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Exposure Of Stored Erythrocytes To Nitric Oxide Prevents Transfusion–Associated Pulmonary Hypertension

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Abstract

Background—Transfusion of erythrocytes stored for a long duration is associated with increased pulmonary arterial pressure and vascular resistance. Prolonged storage decreases erythrocyte deformability and older erythrocytes are rapidly removed from the circulation after transfusion. We studied whether treating stored ovine erythrocytes with nitric oxide prior to transfusion could prevent pulmonary vasoconstriction, enhance erythrocyte deformability and prolong erythrocyte survival after transfusion.

Methods—Ovine leukoreduced erythrocytes were treated prior to transfusion with either nitric oxide gas or a short-lived nitric oxide-donor. Sheep were transfused with autologous erythrocytes, which were stored at 4°C for either 2 days (“fresh blood”) or 40 days (“stored blood”). Pulmonary

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Disclosures:

The Massachusetts General Hospital (Boston, MA, USA) has received royalties on the sale of nitric oxide for inhalation from Linde Corporation (Munich, Germany) and Ikaria (Hampton, NJ, USA), and WMZ received a portion of these royalties. The Massachusetts General Hospital has filed patents on NO gas-treatment to prevent transfusion-associated pulmonary hypertension. All other authors have no conflicts of interest.

and systemic hemodynamic parameters were monitored before, during and after transfusion. Transfused erythrocytes were labeled with biotin to measure their circulating lifespan. Erythrocyte deformability was assessed before and after nitric oxide-treatment using a microfluidic device.

Results—Nitric oxide-treatment improved the deformability of stored erythrocytes and increased the number of stored erythrocytes circulating at 1 and 24 h after transfusion. Nitric oxide-treatment prevented transfusion-associated pulmonary hypertension (PH) (mean pulmonary arterial pressure at 30 min of 21 ± 1 mmHg vs. 15 ± 1 mmHg in control and nitric oxide-treated erythrocytes, $p < 0.0001$). Washing stored erythrocytes prior to transfusion did not prevent PH.

Conclusion—Nitric oxide-treatment of stored erythrocytes prior to transfusion oxidizes cell-free oxy-Hb to met-Hb, prevents subsequent nitric oxide scavenging in the pulmonary vasculature and limits PH. Nitric oxide-treatment increases erythrocyte deformability and erythrocyte survival after transfusion. Nitric oxide-treatment might provide a promising therapeutic approach to prevent PH and extend erythrocyte survival.

Introduction

Transfusion of packed erythrocytes (RBCs) is a life-saving therapy for resuscitation from hemorrhage after trauma or surgery. Because supplies of fresh RBCs are limited, the U.S. Food and Drug Administration (FDA) allows transfusion of human RBCs that have been stored for up to 42 days¹. Each year approximately 326,000 patients in the United States receive one or more RBC units that have been stored for more than 30 days^{1,2}.

The safety of transfusing RBCs that have been stored for prolonged periods is controversial. Observational trials linked the transfusion of RBCs stored for longer than 14 days to an increase in morbidity and mortality³. Recent prospective clinical studies found that transfusion of RBCs stored for longer than 21 days was not associated with a greater frequency of adverse outcomes compared to transfusion of RBCs stored for fewer than 10 days^{2,4}. However, these studies did not include enough patients to establish the safety of transfusing blood stored for 30-42 days⁵.

RBCs undergo numerous morphological and biochemical changes during extended storage, collectively referred to as the 'RBC storage lesion'⁶⁻⁸. Ex vivo storage of RBCs reduces their deformability and viability and leads to hemolysis^{9,10}. The rate of hemolysis accelerates after 30 days of storage and free hemoglobin (Hb) and microvesicles containing Hb accumulate in the supernatant of the stored unit^{8,9}. After transfusion of stored RBCs, extracellular Hb scavenges nitric oxide produced by endothelial cells⁹. Depletion of endothelium-derived nitric oxide by cell-free Hb is believed to be the mechanism underlying the pulmonary vasoconstriction that occurs in sheep and humans transfused with stored RBCs¹¹⁻¹³.

To establish the validity of an ovine model of autologous blood transfusion, Baron and coworkers investigated the pathophysiological effects of transfusing sheep with stored RBCs. Ovine and human blood stored for 40 days prior to transfusion exhibited similar characteristics. In both species less than 1% of the pre-transfused blood was hemolyzed during storage, and by 24 h after transfusion 25% of the transfused RBCs were removed

from the circulation^{11,14}. Transfusion of stored RBCs in sheep was associated with a transiently increased mean pulmonary arterial pressure (PAP) and vascular resistance index (PVRI) during and after transfusion^{11,12}. Recent studies in obese human volunteers with evidence of endothelial dysfunction demonstrated that transfusion of autologous stored RBCs increased the PAP¹³. Thus, both humans and sheep exhibit pulmonary hypertension after receiving stored autologous RBCs. Inhalation of nitric oxide gas, a selective pulmonary vasodilator¹⁵ (as opposed to intravenously administered nitric oxide-releasing drugs that lower both pulmonary and systemic arterial pressures), prevented the pulmonary hypertension associated with transfusion of stored RBCs in humans¹³.

We hypothesized that ex vivo nitric oxide-treatment before transfusion would (1) prevent pulmonary hypertension in lambs during and after transfusion; (2) could improve the survival of stored RBCs in the circulation for up to 7 days after transfusion and (3) would increase the in vitro deformability of stored RBCs. We further hypothesized that washing stored RBCs prior to transfusion might also ameliorate transfusion-associated pulmonary hypertension.

Materials and Methods

Storage and processing of ovine autologous RBCs

All experiments were approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital, Boston, MA. We studied 3- to 4-month-old Polypay lambs, weighing 33 ± 2 kg (mean $\pm$ SD) from a caesarean-derived, specific pathogen-free sheep flock (New England Ovis, Dover, NH). For autologous blood collection, lambs were anesthetized with an intramuscular injection of ketamine (20mg/kg; Hospira, Lake Forest, IL) and whole blood (450ml) was collected from the left jugular vein into a Double Blood-Pack Unit (Fenwal, Inc., Lake Zurich, IL), containing citrate-phosphate-dextrose-adenine (CPDA) solution¹¹. Erythrocytes were leukoreduced using a leukoreduction filter (RS2000, Fenwal, Inc., Lake Zurich, IL), separated from plasma by centrifugation at 2,600 g and resuspended with a solution containing saline, adenine, glucose and mannitol (AS-1, Adsol Solution, Fenwal) to produce a final hematocrit of approximately 60%. Erythrocytes were stored for either 2 or 40 days at 4°C. In this study, RBCs stored for 2 days are designated as “fresh”; RBCs refrigerated for 40 days are considered “stored”. Lambs were transfused over 30 min with one autologous RBC unit (300ml), of either fresh or stored erythrocytes, warmed to 37°C. Assuming blood volume is equal to 6.5% of the animal's weight, the volume of transfused blood was equivalent to 14% of the lamb's total blood volume¹⁶. To evaluate two novel methods of treating RBCs with nitric oxide prior to transfusion, erythrocyte units were treated either by nitric oxide gas exposure or incubation with the nitric oxide-donor Methylaminehexamethylenemethylamine nonoate (MAHMA NONOate). In addition, some lambs were transfused with washed stored RBCs.

Although RBCs stored for 2 days are technically not “fresh”, the design of this study mimicked common hospital practice in which “fresh” RBC units are available in the inventory of a blood bank after appropriate immunological tests are performed, approximately 48-72 h after collection.

Ex vivo exposure of RBCs to nitric oxide

Erythrocytes were passed through an extracorporeal membrane oxygenator (surface area 0.8 m²) (Quadrox iD pediatric, Maquet, Wayne NJ, USA) using a gravity feed with 30 cm H₂O driving pressure, resulting in a blood flow rate of 8-10 ml/min. RBCs were exposed to either 300 ppm nitric oxide (NO) in 90% nitrogen (N₂)/10% oxygen (O₂) or 90% N₂/10% O₂ (control gas) with a gas flow rate of 700 ml/min. Ten percent O₂ in the gas mixture converted deoxy-Hb to oxy-Hb, a moiety that oxidizes met-Hb. Because nitrogen dioxide is produced when nitric oxide is diluted in oxygen¹⁷, a scavenger reservoir containing 90 grams of SodaLime (Biodex, Shirley, NY) was placed immediately before the gas exchanger to remove nitrogen dioxide¹⁸. Nitric oxide gas levels were measured using a nitric oxide chemiluminescence analyzer (Sievers 280i, Boulder, CO). The Sievers 280i with an M&C nitrogen dioxide to nitric oxide converter (AMP Cherokee) was used to measure nitric oxide levels.

As an alternative approach to pre-treating RBCs ex vivo with nitric oxide gas to oxidize plasma free Hb, the nitric oxide donor compound MAHMA NONOate (Cayman Chemical, Ann Arbor, MI, USA) was added to the RBCs. Preliminary in vitro pilot experiments on stored ovine RBCs suggested a 1:4 ratio of cell-free Hb to nitric oxide was needed to reduce nitric oxide-scavenging by cell-free Hb and to improve in vitro RBC flexibility. The average cell-free Hb concentration after 40 days of storage was 100 µM (in terms of heme). Breakdown of MAHMA NONOate produces methylaminehexamethylenemethylamine and 2 nitric oxide molecules. Therefore, RBC units were incubated with MAHMA NONOate at a final concentration of 200 µM.

Either fresh or stored ovine RBCs were incubated for 20 min with 200 µM of the nitric oxide-donor at 25°C. MAHMA NONOate was first reconstituted in ice-cold sodium chloride (0.9%). Because MAHMA NONOate has a short half-life (2-3 min at 25°C), a 20 min RBC incubation period at 25°C was allowed to ensure the release of all nitric oxide from the nitric oxide-donor; no additional nitric oxide was available to be released during and after transfusion. To avoid additional hemolysis that might be associated with mechanical or osmotic stress to erythrocytes, RBCs were not washed after exposure to MAHMA NONOate. The nitric oxide-donor-treated RBC units were transfused immediately after the 20 min incubation period over 30 min in awake lambs. We used the in vitro and in vivo results from experiments using fresh and stored RBC units exposed to the control gas as controls for the MAHMA NONOate study.

Washing stored RBCs prior to transfusion

In some experiments, stored RBCs were washed using the FDA-licensed COBE 2991 blood cell processor (TERUMOBCT, Lakewood, CO). In these studies, an aseptic closed system circuit (COBE 2991 Cell Processing Set, TERUMOBCT, Lakewood, CO) was used to centrifuge a unit of stored RBC (300 ml). After separation of RBCs and plasma, plasma was discarded and one liter of 0.2% Dextrose and 0.9% Sodium Chloride Processing Solution (Baxter, Deerfield, IL) was added to the stored RBCs spun at 3000 rpm/min for one hour. After centrifugation, washing solution was discarded and RBCs were stored in the container until transfused into the lamb.

Animal preparation and hemodynamic monitoring

Anesthesia and surgical procedures, hemodynamic measurements and perioperative care were performed as previously described^{11,12} (see Supplemental Material).

Biochemical analyses of blood samples

Ovine blood samples were obtained through a central venous catheter immediately before, during (10, 20, and 30 min), and after (1 and 4 h) transfusion. Samples (1.5 ml) of transfused autologous RBC units were obtained immediately before transfusion. Extracellular free-Hb concentrations in the transfused RBC supernatant and plasma were measured using a QuantiChrom hemoglobin assay kit (BioAssay Systems, Hayward, CA). The fraction of extracellular met-Hb in the supernatant of the transfusate was determined by spectral deconvolution¹². To measure intracellular met-Hb levels, a sample from the RBC unit was centrifuged (800 g) for 10 min, the supernatant was discarded, and the cell pellet was re-suspended in fresh AS-1 solution. Intracellular met-Hb was measured using an ABL 800 Flex blood gas analyzer (Radiometer Medical, Copenhagen, Denmark). A nitric oxide-consumption assay¹⁹ was performed on samples of circulating plasma during and after transfusion and from the supernatant of the RBC units before and after nitric oxide-treatment (see Supplemental Methods).

Blood gas tensions (PaO_2 , PaCO_2), oxygen saturation of Hb (SaO_2) and pHa were measured in arterial and mixed-venous blood samples with a blood gas analyzer 30 min before transfusion, as well as during transfusion (10, 20, 30 min), and every 30 min up to 4 h thereafter.

Lifespan measurements of transfused RBCs

To determine the half-life of transfused RBCs, biotin was conjugated to fresh or stored RBCs using sulfo-N-hydroxysuccinimide-biotin (10 $\mu\text{g/ml}$ Thermo Fisher Scientific, Rockford, IL). After transfusion, blood samples were obtained at 15, 30, and 60 min, 24 hrs and 7 days after transfusion. A 50 μl aliquot of the blood sample was incubated with fluorescein isothiocyanate-conjugated streptavidin (Biolegend, San Diego, CA) and analyzed by flow cytometry to determine the fraction of biotinylated circulating RBCs present in each sample. The fraction of biotinylated RBCs present immediately after transfusion (time 0) was determined by extrapolation from measurements at 15 and 30 min. The ratio of the percentage of biotinylated erythrocytes in the samples obtained after transfusion to the percentage of biotinylated erythrocytes calculated to be present at time 0 was determined and represents the number of transfused RBCs that remain in the circulation at each sampling time. The method is free of an effect of volume of blood infused because we measured the decrease in percentage of transfused (to total number of) circulating cells over time. Error in the percentage at the zero time point can be estimated by adding the errors at the 15 and 30 minutes time points in quadrature²⁰. In our study, the standard error of the zero time point is 1.7-2.7% (out of 100%) for the four groups. We believe this to be a reasonable amount of error, and less than the significance threshold of 5%.

Indirect measurement of the nitric oxide-treated RBC deformability using microfluidics

The microfluidic device used in these studies is an optimized design of the device described by Bow et al²¹. RBCs flow through a microchannel (1 mm wide and 5 mm long), which distributes RBCs via an inverted trapezoidal flow entrance into three periodic sets of rectangular capillaries. The cross section of the capillaries or “slits” is 5 μm high and 2 μm wide. “Individual transit RBC velocity” is defined as the velocity of the RBC within each slit, averaged over three periodic slit sets. Average velocity for each sample is comprised of the average of 134 individual RBC transit velocities. The transit velocity of individual RBCs is an indirect measure of RBC deformability²¹. A higher transit velocity through the capillaries indicates greater deformability.

Statistical analyses

All data are expressed as mean $\pm$ SD. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). Variables were tested for normality using the Shapiro-Wilk test. Comparisons of the change in cell-free Hb, nitric oxide consumption, and met-Hb in nitric oxide-treated RBCs versus control-treated RBCs were performed using either an unpaired *t*-test or Mann-Whitney test, as appropriate (Figure 1 and Supplemental Figure 1, $p < 0.025$ was considered significant when adjusting for multiple comparisons). For *in vivo* transfusion experiments (Figure 2 and Supplemental Figure 2), initially a repeated measures ANOVA was performed. However, because the interaction between time and treatment group was significant ($p < 0.05$), a one-way ANOVA with Bonferroni-adjusted post-hoc testing at each time point was performed. Since 5 comparisons were made at each time point, a p -value < 0.01 was considered significant. Changes in circulating biotinylated RBCs over time (Figure 3, and Supplemental Figure 3) were compared between groups using a repeated measures ANOVA. However, because the interaction between time and treatment was significant ($p < 0.05$), a one-way ANOVA with Bonferroni-adjusted post-hoc testing was performed at each time point. Two comparisons were made at each time point, so the Bonferroni adjusted p -value of significance was < 0.025 . Comparison of RBC transit velocity between groups (Figure 4 and Supplemental Figure 4) was performed using a one-way ANOVA with Bonferroni-adjusted post-hoc testing. Since 6 comparisons were made, a p -value < 0.008 was considered significant. When comparing washed SRBCs versus control FRBCs (Figure 5), a Mann-Whitney test was performed with threshold p -value of < 0.05 . To compare changes in circulating biotinylated stored washed RBCs over time (Figure 6), a one-way ANOVA with Bonferroni-adjusted post-hoc testing was performed at each time point. Two comparisons were made at each time point, so the Bonferroni adjusted p -value of significance was < 0.025 . For each test, exact unadjusted p values are provided in the figures. Coefficients of variation (CV) were determined for the following assays: 4.0% for cell-free Hb in RBC units, 3.4% for cell-free Hb in plasma, 5.5% for nitric oxide consumption in RBC units, and 5.6% for nitric oxide consumption in plasma, indicating adequate reproducibility. Measurements of biotinylated RBCs were highly reproducible at the 15 min and 30 min time points with technical replicates exhibiting a coefficient of variation of 3.4%.

Power analysis

The primary endpoint is to compare the change in PAP from baseline to post-transfusion in the control-treated stored RBC group versus the nitric oxide-treated stored RBC group. The anticipated change in PAP in the control-treated stored RBC group is 5 ± 1.4 mmHg (mean $\pm$ SD) whereas the expected change in the nitric oxide-treated stored RBC group is 2 ± 1.4 mmHg (mean $\pm$ SD), based on previously published work^{11,12}. Using a level of significance of 0.05, we determined that at least 6 animals per group were needed to obtain a power of >96% to detect a difference. Levels of significance of 0.025 and 0.01 leads to a power of 92.9% and 87.2% respectively when 6 animals per group are used.

Results

Transient exposure of stored blood to nitric oxide reduces nitric oxide consumption by extracellular Hb

To determine whether in vitro treatment of stored RBCs with nitric oxide could reduce scavenging by extracellular Hb, RBC units were exposed to nitric oxide and the ability of supernatant to scavenge nitric oxide was measured. In pilot studies we examined the effect of different nitric oxide gas levels or MAHMA NONOate doses on either outdated stored human RBC units obtained from the blood bank (nitric oxide gas) or outdated stored ovine RBCs (MAHMA NONOate) (data not shown). We identified the optimal nitric oxide doses that produced low intracellular met-Hb and high extracellular met-Hb levels. Also, nitric oxide-consumption by extracellular Hb was markedly reduced. Comparisons were performed on the change in cell-free Hb, nitric oxide consumption, and met-Hb in nitric oxide-treated RBCs versus control-treated RBCs.

Cell-free Hb levels in fresh and stored RBC supernatants were measured before and after nitric oxide-treatment. The amount of extracellular Hb in the supernatant was greater in stored as compared to fresh RBC units (Figure 1A, increase in extracellular Hb: 104.9 ± 67.09 μ M vs. 15.86 ± 5.38 μ M, $p=0.0002$). Pre-treatment of fresh or stored RBCs with 300 ppm NO did not alter supernatant cell-free Hb levels.

A nitric oxide-consumption assay¹⁹ was used to measure the nitric oxide-scavenging capacity of extracellular Hb in the supernatant before and after exposing RBC units to nitric oxide in a gas exchanger. The supernatant from fresh RBC units scavenged less nitric oxide than the supernatant from stored RBC units. Exposure of stored RBC units to nitric oxide gas reduced the nitric oxide-consumption of the stored supernatant when compared to treatment with the control gas, which did not contain nitric oxide (Figure 1B, reduction of nitric oxide-consumption: 80.1 ± 70.1 μ M vs. 7.4 ± 10.2 μ M, respectively, $p=0.005$), whereas nitric oxide exposure of fresh RBC units did not reduce plasma nitric oxide-consumption.

Exposure of RBCs to nitric oxide gas increased the percentage of extracellular methemoglobin (met-Hb) in fresh and stored blood units when compared to fresh controls (Figure 1C, increase of extracellular met-Hb by $64.45 \pm 14.7\%$ vs. $2.13 \pm 2.14\%$, $p=0.0004$) or stored controls respectively (Figure 1C, increase of extracellular met-Hb by $75.54 \pm 14.47\%$ vs. $2.39 \pm 2.9\%$, $p<0.0001$).

The intracellular percentage of met-Hb was measured before and after nitric oxide-treatment to determine whether RBC oxygen transport capacity was reduced after nitric oxide exposure. Intracellular RBC met-Hb levels were elevated to 12% in the RBC unit when either fresh (Figure 1D, increase of intracellular met-Hb by $10.31 \pm 3.79\%$ vs. $0.05 \pm 0.06\%$, $p=0.0004$) or stored RBCs were transiently exposed to 300 ppm NO (Figure 1D, increase of intracellular met-Hb by $9.19 \pm 2.3\%$ vs. $0.13 \pm 0.13\%$, $p<0.0001$). Incubation of nitric oxide-treated RBCs at 37°C for two hours decreased the intracellular met-Hb by 50% and met-Hb returned to baseline levels 12 h after nitric oxide-treatment (data not shown).

Taken together, these results show that exposing RBCs to 300 ppm NO gas in a gas exchanger did not cause RBC hemolysis. Exposure to nitric oxide gas reduced nitric oxide-scavenging presumably by converting extracellular oxy-Hb to met-Hb. The level of intracellular met-Hb during incubation increased to 12% and returned to baseline upon incubation after 12 hours, however nitric oxide-treatment did not significantly alter the RBC oxygen transport capacity of the recipient lamb's blood (due to a 7 fold dilution with untreated circulating RBCs).

Exposure of stored RBCs to nitric oxide prevents transfusion-associated pulmonary hypertension in awake lambs

To determine whether treatment of stored RBCs with nitric oxide prevents transfusion-associated pulmonary hypertension, the hemodynamic consequences of transfusing nitric oxide-treated fresh and stored autologous RBCs were assessed in the pulmonary circulation of awake sheep. Lambs receiving stored RBCs had an increased PAP during and after transfusion to a maximum of 22.7 ± 2.2 mmHg from a baseline of 13.4 ± 0.8 mmHg (Figure 2A). In contrast, transfusion of stored RBCs that were pre-treated with 300 ppm NO gas did not increase the PAP when compared to control-treated fresh and nitric oxide-treated fresh RBCs during and after the transfusion (Figure 2A, PAP at 20 min of SRBC+NO: 14.5 ± 1.4 mmHg, FRBC+O₂: 13.9 ± 0.6 mmHg, FRBC+NO: 14 ± 1.2 mmHg, $p>0.01$). Transfusion of fresh RBCs, whether treated ex vivo with or without nitric oxide gas, did not alter the PAP during or after transfusion. To determine whether increases of PAP in lambs receiving stored RBCs treated with control gas were caused by vasoconstriction, the PVRI was measured before, during and after transfusion. Transfusion of stored RBCs caused a transient increase in the PVRI from 10 to 30 min after commencing transfusion. Pre-treatment of stored RBCs with nitric oxide gas prevented the increase in PVRI when compared to control-treated stored RBCs (Figure 2B, PVRI at 20 min of 114.6 ± 18.9 dyn $\cdot\text{sec}\cdot\text{cm}^{-5}\cdot\text{m}^{-2}$ vs. 211.1 ± 44.4 dyn $\cdot\text{sec}\cdot\text{cm}^{-5}\cdot\text{m}^{-2}$, $p<0.0001$). Transfusion of fresh RBCs, with or without nitric oxide exposure, did not alter the PVRI. These results demonstrate that ex vivo treatment of stored blood with 300 ppm NO prevents pulmonary hypertension and vasoconstriction during transfusion of stored blood.

Previous studies suggested that transfusion of stored RBCs reduced the bioavailability of nitric oxide in the sheep's pulmonary vasculature and thereby induced pulmonary vasoconstriction^{11,12}. To determine whether exposure of stored RBCs to nitric oxide could reduce nitric oxide-consumption by plasma Hb during and after transfusion, the levels of circulating plasma Hb and plasma nitric oxide-consumption were measured. Plasma Hb

concentrations were greater in sheep transfused with stored, compared with fresh RBCs at 10, 20, 30 and 60 min after commencing transfusion. Transfusing nitric oxide gas-treated or control gas-treated stored RBCs increased the level of plasma free Hb to a similar extent. Transfusing either nitric oxide gas-treated or control gas-treated fresh RBCs did not increase the circulating level of plasma free Hb.

Transfusion of stored RBCs increased the consumption of nitric oxide by circulating plasma Hb at 10, 20, 30, and 60 min after commencing transfusion as compared to animals transfused with fresh RBCs (Figure 2D, nitric oxide-consumption at 30 min of $15.6 \pm 8.2 \mu\text{M}$ vs. $3.1 \pm 1.8 \mu\text{M}$, $p=0.0003$). Pre-treatment of stored RBCs with nitric oxide gas resulted in significantly less plasma nitric oxide-consumption between 10 and 60 min after commencing transfusion when compared to transfusion of control gas treated stored RBCs (Figure 2D, nitric oxide-consumption at 30 min of 5.6 ± 3.6 vs. $15.6 \pm 8.2 \mu\text{M}$, $p=0.0003$). Plasma nitric oxide-consumption levels did not increase when fresh RBCs were transfused after treatment with either nitric oxide or control gas.

These results show that exposure of either fresh or stored RBCs to nitric oxide gas does not increase circulating plasma free Hb levels after transfusion. Treatment of stored RBCs with nitric oxide gas prior to transfusion reduces scavenging of nitric oxide by plasma Hb.

Pre-treatment with nitric oxide gas improves the survival of stored RBCs 1 and 24 h after transfusion

The survival of stored RBCs circulating after transfusion is markedly reduced when compared to the survival of fresh RBCs¹¹. To investigate the potential beneficial effect of nitric oxide gas-treatment on stored RBC survival after transfusion, we biotin-labeled fresh and stored RBCs, exposed them to 300 ppm NO gas and measured the fraction of biotinylated RBCs that remained in the circulation for up to 7 days. Blood samples were collected at 15, 30, 60 min, 24 h, and 7 days after transfusion and the ratio of biotinylated RBCs to total RBCs was measured by flow cytometry.

When fresh RBCs were transfused into sheep, $94.2 \pm 4.6\%$ of the RBCs remained in the circulation at 1 h after transfusion (Figure 3). In contrast, the percentage of circulating stored RBCs treated with control gas (without nitric oxide) was reduced to $75.3 \pm 5.8\%$ at 1 h after transfusion. In sheep transfused with stored RBCs pre-treated with NO gas, $86.8 \pm 8.1\%$ of biotinylated RBCs remained in the circulation 1 hour after transfusion. After 24 h, $90.8 \pm 4.1\%$ of control- and $91.4 \pm 1.4\%$ of nitric oxide gas-treated fresh RBCs remained in the circulation, while $78.3 \pm 6.3\%$ of nitric oxide gas-treated stored RBCs and $73.4 \pm 3.8\%$ of control gas-treated stored RBCs were circulating. Seven days after transfusion, there was no difference in the percentage of nitric oxide-treated, as compared to control-treated, stored RBCs remaining in the circulation. These results show that nitric oxide gas-treatment improved early stored RBC survival measured at 1 and 24 h after transfusion.

Pre-treatment of stored RBCs with nitric oxide gas may increase RBC deformability

To determine whether nitric oxide gas-treatment increases RBC deformability, we used a customized microfluidic device to indirectly measure RBC deformability as the RBC transit velocity through the device²¹. The average transit velocity of fresh ovine RBCs, whether

treated with nitric oxide gas or control gas, was greater than the average RBC transit velocity of stored RBCs (Figure 4). The average RBC transit velocity of nitric oxide gas-treated, stored RBCs was greater than that of control gas-treated, stored RBCs (Figure 4, RBC transit velocity of $103 \pm 5 \mu\text{m/s}$ vs. $94 \pm 3 \mu\text{m/s}$, $p=0.006$). These findings suggest that ex vivo exposure of stored RBCs to nitric oxide may increase RBC deformability.

The effects of treating stored RBCs with MAHMA NONOate prior to transfusion in awake lambs

Treatment of stored RBCs, prior to transfusion, with the nitric oxide-donor compound MAHMA NONOate was studied as an alternative approach to nitric oxide gas exposure of stored RBCs. Incubating either fresh or stored RBC with MAHMA NONOate had similar effects on the oxidation of supernatant cell-free oxy-Hb to met-Hb, in vitro nitric oxide consumption, intracellular and extracellular met-Hb levels as exposing fresh or stored RBCs to nitric oxide gas (Supplemental Digital Content 1, Figure 1A-D, which presents cell-free Hb levels, nitric oxide-consumption of extracellular Hb, extracellular and intracellular met-Hb percent before and after MAHMA NONOate treatment). In addition, transfusion of stored MAHMA NONOate-treated RBCs prevented transfusion-associated pulmonary hypertension and pulmonary vasoconstriction and reduced plasma nitric oxide consumption without causing systemic vasodilation (Supplemental Digital content 1, Figure 2 A-D which presents the mean pulmonary arterial pressure, pulmonary vascular resistance index, plasma hemoglobin levels, and plasma nitric oxide-consumption before, during and after transfusion of RBC units treated with $200 \mu\text{M}$ MAHMA NONOate). Similar to nitric oxide gas exposure before transfusion, pre-treatment of stored RBCs with MAHMA NONOate improved the 1 and 24 h RBC survival, but there was no difference in stored RBC lifespan at 7 days after transfusion (Supplemental Digital Content 1, Figure 3, which shows the lifespan of circulating biotinylated fresh (FRBC) or stored erythrocytes (SRBC), treated with $200 \mu\text{M}$ MAHMA NONOate). Furthermore, as with exposure to nitric oxide gas, ex vivo exposure of stored RBCs to a chemical nitric oxide-donor increased the indirectly measured RBC deformability (Supplemental Digital Content 1, Figure 4 which is a indirect measure of the erythrocyte deformability).

Washing stored RBCs prior to transfusion does not prevent transfusion-associated pulmonary hypertension in awake lambs

Thus far in this study, we have shown that ex vivo exposure of stored RBCs to nitric oxide reduced nitric oxide-consumption by extracellular Hb and prevented transfusion-associated pulmonary hypertension. We then considered the possibility that removing extracellular Hb by washing stored RBCs, using standard blood banking techniques, would be as effective as nitric oxide-treatment.

Transfusion of washed, stored RBCs increased the PAP to a maximum of $18.4 \pm 2.1 \text{ mmHg}$ at 30 min after starting the transfusion when compared to fresh RBCs (Figure 5A, PAP at 30 min of $18.4 \pm 2.1 \text{ mmHg}$ vs. $13.9 \pm 0.6 \text{ mmHg}$, $p=0.02$). In addition, transfusion of washed, stored RBCs caused a significant increase in PVRI compared to fresh RBCs beginning at 20 min after commencing transfusion (Figure 5B, PVRI at 20 min of $153.8 \pm 12.5 \text{ dyn}\cdot\text{sec}\cdot\text{cm}^{-5}\cdot\text{m}^{-2}$ vs. $107.5 \pm 17.8 \text{ dyn}\cdot\text{sec}\cdot\text{cm}^{-5}\cdot\text{m}^{-2}$, $p=0.02$). Washing stored RBCs did

not prevent the transfusion-associated increase in plasma hemoglobin (Figure 5C, Hb in plasma at 30 min of $11.7 \pm 6.0 \mu\text{M}$ vs. $2.5 \pm 1.6 \mu\text{M}$, $p=0.03$). Similarly, plasma NO consumption increased 20 min after starting the transfusion of washed, stored RBCs when compared to the transfusion of fresh RBCs (Figure 5D, plasma NO-consumption at 20 min of $10.9 \pm 9.9 \mu\text{M}$ vs. $1.7 \pm 0.8 \mu\text{M}$, $p=0.03$). These findings demonstrate that washing stored RBCs prior to transfusion did not eliminate the adverse effects associated with the transfusion of stored RBCs.

Discussion

We evaluated two novel methods of treating stored ovine RBC units to prevent transfusion-associated pulmonary vasoconstriction and hypertension and increase the survival of stored RBCs. Stored RBC units were treated *ex vivo* with nitric oxide either by exposing the RBCs to 300 ppm NO gas using a membrane gas exchanger or by a brief incubation with the short-lived nitric oxide donor MAHMA NONOate. We report that nitric oxide-treatment with either nitric oxide gas or MAHMA NONOate prevented nitric oxide-scavenging by extracellular Hb in the stored RBC unit supernatant. Nitric oxide-treatment of stored RBCs by either method prevented pulmonary vasoconstriction and hypertension during and after transfusion, and did not cause systemic vasodilation. Both nitric oxide-treatments increased the 1 and 24 h survival rate of transfused stored RBCs.

Erythrocyte viability is reduced following extended storage and leads to the release of Hb into the RBC unit supernatant^{8,22}. Donadee and co-workers reported increased extracellular Hb in stored RBC units after 30 days of storage compared to 4 days of storage⁹. Circulating cell-free Hb and Hb-containing microvesicles scavenge nitric oxide, which is generated by endothelial cells. Nitric oxide-scavenging by extracellular Hb appears to be the cause of the pulmonary vasoconstriction that occurs after transfusion of stored blood into obese human volunteers¹³.

Inhaled nitric oxide is a selective pulmonary vasodilator that has been used for over two decades to treat pulmonary hypertension of the newborn and other cardiopulmonary diseases¹⁵. Baron and coworkers reported that *inhaled* nitric oxide prevented pulmonary vasoconstriction and hypertension in sheep transfused with stored autologous RBCs^{11,12}. We attempted to simplify and better understand this treatment strategy by exposing the stored RBC units *ex vivo* to nitric oxide gas or a nitric oxide-donor molecule. We studied whether pulmonary hypertension and pulmonary vasoconstriction could be prevented without requiring the transfusion recipient to breathe nitric oxide.

In this study, we observed that intracellular met-Hb levels increased up to 12% after the *ex vivo* treatment with nitric oxide gas but returned to baseline levels after 12h. Because the intracellular met-Hb in the RBC unit was subsequently diluted 7-fold in the lamb's total circulating blood volume, we estimate that the final concentration of met-Hb in the animal was approximately 1.7%.

The transient *in vitro* exposure of either fresh or stored blood to either nitric oxide gas or MAHMA NONOate did not harm the stored RBCs; there was no increase in RBC hemolysis

nor was there a reduction in RBC lifespan after transfusion. There was an important reduction of nitric oxide-consumption by the RBC supernatant fluid when stored RBC units were treated with nitric oxide, which may be attributable to the conversion of extracellular oxy-Hb to met-Hb. Met-Hb is unable to scavenge nitric oxide²³. Inhibition of nitric oxide-scavenging by oxidation of extracellular ferrous Hb is likely to be the underlying mechanism by which pre-treatment with nitric oxide prevents transfusion-associated pulmonary vasoconstriction and hypertension^{11-13,24,25}.

Transfusion of stored RBCs increases the pulmonary artery pressure in sheep, dogs and humans^{11-13,26}. The increase in PAP after transfusion is more pronounced when endothelial dysfunction (caused by systemic diseases such as diabetes, or cardiopulmonary bypass) reduces nitric oxide bioavailability^{11,13,27}. In addition, stored RBCs themselves may have inhibitory effects on nitric oxide-mediated vasodilation through nitric oxide scavenging²⁸⁻³⁰. Solomon and co-workers demonstrated in an exchange transfusion model in septic dogs that transfusion of stored blood produces persistent pulmonary hypertension that was linked to severe lung damage including necrosis, hemorrhage and thrombosis²⁶. Increased PAP raises transcapillary pressure and can enhance the development of alveolar edema^{31,32}. An increased risk of acute respiratory distress syndrome after transfusion of stored RBCs has been reported³³. Treating stored RBCs with nitric oxide may improve the safety of stored RBC transfusion.

When RBCs traverse small capillaries, they must change shape³⁴. However, extended storage decreases RBC deformability^{8,22}. During storage, erythrocyte membrane permeability to calcium (Ca^{2+}) ions increases over time, leading to increased intracellular Ca^{2+} levels. Intracellular Ca^{2+} activates calcium-sensitive KCa3.1 ion channels, which are located in the erythrocyte membrane³⁵. The resulting potassium ion efflux causes RBCs to shrink and makes their cell membranes less flexible³⁶. Previous studies showed that nitric oxide has a crucial role in enhancing RBC deformability and that reduced RBC nitric oxide levels decrease deformability^{35,37}. Loss of RBC deformability may lead to an increased rate of intravascular hemolysis and a reduced lifespan of transfused stored RBCs. In this study, we used a microfluidic device to measure the in vitro transit velocity of nitric oxide-treated RBCs. The transit velocity of RBCs through the synthetic capillaries in this device is an indirect measure of RBC deformability²¹. When stored ovine RBCs were treated with nitric oxide (either nitric oxide gas or MAHMA NONOate), their transit velocity was increased as compared to untreated stored RBCs. Other studies have described similar improvement in RBC deformability with nitric oxide-donor treatment^{37,38}. Our results suggest that nitric oxide-treatment augments the deformability of RBCs. The precise mechanism by which nitric oxide-treatment increases deformability is unknown.

Recent evidence suggests that washing stored RBCs, using standard blood banking methods, prior to transfusion might be another strategy to prevent the adverse effects of transfusing stored blood³⁹. Washing stored RBCs might remove extracellular Hb and thereby prevent transfusion-associated pulmonary hypertension and vasoconstriction. In this study, washing stored blood delayed, but did not prevent pulmonary vasoconstriction during transfusion. Although there is likely to be less extracellular Hb at the beginning of transfusion, subsequent increased intravascular hemolysis may explain the delayed pulmonary

hypertension. The reduction in RBC lifespan of washed stored RBCs compared to fresh and untreated stored RBCs, and the corresponding increase in plasma Hb, support this hypothesis.

In this study, normovolemic lambs were transfused with RBCs, making this a “top-load” transfusion model. The transfusion of RBCs, representing a 14% increase in the lambs total blood volume, did not appear to affect the pulmonary vasculature. In lambs receiving fresh RBC transfusion, the increased volume did not alter either PAP or PVRI. However, because we used a “top-load” transfusion model, we cannot completely exclude the possibility that some of the effects of stored blood transfusion were the result of combined effects of stored blood and increased blood volume.

A limitation of this study is that MAHMA NONOate is not an FDA-approved drug and the potential toxicity of the donor residual molecule (MAHMA) is unknown and would need to be studied before testing in humans. A second potential limitation is that the RBC storage lesion of laboratory animals and humans is similar but not identical. We demonstrated in a previous study that the oxygen affinity of ovine RBCs did not change during storage (as opposed to human RBCs) and that ovine RBC 2,3 DPG levels were lower than in humans¹¹. However, with the exception of the P₅₀ and 2,3 DPG concentrations, ovine RBCs have similar properties to those measured in human RBCs when both are stored for 40 days, including similar cell free hemoglobin levels *in vitro*. The *in vivo* lifespan of ovine erythrocytes is closer to that of humans (100-120 days) than that of mice (55-60 days)⁴⁰⁻⁴². In this study, the mean 24h post-transfusion survival rate of ovine RBCs stored in a standard preservative (AS-1 solution) was lower than the survival rate of human RBCs (73±1% at 24h after transfusion in lambs vs. 82.1±7% in humans at 24h)¹⁴.

In summary, we have developed and tested two methods for treating 40 day stored ovine RBC units with nitric oxide prior to transfusion to prevent transfusion-associated pulmonary hypertension. We found that transiently exposing stored blood to either nitric oxide gas or a nitric oxide-donor compound reduced nitric oxide-scavenging by cell-free Hb, prevented pulmonary vasoconstriction and hypertension, and increased *in vitro* RBC velocity, an indirect measure of RBC deformability. In addition, nitric oxide exposure increased the survival of stored ovine RBCs at 1 and 24 hours after starting transfusion. Further studies with human stored RBCs are required to confirm the beneficial effects of *ex vivo* nitric oxide exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Reference List

1. The U.S. Department of Health and Human Services. [July 10, 2016] National Blood Collection and Utilization Survey Report. 2011. Available from: <http://www.hhs.gov/ash/bloodsafety/nbcus/>
2. Steiner ME, Ness PM, Assmann SF, Triulzi DJ, Sloan SR, Delaney M, Granger S, Bennett-Guerrero E, Blajchman MA, Scavo V, Carson JL, Levy JH, Whitman G, D'Andrea P, Pulkrabek S, Ortel TL, Bornikova L, Raife T, Puca KE, Kaufman RM, Nuttall GA, Young PP, Youssef S, Engelman R, Greulich PE, Miles R, Josephson CD, Bracey A, Cooke R, McCullough J, Hunsaker R, Uhl L, McFarland JG, Park Y, Cushing MM, Klodell CT, Karanam R, Roberts PR, Dyke C, Hod EA, Stowell CP. Effects of red-cell storage duration on patients undergoing cardiac surgery. *N Engl J Med*. 2015; 372:1419–29. [PubMed: 25853746]
3. Wang D, Sun J, Solomon SB, Klein HG, Natanson C. Transfusion of older stored blood and risk of death: a meta-analysis. *Transfusion*. 2012; 52:1184–95. [PubMed: 22188419]
4. Lacroix J, Hebert PC, Fergusson DA, Tinmouth A, Cook DJ, Marshall JC, Clayton L, McIntyre L, Callum J, Turgeon AF, Blajchman MA, Walsh TS, Stanworth SJ, Campbell H, Capellier G, Tiberghien P, Bardiaux L, van de Wetering L, van der Meer NJ, Sabri E, Vo D, Investigators A. Canadian Critical Care Trials G: Age of transfused blood in critically ill adults. *N Engl J Med*. 2015; 372:1410–8. [PubMed: 25853745]
5. Klein HG, Cortes-Puch I, Natanson C. More on the Age of Transfused Red Cells. *N Engl J Med*. 2015; 373:283.
6. Vandromme MJ, McGwin G Jr, Weinberg JA. Blood transfusion in the critically ill: does storage age matter? *Scand J Trauma Resusc Emerg Med*. 2009; 17:35. [PubMed: 19678943]
7. Relevy H, Koshkaryev A, Manny N, Yedgar S, Barshtein G. Blood banking-induced alteration of red blood cell flow properties. *Transfusion*. 2008; 48:136–46. [PubMed: 17900281]
8. Bennett-Guerrero E, Veldman TH, Doctor A, Telen MJ, Ortel TL, Reid TS, Mulherin MA, Zhu H, Buck RD, Califf RM, McMahon TJ. Evolution of adverse changes in stored RBCs. *Proc Natl Acad Sci U S A*. 2007; 104:17063–8. [PubMed: 17940021]
9. Donadee C, Raat NJ, Kanas T, Tejero J, Lee JS, Kelley EE, Zhao X, Liu C, Reynolds H, Azarov I, Frizzell S, Meyer EM, Donnenberg AD, Qu L, Triulzi D, Kim-Shapiro DB, Gladwin MT. Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. *Circulation*. 2011; 124:465–76. [PubMed: 21747051]
10. Berezina TL, Zaets SB, Morgan C, Spillert CR, Kamiyama M, Spolarics Z, Deitch EA, Machiedo GW. Influence of storage on red blood cell rheological properties. *J Surg Res*. 2002; 102:6–12. [PubMed: 11792145]
11. Baron DM, Yu B, Lei C, Bagchi A, Beloiartsev A, Stowell CP, Steinbicker AU, Malhotra R, Bloch KD, Zapol WM. Pulmonary hypertension in lambs transfused with stored blood is prevented by breathing nitric oxide. *Anesthesiology*. 2012; 116:637–47. [PubMed: 22293717]
12. Baron DM, Beloiartsev A, Nakagawa A, Martyn T, Stowell CP, Malhotra R, Mayeur C, Bloch KD, Zapol WM. Adverse effects of hemorrhagic shock resuscitation with stored blood are ameliorated by inhaled nitric oxide in lambs*. *Crit Care Med*. 2013; 41:2492–501. [PubMed: 23887236]
13. Berra L, Pinciroli R, Stowell CP, Wang L, Yu B, Fernandez BO, Feelisch M, Mietto C, Hod EA, Chipman D, Scherrer-Crosbie M, Bloch KD, Zapol WM. Autologous transfusion of stored red blood cells increases pulmonary artery pressure. *Am J Respir Crit Care Med*. 2014; 190:800–7. [PubMed: 25162920]
14. Dumont LJ, AuBuchon JP. Evaluation of proposed FDA criteria for the evaluation of radiolabeled red cell recovery trials. *Transfusion*. 2008; 48:1053–60. [PubMed: 18298603]
15. Frostell C, Fratacci MD, Wain JC, Jones R, Zapol WM. Inhaled nitric oxide. A selective pulmonary vasodilator reversing hypoxic pulmonary vasoconstriction. *Circulation*. 1991; 83:2038–47. [PubMed: 2040056]
16. Hansard SL. Residual organ blood volume of cattle, sheep and swine. *Proc Soc Exp Biol Med*. 1956; 91:31–4. [PubMed: 13297700]

17. Sokol GM, Van Meurs KP, Wright LL, Rivera O, Thorn WJ 3rd, Chu PM, Sams RL. Nitrogen dioxide formation during inhaled nitric oxide therapy. *Clin Chem*. 1999; 45:382–7. [PubMed: 10053039]
18. Weimann J, Hagenah JU, Motsch J. Reduction in nitrogen dioxide concentration by soda lime preparations during simulated nitric oxide inhalation. *Br J Anaesth*. 1997; 79:641–4. [PubMed: 9422905]
19. Wang X, Tanus-Santos JE, Reiter CD, Dejam A, Shiva S, Smith RD, Hogg N, Gladwin MT. Biological activity of nitric oxide in the plasmatic compartment. *Proc Natl Acad Sci U S A*. 2004; 101:11477–82. [PubMed: 15258287]
20. Wake Forest University. [July 10, 2016] Handout on Statistics. Available from: <http://www.users.wfu.edu/ecarlson/skeptic/statistics.pdf>
21. Bow H, Pivkin IV, Diez-Silva M, Goldfless SJ, Dao M, Niles JC, Suresh S, Han J. A microfabricated deformability-based flow cytometer with application to malaria. *Lab Chip*. 2011; 11:1065–73. [PubMed: 21293801]
22. Almizraq R, Tchir JD, Holovati JL, Acker JP. Storage of red blood cells affects membrane composition, microvesiculation, and in vitro quality. *Transfusion*. 2013; 53:2258–67. [PubMed: 23320518]
23. Yu B, Lei C, Baron DM, Steinbicker AU, Bloch KD, Zapol WM. Diabetes augments and inhaled nitric oxide prevents the adverse hemodynamic effects of transfusing syngeneic stored blood in mice. *Transfusion*. 2012; 52:1410–22. [PubMed: 22233290]
24. Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO 3rd, Schechter AN, Gladwin MT. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med*. 2002; 8:1383–9. [PubMed: 12426562]
25. Minneci PC, Deans KJ, Zhi H, Yuen PS, Star RA, Banks SM, Schechter AN, Natanson C, Gladwin MT, Solomon SB. Hemolysis-associated endothelial dysfunction mediated by accelerated NO inactivation by decompartmentalized oxyhemoglobin. *J Clin Invest*. 2005; 115:3409–17. [PubMed: 16294219]
26. Solomon SB, Wang D, Sun J, Kanas T, Feng J, Helms CC, Solomon MA, Alimchandani M, Quezado M, Gladwin MT, Kim-Shapiro DB, Klein HG, Natanson C. Mortality increases after massive exchange transfusion with older stored blood in canines with experimental pneumonia. *Blood*. 2013; 121:1663–72. [PubMed: 23255558]
27. Wessel DL, Adatia I, Giglia TM, Thompson JE, Kulik TJ. Use of inhaled nitric oxide and acetylcholine in the evaluation of pulmonary hypertension and endothelial function after cardiopulmonary bypass. *Circulation*. 1993; 88:2128–38. [PubMed: 8222107]
28. Stapley R, Rodriguez C, Oh JY, Honavar J, Brandon A, Wagener BM, Marques MB, Weinberg JA, Kerby JD, Pittet JF, Patel RP. Red blood cell washing, nitrite therapy, and antiheme therapies prevent stored red blood cell toxicity after trauma-hemorrhage. *Free Radic Biol Med*. 2015; 85:207–18. [PubMed: 25933588]
29. Roback JD, Neuman RB, Quyyumi A, Sutliff R. Insufficient nitric oxide bioavailability: a hypothesis to explain adverse effects of red blood cell transfusion. *Transfusion*. 2011; 51:859–66. [PubMed: 21496047]
30. Neuman R, Hayek S, Rahman A, Poole JC, Menon V, Sher S, Newman JL, Karatela S, Polhemus D, Lefer DJ, De Staercke C, Hooper C, Quyyumi AA, Roback JD. Effects of storage-aged red blood cell transfusions on endothelial function in hospitalized patients. *Transfusion*. 2015; 55:782–90. [PubMed: 25393772]
31. Ochoa CD, Stevens T. Studies on the cell biology of interendothelial cell gaps. *Am J Physiol Lung Cell Mol Physiol*. 2012; 302:L275–86. [PubMed: 21964402]
32. Chetham PM, Babal P, Bridges JP, Moore TM, Stevens T. Segmental regulation of pulmonary vascular permeability by store-operated Ca²⁺ entry. *Am J Physiol*. 1999; 276:L41–50. [PubMed: 9887054]
33. Zilberberg MD, Carter C, Lefebvre P, Raut M, Vekeman F, Duh MS, Shorr AF. Red blood cell transfusions and the risk of acute respiratory distress syndrome among the critically ill: a cohort study. *Crit Care*. 2007; 11:R63. [PubMed: 17553147]

34. Guest MM, Bond TP, Cooper RG, Derrick JR. Red Blood Cells: Change in Shape in Capillaries. *Science*. 1963; 142:1319–21. [PubMed: 14074848]
35. Barodka V, Mohanty JG, Mustafa AK, Santhanam L, Nyhan A, Bhunia AK, Sikka G, Nyhan D, Berkowitz DE, Rifkind JM. Nitroprusside inhibits calcium-induced impairment of red blood cell deformability. *Transfusion*. 2014; 54:434–44. [PubMed: 23781865]
36. Romero JR, Fabry ME, Suzuka S, Nagel RL, Canessa M. Red blood cells of a transgenic mouse expressing high levels of human hemoglobin S exhibit deoxy-stimulated cation flux. *J Membr Biol*. 1997; 159:187–96. [PubMed: 9312208]
37. Bor-Kucukatay M, Wenby RB, Meiselman HJ, Baskurt OK. Effects of nitric oxide on red blood cell deformability. *Am J Physiol Heart Circ Physiol*. 2003; 284:H1577–84. [PubMed: 12521942]
38. Horn P, Cortese-Krott MM, Keymel S, Kumara I, Burghoff S, Schrader J, Kelm M, Kleinbongard P. Nitric oxide influences red blood cell velocity independently of changes in the vascular tone. *Free Radic Res*. 2011; 45:653–61. [PubMed: 21480762]
39. Cortes-Puch I, Wang D, Sun J, Solomon SB, Remy KE, Fernandez M, Feng J, Kanas T, Bellavia L, Sinchar D, Perlegas A, Solomon MA, Kelley WE, Popovsky MA, Gladwin MT, Kim- Shapiro DB, Klein HG, Natanson C. Washing older blood units before transfusion reduces plasma iron and improves outcomes in experimental canine pneumonia. *Blood*. 2014; 123:1403–11. [PubMed: 24366359]
40. Mock DM, Lankford GL, Widness JA, Burmeister LF, Kahn D, Strauss RG. Measurement of red cell survival using biotin-labeled red cells: validation against ⁵¹Cr-labeled red cells. *Transfusion*. 1999; 39:156–62. [PubMed: 10037125]
41. Shemin D, Rittenberg D. The life span of the human red blood cell. *J Biol Chem*. 1946; 166:627–36. [PubMed: 20276177]
42. Giles RC Jr, Berman A, Hildebrandt PK, McCaffrey RP. The use of ⁵¹Cr for sheep red blood cell survival studies. *Proc Soc Exp Biol Med*. 1975; 148:795–8. [PubMed: 1129303]

Figure 1.

(A) Cell-free hemoglobin, (B) Nitric oxide-consumption of extracellular hemoglobin, (C) extracellular and (D) intracellular met-hemoglobin percent in both fresh (FRBC) and stored erythrocytes (SRBC) supernatant before and after gas exposure to 300 ppm NO in 90% nitrogen (N₂)/10% oxygen (O₂) or to 90% N₂/10% O₂ using a gas exchanger. The comparison of interest is whether the pre-post change with nitric oxide-treatment differed from the pre-post change under control. (A) pre-gas exposure SRBC+O₂: 104.9±67.09 μM vs. pre-gas exposure FRBC+O₂: 15.86±5.38 μM, p=0.0002. (B) SRBC+O₂: 7.4±10.2 μM vs. SRBC+NO: 80.1±70.1 μM, p=0.005. (C) FRBC+O₂: 2.13±2.14% vs. FRBC+NO: 64.45±14.7%, p<0.0001 and SRBC+O₂: 2.39±2.9% vs. SRBC+NO: 75.54±14.47%, p<0.01.

(D) FRBC+O₂: 0.05±0.06% vs. FRBC+NO: 10.31±3.79%, p=0.0004 and SRBC+O₂: 0.13±0.13% vs. SRBC+NO: 9.19±2.3%, p<0.0001. Data are presented as mean ± SD throughout. Hb=hemoglobin; RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; O₂=oxygen; NO=nitric oxide.

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Figure 2.

(A) Mean pulmonary arterial pressure, (B) pulmonary vascular resistance index, (C) plasma hemoglobin levels, and (D) plasma nitric oxide-consumption before, during and after transfusion of fresh (FRBC) and stored (SRBC) RBC units after exposure to 300 ppm NO in 90% nitrogen (N₂)/10% oxygen (O₂) or to 90% N₂/10% O₂ (without nitric oxide) using a gas exchanger. *p<0.01 values of SRBC+10% O₂ differ from FRBC+10% O₂, FRBC+NO and SRBC+NO. +p<0.01 values of both SRBC+10% O₂ and SRBC+NO differ from FRBC +10% O₂ and FRBC+NO. Data are presented as mean ± SD throughout. Hb=hemoglobin;

RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; O₂=oxygen; NO=nitric oxide; PAP=mean pulmonary arterial pressure; PVRI=pulmonary vascular resistance index.

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Figure 3.

Fresh (FRBC) or stored (SRBC) erythrocytes were either exposed to 300 ppm NO in 90% nitrogen (N_2)/10% oxygen (O_2) or to 90% N_2 /10% O_2 (without nitric oxide) and the lifespan of circulating biotinylated erythrocytes was measured for up to 7 days. * values of SRBC +NO differ from SRBC+10% O_2 , Bonferroni-adjusted p-values: 15 min: $p=0.002$, 30 min: $p=0.003$, 60 min: $p=0.002$, 24 h: $p=0.046$. Data are presented as mean $\pm$ SD throughout. RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; O_2 =oxygen; NO=nitric oxide.

Figure 4.

Average velocity of fresh (FRBC) and stored erythrocytes (SRBC), with and without exposure to 300 ppm NO, traveling across the microfluidic synthetic capillaries. Data are presented as mean $\pm$ SD throughout. RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; NO=nitric oxide.

Figure 5.

(A) Mean pulmonary arterial pressure, (B) pulmonary vascular resistance index, (C) plasma hemoglobin levels, and (D) plasma nitric oxide-consumption before, during and after transfusion of washed stored erythrocytes (SRBC) units. Fresh RBCs that were exposed to the control gas are as shown as in Figure 2 and are superimposed in panels A to D. * $p < 0.05$ values of washed SRBC differ from FRBC controls. Data are presented as mean $\pm$ SD throughout. Hb=hemoglobin; RBC=packed erythrocytes; FRBC= fresh packed erythrocytes;

SRBC=stored packed erythrocytes; O₂=oxygen; NO=nitric oxide; PAP=mean pulmonary arterial pressure; PVRI=pulmonary vascular resistance index.

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Figure 6.

The RBC-lifespan of circulating biotin-labeled washed stored RBCs was measured for up to 24 h. The RBC-lifespan of RBCs that were exposed to nitric oxide or the control gas are as shown as in Figure 3 and are superimposed. * values of washed stored RBCs differ from nitric oxide-treated stored RBCs, Bonferroni-adjusted p-values: 15min: p=0.012, 30min: p=0.015, 60min: p=0.002, 24h: p=0.0006. # values of washed stored RBCs differ from untreated stored RBCs, Bonferroni-adjusted p-values: 60min: p=0.073, 24h: p=0.02. Data are presented as mean ± SD throughout. RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; O₂=oxygen; NO=nitric oxide.