$ 0.40) % in n=7 samples. These results are consistent with the relatively low stoichiometry of NO:Hb, and with the presence in RBCs of reductive enzyme systems such as metHb reductase. Because the deoxygenation/reoxygenation protocol did not affect the Hb-bound NO levels in banked RBCs (Figure S2), stored RBCs rather than sham RBCs were used as the control RBCs for subsequent comparisons except where otherwise noted.

SNO-Hb restoration in banked RBCs: Influence of the NO:Hb stoichiometry and the NO-donor release kinetics

The instantaneous ratio of NO ligated to Hb tetramer during NO donor incubation is dictated in part by the NO release kinetics of the NO donor employed. To evaluate the stoichiometry of total NO to Hb in reinitrosylation of banked blood, we exploited the well-defined release kinetics of NONOates to deliver varying molar loading ratios of NO over a 15-min anaerobic incubation with RBCs (Figure 2). For each NO donor, both total Hb-NO and Hb[Fe]NO levels significantly increased as the NO:Hb loading ratio was increased (i.e., 1:250 to 1:62.5 to 1:25) (Figure 2B–C; two-way ANOVA, $P < 0.0001$ for dose-dependence). The levels of SNO-Hb tended to increase similarly within each group as the NO:Hb loading ratio increased (Figure 2A), and were comparable to or exceeded SNO-Hb concentrations reported by multiple lab groups studying healthy human RBCs.^{10,44,45} As expected, incubating at the greatest NO:Hb ratio, 1:25, resulted in SNO-Hb levels exceeding all other ratios for both NO donors (ANOVA, $P < 0.05$). When compared to an unmatched cohort of control stored RBCs, incubation with the NONOates tended to increase SNO-Hb, Hb[Fe]NO, and total Hb-NO. Again, the greatest NO:Hb ratio significantly elevated SNO-Hb when employing DEA/NO. The 1:25 ratio also significantly increased Hb[Fe]NO and total Hb-NO versus control stored RBCs for both NONOates. Additionally, the use of DEA/NO with a 1:62.5 NO:Hb ratio significantly increased Hb[Fe]NO and total Hb-NO. Compared to PROLI/NO, using DEA/NO (within matched NO:Hb incubation ratios) led to higher levels of Hb[Fe]NO and total Hb-NO across all loading ratios. The SNO-Hb yield was also significantly higher when using DEA/NO (versus PROLI/NO) at NO:Hb ratios of 1:250 and 1:25. Renitrosylation-induced increases in SNO-Hb and Hb[Fe]NO lasted at least 4 hours (See Figure S2).

The NO content of the RBC membrane fraction was also assayed under the various renitrosylation conditions (Figure 3). Average SNO normalized to membrane protein rose within each NONOate group as the NO:Hb ratio was increased, but reached significance only after incubation with DEA/NO at the highest NO:Hb ratio (1:25). Membrane SNO did not differ between PROLI/NO and DEA/NO groups when comparing matched NO:Hb ratios ($P > 0.05$ by two-way ANOVA).

Deformability of banked RBCs exposed to NO donors

To examine the functional effects of renitrosylation, deformability of banked RBCs was assessed across all NO:Hb loading ratios and for both NONOate treatments (Figure 4). As expected, increasing shear stress (from 3 to 30 Pa) induced greater RBC deformation. As compared to control stored RBCs (that were unexposed to the deoxygenation-reoxygenation procedure), sham stored RBCs did not differ significantly at shear stresses of 3 or 30 Pa (Figure S3). Deformability tended to increase for all NO treatments (vs. control). Specifically, a ratio of 1:62.5 NO:Hb yielded significantly greater deformability in banked RBCs at both shear stresses of 3 and 30 Pa when either NONOate was used for SNO restoration. Of note, 3 and 30 Pa were chosen as shear stresses physiologically relevant to RBC deformation within the circulation *in vivo*.^{5,46} Incubating with DEA/NO at 1:250 NO:Hb and with PROLI/NO at 1:25 NO:Hb also significantly increased the deformability of

banked RBCs at shear stresses of 3 and 30 Pa, respectively. Similar findings were seen at shear stresses of 1 and 10 Pa (not shown).

SNO-RAC analysis of S-nitrosylated RBC-membrane proteins

The identification of RBC protein targets of NONOate-dependent *S*-nitrosylation may be useful in determining how NO incubation ameliorates banked RBC dysfunction. However, the identities of RBC membrane SNO-proteins are not well known.⁴⁰ We chose to screen candidates (ankyrin, band 3, spectrin α , and spectrin β) as possible targets of *S*-nitrosylation because they are abundant within RBC membranes,⁴⁷ implicated in RBC deformability,^{47–49} and/or exhibit sites of known *S*-nitrosylation.^{40,50} Using resin-assisted capture of *S*-nitrosylated proteins (SNO-RAC), we assessed *S*-nitrosylation of these proteins following treatment of banked RBCs with DEA/NO at a NO:Hb ratio of 1:62.5 (Figure 5A). Compared to the untreated controls, ankyrin *S*-nitrosylation was undetectable under these conditions, whereas band 3 and spectrin α showed a modest trend toward increase in *S*-nitrosylation. Additionally, spectrin β *S*-nitrosylation was significantly increased, at approximately 4-fold versus controls (Figure 5B).

Endothelial cell (EC) adhesion of banked RBCs exposed to NO donors

The enhanced RBC deformability achieved after incubation with DEA/NO at a 1:62.5 NO:Hb ratio suggested that this particular NO incubation strategy may ameliorate banked-RBC dysfunction. After reinitrosylation, banked RBCs were significantly less adherent to cultured ECs at a shear stress of 1 dyne cm⁻² than control stored RBCs (Figure 6). Nonsignificant trends toward decreased adhesion were also seen at higher shear stresses.

Influence of banked-RBC exposure to NO-donor in a murine model of transfusion

Transfusion-related acute lung injury (TRALI) is now the most frequent serious complication of RBC transfusion.⁵¹ To determine whether effects on banked RBC deformability and adhesivity by DEA/NO (1:62.5 NO:Hb) translated into improved transfusion outcome, transfused mice were assessed with attention to RBC sequestration in the lungs and absolute changes (peak decreases) in blood oxygenation (SaO₂). Basal SaO₂ was 96.7 ± 0.4% (mean ± SEM; n=16; min. 92.1%). These pulmonary sequelae of transfusing older RBCs may represent downstream effects of rigid and adherent banked RBCs, and are minimal or absent when mice are transfused with fresh, human RBCs.²⁶ Therefore, the species difference alone does not account for this phenotype.²⁶ Indeed, NO incubation of banked RBCs significantly decreased post-transfusion sequestration of RBCs (Figure 7A–C), as compared either to RBCs that were deoxygenated and then reoxygenated following 35–42 days of storage (“Sham,” as a control that recapitulates those steps in the NO-loading protocol other than NO exposure itself), or to untreated control stored RBCs. Given that the lung fluorescence changes (reflecting adhesion of RBCs) were attenuated by prior NO loading as compared to those after transfusion of either control or sham (deoxygenated, then reoxygenated) RBCs, we present the data from these two control series grouped together (Fig. 7C, “Con”); the data from the subgroups are depicted in SI (Figure S5). The mild SaO₂ depressions after transfusing banked RBCs²⁹ were significantly

attenuated and shortened by reinitrosylation (Figure 7D–F). Stored-RBC transfusion did not significantly alter heart rate (not shown), irrespective of reinitrosylation.

Discussion

Recently published randomized controlled clinical trials have indicated a lack of benefit of restricting the transfusion of RBCs to units stored for shorter periods (e.g., ~10 days or less) when directly compared to conventional practice in critically ill adults or neonates, or in complex cardiac surgery patients.^{52–54} However these studies did not answer the question of whether transfusing RBCs stored for longer periods (for example, >22 days) may promote adverse outcomes.^{8,55} Moreover, RBCs stored only hours may be dysfunctional compared to fresh RBCs, given the substantial biochemical change during the first days of storage, such as the marked fall in pH and loss of bioactive NO adducts.⁵ In this study, reinitrosylation of banked RBCs (i.e., 35–42 d stored) was accomplished through anaerobic incubation of the RBCs with NONOates. We studied reinitrosylation of Hb in particular, because of its abundance in RBCs and its ability to transnitrosylate other thiol targets to effect vascular signaling.⁴⁰ The increase in NO ligation to heme iron (i.e., Hb[Fe]NO) that accompanies anaerobic incubation was expected because deoxygenated (T-state) Hb has a high affinity for NO at the heme.³⁵ Hb[Fe]NO is a reservoir for subsequent SNO-Hb formation when Hb allosterically transitions to R-state upon reoxygenation.^{10,16,17} While aerobic NONOate incubation conditions yielded comparable levels of SNO-Hb, the total NO stored by RBC Hb, and hence the potential bioactive store via conversion of Hb[Fe]NO to SNO-Hb, were lower. Thus, maintaining banked RBCs deoxygenated during incubation with NONOates yielded more NO/SNO. The stoichiometry of NO with free or intracellular Hb influences the identities and yields of reaction products (e.g., SNO-Hb, metHb, and Hb[Fe]NO) and is critical when mimicking in vivo conditions to demonstrate physiological relevance.³⁵ The implementation of of banked blood reinitrosylation as a therapeutic approach warrants evaluation across varying NO:Hb ratios to reveal unforeseen therapeutic or functional benefit. Thus, we examined three loading ratios, bracketing and exceeding the physiological range, from high nanomolar to low micromolar NO, across both NONOates to determine their impact on the restoration of SNO in banked blood (Figures 2, 3). The levels of SNO-Hb and Hb[Fe]NO were easily tuned by adjusting this ratio. The greatest improvement in SNO-Hb compared to control stored RBCs was seen after incubation with DEA/NO at the NO:Hb ratio of 1:25. However, the SNO-Hb levels attained across all ratios and both NONOates were all between 2- and 70-fold higher than those in 6-week-old banked RBCs.^{5,14} Of note, SNO-Hb was reported to be $\sim 7 \times 10^{-4}$ moles (S)NO/mole Hb.⁵ Importantly, therefore, the SNO restoration yields achieved here encompass and exceed SNO levels reported in fresh RBCs.^{10,44,45} Supraphysiological RBC SNO concentrations are not expected to adversely influence the response to a transfusion, because RBCs release (S)NO-related bioactivity only in graded proportion to tissue metabolic need (e.g., secondary to tissue hypoxia).

Beyond stoichiometry, the kinetics of NO addition to Hb also determines the products. We compared reinitrosylation distribution after exposure to otherwise similar NO donors with differing kinetics of decomposition and NO release. We observed different Hb[Fe]NO yields between higher (i.e., bolus release from PROLI/NO) vs. lower (i.e., sustained release from

DEA/NO) instantaneous local concentrations of liberated NO. Low, sustained NO release yielded higher Hb[Fe]NO, translating into a larger reservoir for SNO-Hb formation upon reoxygenation as corroborated by higher SNO-Hb levels for stored RBCs renitrosylated with DEA/NO versus PROLI/NO. While RBC Hb[Fe]NO is unlikely to “release” NO, it can act as a precursor to bioactive SNO-Hb.^{56,57} Possible mechanistic reasons for the influence on renitrosylation of lower, sustained NO fluxes vs. that from rapid bolus NO are discussed in more detail in SI. We did not comprehensively assay for other possible products of NO’s reactions with RBC constituents, such as nitrate, nitrite, peroxynitrite, or S-nitrosoglutathione (GSNO), but their formation would be disfavored given the avidity of NO for both oxy- and deoxyHb.

To determine the impact of renitrosylation on banked RBC function, RBC deformability and adhesivity to endothelium were assessed because dysfunction of these traits may contribute to morbidity in patients transfused with stored RBCs.^{5–7} Such RBC dysfunction may be especially deleterious in transfused patients with preexisting inflammation, where endothelium may be activated and RBC rheology compromised.^{8,58–60} RBC membrane components, namely a cholesterol and phospholipid bilayer, are tethered to the cytoskeletal network, mainly the protein spectrin.^{61,62} While banked-RBC deformability was improved by renitrosylation strategies across both NONOates and both shear stresses (Figure 4), differences were only seen with 1:62.5 NO:Hb. Consistent with a report in fresh RBCs,²² rheologic changes were biphasic, with deformability peaking at mid-range NO exposure. The reason for this biphasic relationship is unknown, but may reflect differences in the set of RBC lipids or proteins susceptible to nitrosation or transnitrosylation as [NO] rises, or dose-dependent saturation of denitrosylation mechanisms. While the increased RBC deformability here is not a complete return to that of fresh RBCs,⁵ the overall favorable relationship between RBC-SNO and improved RBC deformability is clear. The degree to which the treatment-induced improvement in deformability under these clinically relevant shear stresses is physiologically significant is uncertain, particularly given the parallel changes in other properties (e.g., adhesivity), and merits further testing in other preclinical models. Enhanced fresh-RBC deformability after NOS activation or exogenous NO exposure was previously correlated with S-nitrosylation of specific RBC cytoskeletal proteins (α - and β -spectrins).⁵⁰ Taken together, these findings indicate that the deficient NO-related bioactivity in banked RBCs can be mitigated by NO exposure and formation of SNO groups on RBC proteins.

To examine whether the favorable effects of NO on stored-RBC deformability involved the same target proteins (or other critical RBC membrane proteins) as for fresh RBCs,⁵⁰ SNO-RAC analysis was performed on banked RBCs treated with DEA/NO (1:62.5 NO:Hb). Not all of the screened proteins are nitrosylated under these conditions, but some key cytoskeletal proteins crucial to RBC deformability, specifically spectrin β , sustained significant protein S-nitrosylation (Figure 5). Ankyrin did not gain SNO under these conditions, but direct incubation of RBC “ghosts” with S-nitrosocysteine (a S-nitrosylating agent) revealed its susceptibility to S-nitrosylation (Figure S3), highlighting that renitrosylation methods may differentially modify individual RBC proteins or Cys residues, with varied functional consequences. As renitrosylation with DEA/NO at 1:62.5 NO:Hb

yielded high levels of RBC-bound SNO, elevations in Hb[Fe]NO and total Hb-NO, and improved deformability, it was chosen as the strategy for reducing banked RBC-endothelial adhesion. Attenuated adhesion was seen at low shear stress (Figure 6). Inhibiting RBC-endothelial adhesion may improve blood flow,⁹ deter endothelial injury, and reduce harmful RBC sequestration in the lungs, all of which are recognized as transfusion-related complications.²⁶ In our transfusion model,²⁶ lung dysfunction is evident even without a “first hit,” underscoring the potentially injurious effects of older RBCs alone, and suggesting the model’s relevance. Although the phenotype in this model is mild, it may be relevant in critically ill patients whose blood oxygenation is marginal at the time of transfusion, and may be additive with other processes and even treatments that could worsen gas exchange or promote lung injury. In transfused mice, SNO-restoring RBC incubation with DEA/NO significantly improved respiratory outcomes after transfusion of banked RBCs (Figure 7). The attenuated adhesivity and increased deformability did not represent a full correction of these in vitro RBC properties, but may nevertheless have contributed to the favorable post-transfusion respiratory phenotype. These findings illustrate the effectiveness of renitrosylation in ameliorating *established* RBC dysfunction associated with blood banking, and the improved transfusion outcome underscores the translational potential of SNO-restoring exposure to NONOates.

Whether untreated RBCs transfused after storage are renitrosylated in the patient is unknown. However, we reason that ex vivo renitrosylation of RBC transfusates may have advantages over in vivo renitrosylation (alone), because injurious events might take place before the transfused RBCs have processed and converted any imported, endogenous NO, consistent with the post-transfusion phenotype that we and others have demonstrated. We did not examine the stability of the augmented SNO bound to Hb and other proteins beyond 4 hours. But we reason that once the treated, stored RBCs are transfused and resist the initial tendencies to adhere to endothelium and/or occlude microvessels due to poor deformability, they may support cycles of renitrosylation and SNO-offloading in vivo, in concert with normal cardiopulmonary cycling.

In summary, we demonstrate the benefit of SNO-restoring NO-donor exposure of RBC transfusates as a novel therapeutic that ameliorates two critical parameters of banked RBC (dys)function: deformability and adhesivity to endothelium. Similar strategies for renitrosylation of banked RBCs rectified other functions, including deficiencies in vasoactivity, tissue perfusion, and oxygenation.^{14,32} Our data suggest that poor RBC deformability and elevated adhesivity may impair blood and tissue oxygenation after transfusion, because vessel occlusion by rigid RBCs blocking or adhering to microvessels can impair perfusion and/or promote or cause local injury.⁹ We found efficacious concentrations and kinetics for renitrosylation strategies employing NONOates; this may inform other SNO-restoring strategies (e.g., use of NO solutions or gases).^{14,32} All renitrosylation strategies do not yield the same NO/SNO concentration in banked RBCs, nor will all yields of RBC-associated NO/SNO prove functionally beneficial. Further studies are needed to examine these simple and promising SNO restoration strategies and translate them into improved outcomes in transfused patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Whitaker BI. 2011 Nationwide Blood Collection and Utilization Survey Report. 2011 Nationwide Blood Collection and Utilization Survey Report. 2011
2. Carson JL, Brooks MM, Abbott JD, et al. Liberal versus restrictive transfusion thresholds for patients with symptomatic coronary artery disease. *American Heart Journal*. Jun; 2013 165(6):964–+. [PubMed: 23708168]
3. Hebert PC, Wells G, Blajchman MA, et al. A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. *New England Journal of Medicine*. Feb 11; 1999 340(6): 409–417. [PubMed: 9971864]
4. Lacroix J, Hebert PC, Hutchison JS, et al. Transfusion strategies for patients in pediatric intensive care units. *New England Journal of Medicine*. Apr; 2007 356(16):1609–1619. [PubMed: 17442904]
5. Bennett-Guerrero E, Veldman TH, Doctor A, et al. Evolution of adverse changes in stored RBCs. *Proc Natl Acad Sci U S A*. Oct 23; 2007 104(43):17063–17068. [PubMed: 17940021]
6. Berezina TL, Zaets SB, Morgan C, et al. Influence of storage on red blood cell rheological properties. *J Surg Res*. Jan; 2002 102(1):6–12. [PubMed: 11792145]
7. Luk CS, Gray-Statchuk LA, Cepinkas G, Chin-Yee IH. WBC reduction reduces storage-associated RBC adhesion to human vascular endothelial cells under conditions of continuous flow in vitro. *Transfusion*. Feb; 2003 43(2):151–156. [PubMed: 12559009]
8. Tinmouth A, Fergusson D, Yee IC, Hebert PC. Clinical consequences of red cell storage in the critically ill. *Transfusion*. Nov; 2006 46(11):2014–2027. [PubMed: 17076859]
9. Kaul DK, Koshkaryev A, Artmann G, Barshtein G, Yedgar S. Additive effect of red blood cell rigidity and adherence to endothelial cells in inducing vascular resistance. *American Journal of Physiology-Heart and Circulatory Physiology*. Oct; 2008 295(4):H1788–H1793. [PubMed: 18757485]
10. McMahon TJ, Moon RE, Luschinger BP, et al. Nitric oxide in the human respiratory cycle. *Nature Medicine*. Jul; 2002 8(7):711–717.
11. Karon BS, van Buskirk CM, Jaben EA, Hoyer JD, Thomas DD. Temporal sequence of major biochemical events during Blood Bank storage of packed red blood cells. *Blood Transf*. Oct; 2012 10(4):453–461.
12. Karam O, Tucci M, Toledano BJ, et al. Length of storage and in vitro immunomodulation induced by prestorage leukoreduced red blood cells. *Transfusion*. Nov; 2009 49(11):2326–2334. [PubMed: 19624600]
13. Latham JT, Bove JR, Weirich FL. Chemical and hematologic changes in stored CPDA-1 blood. *Transfusion*. 1982; 22(2):158–159. 1982. [PubMed: 7071919]
14. Reynolds JD, Ahearn GS, Angelo M, Zhang J, Cobb F, Stamler JS. S-nitrosohemoglobin deficiency: a mechanism for loss of physiological activity in banked blood. *Proc Natl Acad Sci U S A*. Oct 23; 2007 104(43):17058–17062. [PubMed: 17940022]
15. Marik PE, Corwin HL. Efficacy of red blood cell transfusion in the critically ill: A systematic review of the literature. *Crit Care Med*. Sep; 2008 36(9):2667–2674. [PubMed: 18679112]
16. Jia L, Bonaventura C, Bonaventura J, Stamler JS. S-nitrosohaemoglobin: A dynamic activity of blood involved in vascular control. *Nature*. Mar 21; 1996 380(6571):221–226. [PubMed: 8637569]

17. Stamler JS, Jia L, Eu JP, et al. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. *Science*. 1997; 276(5321):2034–2037. [PubMed: 9197264]
18. McMahon TJ, Stamler JS. Concerted nitric oxide oxygen delivery by hemoglobin. *Nitric Oxide, Pt C*. 1999; 301:99–114. 1999.
19. Gladwin MT, Lancaster JR, Freeman BA, Schechter AN. Nitric oxide's reactions with hemoglobin: a view through the SNO-storm. *Nature Medicine*. May; 2003 9(5):496–500.
20. Allen BW, Piantadosi CA. How do red blood cells cause hypoxic vasodilation? The SNO-hemoglobin paradigm. *American Journal of Physiology-Heart and Circulatory Physiology*. Oct; 2006 291(4):H1507–H1512. [PubMed: 16751292]
21. Gaston B, May WJ, Sullivan S, et al. Essential role of hemoglobin beta-93-cysteine in posthypoxia facilitation of breathing in conscious mice. *Journal of Applied Physiology*. May; 2014 116(10):1290–1299. [PubMed: 24610531]
22. Bor-Kucukatay M, Wenby RB, Meiselman HJ, Baskurt OK. Effects of nitric oxide on red blood cell deformability. *Am J Physiol Heart Circ Physiol*. May; 2003 284(5):H1577–1584. [PubMed: 12521942]
23. Korbut R, Gryglewski RJ. Nitric oxide from polymorphonuclear leukocytes modulates red blood cell deformability in vitro. *Eur J Pharmacol*. Mar; 1993 234(1):17–22. [PubMed: 8472757]
24. Bor-Kucukatay M, Yalcin O, Gokalp O, et al. Red blood cell rheological alterations in hypertension induced by chronic inhibition of nitric oxide synthesis in rats. *Clin Hemorheol Microcirc*. 2000; 22(4):267–275. [PubMed: 11081464]
25. Chin-Yee IH, Gray-Statchuk L, Milkovich S, Ellis CG. Transfusion of stored red blood cells adhere in the rat microvasculature. *Transfusion*. Nov; 2009 49(11):2304–2310. [PubMed: 19624601]
26. Zhu HM, Zennadi R, Xu BX, et al. Impaired adenosine-5'-triphosphate release from red blood cells promotes their adhesion to endothelial cells: A mechanism of hypoxemia after transfusion. *Crit Care Med*. Nov; 2011 39(11):2478–2486. [PubMed: 21765360]
27. Grossin N, Wautier MP, Wautier JL. Red blood cell adhesion in diabetes mellitus is mediated by advanced glycation end product receptor and is modulated by nitric oxide. *Biorheology*. 2009; 46(1):63–72. [PubMed: 19252229]
28. Serirom S, Raharjo WH, Chotivanich K, Loareesuwan S, Kubes P, Ho M. Anti-adhesive effect of nitric oxide on *Plasmodium falciparum* cytoadherence under flow. *Am J Pathol*. May; 2003 162(5):1651–1660. [PubMed: 12707049]
29. Space SL, Lane PA, Pickett CK, Weil JV. Nitric oxide attenuates normal and sickle red blood cell adherence to pulmonary endothelium. *Am J Hematol*. Apr; 2000 63(4):200–204. [PubMed: 10706764]
30. Wautier MP, Khodabandehlou T, Le Devehat C, Wautier JL. Modulation of RAGE expression influences the adhesion of red blood cells from diabetic patients. *Clin Hemorheol Microcirc*. 2006; 35(3):379–386. [PubMed: 16899960]
31. Baron DM, Yu BL, Lei C, et al. Pulmonary Hypertension in Lambs Transfused with Stored Blood Is Prevented by Breathing Nitric Oxide. *Anesthesiology*. Mar; 2012 116(3):637–647. [PubMed: 22293717]
32. Reynolds JD, Bennett KM, Cina AJ, et al. S-nitrosylation therapy to improve oxygen delivery of banked blood. *Proc Natl Acad Sci U S A*. Jul; 2013 110(28):11529–11534. [PubMed: 23798386]
33. Bateman RM, Jagger JE, Sharpe MD, Ellsworth ML, Mehta S, Ellis CG. Erythrocyte deformability is a nitric oxide-mediated factor in decreased capillary density during sepsis. *American Journal of Physiology-Heart and Circulatory Physiology*. Jun; 2001 280(6):H2848–H2856. [PubMed: 11356644]
34. Hrabie JA, Keefer LK. Chemistry of the nitric oxide-releasing diazeniumdiolate (“nitrosohydroxylamine”) functional group and its oxygen-substituted derivatives. *Chem Rev*. Apr; 2002 102(4):1135–1154. [PubMed: 11942789]
35. Gow AJ, Stamler JS. Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature*. 1998; 391(6663):169–173. [PubMed: 9428761]

36. McMahon TJ, Stone AE, Bonaventura J, Singel DJ, Stamler JS. Functional coupling of oxygen binding and vasoactivity in S-nitrosohemoglobin. *J Biol Chem.* Jun 2; 2000 275(22):16738–16745. [PubMed: 10747928]
37. Johnson RM. EKTACYTOMETRY OF RED BLOOD-CELLS. *Methods in Enzymology.* 1989; 173:35–54. 1989. [PubMed: 2779431]
38. Forrester MT, Thompson JW, Foster MW, Nogueira L, Moseley MA, Stamler JS. Proteomic analysis of S-nitrosylation and denitrosylation by resin-assisted capture. *Nature Biotechnology.* 2009; 27(6):557–559.
39. Zennadi R, Hines PC, De Castro LM, Cartron JP, Parise LV, Telen MJ. Epinephrine acts through erythroid signaling pathways to activate sickle cell adhesion to endothelium via LW- α v β 3 interactions. *Blood.* Dec; 2004 104(12):3774–3781. [PubMed: 15308566]
40. Pawloski JR, Hess DT, Stamler JS. Export by red blood cells of nitric oxide bioactivity. *Nature.* 2001; 409(6820):622–626. [PubMed: 11214321]
41. Weiskopf RB, Yau R, Sanchez R, Lowell C, Toy P. Microarray kit analysis of cytokines in blood product units and segments. *Transfusion.* Nov; 2009 49(11):2269–2275. [PubMed: 19548981]
42. Kurach JD, Hansen AL, Turner TR, Jenkins C, Acker JP. Segments from red blood cell units should not be used for quality testing. *Transfusion.* Feb; 2014 54(2):451–455. [PubMed: 23834158]
43. Roback, JD., editor. Technical manual. 15. Bethesda, MD: AABB; 2011.
44. Funai EF, Davidson A, Seligman SP, Finlay TH. S-Nitrosohemoglobin in the fetal circulation may represent a cycle for blood pressure regulation. *Biochemical and Biophysical Research Communications.* Oct; 1997 239(3):875–877. [PubMed: 9367862]
45. Rogers SC, Khalatbari A, Gapper PW, Frenneaux MP, James PE. Detection of human red blood cell-bound nitric oxide. *Journal of Biological Chemistry.* Jul 22; 2005 280(29):26720–26728. [PubMed: 15879596]
46. van Bommel J, de Korte D, Lind A, et al. The effect of the transfusion of stored RBCs on intestinal microvascular oxygenation in the rat. *Transfusion.* Dec; 2001 41(12):1515–1523. [PubMed: 11778066]
47. van den Akker E, Satchwell TJ, Williamson RC, Toye AM. Band 3 multiprotein complexes in the red cell membrane; of mice and men. *Blood Cells Molecules and Diseases.* Jun 15; 2010 45(1):1–8.
48. Czogalla A, Sikorski AF. Do we already know how spectrin attracts ankyrin? *Cellular and Molecular Life Sciences.* Aug; 2010 67(16):2679–2683. [PubMed: 20411297]
49. Zhang R, Zhang C, Zhao Q, Li D. Spectrin: Structure, function and disease. *Science China-Life Sciences.* Dec; 2013 56(12):1076–1085. [PubMed: 24302288]
50. Grau M, Pauly S, Ali J, et al. RBC-NOS-Dependent S-Nitrosylation of Cytoskeletal Proteins Improves RBC Deformability. *PLoS One.* Feb.2013 8(2)
51. Toy P, Gajic O, Bacchetti P, et al. Transfusion-related acute lung injury: incidence and risk factors. *Blood.* Feb 16; 2012 119(7):1757–1767. [PubMed: 22117051]
52. Fergusson DA, Hebert P, Hogan DL, et al. Effect of fresh red blood cell transfusions on clinical outcomes in premature, very low-birth-weight infants: the ARIPI randomized trial. *JAMA.* Oct 10; 2012 308(14):1443–1451. [PubMed: 23045213]
53. Steiner ME, Ness PM, Assmann SF, et al. Effects of red-cell storage duration on patients undergoing cardiac surgery. *N Engl J Med.* Apr 9; 2015 372(15):1419–1429. [PubMed: 25853746]
54. Lacroix J, Hebert PC, Fergusson DA, et al. Age of transfused blood in critically ill adults. *N Engl J Med.* Apr 9; 2015 372(15):1410–1418. [PubMed: 25853745]
55. Koch CG, Li L, Sessler DI, et al. Duration of red-cell storage and complications after cardiac surgery. *N Engl J Med.* Mar 20; 2008 358(12):1229–1239. [PubMed: 18354101]
56. Luchsinger BP, Rich EN, Yan Y, Williams EM, Stamler JS, Singel DJ. Assessments of the chemistry and vasodilatory activity of nitrite with hemoglobin under physiologically relevant conditions. *Journal of Inorganic Biochemistry.* Apr; 2005 99(4):912–921. [PubMed: 15811508]
57. McMahon TJ, Stone AE, Bonaventura J, Singel DJ, Stamler JS. Functional coupling of oxygen binding and vasoactivity in S-nitrosohemoglobin. *Journal of Biological Chemistry.* Jun 2; 2000 275(22):16738–16745. [PubMed: 10747928]

58. Annis AM, Sparrow RL. Variable adhesion of different red blood cell products to activated vascular endothelium under flow conditions. *Am J Hematol.* Jun; 2007 82(6):439–445. [PubMed: 17133424]
59. Tissot Van Patot MC, MacKenzie S, Tucker A, Voelkel NF. Endotoxin-induced adhesion of red blood cells to pulmonary artery endothelial cells. *Am J Physiol.* Jan; 1996 270(1 Pt 1):L28–36. [PubMed: 8772524]
60. Baskurt OK, Gelmont D, Meiselman HJ. Red blood cell deformability in sepsis. *Am J Respir Crit Care Med.* Feb; 1998 157(2):421–427. [PubMed: 9476853]
61. Mohandas N, Gallagher PG. Red cell membrane: past, present, and future. *Blood.* Nov 15; 2008 112(10):3939–3948. [PubMed: 18988878]
62. Piagnerelli M, Boudjeltia KZ, Vanhaeverbeek M, Vincent JL. Red blood cell rheology in sepsis. *Intensive Care Medicine.* Jul; 2003 29(7):1052–1061. [PubMed: 12802488]

Figure 1. Aeration status and timing influence SNO-Hb restoration yields

Levels of Hb nitrosylation in the forms of SNO-Hb (A), Hb[Fe]NO (B) and total Hb-NO (C) as determined by MPC assay in banked (35–42 days) RBCs incubated with either PROLI/NO or DEA/NO (NO:Hb ratio of 1:250) under either 15 min of ambient air exposure (open bars), 5 min in the anaerobic state followed by 10 min of air exposure (gray bars), or 15 min in the anaerobic state (black bars). Results from control (untreated) and sham-treated (deoxygenation then reoxygenation) RBCs (35–42 d) are also shown). Data are mean $\pm$ SEM from 3 samples within each NO-donor (n=3 each) or Con/Sham (n=6) group. Results

were compared as a function of the (an)aerobic state during incubation and NO donor type only. *, $P < 0.05$ by two-way ANOVA with Tukey's post-test comparison of means. #, $P < 0.05$ for Con or Sham vs all DEA/NO or PROLINO treatment groups (ANOVA with Dunnett's post-test; $P < 0.05$). ##, $P < 0.05$ for Con (SNO-Hb) vs all DEA/NO or PROLINO treatment groups except the 15-min air PROLI/NO group (ANOVA with Dunnett's post-test; $P < 0.05$).

Figure 2. The NO:Hb stoichiometry influences SNO-Hb restoration yields

Yields of nitrosylated Hb in the forms of SNO-Hb (A), Hb[Fe]NO (B) and total Hb-NO (C) as determined by MPC assay for banked (35–42 days) RBCs incubated with either PROLI/NO or DEA/NO at NO:Hb ratios of 1:250 (open bars), 1:62.5 (gray bars), and 1:25 (black bars). Data are mean $\pm$ SEM for PROLI/NO (n=6) and DEA/NO (n=5). *, $P < 0.05$ for DEA/NO versus PROLI/NO at matched NO:Hb ratios by ANOVA with Tukey's post-test. ‡, $P < 0.05$ versus Control (striped bars; n=6) by one-way ANOVA with Dunnett's post-test comparison of means.

Figure 3. The NO:Hb stoichiometry influences membrane-bound SNO restoration yields
 SNO bound to RBC membrane proteins was assayed by MPC assay in banked (35–42 days) RBCs incubated with either PROLI/NO or DEA/NO at NO:Hb ratios of 1:250 (open bars; n=4 and 3, respectively), 1:62.5 (gray bars; n=5 and 4), and 1:25 (black bars; n=5 each). Data are mean $\pm$ SEM. ‡, $P < 0.05$ versus Control (striped bars; n=3) by two-way ANOVA with Dunnett's post-test comparison of means.

Figure 4. SNO restoration improves banked RBC deformability

Deformability of RBCs as represented by elongation index for banked (35–42 days) RBCs incubated with either PROLI/NO (n=4) or DEA/NO (n=4) at NO:Hb ratios of 1:250 (white bars), 1:62.5 (gray bars), and 1:25 (black bars) at shear stresses of 3 (A) and 30 Pa (B). Data are mean $\pm$ SEM. *, $P < 0.05$, versus Control RBCs (striped bars; n=8) by two-way ANOVA with Dunnett's post-test comparison of means.

Figure 5. Critical RBC cytoskeletal proteins exhibit enhanced S-nitrosylation after controlled stored-RBC exposure to NO donors

Western blotting was performed on lysates from control and DEA/NO-treated stored RBCs after SNO-RAC analysis of selected cytoskeletal membrane proteins and the degree of S-nitrosylation at the candidate proteins was determined by band densitometry for control stored RBCs (striped bars) and stored RBCs after SNO restoration with DEA/NO (NO:Hb ratio = 1:62.5; gray bars). Data are mean $\pm$ SD normalized for input protein to average respective control. Spectrin β : n=3 pairs. Others: n=4 pairs each. *, $P < 0.05$ via paired t-test.

Figure 6. SNO restoration mitigates banked RBC adhesivity

Representative adhesion (A) of control (black, solid) and DEA/NO-treated (gray, dotted) stored RBCs to HUVECS as a function of shear stress. (B), Percent adhesion of RBCs to HUVECS at nominal shear stresses of 1, 2, and 5 dyne cm⁻² for control stored RBCs (striped bars) and stored RBCs after SNO restoration with DEA/NO at a NO:Hb ratio of 1:62.5 (gray bars). Data are mean $\pm$ SEM; n = 6. *, $P < 0.05$ via paired t-test.

Figure 7. SNO restoration attenuates pulmonary sequelae of stored-RBC transfusion

Representative fluorescence micrographs of murine lung tissue post transfusion of labeled (red) (A) control and (B) DEA/NO-treated stored human RBCs and (C) fluorescence intensity of transfused lungs. Data are mean $\pm$ SEM; $n = 7$. *, $P < 0.05$ via paired t-test. (D–F) Averaged curves for changes in absolute blood oxygen saturation (SaO₂) in response to transfusion (time = 0) of control (red; D,F) and DEA/NO-treated (black; E,F) banked RBCs. Mean $\pm$ SEM; $n = 7$ (Con) and 9. *, $P < 0.0005$ via two-way ANOVA testing of significance between the two pulse oximetry curves. Paired comparison of the areas under the curves yielded a similar result ($P < 0.001$, t-test).