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 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 days[1]. 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 mortality[3]. 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 days[5].

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 cells[9]. 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 RBCs[11-13].

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 PAP[13]. Thus, both humans and sheep exhibit pulmonary hypertension after receiving stored autologous RBCs. Inhalation of nitric oxide gas, a selective pulmonary vasodilator[15] (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 humans[13].

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 ± 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) solution[11]. 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 volume[16]. 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 m^2) (Quadrox iD pediatric, Maquet, Wayne NJ, USA) using a gravity feed with 30 cm H_2O 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 (N_2)/10% oxygen (O_2) or 90% N_2/10% O_2 (control gas) with a gas flow rate of 700 ml/min. Ten percent O_2 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 oxygen[17], a scavenger reservoir containing 90 grams of SodaLime (Biodex, Shirley, NY) was placed immediately before the gas exchanger to remove nitrogen dioxide[18]. 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 deconvolution[12]. 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 assay[19] 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 μg/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 quadrature[20]. 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 al[21]. 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 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, 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 ± SD) whereas the expected change in the nitric oxide-treated stored RBC 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 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 μM vs. 15.86±5.38 μM, 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 assay[19] 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 μM vs. 7.4±10.2 μM, 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±14.7% vs. 2.13±2.14%, p=0.0004) or stored controls respectively (Figure 1C, increase of extracellular met-Hb by 75.54±14.47% vs. 2.39±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±3.79% vs. 0.05±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±2.3% vs. 0.13±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+ O_2: 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•sec•cm^{-5}•m^{-2} vs. 211.1±44.4

dyn•sec•cm^{-5}•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±8.2 μM vs. 3.1±1.8 μM, 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±8.2 μM, 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 RBCs[11]. 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±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±5.8% at 1 h after transfusion. In sheep transfused with stored RBCs pre-treated with NO gas, 86.8%±8.1 of biotinylated RBCs remained in the circulation 1 hour after transfusion. After 24 h, 90.8±4.1% of control- and 91.4±1.4% of nitric oxide gas-treated fresh RBCs remained in the circulation, while 78.3±6.3% of nitric oxide gas-treated

stored RBCs and 73.4±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 device[21]. 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±5 μm/s vs. 94±3 μm/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 μM 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 μM 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±2.1 mmHg at 30 min after starting the transfusion when compared to fresh RBCs (Figure 5A, PAP at 30 min of 18.4±2.1 mmHg vs. 13.9±0.6 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±12.5 dyn•sec•cm^{-5}•m^{-2} vs. 107.5±17.8 dyn•sec•cm^{-5}•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±6.0 μM vs. 2.5±1.6 μM, 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±9.9 μM vs. 1.7±0.8 μM, 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 storage[9]. 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 volunteers[13].

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 diseases[15]. 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 oxide[23]. 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 scavenging[28-30]. 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 thrombosis[26]. 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 reported[33]. Treating stored RBCs with nitric oxide may improve the safety of stored RBC transfusion.

When RBCs traverse small capillaries, they must change shape[34]. 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 membrane[35]. The resulting potassium ion efflux causes RBCs to shrink and makes their cell membranes less flexible[36]. 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 deformability[21]. 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 blood[39]. 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 humans[11]. However, with the exception of the P_{50} 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 vitr*o. 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.

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Figure Legends

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 (N_2)/10% oxygen (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 nitric oxide-treatment differed from the pre-post change under control. **(A)** pre-gas exposure SRBC+O_2: 104.9±67.09 μM vs. pre-gas exposure FRBC+O_2: 15.86±5.38 μM, p=0.0002. **(B)** SRBC+O_2: 7.4±10.2 μM vs. SRBC+NO: 80.1±70.1 μM, p=0.005. **(C)** FRBC+O_2: 2.13±2.14% vs. FRBC+NO: 64.45±14.7%, p<0.0001 and SRBC+O_2: 2.39±2.9% vs. SRBC+NO: 75.54±14.47%, p<0.01. **(D)** FRBC+O_2: 0.05±0.06% vs. FRBC+NO: 10.31±3.79%, p=0.0004 and SRBC+O_2: 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; O_2=oxygen; NO=nitric oxide.

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 (N_2)/10% oxygen (O_2) or to 90% N_2/10% O_2 (without nitric

oxide) 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. Hb=hemoglobin; RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; O_2=oxygen; NO=nitric oxide; PAP=mean pulmonary arterial pressure; PVRI=pulmonary vascular resistance index.

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 ± 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 ± 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 ± SD throughout. Hb=hemoglobin; RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; O_2=oxygen; NO=nitric oxide; PAP=mean pulmonary arterial pressure; PVRI=pulmonary vascular resistance index.

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; O_2=oxygen; NO=nitric oxide.

Figure 1:

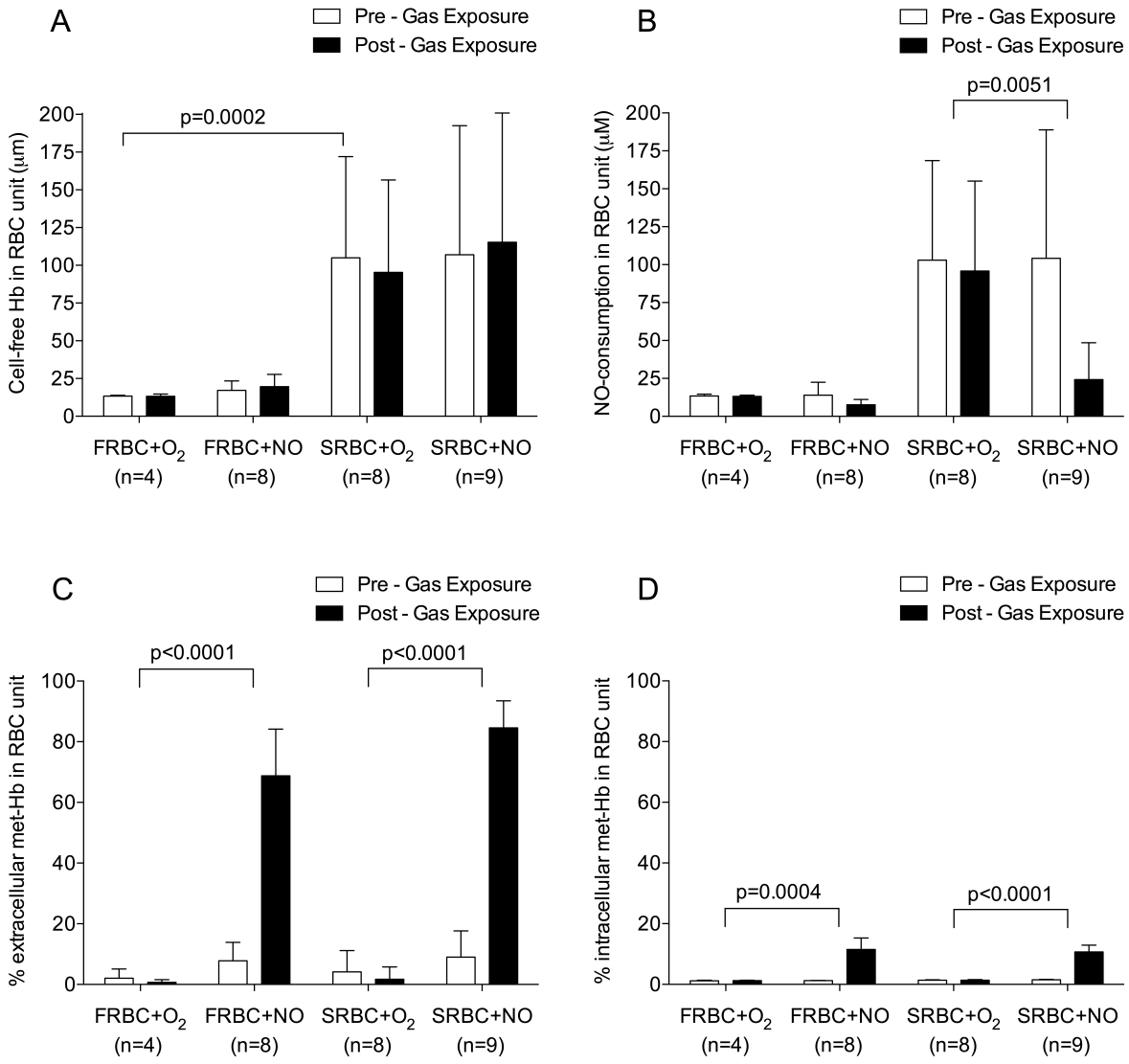

A
Pre - Gas Exposure
Post - Gas Exposure
p=0.0002
Cell-free Hb in RBC unit (μm)
200
175
150
125
100
75
50
25
0
FRBC+O2 (n=4)
FRBC+NO (n=8)
SRBC+O2 (n=8)
SRBC+NO (n=9)
B
Pre - Gas Exposure
Post - Gas Exposure
p=0.0051
NO-consumption in RBC unit (μM)
200
175
150
125
100
75
50
25
0
FRBC+O2 (n=4)
FRBC+NO (n=8)
SRBC+O2 (n=8)
SRBC+NO (n=9)
C
Pre - Gas Exposure
Post - Gas Exposure
p<0.0001
p<0.0001
% extracellular met-Hb in RBC unit
100
80
60
40
20
0
FRBC+O2 (n=4)
FRBC+NO (n=8)
SRBC+O2 (n=8)
SRBC+NO (n=9)
D
Pre - Gas Exposure
Post - Gas Exposure
p=0.0004
p<0.0001
% intracellular met-Hb in RBC unit
100
80
60
40
20
0
FRBC+O2 (n=4)
FRBC+NO (n=8)
SRBC+O2 (n=8)
SRBC+NO (n=9)

Figure 2:

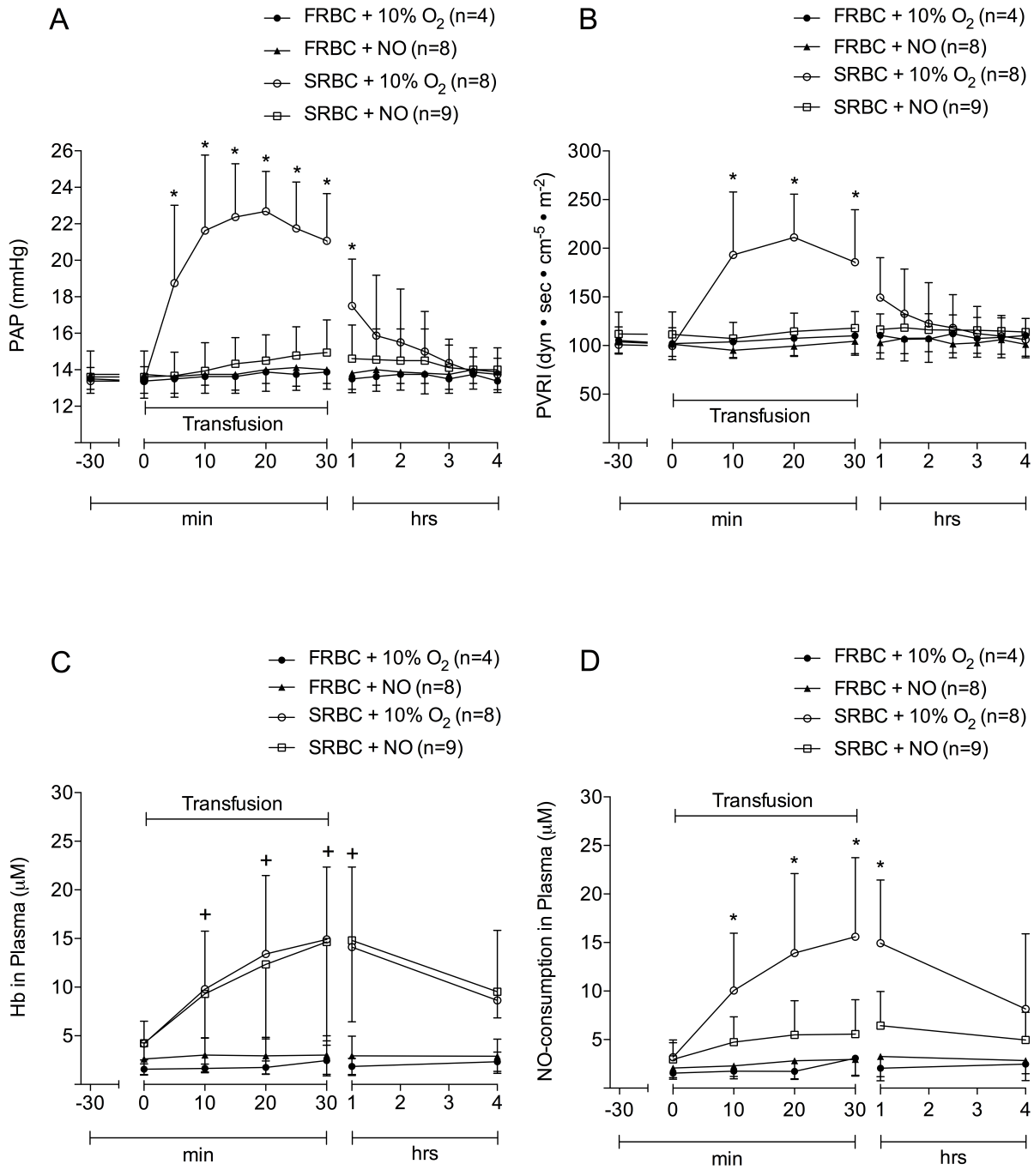

A
FRBC + 10% O2 (n=4)
FRBC + NO (n=8)
SRBC + 10% O2 (n=8)
SRBC + NO (n=9)
PAP (mmHg)
Transfusion
min
hrs
B
PVRI (dyn • sec • cm-5 • m-2)
C
Hb in Plasma (µM)
D
NO-consumption in Plasma (µM)

Figure 3:

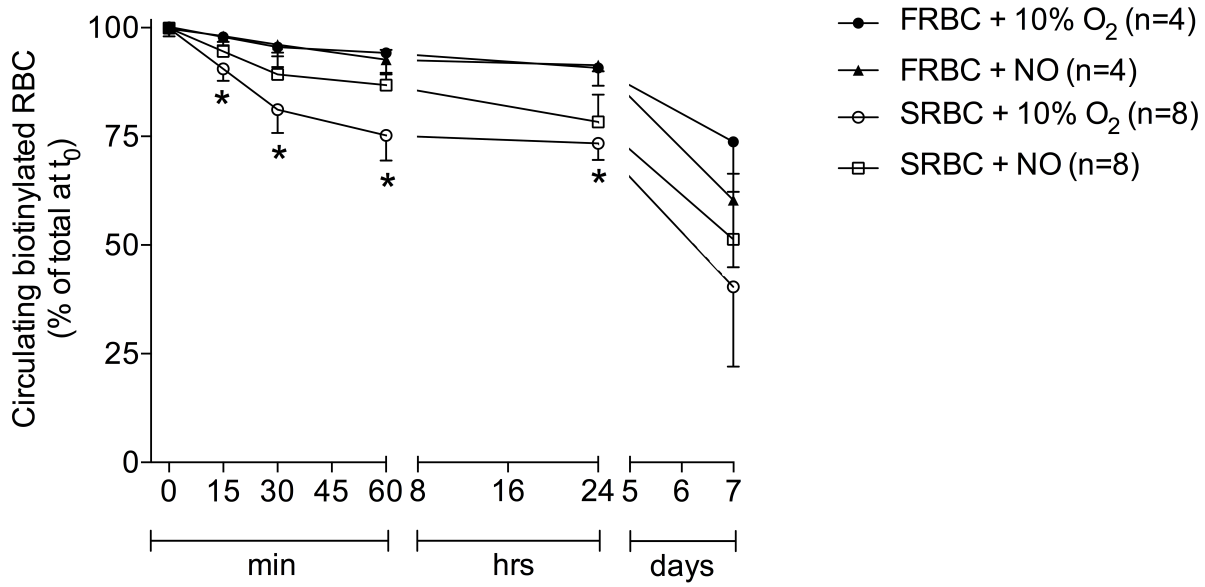

Circulating biotinylated RBC
(% of total at t_0)
100
75
50
25
0
0 15 30 45 60 8 16 24 5 6 7
min
hrs
days
*
*
*
*
FRBC + 10% O_2 (n=4)
FRBC + NO (n=4)
SRBC + 10% O_2 (n=8)
SRBC + NO (n=8)

Figure 4:

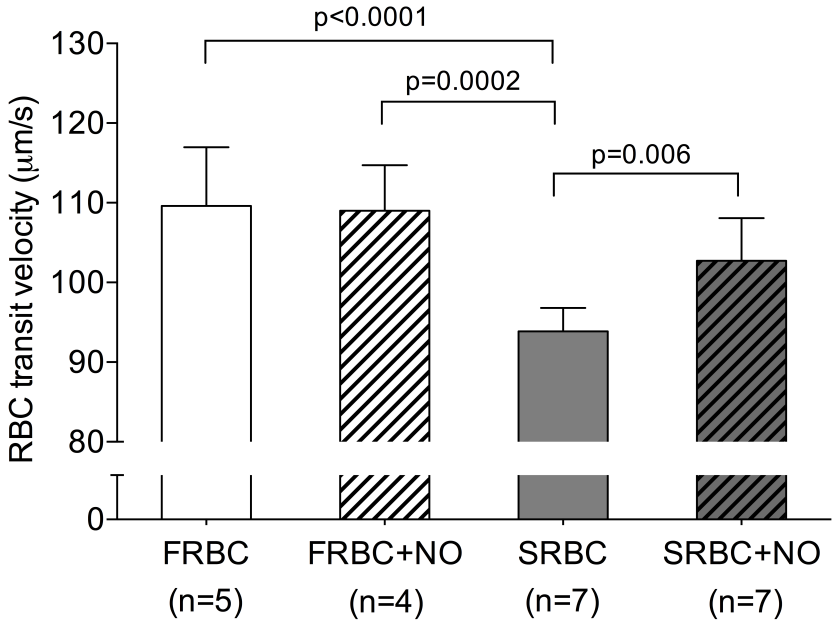

p<0.0001
p=0.0002
p=0.006
RBC transit velocity (μm/s)
130
120
110
100
90
80
0
FRBC
(n=5)
FRBC+NO
(n=4)
SRBC
(n=7)
SRBC+NO
(n=7)

Figure 5:

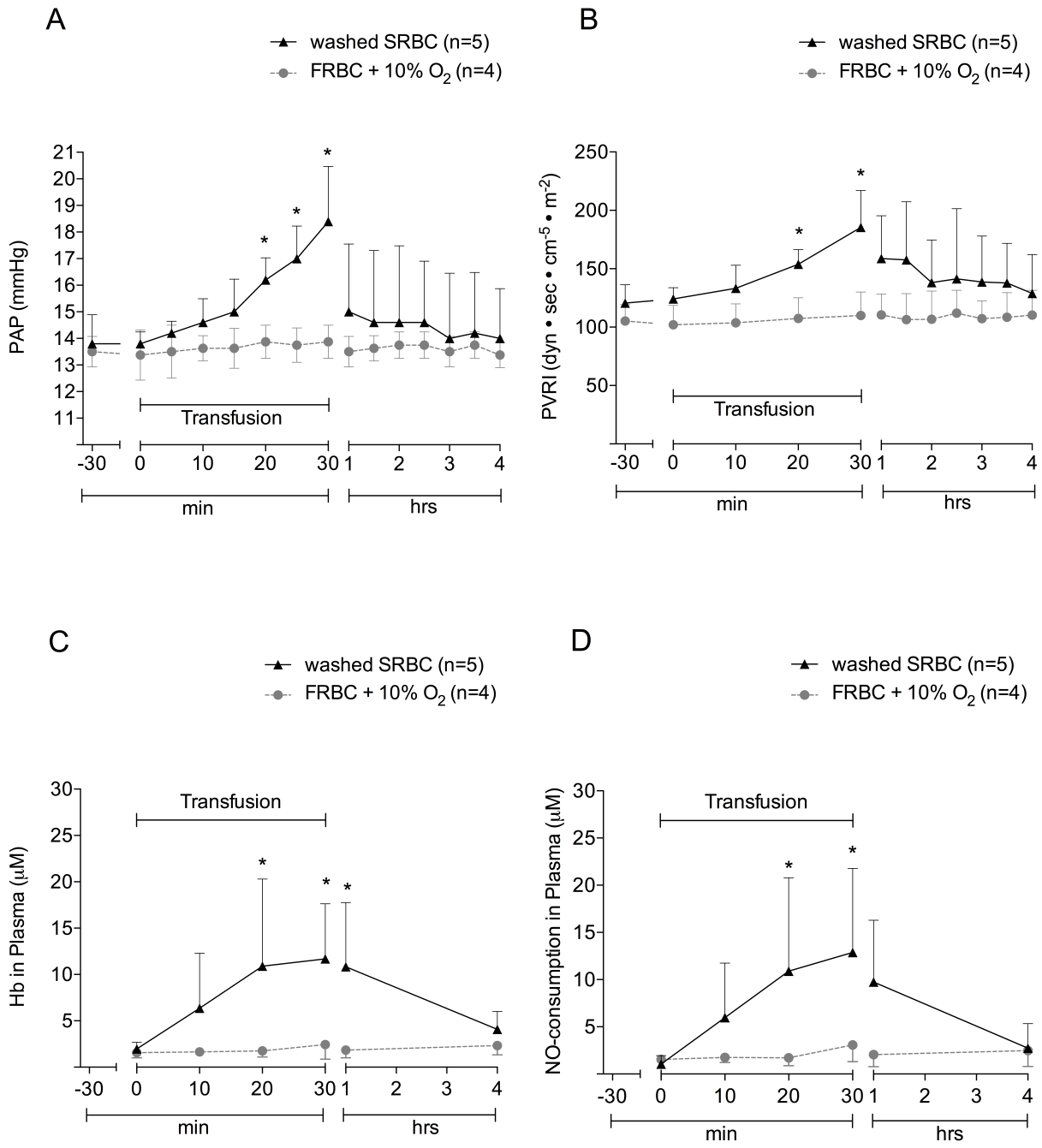
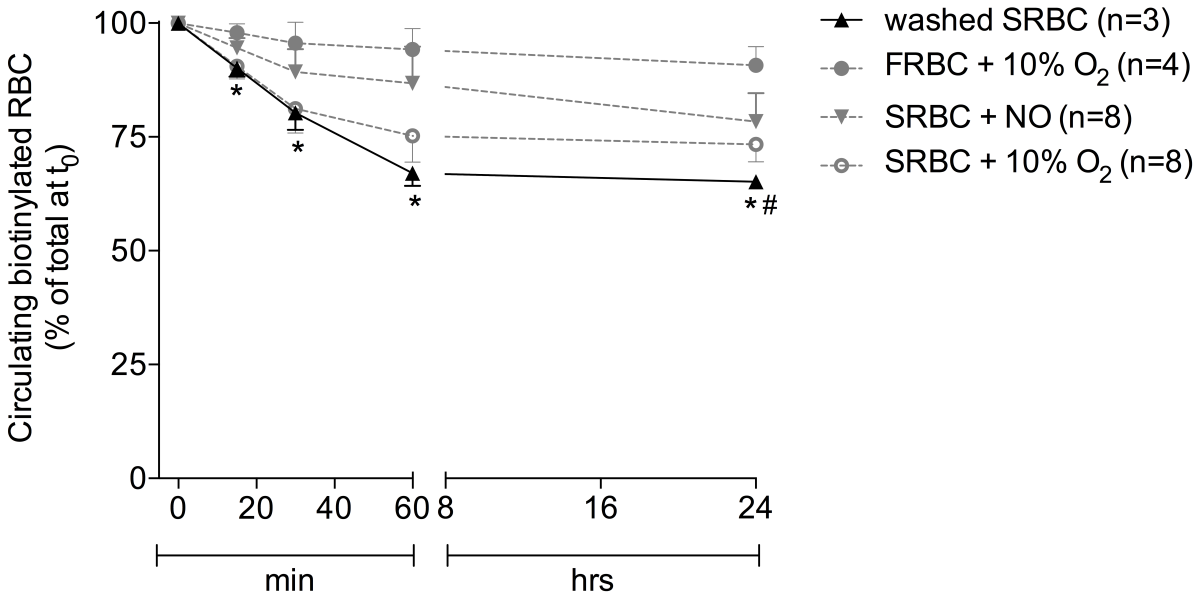

A
washed SRBC (n=5)
FRBC + 10% O2 (n=4)
PAP (mmHg)
Transfusion
min
hrs
B
washed SRBC (n=5)
FRBC + 10% O2 (n=4)
PVRI (dyn • sec • cm-5 • m-2)
Transfusion
min
hrs
C
washed SRBC (n=5)
FRBC + 10% O2 (n=4)
Hb in Plasma (µM)
Transfusion
min
hrs
D
washed SRBC (n=5)
FRBC + 10% O2 (n=4)
NO-consumption in Plasma (µM)
Transfusion
min
hrs

Figure 6:


Circulating biotinylated RBC
(% of total at t_0)
100
75
50
25
0
0
20
40
60
8
16
24
min
hrs
*
*
*
*#
washed SRBC (n=3)
FRBC + 10% O_2 (n=4)
SRBC + NO (n=8)
SRBC + 10% O_2 (n=8)

SUPPLEMENTAL DIGITAL CONTENT

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

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Supplemental Methods:

Animal preparation and hemodynamic monitoring

Seven groups of awake lambs were studied and randomized to the different study groups. One group of lambs (n=4) received control-treated fresh RBCs, a second group (n=8) was transfused with nitric oxide gas-treated fresh RBCs, in a third group (n=8) transfusion was performed with untreated stored RBCs and a fourth group (n=9) received nitric oxide gas-treated stored RBCs. Two additional groups were transfused with either fresh (n=4) or stored (n=6) MAHMA NONOate-treated RBCs whereas a

seventh group (n=5) received washed stored RBCs. The researcher who performed the hemodynamic measurements and analyses was blinded to the group and treatment assignment.

Animals were anesthetized with 2-3% isoflurane (Piramal Critical Care, Inc., Bethlehem, PA) in oxygen via a mask. After endotracheal intubation of the lamb, an 18 G catheter was placed in the right carotid artery. A 7 FR Swan-Ganz catheter was inserted in the pulmonary artery using a 8.5 Fr sheath introducer set with an integral hemostasis port (ID 2.8 mm, placed in the right jugular vein) that was used for the blood transfusion. Cefazolin (10 mg/kg) was given intravenously for perioperative antibiotic prophylaxis. After surgery, animals were extubated and allowed to recover from anesthesia in a Babraham metabolic cage for 2 h[1].

The lambs were gently restrained to prevent them from inadvertently removing invasive catheters, but the restraints allowed the animals to stand up or sit at will. On rare occasions, if the lambs appeared to be distressed, the animals were on rare occasion sedated with an IV bolus of 0.01-0.02 mg/kg midazolam.

Mathers and colleagues demonstrated in dogs that 1.0 minimal alveolar concentration (MAC) of isoflurane is needed to lower PAP and 2.0 MAC to lower pulmonary vascular resistance[2]. Our lambs were extubated after surgery when levels of isoflurane were below 0.17 MAC. We believe that, after 2 h of air breathing during the recovery period, there were insufficient isoflurane levels remaining to influence the pulmonary vasomotor response to transfusion in our awake lambs.

Hemodynamic parameters, including mean systemic arterial blood pressure (MAP), heart rate (HR), central venous pressure (CVP), and mean pulmonary arterial

pressure (PAP) were monitored continuously and the pulmonary capillary wedge pressure (PCWP) was measured intermittently every 10-30 min for 4 h after beginning RBC transfusion. Cardiac output was assessed by thermodilution as the average of three measurements after intravenous bolus injection of 10 ml of 0°C saline. Systemic vascular resistance index (SVRI), pulmonary vascular resistance index (PVRI) and cardiac index were calculated using standard formulae.

After all hemodynamic measurements were completed, lambs were anesthetized with 2% isoflurane in oxygen, all catheters were removed, and the right carotid artery was ligated. After recovery from anesthesia, sheep were housed and blood was sampled by venipuncture in the animal facility on days 1 and 7 to determine the lifespan of transfused RBCs.

Nitric oxide consumption assay

The nitric oxide consumption assay[3] is an *in vitro* test to measure the amount of nitric oxide that can be scavenged by a solution containing cell-free Hb and microvesicles containing Hb. Briefly, 0.01mM DETA NONOate, a nitric oxide donor with a half-life of 56 h at 25°C, was equilibrated in an anaerobic purge vessel flushed with helium gas entering a nitric oxide chemiluminescence analyzer (Sievers, Boulder CO). When the nitric oxide concentration achieved a steady state (after 20-30min), samples of supernatant from the stored blood units are injected into the solution and the instantaneous decrease in nitric oxide concentration is quantified.

Experimental assay of deformability of nitric oxide-treated RBC using microfluidics

Blood samples (1.5 ml) were obtained from fresh and stored ovine RBC units after transient *ex vivo* exposure to 300 ppm NO in 90% N_2/10% O_2 or a control gas mixture (90% N_2/10% O_2). One microliter of the RBC sample was pelleted and re-suspended in 200 µl PBS solution containing 1% w/v bovine serum albumin (BSA). The addition of BSA prevented cell adhesion to walls of the container, while the low cell density prolonged the time that cells remained in a single-cell suspension.

The microfluidic device is an optimized design of the device described by Bow and co-workers[4] to study the deformability of individual RBCs. Standard microfabrication and soft lithography were used to fabricate poly-dimethylsiloxane (PDMS) microfluidic devices as described[4]. In brief, silicon wafers with the desired pattern were created using SU-8 photoresist and UV exposure. We then silanized wafers using trichloro(1H,1H,2H,2H-perfluorooctyl)silane (FOTS). PDMS prepolymer (Sylgard 194, Dow Corning, Midland, MI) was mixed with curing agent in a ratio of 10:1 and then cast on patterned wafers with 2 h of curing at 80ºC. Inlets and outlets were created in the PDMS device using a 1.5mm biopsy punch. The patterned PDMS then was bonded to a glass slide using oxygen plasma treatment via an RF source (Harrick Plasma).

The RBCs flowed through a large microchannel (1mm wide and 5mm long) facing three periodic sets of rectangular capillaries (Figure 10). The cross section of capillaries or “slits” were 5 µm high and 2 µm wide with an inverted trapezoidal shaped flow entrance. The trajectories of individual RBCs were monitored through an inverted

microscope (Zeiss Axiovert 200) equipped with a CCD camera (Hitachi KP-D20AU) at 30 frames per second and fed to a PC via a Labview interface. “Individual transit RBC velocity” was defined as the velocity of the RBC within each slit; averaged over three periodic slit sets. This velocity was obtained by post-imagining analysis ImageJ software (NIH, Bethesda, MD). Average RBC velocity for each sample was computed from 134 individual RBC transit velocities.

Supplemental Results

Treatment of stored RBCs with MAHMA NONOate reduces nitric oxide consumption by supernatant Hb

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. We tested whether in vitro treatment of stored RBC units with MAHMA NONOate would reduce scavenging of nitric oxide by Hb in storage unit supernatant. Comparisons were performed on the change in cell-free Hb, nitric oxide consumption, and met-Hb in MAHMA NONOate-treated RBCs versus control-treated RBCs.

As previously noted, the Hb concentration in the supernatant of stored RBCs was greater than that in the supernatant of fresh RBCs. Treatment of stored RBCs with MAHMA NONOate (at a final concentration of 200 μM) did not increase the level of supernatant Hb, suggesting that MAHMA NONOate treatment did not induce hemolysis (Supplemental Figure 1A).

To investigate the nitric oxide-scavenging effects of supernatant Hb exposed to MAHMA NONOate, we measured the nitric oxide-consumption of RBC unit supernatant before and after incubation with the nitric oxide-donor. We found the fresh RBC unit supernatant scavenged far less nitric oxide than the stored RBC unit supernatant (Supplemental Figure 1B). Incubation of stored RBC supernatant with MAHMA NONOate reduced nitric oxide consumption as compared with untreated stored RBC

supernatant (Supplemental Figure 1B, reduction in nitric oxide-consumption by 81.2±14.8 μM vs. 7.4±10.2 μM, p=0.0007).

To test whether the reduction in nitric oxide-consumption might be related to an increase in extracellular met-Hb, we measured the extracellular fraction of met-Hb by spectral deconvolution[5]. Pre-treatment with the nitric oxide-donor compound increased the percent of extracellular met-Hb in RBC storage supernatant from 1±0.4% to 32±5%, p=0.001 (fresh blood) and from 5±3% to 68±4%, p<0.0001 (stored blood) respectively (Supplemental Figure 1C).

The percentage of intracellular met-Hb was measured to evaluate the RBC oxygen transport capacity after incubation with MAHMA NONOate. Intracellular met-Hb levels increased to 3±0.2% when either fresh or stored RBCs were incubated with MAHMA NONOate for 20 min (Supplemental Figure 1D). The intracellular met-Hb concentrations did not differ from baseline levels at 2 h after treatment and incubation with MAHMA NONOate (data not shown).

These findings demonstrate that the ability of the supernatant of stored RBC units to consume nitric oxide was reduced after MAHMA NONOate treatment, via conversion of oxy-Hb to met-Hb. Treatment with the nitric oxide-donor compound MAHMA NONOate did not cause hemolysis of stored RBCs. Furthermore, because the level of intracellular met-Hb was increased to only 3% and rapidly returned to normal, the oxygen transport capacity of stored RBCs was not permanently impaired by MAHMA NONOate treatment.

Incubating stored RBCs with the nitric oxide-donor compound MAHMA NONOate prevents transfusion-associated pulmonary hypertension

Pulmonary hemodynamic parameters were measured in awake lambs before, during and after transfusion of one unit of fresh or stored MAHMA NONOate-treated RBCs. When stored RBCs were exposed to the nitric oxide-donor compound before transfusion, PAP did not increase when compared to fresh RBCs (Supplemental Figure 2A, PAP at 20 min of 14.5±0.8 mmHg vs. 13.9±0.6 mmHg, $p>0.01$), indicating that treatment with MAHMA NONOate prevented the transfusion-related increase in PAP. Transfusion of fresh RBCs, whether treated with the nitric oxide-donor compound or not, did not change the PAP (Supplemental Figure 2A).

Pre-treatment of a stored RBC unit with the nitric oxide-donor compound prevented the increase in PVRI (Supplemental Figure 2B, PVRI at 20 min of 118.5±12 $dyn•sec•cm^{-5}•m^{-2}$ vs. 107.5±17.8 $dyn•sec•cm^{-5}•m^{-2}$, $p>0.05$). Transfusion of a fresh RBC unit with or without adding MAHMA NONOate did not alter PVRI (Supplemental Figure 2B). These results show that, similar to ex vivo nitric oxide gas exposure, pre-treatment with MAHMA NONOate prevents the pulmonary vasoconstriction and hypertension associated with transfusion of stored RBCs.

Plasma Hb concentrations, measured 10 to 60 min after commencing transfusion, were greater in sheep transfused with stored RBCs and MAHMA NONOate-treated stored RBCs, as compared to sheep transfused with fresh RBCs (Supplemental Figure 2C). However, treatment with the nitric oxide-donor compound did not further increase the level of circulating cell-free Hb in the plasma of sheep that received MAHMA NONOate-treated, as compared to untreated, stored RBCs.

Treatment of a stored RBC unit with MAHMA NONOate prior to transfusion markedly decreased the ability of circulating plasma to scavenge nitric oxide (Supplemental Figure 2D). Transfusion of fresh RBCs was not associated with increased plasma nitric oxide-consumption levels. Nitric oxide-scavenging did not differ between fresh RBCs with or without MAHMA NONOate treatment (Supplemental Figure 2D).

Pre-treatment of the stored RBC unit with MAHMA NONOate and subsequent transfusion did not produce systemic vasodilation or alter systemic hemodynamic parameters, including heart rate, mean arterial pressure, systemic vascular resistance index, cardiac index and central venous pressure. Transfusion of a MAHMA NONOate-treated RBC unit also had no effect on arterial or mixed venous blood gas tensions (data not shown).

Taken together, the results show that treatment of a stored RBC unit with the short-lived nitric oxide-donor compound MAHMA NONOate prevents transfusion-associated pulmonary vasoconstriction and pulmonary hypertension without causing systemic vasodilation.

Pre-treatment with MAHMA NONOate improves 1 and 24 h RBC survival

The number of MAHMA NONOate-treated, biotin-labeled circulating RBCs was measured after transfusion of fresh or stored RBCs. When fresh RBCs were pre-treated with MAHMA NONOate, 94.2±2.3% of the cells were circulating after 1 h (Supplemental Figure 3). After 24 h, 93±2% of MAHMA NONOate-treated fresh RBCs remained in the circulation. In addition, the number of MAHMA NONOate-treated stored RBC which

were circulating after 24 h was higher when compared to untreated stored RBCs (Supplemental Figure 3). Seven days after transfusion, there was still a difference in the percentage of MAHMA NONOate-treated, compared to control-treated, stored RBCs remaining in the circulation.

These findings demonstrate that, similar to NO gas exposure before transfusion, pre-treatment of stored RBCs with MAHMA NONOate improved the 24 h and 7 day RBC survival.

Pre-treatment of stored RBCs with MAHMA NONOate increases RBC deformability

To investigate whether the nitric oxide-donor compound increases RBC deformability, the average transit velocity of MAHMA NONOate-treated fresh and stored RBCs through the microfluidic cytometer was measured. The average transit velocity of MAHMA NONOate-treated stored RBCs was higher than untreated stored RBCs (MAHMA NONOate-treated stored RBCs vs. control-treated stored RBCs, 95±5 μm/s vs. 115±5 μm/s, Supplemental Figure 4). These results suggest that, as with exposure to nitric oxide gas, ex vivo exposure of stored RBCs to a chemical nitric oxide-donor increases RBC deformability.

Supplemental Figure Legends

Supplemental 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 erythrocyte (SRBC) supernatants before and after treatment with 200 μM MAHMA NONOate. Fresh and stored RBC controls that were exposed to the control gas (90% nitrogen (N_2)/10% oxygen (O_2)) are superimposed (grey dashed lines) as historic controls in panels A-D. All data mean±SD. Hb=hemoglobin; RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; O_2=oxygen; MAHMA=Methylaminehexamethylenemethylamine nonoate.

Supplemental 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 both fresh (FRBC) and stored erythrocyte (SRBC) units after treatment with 200 μM MAHMA NONOate. Fresh and stored RBC controls that were exposed to the control gas (90% nitrogen (N_2)/10% oxygen (O_2)) are superimposed (grey dashed lines) as historic controls in panels A-D. *$p<0.01$ values of SRBC+10% O_2 differ from FRBC+10% O_2, FRBC+MAHMA NONOate and SRBC+MAHMA NONOate. +$p<0.01$ values of both SRBC+10% O_2 and SRBC+MAHMA NONOate differ from FRBC+10 % O_2 and FRBC+MAHMA NONOate. All data mean±SD. Hb=hemoglobin; RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed

erythrocytes; O_2=oxygen; MAHMA=Methylaminehexamethylenemethylamine nonoate; PAP=mean pulmonary arterial pressure; PVRI=pulmonary vascular resistance index.

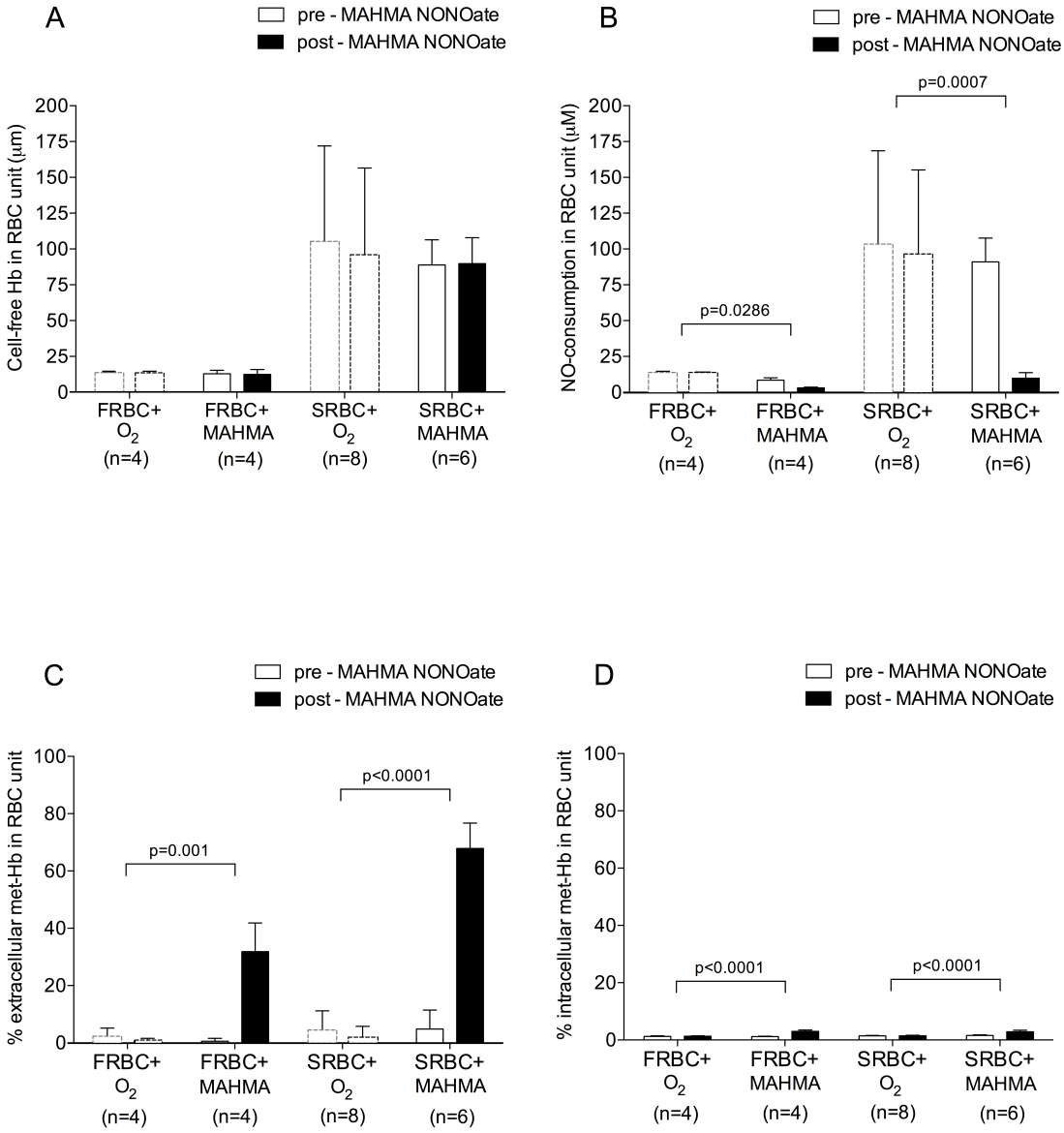
Supplemental Figure 3:

Fresh (FRBC) or stored erythrocytes (SRBC) were treated with 200 µM MAHMA NONOate and the lifespan of circulating biotinylated erythrocytes was measured up to 7 days after transfusion. Fresh and stored RBC controls that were exposed to the control gas (90% nitrogen (N_2)/10% oxygen (O_2)) are superimposed (grey dashed lines) as historic controls. * values of SRBC+MAHMA NONOate differ from SRBC+10% O_2, Bonferroni-adjusted p-values: 60min: p=0.074, 24h: p=0.005, 7 days: p=0.005. All data mean±SD. RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; O_2=oxygen; MAHMA=Methylaminehexamethylenemethylamine nonoate.

Supplemental Figure 4:

Average velocity to travel across the microfluidic synthetic capillaries of fresh (FRBC) and stored erythrocytes (SRBC) treated with 200 µM MAHMA NONOate. All data mean±SD. RBC=packed erythrocytes; FRBC= fresh packed erythrocytes; SRBC=stored packed erythrocytes; MAHMA=Methylaminehexamethylenemethylamine nonoate.

Supplemental Figure 1:



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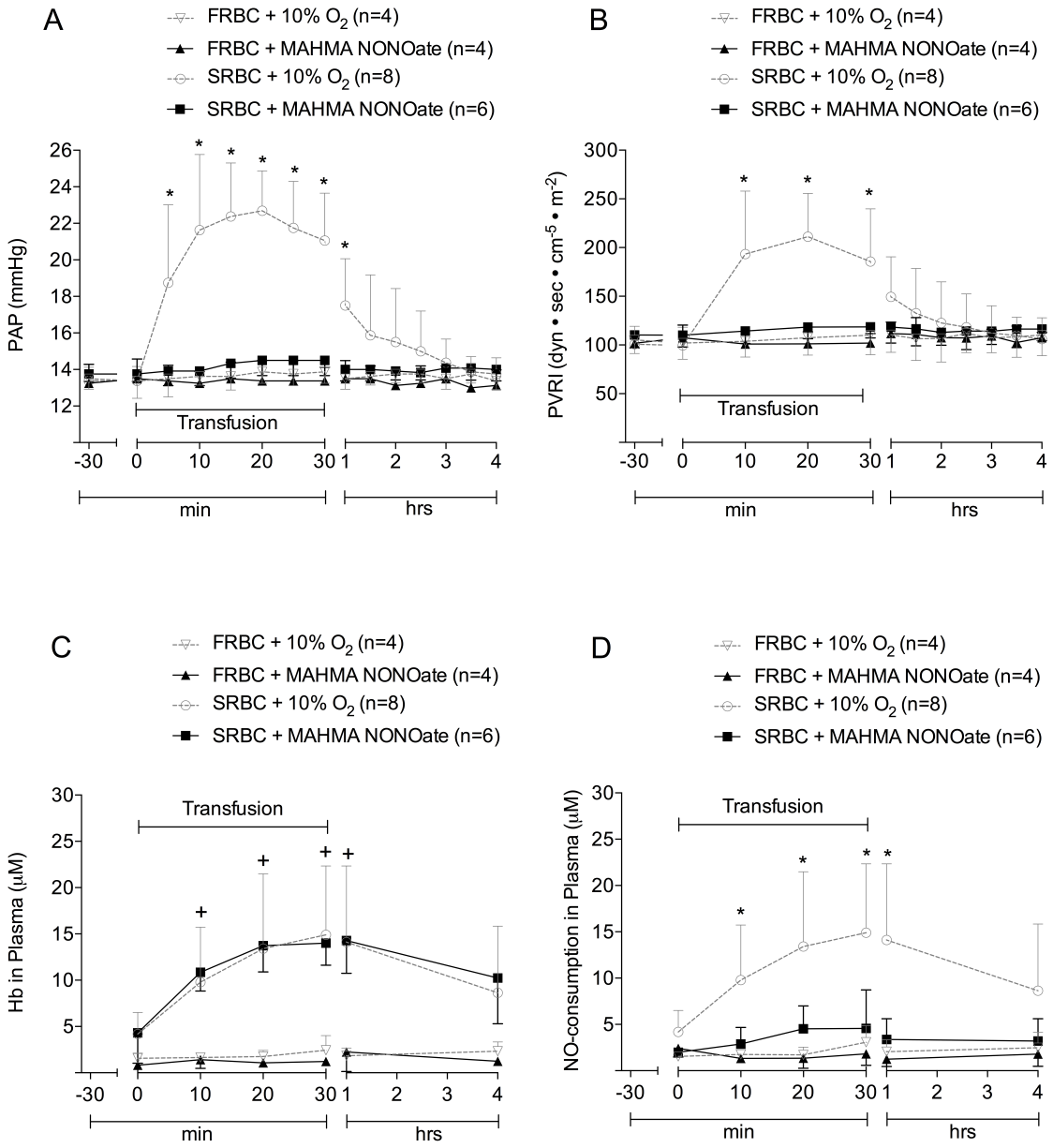

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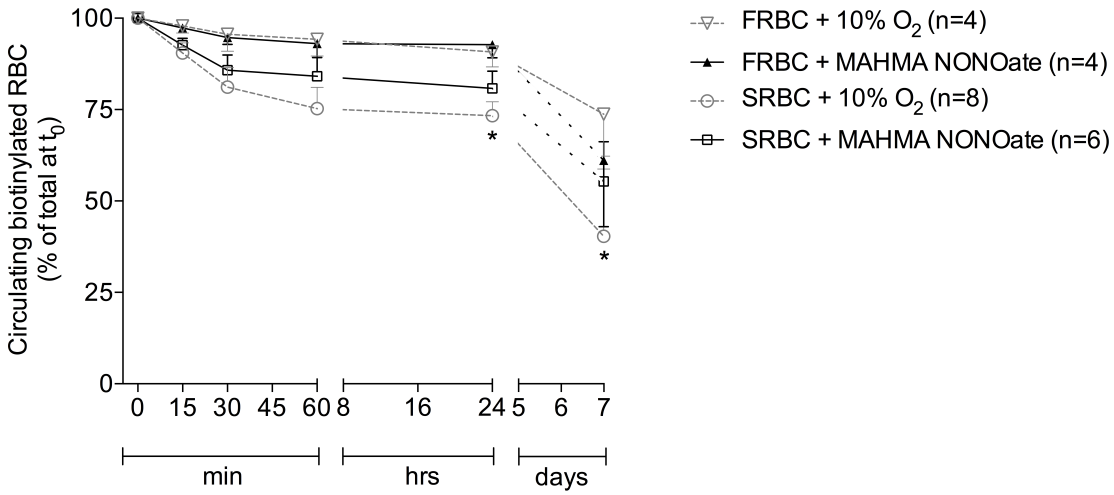

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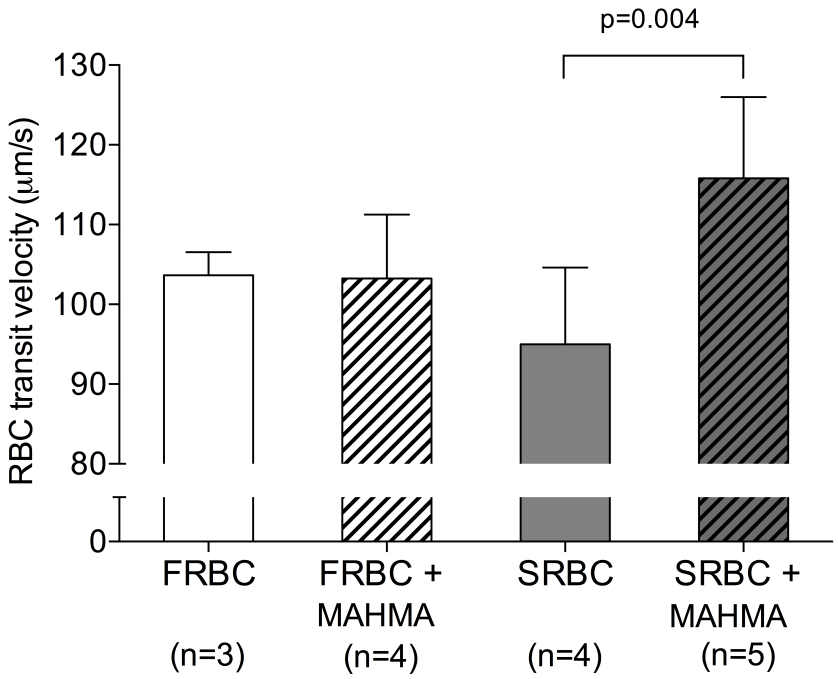

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