Exposure of Stored Packed Erythrocytes to Nitric Oxide Prevents Transfusion-associated Pulmonary Hypertension

Stefan Muenster, M.D., Arkadi Beloiartsev, M.D., Binglan Yu, Ph.D., E Du, Ph.D., Sabia Abidi, Ph.D., Ming Dao, Ph.D., Gregor Fabry, B.S., Jan A. Graw, M.D., Martin Wepler, M.D., Rajeev Malhotra, M.D., Bernadette O. Fernandez, Ph.D., Martin Feelisch, Ph.D., Kenneth D. Bloch, M.D.,† Donald B. Bloch, M.D., Warren M. Zapol, M.D.

ABSTRACT

Background: Transfusion of packed 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. The authors studied whether treating stored packed ovine erythrocytes with NO before transfusion could prevent pulmonary vasoconstriction, enhance erythrocyte deformability, and prolong erythrocyte survival after transfusion.

Methods: Ovine leukoreduced packed erythrocytes were treated before transfusion with either NO gas or a short-lived NO donor. Sheep were transfused with autologous packed erythrocytes, which were stored at 4°C for either 2 ("fresh blood") or 40 days ("stored blood"). Pulmonary 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 NO treatment using a microfluidic device.

Results: NO treatment improved the deformability of stored erythrocytes and increased the number of stored erythrocytes circulating at 1 and 24 h after transfusion. NO treatment prevented transfusion-associated pulmonary hypertension (mean pulmonary arterial pressure at 30 min of 21 ± 1 *vs.* 15 ± 1 mmHg in control and NO–treated packed erythrocytes, $P < 0.0001$). Washing stored packed erythrocytes before transfusion did not prevent pulmonary hypertension.

Conclusions: NO treatment of stored packed erythrocytes before transfusion oxidizes cell-free oxyhemoglobin to methemoglobin, prevents subsequent NO scavenging in the pulmonary vasculature, and limits pulmonary hypertension. NO treatment increases erythrocyte deformability and erythrocyte survival after transfusion. NO treatment might provide a promising therapeutic approach to prevent pulmonary hypertension and extend erythrocyte survival. **(ANESTHESIOLOGY 2016; 125:952-63)**

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

The safety of transfusing packed erythrocytes that have been stored for prolonged periods is controversial. Observational trials linked the transfusion of packed erythrocytes stored for longer than 14 days to an increase in morbidity and mortality.[3] Recent prospective clinical studies found that transfusion of packed erythrocytes stored for longer than 21 days was not associated with a greater frequency

What We Already Know about This Topic

- Transfusion of stored packed erythrocytes has many adverse effects including decreased erythrocyte survival and an increased pulmonary vascular resistance

What This Article Tells Us That Is New

- In an ovine model, pretreatment of stored packed erythrocytes with NO before transfusion increases their survival after transfusion, prevents increases in pulmonary vascular resistance, and may provide a potential approach to mitigate the adverse effects of stored packed erythrocyte transfusions

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†Deceased. From the Department of Anesthesia, Critical Care and Pain Medicine, Anesthesia Center for Critical Care Research, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts (S.M., A.B., B.Y., G.F., J.A.G., M.W., K.D.B., D.B.B., W.M.Z.); Department of Anesthesiology and Critical Care Medicine, University Hospital Bonn, Bonn, Germany (S.M.); Department of Materials Science and Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts (E.D., S.A., M.D.); Cardiovascular Research Center and Cardiology Division, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts (R.M., K.D.B.); Faculty of Medicine, Clinical and Experimental Sciences, University of Southampton, Southampton, United Kingdom (B.O.F., M.F.); and The Center for Immunology and Inflammatory Diseases and the Division of Rheumatology, Allergy and Immunology of the General Medical Services, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts (D.B.B.).

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of adverse outcomes compared to transfusion of packed erythrocytes 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 to 42 days.[5]

Packed erythrocytes undergo numerous morphologic and biochemical changes during extended storage, collectively referred to as the "packed erythrocyte storage lesion."[6–8] *Ex vivo* storage of packed erythrocytes reduces their deformability and viability and leads to hemolysis.[9,10] The rate of hemolysis accelerates after 30 days of storage, and free hemoglobin and microvesicles containing hemoglobin accumulate in the supernatant of the stored unit.[8,9] After transfusion of stored packed erythrocytes, extracellular hemoglobin scavenges NO produced by endothelial cells.[9] Depletion of endothelium-derived NO by cell-free hemoglobin is believed to be the mechanism underlying the pulmonary vasoconstriction that occurs in sheep and humans transfused with stored packed erythrocytes.[11–13]

To establish the validity of an ovine model of autologous blood transfusion, Baron *et al.*[11] investigated the pathophysiologic effects of transfusing sheep with stored packed erythrocytes. Ovine and human blood stored for 40 days before transfusion exhibited similar characteristics. In both species, less than 1% of the pretransfused blood was hemolyzed during storage, and by 24 h after transfusion, 25% of the transfused packed erythrocytes were removed from the circulation.[11,14] Transfusion of stored packed erythrocytes in sheep was associated with a transiently increased mean pulmonary arterial pressure (PAP) and pulmonary 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 packed erythrocytes increased the PAP.[13] Thus, both humans and sheep exhibit pulmonary hypertension after receiving stored autologous packed erythrocytes. Inhalation of NO gas, a selective pulmonary vasodilator[15] (as opposed to intravenously administered NO–releasing drugs that lower both pulmonary and systemic arterial pressures), prevented the pulmonary hypertension associated with transfusion of stored packed erythrocytes in humans.[13]

We hypothesized that *ex vivo* NO treatment before transfusion would (1) prevent pulmonary hypertension in lambs during and after transfusion; (2) could improve the survival of stored erythrocytes in the circulation for up to 7 days after transfusion; and (3) would increase the *in vitro* deformability of stored erythrocytes. We further hypothesized that washing stored packed erythrocytes before transfusion might also ameliorate transfusion-associated pulmonary hypertension.

Materials and Methods

Storage and Processing of Ovine Autologous Packed Erythrocytes

All experiments were approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital, Boston, Massachusetts. We studied 3- to 4-month-old Polypay lambs, weighing 33 ± 2 kg (mean ± SD) from a cesarean-derived, specific pathogen-free sheep flock (New England Ovis, USA). For autologous blood collection, lambs were anesthetized with an intramuscular injection of ketamine (20 mg/kg; Hospira, USA), and whole blood (450 ml) was collected from the left jugular vein into a Double Blood-Pack Unit (Fenwal, Inc., USA), containing a citrate-phosphate-dextrose-adenine solution.[11] Erythrocytes were leukoreduced using a leukoreduction filter (RS2000, Fenwal, Inc.), 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, Inc.) to produce a final hematocrit of approximately 60%. Packed erythrocytes were stored for either 2 or 40 days at 4°C. In this study, packed erythrocytes stored for 2 days are designated as "fresh"; packed erythrocytes refrigerated for 40 days are considered "stored." Lambs were transfused for 30 min with 1 autologous packed erythrocyte unit (300 ml), 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 volume.[16] To evaluate two novel methods of treating packed erythrocytes with NO before transfusion, packed erythrocyte units were treated either with NO gas exposure or with incubation with the NO donor methylamine hexamethylene methylamine NONOate (MAHMA NONOate). In addition, some lambs were transfused with washed stored packed erythrocytes.

Although packed erythrocytes stored for 2 days are technically not " fresh," the design of this study mimicked common hospital practice in which "fresh" packed erythrocyte units are available in the inventory of a blood bank after appropriate immunologic tests are performed, approximately 48 to 72 h after collection.

Ex Vivo *Exposure of Packed Erythrocytes to NO*

Packed erythrocytes were passed through an extracorporeal membrane oxygenator (surface area, 0.8 m^2) (Quadrox iD Pediatric, Maquet, USA) using a gravity feed with 30 cm H_2O driving pressure, resulting in a blood flow rate of 8 to 10 ml/min. Packed erythrocytes were exposed to either 300 ppm NO in 90% N_2/10% O_2 or 90% N_2/10% O_2 (control gas) with a gas flow rate of 700 ml/min. Ten percent oxygen in the gas mixture converted deoxyhemoglobin to oxyhemoglobin, a moiety that oxidizes methemoglobin. Because nitrogen dioxide is produced when NO is diluted in oxygen,[17] a scavenger reservoir containing 90 g of SodaLime (Biodex, USA) was placed immediately before the gas exchanger to remove nitrogen dioxide.[18] NO gas levels were measured using a NO chemiluminescence analyzer (Sievers 280i, USA). The Sievers 280i with an M&C nitrogen dioxide to NO converter (AMP Cherokee, USA) was used to measure NO levels.

As an alternative approach to pretreating packed erythrocytes *ex vivo* with NO gas to oxidize plasma-free hemoglobin, the NO donor compound MAHMA NONOate (Cayman Chemical, USA) was added to the packed erythrocytes. Preliminary *in vitro* pilot experiments on stored ovine packed erythrocytes suggested that a 1:4 ratio of cell-free hemoglobin to NO was needed to reduce NO scavenging by cell-free hemoglobin and to improve *in vitro* erythrocyte flexibility. The average cell-free hemoglobin concentration after 40 days of storage was 100 μM (in terms of heme). Breakdown of MAHMA NONOate produces ethylamine hexamethylene methylamine and 2 NO molecules. Therefore, packed erythrocyte units were incubated with MAHMA NONOate at a final concentration of 200 μM.

Either fresh or stored ovine packed erythrocytes were incubated for 20 min with 200 μM of the NO 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 to 3 min at 25°C), a 20-min packed erythrocyte incubation period at 25°C was allowed to ensure the release of all NO from the NO donor; no additional NO was available to be released during and after transfusion. To avoid additional hemolysis that might be associated with mechanical or osmotic stress to erythrocytes, packed erythrocytes were not washed after exposure to MAHMA NONOate. The NO donor–treated packed erythrocyte units were transfused immediately after the 20-min incubation period for 30 min in awake lambs. We used the *in vitro* and *in vivo* results from experiments using fresh and stored packed erythrocyte units exposed to the control gas as controls for the MAHMA NONOate study.

Washing Stored Packed Erythrocytes before Transfusion

In some experiments, stored packed erythrocytes were washed using the FDA-licensed COBE 2991 blood cell processor (TERUMOBCT, USA). In these studies, an aseptic closed system circuit (COBE 2991 Cell Processing Set, TERUMOBCT) was used to centrifuge a unit of stored packed erythrocytes (300 ml). After separation of packed erythrocytes and plasma, plasma was discarded, and 11 of 0.2% Dextrose and 0.9% Sodium Chloride Processing Solution (Baxter, USA) was added to the stored packed erythrocytes spun at 3,000 rpm/min for 1 h. After centrifugation, washing solution was discarded, and packed erythrocytes 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, http://links.lww.com/ALN/B308).

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 packed erythrocyte units were obtained immediately before transfusion. Extracellular free hemoglobin concentrations in the transfused packed erythrocyte supernatant and plasma were measured using a QuantiChrom hemoglobin assay kit (BioAssay Systems, USA). The fraction of extracellular methemoglobin in the supernatant of the transfusate was determined by spectral deconvolution.[12] To measure intracellular methemoglobin levels, a sample from the packed erythrocyte unit was centrifuged (800*g*) for 10 min, the supernatant was discarded, and the cell pellet was resuspended in fresh AS-1 solution. Intracellular methemoglobin was measured using an ABL 800 Flex blood gas analyzer (Radiometer Medical, Denmark). A NO consumption assay[19] was performed on samples of circulating plasma during and after transfusion and from the supernatant of the packed erythrocyte units before and after NO treatment (see Supplemental Methods, http://links.lww.com/ALN/B308).

Blood gas tensions (Pa_{O_2} and Pa_{CO_2}), oxygen saturation of hemoglobin (Sa_{O_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, and 30 min), and every 30 min up to 4 h thereafter.

Lifespan Measurements of Transfused Packed Erythrocytes

To determine the half-life of transfused packed erythrocytes, biotin was conjugated to fresh or stored packed erythrocytes using sulfo-*N*-hydroxysuccinimide-biotin (10 μg/ml; Thermo Fisher Scientific, USA). After transfusion, blood samples were obtained at 15, 30, and 60 min; 24 h; and 7 days after transfusion. A 50-μl aliquot of the blood sample was incubated with fluorescein isothiocyanate–conjugated streptavidin (Biolegend, USA) and analyzed by flow cytometry to determine the fraction of biotinylated circulating erythrocytes present in each sample. The fraction of biotinylated erythrocytes 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 erythrocytes 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-min time points in quadrature.[20] In our study, the standard error of the zero time point is 1.7 to 2.7% (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 NO–treated Erythrocyte Deformability Using Microfluidics

The microfluidic device used in these studies is an optimized design of the device described by Bow *et al.*[21] Erythrocytes flow through a microchannel (1 mm wide and 5 mm long), which distributes erythrocytes *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 erythrocyte velocity" is defined as the velocity of the erythrocyte within each slit, averaged over three periodic slit sets. Average velocity for each sample is composed of the average of 134 individual erythrocyte transit velocities. The transit velocity of individual erythrocytes is an indirect measure of erythrocyte deformability.[21] A higher transit velocity through the capillaries indicates greater deformability.

Statistical Analyses

All data are expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, USA). Variables were tested for normality using the Shapiro–Wilk test. Comparisons of the change in cell-free hemoglobin, NO consumption, and methemoglobin in NO–treated packed erythrocytes *versus* control-treated packed erythrocytes were performed using either an unpaired Student's *t* test or Mann–Whitney U test, as appropriate (fig. 1 and Supplemental fig. 1, http://links.lww.com/ALN/B308; $P < 0.025$ was considered significant when adjusting for multiple comparisons). For *in vivo* transfusion experiments (fig. 2 and Supplemental fig. 2, http://links.lww.com/ALN/B308), 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. Because five comparisons were

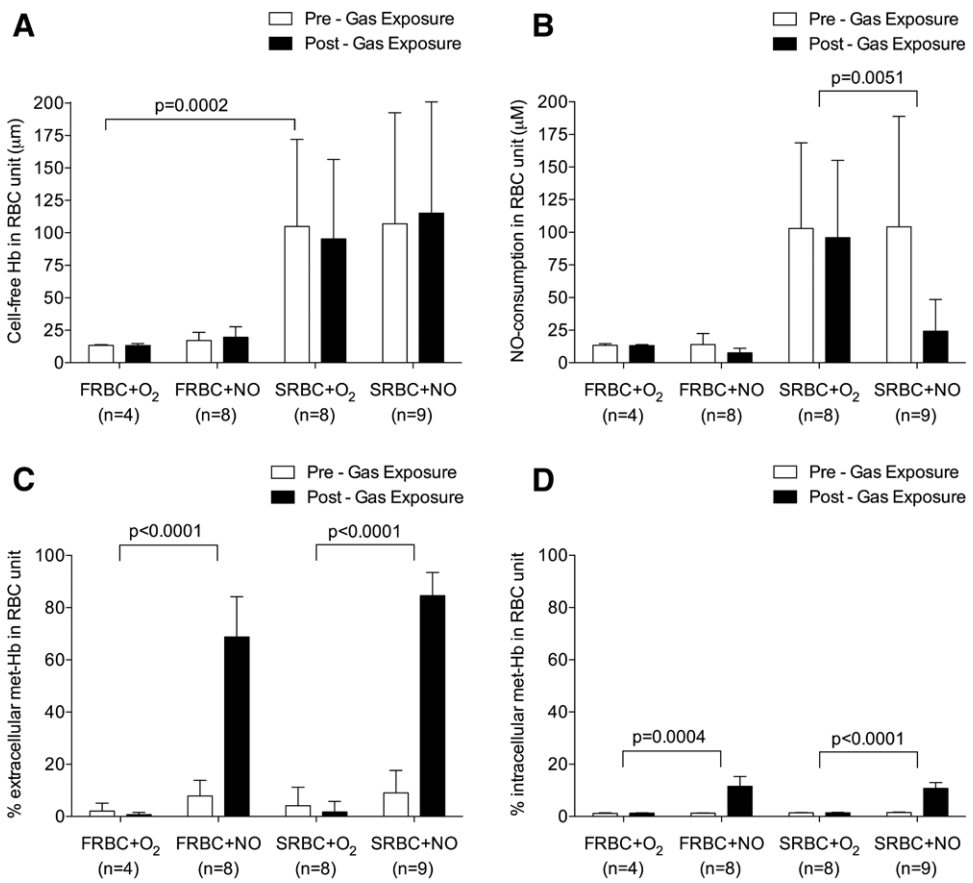

Fig. 1. (*A*) Cell-free hemoglobin, (*B*) NO consumption of extracellular hemoglobin, (*C*) extracellular, and (*D*) intracellular methemoglobin (met-Hb) percent in both fresh packed erythrocytes (FRBCs) and stored packed erythrocytes (SRBC) supernatant before and after gas exposure to 300 ppm NO in 90% nitrogen (N_2)/10% O_2 or to 90% N_2/10% O_2 using a gas exchanger. The comparison of interest is whether the pre–post change with NO treatment differed from the pre–post change under control. (*A*) Pregas exposure SRBC + O_2: 104.9 ± 67.09 μM *versus* pregas exposure FRBC + O_2: 15.86 ± 5.38 μM, $P = 0.0002$. (*B*) SRBC + O_2: 7.4 ± 10.2 μM *versus* SRBC + NO: 80.1 ± 70.1 μM, $P = 0.005$. (*C*) FRBC + O_2: 2.13 ± 2.14% *versus* FRBC + NO: 64.45 ± 14.7%, $P < 0.0001$ and SRBC + O_2: 2.39 ± 2.9% *versus* SRBC + NO: 75.54 ± 14.47%, $P < 0.01$. (*D*) FRBC + O_2: 0.05 ± 0.06% *versus* FRBC + NO: 10.31 ± 3.79%, $P = 0.0004$ and SRBC + O_2: 0.13 ± 0.13% *versus* SRBC + NO: 9.19 ± 2.3%, $P < 0.0001$. Data are presented as mean ± standard deviation throughout. RBC = packed erythrocytes.

made at each time point, *P* value of less than 0.01 was considered significant. Changes in circulating biotinylated erythrocytes over time (fig. 3 and Supplemental fig. 3, http://links.lww.com/ALN/B308) 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 less than 0.025. Comparison of erythrocyte transit velocity between groups (fig. 4 and Supplemental fig. 4, http://links.lww.com/ALN/B308) was performed using a one-way ANOVA with Bonferroni-adjusted *post hoc* testing. Because six comparisons were made, *P* value of less than 0.008 was considered significant. When comparing washed stored packed erythrocytes *versus* control fresh packed erythrocytes (fig. 5), a Mann–Whitney U test was performed with a threshold *P* value of less than 0.05. To compare changes in circulating biotinylated stored washed erythrocytes over time (fig. 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 less than 0.025. For each test, exact unadjusted *P* values are provided in the figures. Coefficients of variation were determined for the following assays: 4.0% for cell-free hemoglobin in packed erythrocyte units, 3.4% for cell-free hemoglobin in plasma, 5.5% for NO consumption in packed erythrocyte units, and 5.6% for NO consumption in plasma, indicating adequate reproducibility. Measurements of biotinylated erythrocytes were highly reproducible

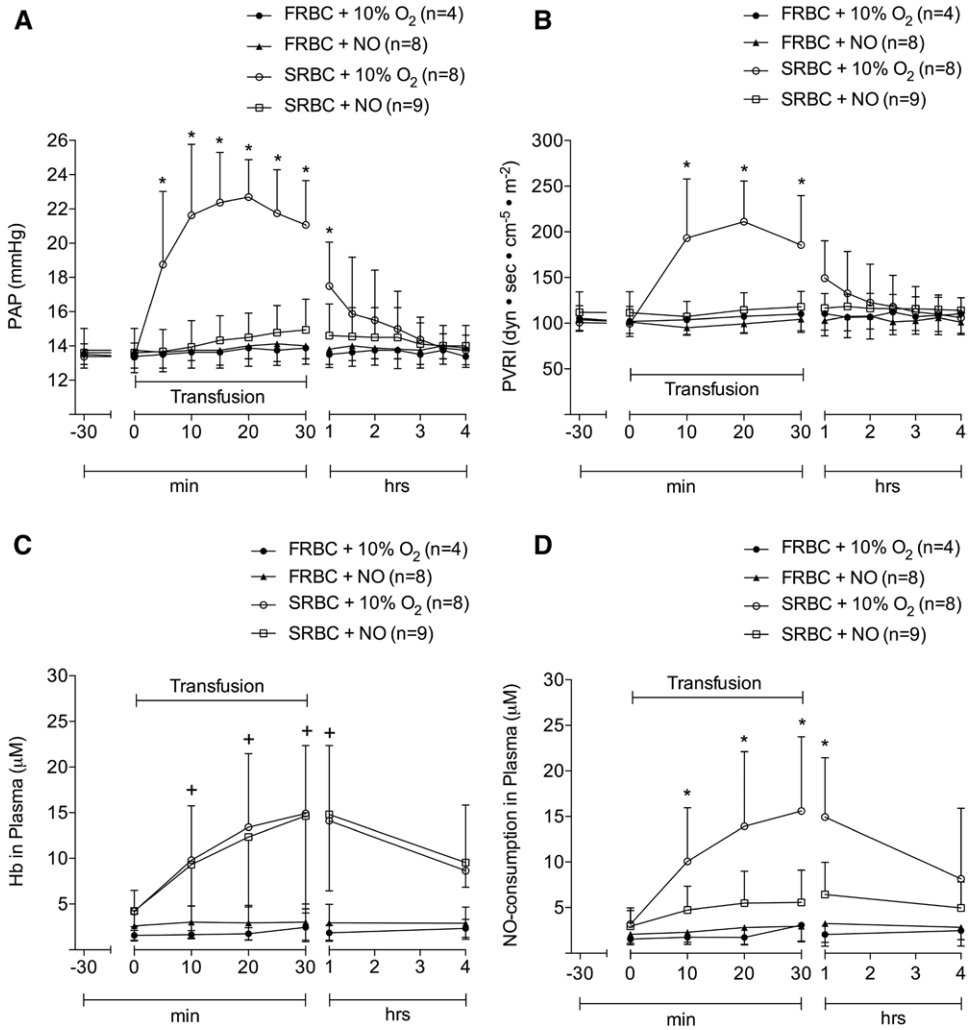

Fig. 2. (*A*) Mean pulmonary arterial pressure (PAP), (*B*) pulmonary vascular resistance index (PVRI), (*C*) plasma hemoglobin (Hb) levels, and (*D*) plasma NO consumption before, during, and after transfusion of fresh (FRBC) and stored (SRBC) erythrocyte units after exposure to 300 ppm NO in 90% nitrogen (N_2)/10% O_2 or to 90% N_2/10% O_2 (without NO) using a gas exchanger. *$P < 0.01$ values of SRBC + 10% O_2 differ from FRBC + 10% O_2, FRBC + NO, and SRBC + NO. +$P < 0.01$ values of both SRBC + 10% O_2 and SRBC + NO differ from FRBC + 10% O_2 and FRBC + NO. Data are presented as mean ± SD throughout.

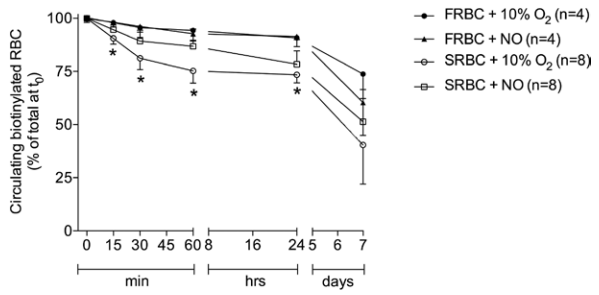

Fig. 3. Fresh packed erythrocytes (FRBCs) or stored packed erythrocytes (SRBCs) were either exposed to 300 ppm NO in 90% nitrogen (N_2)/10% O_2 or to 90% N_2/10% O_2 (without NO) and the lifespan of circulating biotinylated erythrocytes was measured for up to 7 days. *Values of SRBC + NO differ from 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 ± standard deviation throughout. RBC = packed erythrocytes.

at the 15- 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 posttransfusion in the control-treated stored packed erythrocyte group *versus* the NO–treated stored packed erythrocyte group. The anticipated change in PAP in the control-treated stored packed erythrocyte group is 5 ± 1.4 mmHg (mean ± SD), whereas the expected change in the NO–treated stored packed erythrocyte group is 2 ± 1.4 mmHg (mean ± SD), based on previously published work.[11,12] Using a level of significance of 0.05, we determined that at least six animals per group were needed to obtain a power of more than 96% to detect a difference. Levels of significance of 0.025 and 0.01 lead to a power of 92.9 and 87.2%, respectively, when six animals per group are used.

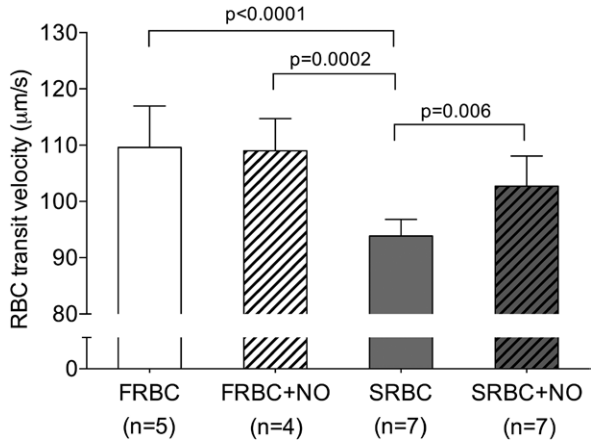

Fig. 4. Average velocity of fresh erythrocyte (FRBC) and stored erythrocyte (SRBC), with and without exposure to 300 ppm NO, traveling across the microfluidic synthetic capillaries. Data are presented as mean ± SD throughout. RBC = erythrocytes.

Results

Transient Exposure of Stored Blood to NO Reduces NO Consumption by Extracellular Hemoglobin

To determine whether *in vitro* treatment of stored packed erythrocytes with NO could reduce scavenging by extracellular hemoglobin, packed erythrocyte units were exposed to NO, and the ability of supernatant to scavenge NO was measured. In pilot studies, we examined the effect of different NO gas levels or MAHMA NONOate doses on either outdated stored human packed erythrocyte units obtained from the blood bank (NO gas) or outdated stored ovine packed erythrocytes (MAHMA NONOate) (data not shown). We identified the optimal NO doses that produced low intracellular methemoglobin and high extracellular methemoglobin levels. Also, NO consumption by extracellular hemoglobin was markedly reduced. Comparisons were performed on the change in cell-free hemoglobin, NO consumption, and methemoglobin in NO–treated packed erythrocytes *versus* control-treated packed erythrocytes.

Cell-free hemoglobin levels in fresh and stored packed erythrocyte supernatants were measured before and after NO treatment. The amount of extracellular hemoglobin in the supernatant was greater in stored as compared to fresh packed erythrocyte units (fig. 1A, increase in extracellular hemoglobin: 104.9 ± 67.09 *vs.* 15.86 ± 5.38 μM, *P* = 0.0002). Pretreatment of fresh or stored packed erythrocytes with 300 ppm NO did not alter supernatant cell-free hemoglobin levels.

A NO consumption assay[19] was used to measure the NO–scavenging capacity of extracellular hemoglobin in the supernatant before and after exposing packed erythrocyte units to NO in a gas exchanger. The supernatant from fresh packed erythrocyte units scavenged less NO than the supernatant from stored packed erythrocyte units. Exposure of stored packed erythrocyte units to NO gas reduced the NO consumption of the stored supernatant when compared to treatment with the control gas, which did not contain NO (fig. 1B, reduction of NO consumption: 80.1 ± 70.1 *vs.* 7.4 ± 10.2 μM, respectively, *P* = 0.005), whereas NO exposure of fresh packed erythrocyte units did not reduce plasma NO consumption.

Exposure of packed erythrocytes to NO gas increased the percentage of extracellular methemoglobin in fresh and stored blood units when compared to fresh controls (fig. 1C, increase of extracellular methemoglobin by 64.45 ± 14.7 *vs.* 2.13 ± 2.14%, *P* = 0.0004) or stored controls, respectively (fig. 1C, increase of extracellular methemoglobin by 75.54 ± 14.47 *vs.* 2.39 ± 2.9%, *P* < 0.0001).

The intracellular percentage of methemoglobin was measured before and after NO treatment to determine whether erythrocyte oxygen transport capacity was reduced after NO exposure. Intracellular packed erythrocyte methemoglobin levels were increased to 12% in the packed erythrocyte unit when either fresh (fig. 1D, increase of intracellular methemoglobin by 10.31 ± 3.79 *vs.* 0.05 ± 0.06%, *P* = 0.0004) or stored packed

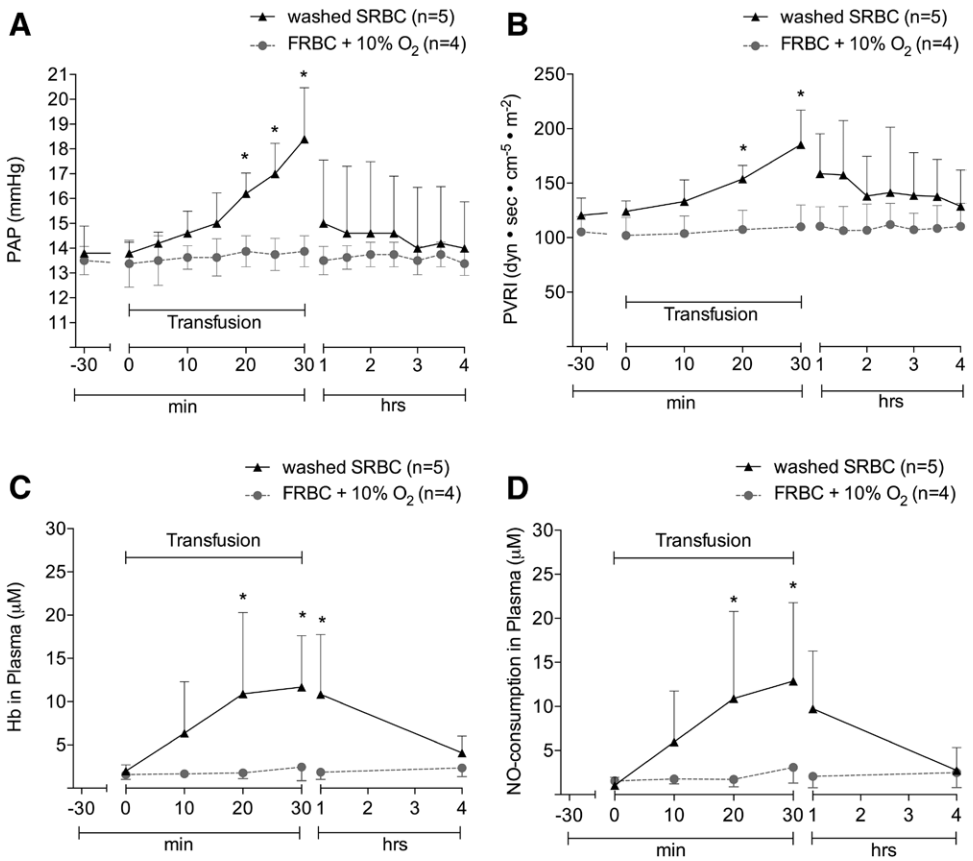

Fig. 5. (*A*) Mean pulmonary arterial pressure (PAP), (*B*) pulmonary vascular resistance index (PVRI), (*C*) plasma hemoglobin (Hb) levels, and (*D*) plasma NO consumption before, during, and after transfusion of washed stored packed erythrocytes (SRBC) units. Fresh packed erythrocytes (FRBCs) that were exposed to the control gas are shown as in figure 2 and are superimposed in (*A*) to (*D*). *$P < 0.05$ values of washed SRBC differ from FRBC controls. Data are presented as mean ± SD throughout.

erythrocytes were transiently exposed to 300 ppm NO (fig. 1D, increase of intracellular methemoglobin by 9.19 ± 2.3 *vs.* 0.13 ± 0.13%, $P < 0.0001$). Incubation of NO–treated packed erythrocytes at 37°C for 2 h decreased the intracellular methemoglobin by 50%, and methemoglobin returned to baseline levels 12 h after NO treatment (data not shown).

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

Exposure of Stored Packed Erythrocytes to NO Prevents Transfusion-associated Pulmonary Hypertension in Awake Lambs

To determine whether treatment of stored packed erythrocytes with NO prevents transfusion-associated pulmonary hypertension, the hemodynamic consequences of transfusing NO–treated fresh and stored autologous packed erythrocytes were assessed in the pulmonary circulation of awake sheep. Lambs receiving stored packed erythrocytes 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 (fig. 2A). In contrast, transfusion of stored packed erythrocytes that were pretreated with 300 ppm NO gas did not increase the PAP when compared to control-treated fresh packed erythrocytes and NO–treated fresh packed erythrocytes during and after the transfusion (fig. 2A, PAP at 20 min of stored packed erythrocyte plus NO, 14.5 ± 1.4 mmHg; fresh packed erythrocyte plus O_2, 13.9 ± 0.6 mmHg; fresh packed erythrocyte plus NO, 14 ± 1.2 mmHg; $P > 0.01$). Transfusion of fresh packed erythrocytes, whether treated *ex vivo* with or without NO gas, did not alter the PAP during or after transfusion.

To determine whether increases of PAP in lambs receiving stored packed erythrocytes treated with control gas were caused by vasoconstriction, the PVRI was measured before, during, and after transfusion. Transfusion of stored packed erythrocytes caused a transient increase in the PVRI from 10 to 30 min after commencing transfusion. Pretreatment of stored packed erythrocytes with NO gas prevented the increase in

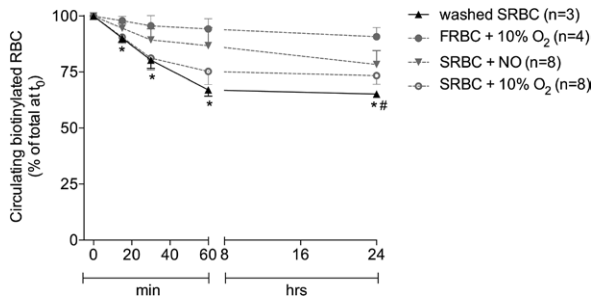

Fig. 6. The packed erythrocyte (RBC) lifespan of circulating biotin-labeled washed stored packed erythrocytes (SRBCs) was measured for up to 24 h. The RBC-lifespan of RBCs that were exposed to NO or the control gas are shown as in figure 3 and are superimposed. *Values of washed SRBCs differ from NO–treated SRBCs, Bonferroni-adjusted *P* values: 15 min: $P = 0.012$; 30 min: $P = 0.015$; 60 min: $P = 0.002$; 24 h: $P = 0.0006$. #Values of washed SRBCs differ from untreated SRBCs, Bonferroni-adjusted *P* values: 60 min: $P = 0.073$; 24 h: $P = 0.02$. Data are presented as mean ± standard deviation throughout. FRBC = fresh packed erythrocytes.

PVRI when compared to control-treated stored packed erythrocytes (fig. 2B, PVRI at 20 min of 114.6 ± 18.9 *vs.* 211.1 ± 44.4 dyn·sec·cm^{-5}·m^{-2}, $P < 0.0001$). Transfusion of fresh packed erythrocytes, with or without NO 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 packed erythrocytes reduced the bioavailability of NO in the sheep's pulmonary vasculature and thereby induced pulmonary vasoconstriction.[11,12] To determine whether exposure of stored packed erythrocytes to NO could reduce NO consumption by plasma hemoglobin during and after transfusion, the levels of circulating plasma hemoglobin and plasma NO consumption were measured. Plasma hemoglobin concentrations were greater in sheep transfused with stored compared with fresh packed erythrocytes at 10, 20, 30, and 60 min after commencing transfusion. Transfusing NO gas–treated or control gas–treated stored packed erythrocytes increased the level of plasma-free hemoglobin to a similar extent. Transfusing either NO gas–treated or control gas–treated fresh packed erythrocytes did not increase the circulating level of plasma-free hemoglobin.

Transfusion of stored packed erythrocytes increased the consumption of NO by circulating plasma hemoglobin at 10, 20, 30, and 60 min after commencing transfusion as compared to animals transfused with fresh packed erythrocytes (fig. 2D, NO consumption at 30 min of 15.6 ± 8.2 *vs.* 3.1 ± 1.8 μM, $P = 0.0003$). Pretreatment of stored packed erythrocytes with NO gas resulted in significantly less plasma NO consumption between 10 and 60 min after commencing transfusion when compared to transfusion of control gas–treated stored packed erythrocytes (fig. 2D, NO consumption at 30 min of 5.6 ± 3.6 *vs.* 15.6 ± 8.2 μM, $P = 0.0003$). Plasma NO consumption levels did not increase when fresh packed erythrocytes were transfused after treatment with either NO or control gas.

These results show that exposure of either fresh or stored packed erythrocytes to NO gas does not increase circulating plasma-free hemoglobin levels after transfusion. Treatment of stored packed erythrocytes with NO gas before transfusion reduces scavenging of NO by plasma hemoglobin.

Pretreatment with NO Gas Improves the Survival of Stored Erythrocytes 1 and 24 h after Transfusion

The survival of stored erythrocytes circulating after transfusion is markedly reduced when compared to the survival of fresh erythrocytes.[11] To investigate the potential beneficial effect of NO gas treatment on stored packed erythrocyte survival after transfusion, we biotin-labeled fresh and stored packed erythrocytes, exposed them to 300 ppm NO gas, and measured the fraction of biotinylated erythrocytes that remained in the circulation for up to 7 days. Blood samples were collected at 15, 30, and 60 min; 24 h; and 7 days after transfusion, and the ratio of biotinylated erythrocytes to total erythrocytes was measured by flow cytometry.

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

Pretreatment of Packed Stored Erythrocytes with NO Gas May Increase Erythrocyte Deformability

To determine whether NO gas treatment increases erythrocyte deformability, we used a customized microfluidic device to indirectly measure erythrocyte deformability as the erythrocyte transit velocity through the device.[21] The average transit velocity of fresh ovine erythrocytes, whether treated with NO gas or control gas, was greater than the average erythrocyte transit velocity of stored erythrocytes (fig. 4). The average erythrocyte transit velocity of NO gas-treated stored packed erythrocytes was greater than that of control gas-treated stored packed erythrocytes (fig. 4, erythrocyte transit velocity of 103 ± 5 *vs.* 94 ± 3 μm/s, $P = 0.006$). These findings suggest that *ex vivo* exposure of stored packed erythrocytes to NO may increase erythrocyte deformability.

The Effects of Treating Stored Packed Erythrocytes with MAHMA NONOate before Transfusion in Awake Lambs

Treatment of stored packed erythrocytes, before transfusion, with the NO donor compound MAHMA NONOate was studied as an alternative approach to NO gas exposure of stored packed erythrocytes. Incubating either fresh or stored packed erythrocyte with MAHMA NONOate had similar effects on the oxidation of supernatant cell-free oxyhemoglobin to methemoglobin, *in vitro* NO consumption, and intracellular and extracellular methemoglobin levels as exposing fresh or stored packed erythrocytes to NO gas (Supplemental Digital Content 1, fig. 1, A to D, http://links.lww.com/ALN/B308, which presents cell-free hemoglobin levels, NO consumption of extracellular hemoglobin, and extracellular and intracellular methemoglobin percent before and after MAHMA NONOate treatment). In addition, transfusion of stored MAHMA NONOate–treated packed erythrocytes prevented transfusion-associated pulmonary hypertension and pulmonary vasoconstriction and reduced plasma NO consumption without causing systemic vasodilation (Supplemental Digital content 1, fig. 2, A to D, http://links.lww.com/ALN/B308, which presents the PAP; PVRI; plasma hemoglobin levels; and plasma NO consumption before, during, and after transfusion of packed erythrocyte units treated with 200 μM MAHMA NONOate). Similar to NO gas exposure before transfusion, pretreatment of stored packed erythrocytes with MAHMA NONOate improved the 1- and 24-h erythrocyte survival, but there was no difference in stored erythrocyte lifespan at 7 days after transfusion (Supplemental Digital Content 1, fig. 3, http://links.lww.com/ALN/B308, which shows the lifespan of circulating biotinylated fresh or stored erythrocytes, treated with 200 μM MAHMA NONOate). Furthermore, as with exposure to NO gas, *ex vivo* exposure of stored packed erythrocytes to a chemical NO donor increased the indirectly measured erythrocyte deformability (Supplemental Digital Content 1, fig. 4, http://links.lww.com/ALN/B308, which is an indirect measure of the erythrocyte deformability).

Washing Stored Packed Erythrocytes before 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 packed erythrocytes to NO reduced NO consumption by extracellular hemoglobin and prevented transfusion-associated pulmonary hypertension. We then considered the possibility that removing extracellular hemoglobin by washing stored packed erythrocytes, using standard blood banking techniques, would be as effective as NO treatment.

Transfusion of washed, stored packed erythrocyte increased the PAP to a maximum of 18.4 ± 2.1 mmHg at 30 min after starting the transfusion when compared to fresh packed erythrocytes (fig. 5A, PAP at 30 min of 18.4 ± 2.1 *vs.* 13.9 ± 0.6 mmHg, $P = 0.02$). In addition, transfusion of washed, stored packed erythrocytes caused a significant increase in PVRI compared to fresh packed erythrocytes beginning at 20 min after commencing transfusion (fig. 5B, PVRI at 20 min of 153.8 ± 12.5 *vs.* 107.5 ± 17.8 dyn·sec·cm^{-5}·m^{-2}, $P = 0.02$). Washing stored packed erythrocytes did not prevent the transfusion-associated increase in plasma hemoglobin (fig. 5C, hemoglobin in plasma at 30 min of 11.7 ± 6.0 *vs.* 2.5 ± 1.6 μM, $P = 0.03$). Similarly, plasma NO consumption increased 20 min after starting the transfusion of washed, stored packed erythrocytes when compared to the transfusion of fresh packed erythrocytes (fig. 5D, plasma NO consumption at 20 min of 10.9 ± 9.9 μM *vs.* 1.7 ± 0.8 μM, $P = 0.03$). These findings demonstrate that washing stored packed erythrocytes before transfusion did not eliminate the adverse effects associated with the transfusion of stored packed erythrocytes.

Discussion

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

Erythrocyte viability is reduced after extended storage and leads to the release of hemoglobin into the packed erythrocyte unit supernatant.[8,22] Donadee *et al.*[9] reported increased extracellular hemoglobin in stored packed erythrocyte units after 30 days of storage compared to 4 days of storage. Circulating cell-free hemoglobin and hemoglobin-containing microvesicles scavenge NO, which is generated by endothelial cells. NO scavenging by extracellular hemoglobin appears to be the cause of the pulmonary vasoconstriction that occurs after transfusion of stored blood into obese human volunteers.[13]

Inhaled NO is a selective pulmonary vasodilator that has been used for more than two decades to treat pulmonary hypertension of the newborn and other cardiopulmonary diseases.[15] Baron *et al.*[11,12] reported that *inhaled* NO prevented pulmonary vasoconstriction and hypertension in sheep transfused with stored autologous packed erythrocytes. We attempted to simplify and better understand this treatment strategy by exposing the stored packed erythrocyte units *ex vivo* to NO gas or a NO donor molecule. We

studied whether pulmonary hypertension and pulmonary vasoconstriction could be prevented without requiring the transfusion recipient to breathe NO.

In this study, we observed that intracellular methemoglobin levels increased up to 12% after the *ex vivo* treatment with NO gas but returned to baseline levels after 12 h. Because the intracellular methemoglobin in the packed erythrocyte unit was subsequently diluted 7-fold in the lamb's total circulating blood volume, we estimate that the final concentration of methemoglobin in the animal was approximately 1.7%.

The transient *in vitro* exposure of either fresh or stored blood to either NO gas or MAHMA NONOate did not harm the stored packed erythrocytes; there was no increase in erythrocyte hemolysis nor was there a reduction in erythrocyte lifespan after transfusion. There was an important reduction of NO consumption by the packed erythrocyte supernatant fluid when stored packed erythrocyte units were treated with NO, which may be attributable to the conversion of extracellular oxyhemoglobin to methemoglobin. Methemoglobin is unable to scavenge NO.[23] Inhibition of NO scavenging by oxidation of extracellular ferrous hemoglobin is likely to be the underlying mechanism by which pretreatment with NO prevents transfusion-associated pulmonary vasoconstriction and hypertension.[11–13,24,25]

Transfusion of stored packed erythrocytes 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 NO bioavailability.[11,13,27] In addition, stored packed erythrocytes themselves may have inhibitory effects on NO–mediated vasodilation through NO scavenging.[28–30] Solomon *et al.*[26] 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 thrombosis. 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 packed erythrocytes has been reported.[33] Treating stored packed erythrocytes with NO may improve the safety of stored packed erythrocyte transfusion.

When erythrocytes traverse small capillaries, they must change shape.[34] However, extended storage decreases erythrocyte deformability.[8,22] During storage, erythrocyte membrane permeability to calcium ions increases over time, leading to increased intracellular calcium levels. Intracellular calcium activates calcium-sensitive KCa3.1 ion channels, which are located in the erythrocyte membrane.[35] The resulting potassium ion efflux causes erythrocytes to shrink and makes their cell membranes less flexible.[36] Previous studies showed that NO has a crucial role in enhancing erythrocyte deformability and that reduced erythrocyte NO levels decrease deformability.[35,37] Loss of erythrocyte deformability may lead to an increased rate of intravascular hemolysis and a reduced lifespan of transfused stored erythrocytes. In this study, we used a microfluidic device to measure the *in vitro* transit velocity of NO–treated erythrocytes. The transit velocity of erythrocytes through the synthetic capillaries in this device is an indirect measure of erythrocyte deformability.[21] When stored ovine packed erythrocytes were treated with NO (either NO gas or MAHMA NONOate), their transit velocity was increased as compared to untreated stored erythrocytes. Other studies have described similar improvement in erythrocyte deformability with NO donor treatment.[37,38] Our results suggest that NO treatment augments the deformability of erythrocytes. The precise mechanism by which NO treatment increases deformability is unknown.

Recent evidence suggests that washing stored packed erythrocytes, using standard blood banking methods, before transfusion might be another strategy to prevent the adverse effects of transfusing stored blood.[39] Washing stored packed erythrocytes might remove extracellular hemoglobin 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 hemoglobin at the beginning of transfusion, subsequent increased intravascular hemolysis may explain the delayed pulmonary hypertension. The reduction in erythrocyte lifespan of washed stored packed erythrocytes compared to fresh and untreated stored packed erythrocytes, and the corresponding increase in plasma hemoglobin, supports this hypothesis.

In this study, normovolemic lambs were transfused with packed erythrocytes, making this a "top-load" transfusion model. The transfusion of packed erythrocytes, representing a 14% increase in the lambs total blood volume, did not appear to affect the pulmonary vasculature. In lambs receiving fresh packed erythrocyte 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 packed erythrocyte storage lesion of laboratory animals and humans is similar but not identical. We demonstrated in a previous study that the oxygen affinity of ovine packed erythrocytes did not change during storage (as opposed to human packed erythrocytes) and that ovine erythrocyte 2,3 diphosphoglycerate levels were lower than in humans.[11] However, with the exception of the P_{50} and 2,3 diphosphoglycerate concentrations, ovine packed erythrocytes have similar properties to those measured in human packed erythrocytes 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 to 120

days) than that of mice (55 to 60 days).[40–42] In this study, the mean 24-h posttransfusion survival rate of ovine erythrocytes stored in a standard preservative (AS-1 solution) was lower than the survival rate of human erythrocytes (73 ± 1% at 24 h after transfusion in lambs *vs.* 82.1 ± 7% in humans at 24 h).[14]

In summary, we have developed and tested two methods for treating 40-day stored ovine packed erythrocyte units with NO before transfusion to prevent transfusion-associated pulmonary hypertension. We found that transiently exposing stored blood to either NO gas or a NO donor compound reduced NO scavenging by cell-free hemoglobin, prevented pulmonary vasoconstriction and hypertension, and increased *in vitro* erythrocyte velocity, an indirect measure of erythrocyte deformability. In addition, NO exposure increased the survival of stored ovine erythrocytes at 1 and 24 h after starting transfusion. Further studies with human stored packed erythrocytes are required to confirm the beneficial effects of *ex vivo* NO exposure.

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Competing Interests

The Massachusetts General Hospital (Boston, Massachusetts) has received royalties on the sale of NO for inhalation from Linde Corporation (Munich, Germany) and Ikaria (Hampton, New Jersey), and Dr. Zapol received a portion of these royalties. The Massachusetts General Hospital has filed patents on NO gas treatment to prevent transfusion-associated pulmonary hypertension. The other authors declare no competing interests.

Correspondence

Address correspondence to Dr. Zapol: Department of Anesthesia, Critical Care and Pain Medicine, Anesthesia Center for Critical Care Research, Massachusetts General Hospital and Harvard Medical School, 55 Fruit Street, Thier 503, Boston, Massachusetts 02114. wzapol@mgh.harvard.edu.

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