Pulmonary Hypertension in Lambs Transfused with Stored Blood Is Prevented by Breathing Nitric Oxide

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ABSTRACT

Background: During extended storage, erythrocytes undergo functional changes. These changes reduce the viability of erythrocytes leading to release of oxyhemoglobin, a potent scavenger of nitric oxide. We hypothesized that transfusion of ovine packed erythrocytes (PRBC) stored for prolonged periods would induce pulmonary vasoconstriction in lambs, and that reduced vascular nitric oxide concentrations would increase this vasoconstrictor effect.

What We Already Know about This Topic

- Storage of packed erythrocytes (PRBC) results in hemolysis and increases the concentration of plasma hemoglobin, a scavenger of nitric oxide
- Transfusion of stored PRBC could therefore reduce nitric oxide bioavailability and produce hemodynamic effects

What This Article Tells Us That Is New

- In sheep, transfusion of stored (40 days) autologous PRBC increased pulmonary vascular resistance and pressure. This effect was worsened in the presence of a nitric oxide synthase inhibitor and ameliorated by inhalation of nitric oxide

Methods: We developed a model of autologous stored blood transfusion in lambs (n = 36). Leukoreduced blood was stored for either 2 days (fresh PRBC) or 40 days (stored PRBC). Fresh or stored PRBC were transfused into donors instrumented for awake hemodynamic measurements. Hemodynamic effects of PRBC transfusion were also studied after infusion of N^G-nitro-L-arginine methyl-ester (25 mg/kg) or during inhalation of nitric oxide (80 ppm).

Results: Cell-free hemoglobin levels were higher in the supernatant of stored PRBC than in supernatant of fresh PRBC (Mean ± SD, 148 ± 20 vs. 41 ± 13 mg/dl, respectively, $P < 0.001$). Pulmonary artery pressure during transfusion of stored PRBC transiently increased from 13 ± 1 to 18 ± 1 mmHg ($P < 0.001$) and was associated with increased plasma hemoglobin concentrations. N^G-nitro-L-arginine methyl-ester potentiated the increase in pulmonary arterial pressure induced by transfusing stored PRBC, whereas inhalation of nitric oxide prevented the vasoconstrictor response.

Conclusions: Our results suggest that patients with reduced vascular nitric oxide levels because of endothelial dysfunction may be more susceptible to adverse effects of transfusing blood stored for prolonged periods. These patients might benefit from transfusion of fresh PRBC, when available, or inhaled nitric oxide supplementation to prevent the pulmo-

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nary hypertension associated with transfusion of stored PRBC.

TRANSFUSION of packed erythrocytes (PRBC) stored for longer than 2 weeks has been associated with increased rates of infection, a prolonged hospital length of stay, and increased mortality rates in intensive care unit patients and patients undergoing cardiovascular surgery (reviewed in reference[1]). Prolonged storage causes marked biochemical, mechanical, and functional alterations in erythrocytes, termed collectively the "storage lesion."[2] However, the precise mechanisms responsible for the adverse effects of transfusing stored blood remain incompletely elucidated.

Erythrocytes lyse during prolonged storage and are more susceptible to *in vivo* lysis after they are transfused.[3,4] Gladwin *et al.* have demonstrated that bioavailability of vascular nitric oxide is reduced when hemolysis causes hemoglobin to be released from erythrocytes into plasma.[5] Similar reductions of vascular nitric oxide bioavailability because of increased plasma hemoglobin concentrations have been reported in patients with hemolytic disorders such as sickle cell disease[6–8] and malaria.[9,10] Other possible mechanisms that can result in a reduction of vascular nitric oxide bioavailability are degradation of L-arginine by erythrocytic arginase after hemolysis or shedding of microparticles containing oxyhemoglobin from the erythrocyte membrane during storage.[11–13] Reduced vascular nitric oxide levels can contribute to vasoconstriction, inflammation, and thrombosis, potentially explaining some of the adverse effects associated with transfusing blood stored for prolonged periods.[14–17] Other nitric oxide carrier molecules such as S-nitroso (SNO)-hemoglobin are also depleted during blood storage and may account for some of the adverse effects after transfusion.[18]

Endothelial dysfunction, commonly associated with cardiovascular and metabolic disorders, is in part characterized by impaired production of nitric oxide by endothelial cells lining blood vessels.[19] We have previously reported that the endothelial dysfunction seen in obese diabetic mice enhances the systemic vasoconstrictor response to infusion of tetrameric hemoglobin and stored murine blood.[17,20]

The pulmonary endothelium produces nitric oxide, and vasoconstriction occurs when the pulmonary endothelium is injured.[21] When inhaled, nitric oxide can selectively dilate the pulmonary circulation and reverse pulmonary hypertension.[22] We have previously demonstrated in lambs that the systemic and pulmonary vasoconstrictor effects of hemoglobin-based oxygen carriers could be prevented by breathing nitric oxide.[17,23]

We hypothesized that (1) transfusion of PRBC stored for prolonged periods would induce pulmonary vasoconstriction in lambs, (2) endothelial dysfunction would markedly increase the vasoconstrictor effects of transfusing stored blood, and (3) breathing nitric oxide would prevent these vasoconstrictor effects. Based upon established human PRBC storage practices, we developed and validated a lamb model for autologous blood storage and transfusion. Ovine PRBC were stored for either 2 or 40 days in an additive solution used for human blood storage. After 2 or 40 days, hemodynamic effects of transfusing autologous stored PRBC were studied in lambs instrumented with carotid artery and pulmonary artery catheters. In order to avoid blunting of vasomotor responses, these animals were studied awake without the influence of anesthetic agents.[24]

The present study reports that transfusion of ovine PRBC stored for 40 days caused pulmonary hypertension associated with increased plasma hemoglobin concentrations. Inhibition of nitric oxide synthase (NOS) sensitized the pulmonary circulation to the vasoconstrictor effects of transfusing blood stored for 40 days. Breathing nitric oxide prevented the pulmonary vasoconstrictor effects of transfusing stored blood.

Materials and Methods

Processing of Blood Products

All experiments were approved by the Subcommittee on Research Animal Care, Massachusetts General Hospital, Boston, MA. We studied thirty-six 3- to 4-month-old Polypay[25] lambs (New England Ovis, Dover, NH) weighing 32 ± 2 kg. Following an intramuscular injection of ketamine HCl (15 mg/kg; Hospira, Inc., Lake Forest, IL), blood (450 ml) was drawn from an external jugular vein into a Double Blood-Pack Unit (Fenwal, Inc., Lake Zurich, IL) containing citrate-phosphate-dextrose solution. Blood was then leukoreduced at room temperature, using an integrated RS2000 leukoreduction filter. Erythrocytes were separated from plasma by centrifugation (600 g for 10 min at 24°C) and stored for either 2 or 40 days at 4°C in an additive solution (110 ml) containing saline, adenine, glucose and mannitol (AS-1, Adsol Solution, Fenwal, Inc.).

Biotinylation of Ovine Erythrocytes

In vivo survival of transfused PRBC was measured in eight lambs using a modified version of the methods of Mock *et al.*[26] After storage in AS-1 for either 2 (n = 4) or 40 days (n = 4), PRBC (60 ml) were withdrawn from the blood storage bag and separated from supernatant by centrifugation at 600 g for 15 min at 4°C. Supernatant was stored at 4°C. The pelleted PRBC were resuspended in washing solution (60 ml; NaCl 0.87%, NaHCO$_3$ 0.2%, dextrose 0.2%, and sodium phosphates 0.1%; Hospira, Inc.). Centrifugation was repeated, and the supernatant was discarded. Washed PRBC were resuspended in washing solution (60 ml) containing sulfo-N-hydroxysuccinimide-biotin (10 μg/ml; Thermo Fisher Scientific, Rockford, IL) and incubated at room temperature for 40 min. To remove unbound sulfo-N-hydroxysuccinimide-biotin, PRBC were washed twice with washing solution, as described above. After the final washing step, PRBC were resuspended in the previously stored supernatant.

Biotinylated PRBC were transfused into sheep donors *via* a 16-G Angiocath (BD Infusion Therapy Systems, Inc.,

Sandy, UT) placed in an external jugular vein. Venous blood samples were drawn into heparinized 4-ml Vacutainers (BD, Franklin Lakes, NJ) at 15 min, 30 min, 60 min, 24 h, and 7 days later.

To assess the survival of biotinylated PRBC, blood samples (50 μl) were incubated for 20 min at room temperature with fluorescein isothiocyanate-tagged streptavidin (250 μl of washing solution containing 20 μg/ml streptavidin-fluorescein isothiocyanate; Biolegend, San Diego, CA). Fluorescein isothiocyanate-labeled PRBC were detected in forward *versus* side scatter (FSC/SSC) on a LSRFortessa flow cytometer (BD Biosciences, San Jose, CA). Sphero rainbow fluorescent particles (3.0–3.4 μm; Spherotech, Inc., Lake Forest, IL) were used to calibrate settings of the flow cytometer on different acquisition days. An extrapolation of the number of biotinylated PRBC at 0 h was performed and equated to 100%. Of 100,000 total events, the ratio of the percentage of biotinylated cells in blood samples obtained after the transfusion to the percentage of biotinylated cells calculated to be present at 0 h was calculated.

Animal Preparation and Hemodynamic Monitoring

Invasive hemodynamic measurements were performed in 28 lambs. Anesthesia was induced by breathing 5% isoflurane (Baxter, Deerfield, IL) in oxygen *via* mask. After tracheal intubation, animals underwent a tracheostomy and were instrumented with indwelling carotid artery and pulmonary artery catheters, as previously described.[17] After a 2-h recovery period from general anesthesia in a large-animal restraint unit (Lomir, Malone, NY), the mean arterial pressure (MAP), mean pulmonary arterial pressure (PAP), and central venous pressure were monitored continuously using a Gould 6600 amplifier system (Gould Electronics, Inc., Eastlake, OH). Pulmonary capillary wedge pressure and heart rate were intermittently measured every 10–30 min. Cardiac output was assessed by thermal dilution as the average of three measurements after intravenous bolus injection of 10 ml ice-cold saline solution. Systemic vascular resistance index and pulmonary vascular resistance index (PVRI), as well as cardiac index, were calculated using standard formulae. Hemodynamic data were collected until 4 h after the end of transfusion.

Hemodynamic Effects of Stored PRBC Transfusion in Awake Lambs

Six groups of awake lambs were studied. All animals received an autologous transfusion of PRBC, equivalent to 14% of their respective total blood volume, assuming 6.5% of body weight was blood volume.[27] PRBC were warmed to 37°C and transfused during 30 min as the lambs breathed spontaneously *via* a tracheostomy at inspired oxygen fraction (FiO$_2$) 0.25.

One group (n = 5) of lambs received PRBC, which were processed and stored for 2 days before transfusion (fresh PRBC). A second group of lambs (n = 6) was transfused with PRBC that were stored for 40 days before transfusion

(stored PRBC). A third group (n = 4) of lambs breathed 80 ppm nitric oxide (Medical-Technical Gases, Medford, MA) at 0.25 FiO$_2$ during transfusion with stored PRBC. nitric oxide breathing was continued for 30 min after the transfusion ended.

Hemodynamic Effects of Transfusing Stored PRBC in a Lamb Model of Endothelial Dysfunction

Three additional groups of lambs were studied after intravenous infusion of the NOS inhibitor, N^G-nitro-L-arginine methyl-ester (L-NAME, Sigma-Aldrich, St. Louis, MO). A bolus of 25 mg/kg L-NAME was injected intravenously 1 h before transfusion.[28] Concomitantly, an infusion of 5 mg/kg/h L-NAME was started and continued throughout the study. Partial inhibition of nitric oxide production was confirmed before, as well as 45 min and 5 h after commencing L-NAME administration by assessing the systemic vasodilation induced by an intravenous bolus injection of acetylcholine (Sigma-Aldrich). NOS-independent systemic vasodilation was also assessed by intravenous injection of sodium nitroprusside (Sigma-Aldrich) at the end of each experiment.

After L-NAME treatment, a fourth group of lambs (n = 4) received fresh PRBC, and a fifth group (n = 5) received stored PRBC. In the sixth group (n = 4), following L-NAME injection, inhalation of 80 ppm nitric oxide at FiO$_2$ 0.25 was commenced 10 min before transfusion of stored PRBC. Nitric oxide breathing continued throughout the transfusion and for 30 min after transfusion ended.

Biochemical Analysis of Blood Samples

Arterial and venous blood samples (10 ml) were drawn immediately before transfusion, as well as 30 min, 2 h, and 4 h after ending transfusion. Samples (10 ml) from the transfusion storage bags were obtained and processed immediately after transfusion in order to avoid bacterial contamination. Blood gas tensions and pH were analyzed using an ABL800 Flex blood gas analyzer (Radiometer Medical, Copenhagen, Denmark).

Cell-free hemoglobin levels in plasma and supernatant were measured using a QuantiChrom hemoglobin assay kit (BioAssay Systems, Hayward, CA). Hematocrit was measured by centrifugation of whole blood at 400 g for 10 min in capillary tubes (Fisher Scientific, Pittsburgh, PA). Hemolysis (in percent) was calculated from the following formula[29]: Cell-free Hemoglobin [g/dl] (100-Hematocrit [%])/Total Hemoglobin [g/dl].

Plasma and supernatant concentrations of nitrate and nitrite were measured with a Nitrate/Nitrite Fluorometric Assay Kit (Cayman Chemical Company, Ann Arbor, MI). Plasma thromboxane B$_2$ (TXB$_2$) levels were determined with an EIA kit (Cayman Chemical Company). The levels of 2,3-diphosphoglycerate (2,3-DPG) were measured with a kit from Roche Diagnostics (Mannheim, Germany). Plasma interleukin-6 (IL-6) levels were measured with a bovine kit from Thermo Fisher Scientific. Haptoglobin (Hp) concen-

trations were assessed using a bovine Hp ELISA kit (Immunology Consultants Lab, Inc., Newberg, OR).

The oxygen dissociation curve of leukoreduced blood stored for either 2 or 40 days was determined using a Hemox-analyzer (TSC Scientific Corp., New Hope, PA). The oxygen tension at which hemoglobin is half-saturated (P_{50}) was calculated from the oxygen dissociation curve.

Quantitation of Messenger RNA (mRNA) Levels

At 4 h after transfusion, animals were euthanized by intravenous injection of 50 ml of 20% KCl solution during isoflurane anesthesia. Tissue samples were obtained from the lung and liver, snap-frozen in liquid nitrogen, and stored at $-80°C$ until further analysis.

RNA was extracted from tissues using Trizol (Invitrogen, Carlsbad, CA), and complementary DNA was synthesized using MMLV-RT (Invitrogen). Real-time amplification of transcripts was detected using a Mastercycler ep Realplex (Eppendorf, Hamburg, Germany). The relative expression of target transcripts was normalized to levels of 18S regulatory RNA. Primer pairs were used to detect transcripts encoding IL-6, CAGAAAATAAGCTGAAACTTCCA, ATGTCAGTGTGTGTGGCTGGAG; tumor necrosis factor-α, GGCTCTCCTGTCTCCCGT, GTTGGCTACAACGTGGGC; and myeloperoxidase, GCTGAGGCGGGACACAACCC, CCCAGTTCCGTTTCCGGGGC.

Statistical Analysis

All data are expressed as mean $\pm$ SD. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). For comparisons of fresh and stored PRBC after AS-1-storage, PRBC-survival experiments, and messenger RNA levels, a two-tailed, two-sample, independent t test was applied to compare differences between two groups, and corrected for multiple comparisons using a Bonferroni correction. For hemodynamic experiments, a two-way ANOVA with repeated measures was used to compare differences between groups at various time points. However, when the interaction P value between time and condition was significant, comparisons were made at each individual time point using a one-way ANOVA with *post hoc* Bonferroni correction-adjusted comparison testing. Within-group comparisons were performed using a two-tailed paired t test. P values <0.05 were considered significant.

Results

Chemical Properties of Leukoreduced and AS-1-stored Lamb PRBC

Storage of human blood products is regulated by the U.S. Food and Drug Administration[30]: Hemolysis of PRBC in the storage bag should not rise above 1%, and residual leukocyte concentrations after leukoreduction should not exceed 5×10^6 leukocytes per transfused unit.

Table 1. Biochemical Properties and Blood Gas Analysis of Leukoreduced Ovine Packed Erythrocytes Stored in Additive Solution-1

	Fresh PRBC (n = 5)	Stored PRBC (n = 6)	P Value
Hemolysis (%)	0.10 ± 0.04	0.50 ± 0.05	<0.001
Free hemoglobin (mg/dl)	41 ± 13	148 ± 20	<0.001
pH	6.97 ± 0.04	6.75 ± 0.05	<0.001
P_{CO_2} (mmHg)	51 ± 7	56 ± 10	0.37
P_{O_2} (mmHg)	80 ± 13	126 ± 22	0.003
Na^+ (mmol/l)	142 ± 3	140 ± 4	0.38
K^+ (mmol/l)	3.7 ± 0.9	7.9 ± 2.2	0.003
Cl^- (mmol/l)	125 ± 2	127 ± 2	0.13
Ca^{2+} (mmol/l)	0.15 ± 0.04	0.12 ± 0.04	0.25
HCO_3^- (mmol/l)	10.3 ± 1.3	8.0 ± 1.2	0.01
Lactate (mmol/l)	1.7 ± 0.4	5.9 ± 2.2	0.002
P_{50} (mmHg)	34 ± 0.1	34 ± 0.1	0.89
2,3-DPG (mmol/l)	0.15 ± 0.05	0.09 ± 0.04	0.07

All data mean $\pm$ SD.
2,3-DPG = 2,3-diphosphoglycerate; P_{50} = oxygen tension at which hemoglobin is half saturated; PRBC = packed erythrocytes.

In order to learn whether or not ovine PRBC fulfilled these criteria, we analyzed the chemical characteristics of leukoreduced PRBC and their supernatants after storage in AS-1 for either 2 or 40 days. Results are summarized in table 1. Cell-free hemoglobin concentrations in the supernatant of fresh PRBC were 41 ± 13 mg/dl, resulting in a calculated level of hemolysis of $0.10 \pm 0.04\%$. In stored PRBC supernatant, cell-free hemoglobin concentrations and hemolysis levels were higher than in fresh PRBC supernatant (148 ± 20 mg/dl and $0.50 \pm 0.05\%$, respectively; $P < 0.001$, values differ *vs.* fresh PRBC for both parameters), but the levels remained below values required by the Food and Drug Administration for stored human blood.

Potassium and lactate concentrations were greater in supernatant of stored PRBC than in fresh PRBC, whereas the pH was less (table 1). The P_{O_2} was greater in stored than in fresh PRBC. As described previously by Bunn,[31] levels of 2,3-DPG measured in ovine erythrocytes were very low. Intraerythrocytic levels of 2,3-DPG did not differ between fresh and stored PRBC (table 1). The P_{50} did not differ between fresh and stored PRBC. After leukoreduction, leukocytes were not detectable in PRBC. Thus, ovine PRBC showed many similar storage properties in AS-1 when compared with previous studies of human PRBC storage.

In Vivo Survival of Leukoreduced and AS-1-stored Lamb PRBC

In addition to the above-mentioned hemolysis criteria, the Food and Drug Administration requires that at least 75% of transfused PRBC are circulating *in vivo* at 24 h after transfusion. To examine whether or not stored ovine PRBC met this requirement, PRBC were biotinylated and transfused after 2 or 40 days of storage. Survival curves for circulating
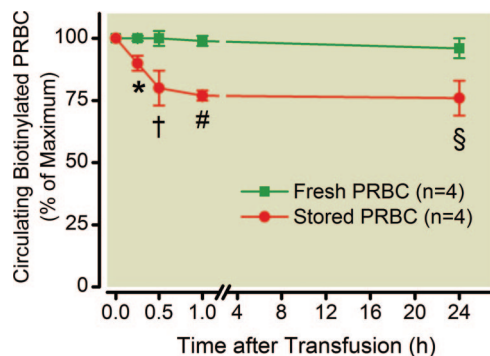
Fig. 1. *In vivo* survival of leukoreduced ovine packed erythrocytes (PRBC) stored in additive solution-1. Circulating biotinylated PRBC are depicted on the y-axis. $96 \pm 4\%$ of biotinylated fresh PRBC survived the first 24 h after transfusion, whereas $76 \pm 7\%$ of biotinylated stored PRBC were circulating 24 h after transfusion. * $P = 0.001$, value differs from fresh PRBC, † $P = 0.002$, value differs from fresh PRBC, # $P < 0.001$, value differs from fresh PRBC, § $P = 0.002$, value differs from fresh PRBC. All data mean $\pm$ SD. PRBC = packed erythrocytes.

biotinylated PRBC are shown in figure 1. When fresh PRBC were biotinylated and transfused, $96 \pm 4\%$ survived for at least 24 h, whereas $76 \pm 7\%$ of stored PRBC remained in the circulation at 24 h after transfusion ($P = 0.002$, values differ). Nearly all of the loss of biotinylated stored PRBC occurred during the first hour after transfusion, with only $77 \pm 2\%$ remaining 1 h after transfusion. Between 1 h and 24 h after transfusion, the circulating levels of labeled fresh and stored PRBC remained stable (stored PRBC: $77 \pm 2\%$ after 1 h and $76 \pm 7\%$ after 24 h, $P = 0.72$; fresh PRBC: $99 \pm 2\%$ after 1 h and $96 \pm 4\%$ after 24 h, $P = 0.26$). One week after transfusion, $80 \pm 8\%$ and $54 \pm 4\%$ of biotinylated fresh and stored PRBC, respectively, remained in circulation (data not shown). Thus, *in vivo* survival properties of ovine PRBC after storage and transfusion are similar to those measured in human PRBC.

Hemodynamic Effects of Transfusing Stored PRBC in Awake Lambs

After developing a new animal transfusion model, we explored the hemodynamic effects of transfusing PRBC after prolonged storage. The effects of stored PRBC transfusion on PAP and PVRI differed from the effects of fresh PRBC transfusion (one-way ANOVA, $P < 0.001$ for PAP and $P = 0.02$ for PVRI, fig. 2). Transfusion of fresh PRBC did not alter any of the measured hemodynamic parameters from baseline (fig. 2 and Supplemental Digital Content 1, fig. 1, http://links.lww.com/ALN/A813, which is a measure of mean systemic arterial pressure, central venous pressure, systemic vascular resistance index, pulmonary capillary wedge pressure, cardiac index, and heart rate during and after transfusion of fresh and stored PRBC with or without concurrent inhalation of nitric oxide in awake lambs). Transfusion of stored PRBC increased PAP (baseline 13 ± 1 *vs.* 18 ± 1 mmHg, $P < 0.001$) and PVRI (baseline
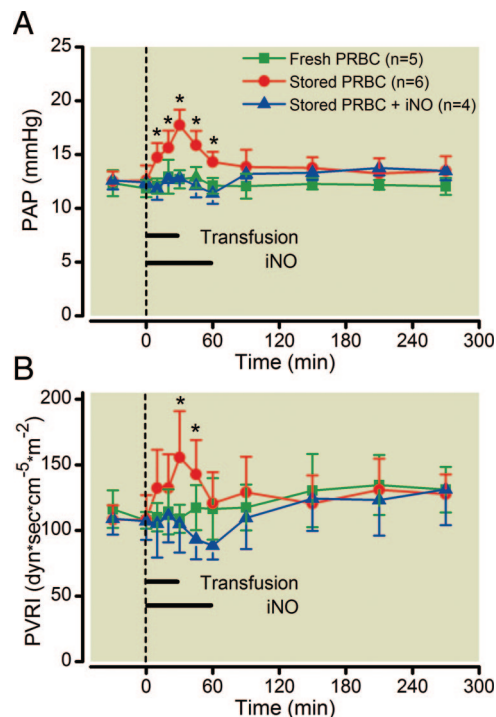

Fig. 2. (*A*) Mean pulmonary arterial pressure and (*B*) pulmonary vascular resistance index measured during and after transfusion of fresh and stored packed erythrocytes (PRBC) in awake lambs. Some lambs received inhaled nitric oxide during and after transfusion of stored PRBC. * $P < 0.05$, stored PRBC value differs from both fresh PRBC and stored PRBC + iNO, ANOVA. All data mean $\pm$ SD. iNO = inhaled nitric oxide; PAP = mean pulmonary arterial pressure; PRBC = packed erythrocytes; PVRI = pulmonary vascular resistance index.

108 ± 19 *vs.* 156 ± 35 dyn $\cdot$ sec $\cdot$ cm^{-5} $\cdot$ m^{-2}, $P = 0.02$), with both parameters peaking at the end of transfusion (fig. 2). Both PAP and PVRI returned to baseline values 60 min after transfusion ended (fig. 2). All other hemodynamic parameters that we measured did not change after transfusion of stored PRBC (Supplemental Digital Content 1, fig. 1, http://links.lww.com/ALN/A813). Therefore, transfusion of stored PRBC increased pulmonary, but not systemic, vascular pressures and resistances in lambs.

Hemodynamic Effects of Stored PRBC Transfusion in a Lamb Model of L-NAME-induced Endothelial Dysfunction

To investigate whether endothelial dysfunction alters the pulmonary vasoconstrictor response to transfusion of stored PRBC, lambs were pretreated with L-NAME. A dose of L-NAME was infused that partially inhibited NOS activity, as reflected by a 50% reduction in the ability of acetylcholine infusion to induce systemic vasodilation (Supplemental Digital Content 1, fig. 2A, http://links.lww.com/ALN/A813, which is a measure of systemic vasodilation in awake lambs after a bolus of 10^{-5} M acetylcholine [10 ml]). As anticipated, L-NAME administration did not alter the vasodilator response to an intravenous bolus infusion of sodium nitro-

prusside (Supplemental Digital Content 1, fig. 2B, http://links.lww.com/ALN/A813, which is a measure of systemic vasodilation in awake lambs after a bolus of 10^{-4} M sodium nitroprusside [10 ml]). Infusion of L-NAME increased MAP, PAP, central venous pressure, and pulmonary capillary wedge pressure, as well as systemic and pulmonary vascular resistance indices, whereas both cardiac index and heart rate decreased (fig. 3 and Supplemental Digital Content 1, fig. 3, http://links.lww.com/ALN/A813, which is a measure of mean systemic arterial pressure, central venous pressure, systemic vascular resistance index, cardiac index, and heart rate during and after transfusion of fresh and stored PRBC). Transfusion of fresh PRBC did not alter any measured hemodynamic parameter. In contrast, transfusion of stored PRBC increased PAP (baseline 17 ± 1 vs. 26 ± 4 mmHg, $P < 0.001$), pulmonary capillary wedge pressure (baseline 7 ± 1 vs. 10 ± 2 mmHg, $P = 0.02$), and PVRI (baseline 170 ± 34 vs. 312 ± 85 dyn · sec · cm^{-5} · m^{-2}, $P = 0.009$) in L-NAME-treated awake lambs (fig. 3). The increase in PAP and PVRI induced by transfusing stored PRBC was greater in the presence of L-NAME than without L-NAME (Δ PAP without L-NAME 5 ± 1 mmHg differs from Δ PAP with L-NAME 9 ± 4 mmHg, $P = 0.03$; Δ PVRI without L-NAME 47 ± 33 dyn · sec · cm^{-5} · m^{-2} differs from Δ PVRI with L-NAME 142 ± 53 dyn · sec · cm^{-5} · m^{-2}, $P = 0.006$). MAP was not statistically different between groups (one-way ANOVA, $P = 0.09$). Hence, partial inhibition of NOS potentiated the vasoconstrictor effect of stored PRBC on the pulmonary, but not the systemic circulation.

Concurrent Inhalation of Nitric Oxide during Transfusion of Stored PRBC

We have previously shown that inhaled nitric oxide prevented pulmonary vasoconstriction during infusion of HBOC-201.[23] To examine whether or not breathing nitric oxide prevents the pulmonary vasoconstrictor response to transfusion of stored PRBC, 80 ppm nitric oxide was administered concurrently during transfusion and for 30 min thereafter. In lambs, which were not pretreated with L-NAME, breathing nitric oxide prevented the increase of PAP (baseline 12 ± 1 vs. 13 ± 1 mmHg, $P = 0.13$) and PVRI (baseline 107 ± 14 vs. 105 ± 22 dyn · sec · cm^{-5} · m^{-2}, $P = 0.87$) associated with transfusion of stored PRBC (fig. 2).

In lambs pretreated with L-NAME, concurrent inhalation of 80 ppm nitric oxide reduced the PAP and PVRI to baseline, and prevented the pulmonary vasoconstriction observed with transfusion of stored PRBC. After nitric oxide breathing ceased at 30 min after transfusion, PAP and PVRI gradually increased, returning to pretransfusion levels 60 min after nitric oxide breathing ended (figs. 3A and 3C). As expected, inhalation of nitric oxide did not alter MAP (one-way ANOVA, $P = 0.89$; Supplemental Digital Content 1, figs. 1A and 3A, http://links.lww.com/ALN/A813). Thus, nitric oxide breathing prevented the pulmonary vasoconstrictor effects associated with transfusion of stored PRBC in lambs.
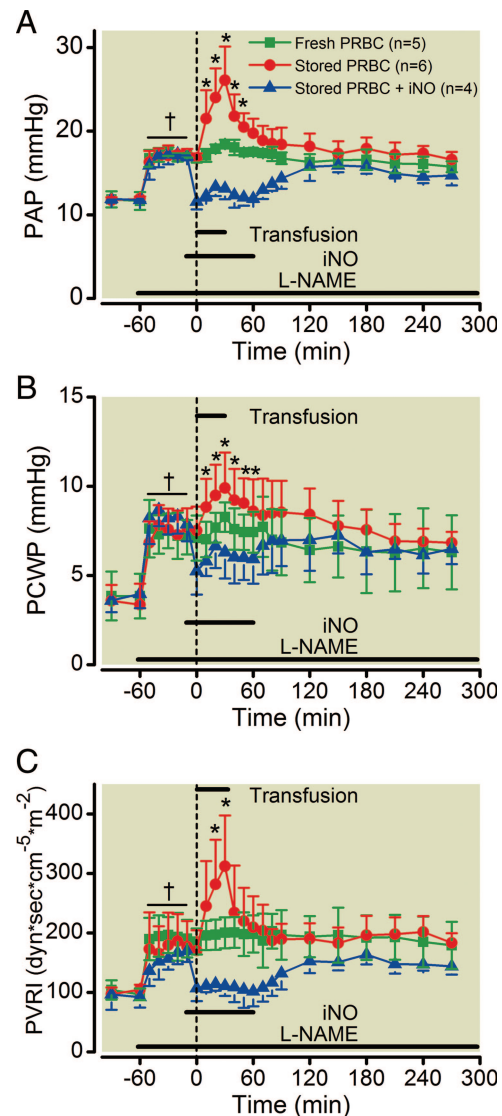

Fig. 3. (A) Mean pulmonary arterial pressure, (B) pulmonary capillary wedge pressure, and (C) pulmonary vascular resistance index measured during and after transfusion of fresh and stored packed erythrocytes (PRBC). Some lambs received inhaled nitric oxide during and after transfusion of stored PRBC. * $P < 0.05$, stored PRBC value differs from both fresh PRBC and stored PRBC + iNO, ANOVA. L-NAME was administered before transfusion in order to induce endothelial dysfunction († $P < 0.05$, measurements at −60 min [before L-NAME] differ from measurements between −50 and −10 min in each group). All data mean ± SD. iNO = inhaled nitric oxide; L-NAME = N^G-nitro-L-arginine methylester; PAP = mean pulmonary arterial pressure; PCWP = pulmonary capillary wedge pressure; PRBC = packed erythrocytes; PVRI = pulmonary vascular resistance index.

Potential Mechanisms for the Pulmonary Hypertension Caused by Transfusing Stored PRBC

To elucidate the mechanisms potentially responsible for the pulmonary vasoconstriction observed after transfusion of stored PRBC, we measured cell-free hemoglobin concentrations in ovine plasma before and after transfusion of fresh
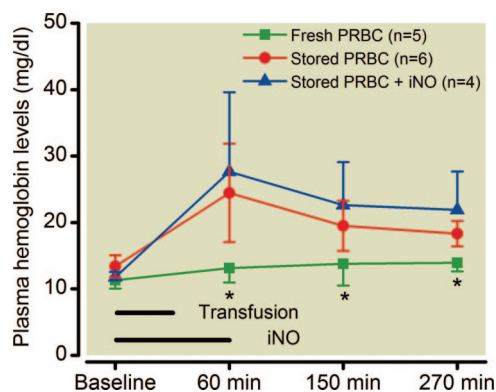
Fig. 4. Plasma hemoglobin levels measured before and after transfusion of fresh and stored packed erythrocytes (PRBC) in healthy awake lambs. Some lambs received inhaled nitric oxide during and after transfusion of stored PRBC. * $P <$ 0.05, fresh PRBC value differs from both stored PRBC and stored PRBC + iNO, ANOVA. All data mean ± SD. iNO = inhaled nitric oxide; PRBC = packed erythrocytes.

and stored PRBC. Transfusion with fresh PRBC did not alter cell-free hemoglobin levels (fig. 4). In contrast, transfusion of stored PRBC increased cell-free hemoglobin levels at 60 min with levels remaining increased for up to 4.5 h. Breathing nitric oxide did not alter the ability of transfusing stored PRBC to increase cell-free hemoglobin.

Compared with humans, sheep have a low plasma concentration of the hemoglobin-scavenger Hp.[32] Plasma levels of Hp decreased after transfusion of either fresh or stored PRBC. However, a more pronounced decrease of Hp was measured after transfusion of stored PRBC (two-way ANOVA comparing fresh PRBC with stored PRBC over time, $P = 0.002$; Supplemental Digital Content 1, fig. 4, http://links.lww.com/ALN/A813, which is a measure of plasma hemoglobin levels before and after transfusion of PRBC in healthy awake lambs).

TXB$_2$ is the stable metabolite of the potent vasoconstrictor thromboxane A$_2$. It has been shown that release of thromboxane metabolites by pulmonary intravascular macrophages can induce pronounced pulmonary hypertension.[33] To determine whether the increase of PAP and pulmonary vascular resistance induced by transfusing stored PRBC was mediated by increased plasma concentrations of thromboxane metabolites, we measured TXB$_2$ levels before and after transfusion of fresh and stored PRBC. Plasma concentrations of TXB$_2$ did not differ before and at 30 min after transfusion of either fresh or stored PRBC (data not shown), providing evidence that thromboxane is unlikely to be responsible for the pulmonary hypertension associated with transfusion of stored PRBC.

Nitrite can be converted to nitric oxide *via* nitrite reductases.[34] Depletion of plasma nitrite after transfusion might therefore be associated with transfusion of stored PRBC. To examine if transfusion of PRBC resulted in changes of plasma nitric oxide metabolites, we measured levels of plasma nitrate and nitrite after transfusion of
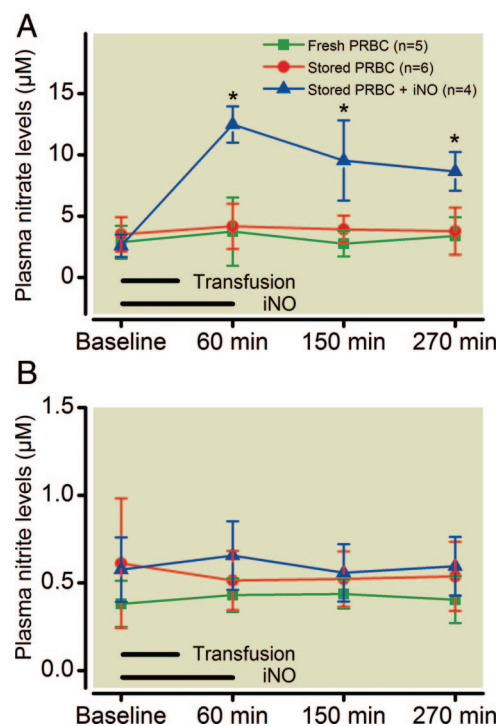

Fig. 5. Plasma (*A*) nitrate and (*B*) nitrite levels measured before and after transfusion of fresh and stored packed erythrocytes (PRBC) in awake lambs. Some lambs received inhaled nitric oxide during and after transfusion of stored PRBC. * $P < 0.05$, stored PRBC + iNO value differs from both fresh PRBC and stored PRBC, ANOVA. All data mean ± SD. iNO = inhaled nitric oxide; PRBC = packed erythrocytes.

fresh and stored PRBC. Plasma nitrate and nitrite levels did not differ before or after transfusion of fresh or stored PRBC (two-way ANOVA, $P = 0.37$ for nitrate and $P = 0.21$ for nitrite; fig. 5).

Transfusion of Leukoreduced and AS-1-stored Lamb PRBC Does Not Induce Systemic Inflammation

In order to determine whether or not transfusion of autologous PRBC resulted in an inflammatory response, we measured plasma IL-6 concentrations and lung and liver levels of messenger RNAs encoding IL-6, tumor necrosis factor-α, and myeloperoxidase before and after transfusion of either fresh or stored PRBC. Neither plasma IL-6 levels (data not shown) nor levels of messenger RNAs encoding inflammatory mediators were altered by transfusing fresh or stored PRBC (Supplemental Digital Content 1, fig. 5, http://links. lww.com/ALN/A813, which is a measure of messenger RNA levels of interleukin-6, tumor necrosis factor-α, and myeloperoxidase in lung and liver 4 h after transfusion of either fresh or stored PRBC). Leukocyte concentrations measured before and after transfusion of fresh or stored PRBC did not differ (data not shown). Thus, transfusion of autologous and leukoreduced fresh or stored PRBC did not induce an inflammatory response in our lamb model within 4 h after transfusion.

Discussion

In the current study, we have established and validated a new model of autologous storage and transfusion of PRBC in lambs. We transfused blood rapidly (300 ml/30 min) to model clinical situations where blood is transfused quickly to treat hemorrhagic shock or life-threatening hemorrhage. Transfusion of fresh PRBC did not produce hemodynamic alterations in the pulmonary circulation, whereas transfusion of stored PRBC transiently increased PAP and PVRI (fig. 2). In contrast, MAP and systemic vascular resistance were not affected by transfusion of either fresh or stored PRBC. The increase in pulmonary vascular tone was associated with increased plasma hemoglobin levels after transfusion of stored PRBC. We further demonstrated that partial blockade of NOS by L-NAME potentiated the pulmonary vasoconstriction produced by transfusion of stored PRBC (fig. 3). The transfusion-induced pulmonary vasoconstriction was prevented by concomitant inhalation of 80 ppm nitric oxide.

In vivo effects of blood transfusion comparing short and long durations of blood storage have been investigated in mice.[20,35,36] Mouse erythrocytes, however, have a shorter lifespan (55–60 days) than human and sheep erythrocytes (100–120 days).[26,37,38] Moreover, the pulmonary circulation of humans is more similar to that of sheep than mice.[39] Thus, we developed a new model of transfusing PRBC in lambs. Autologous transfusion was performed in order to eliminate erythrocyte agglutination or hemolysis because of antibodies and other immune phenomena resulting from blood group incompatibility.[40] Since leukoreduction is commonly performed to reduce transfusion-related febrile reactions, cytomegalovirus transmission, and human leukocyte antigen alloimmunization,[41] leukocytes were depleted from sheep blood before storage using a leukoreduction filter.

In most vertebrates, including humans, the P_{50} of hemoglobin is modulated by intraerythrocytic 2,3-DPG levels. Postneonatal ovine erythrocytes have extremely low concentrations of 2,3-DPG, and because of structural changes in their hemoglobin β-chains, their hemoglobin affinity for 2,3-DPG is very low.[31] Thus, the oxygen affinity of lamb PRBC did not change during prolonged storage. With the exception of 2,3-DPG concentrations and the P_{50}, we found that lamb PRBC have similar properties to those previously measured in human PRBC when both are stored for 40 days (*i.e.*, a similar supernatant hemolysis level *in vitro* and a similar circulating lifespan assessed at 24 h after transfusion). In this regard, our lamb model of PRBC storage and transfusion fulfills the standards required by the Food and Drug Administration for human blood storage and transfusion.[30]

Previous studies have demonstrated vasoconstrictor effects because of increased concentrations of cell-free hemoglobin in large-animal models. Minneci *et al.*[15] induced intravascular hemolysis in dogs by infusing free water (cell-free hemoglobin levels of 320–480 mg/dl) leading to systemic and pulmonary vasoconstriction. Yu *et al.*[23] reported similar vasoconstrictor responses after infusing the hemoglobin-based oxygen carrier HBOC-201 into lambs. Blood *et al.*[42] reported that infusion of erythrocyte hemolysate (cell-free hemoglobin concentrations of 150 mg/dl) induced pulmonary, but not systemic, vasoconstriction in lambs. The concentration of plasma hemoglobin measured in the study by Blood *et al.* was about five times greater than the concentration we measured after transfusion of stored PRBC (approximately 30 mg/dl). Remarkably, the relatively low cell-free plasma hemoglobin concentrations after transfusion of stored PRBC in the current study were associated with pulmonary vasoconstriction, without altering systemic pressure. Our findings suggest that stored PRBC hemolyze after transfusion, especially during the first hour. The consequent increase in plasma concentrations of hemoglobin is associated with an increased PAP and PVRI, suggesting the potent nitric oxide scavenging capacity of cell-free hemoglobin.

In a recently published article, Donadee *et al.* have elegantly shown that hemoglobin can be localized not only in the plasma, but also in microparticles shed from the human erythrocyte membrane during storage.[13] Human microparticle-encapsulated hemoglobin remains in the ferrous form, and can avidly scavenge nitric oxide. Although we do not know if stored ovine erythrocytes shed microparticles during prolonged storage, the formation of microparticles could contribute to the vasoconstrictor effects associated with transfusion of stored PRBC in lambs.

Cell-free hemoglobin is scavenged by Hp, and the resulting complex is removed from the plasma *via* binding to the CD163 receptor on macrophages. Boretti *et al.* reported that increased plasma levels of Hp can efficiently scavenge cell-free oxyhemoglobin, thereby attenuating hemoglobin-induced systemic hypertension in dogs and guinea pigs.[43] Sheep have low plasma levels of Hp (Supplemental Digital Content 1, fig. 4, http://links.lww.com/ALN/A813, and reference[32]). Thus, we cannot exclude the possibility that mammals with higher Hp levels, such as humans, would be protected from the pulmonary vasoconstrictor effects of transfusing stored blood. Whether or not transfusion of stored PRBC induces pulmonary vasoconstriction in humans with or without endothelial dysfunction remains to be determined.

Arginase is an enzyme in erythrocytes that metabolizes arginine to ornithine. When erythrocytes hemolyze, arginase is released into plasma. In adults with sickle cell disease, increased plasma arginase levels are associated with decreased plasma concentrations of arginine, an increased pulmonary artery systolic pressure, and increased mortality. Since arginine is necessary for the biosynthesis of nitric oxide, arginase release appears to reduce nitric oxide bioavailability.[5,11] Thus, increased plasma concentrations of arginase might also be responsible for reducing nitric oxide bioavailability and producing pulmonary vasoconstriction during transfusion of stored PRBC.

Endothelial dysfunction has been observed in a wide variety of disorders including hypertension, atherosclerosis,

and diabetes (for a detailed review, see reference[19]). A reduction in nitric oxide synthesis by endothelial cells is one of the key characteristics of endothelial dysfunction. Yu *et al.* reported that diabetic mice have a greater systemic vasoconstrictor response to an intravenous infusion of murine tetrameric hemoglobin than wild-type mice.[17] In a subsequent study they reported that systemic vasoconstriction was induced by transfusion of stored murine blood into diabetic mice, but not wild-type mice.[20] Based on the observation that impaired endothelial generation of nitric oxide enhances the vasoconstriction produced by cell-free hemoglobin, we developed a model of endothelial dysfunction in lambs. We partially inhibited NOS with an infusion of L-NAME sufficient to reduce the endothelium-dependent vasodilator response to acetylcholine by 50%.[28] Transfusion of stored PRBC induced a more marked pulmonary vasoconstrictor response in sheep treated with L-NAME than in sheep that did not receive L-NAME. Taken together, these results suggest that patients with endothelial dysfunction might have an increased risk of vascular complications when transfused with PRBC stored for prolonged periods.

Studies in lamb models showed that breathing nitric oxide prevents or reverses the pulmonary vasoconstrictor response caused by infusion of hemoglobin-based oxygen carriers[23,44] or hemolyzed erythrocytes.[42] When inhaled, nitric oxide dilates constricted pulmonary vessels. In contrast to intravenously administered nitric oxide-donor compounds, breathing nitric oxide produces pulmonary vasodilation without dilating the systemic circulation.[45] Inhaled nitric oxide can thus be used in patients with systemic hypotension without further decreasing their blood pressure. In the current study, breathing nitric oxide at 80 ppm prevented pulmonary vasoconstriction induced by transfusion of stored PRBC. There are several mechanisms by which inhaled nitric oxide might reduce or prevent pulmonary hypertension after infusing stored PRBC. Inhaled nitric oxide activates pulmonary soluble guanylate cyclase, raising cyclic guanosine monophosphate, which directly causes pulmonary vasodilation. Inhaled nitric oxide can also oxidize plasma oxyhemoglobin to methemoglobin, which cannot scavenge nitric oxide. Minneci *et al.* demonstrated in a canine hemolysis model that plasma methemoglobin increased from less than 10% before inhalation of nitric oxide to almost 100% after inhalation of 80 ppm nitric oxide for 1 h with a concomitant reduction of PAP.[15] Blood *et al.* have shown that inhaling nitrite can reverse hemolysis-induced pulmonary hypertension.[42] It is conceivable that inhaled nitrite could also prevent the pulmonary hypertension caused by transfusion of stored PRBC.

Reduced levels of SNO-hemoglobin can attenuate blood flow in animal models.[18,46] Thus, low SNO-hemoglobin levels might be responsible for pulmonary vasoconstriction after transfusion of stored PRBC. SNO-hemoglobin levels are depleted after 24 h of erythrocyte storage in humans and pigs,[18] but transfusion of fresh PRBC stored for 48 h did not cause pulmonary hypertension in our ovine model. Therefore, although we did not measure sheep SNO-hemoglobin levels, we do not believe that reduced levels of SNO-hemoglobin during storage are the cause of the pulmonary hypertension after transfusion of stored PRBC.

Several research groups have reported that transfusion of stored PRBC into mice induces a pronounced inflammatory reaction.[20,36,47] In the current study, plasma IL-6 concentrations were not altered by transfusion with either fresh or stored PRBC. Moreover, at 4 h after transfusion the levels of messenger RNAs encoding IL-6, tumor necrosis factor α, and myeloperoxidase were similar in lungs and livers of animals transfused with either fresh or stored PRBC. These results indicate that stored PRBC do not trigger systemic inflammation in our lamb model. A possible explanation might be our combination of leukoreduction and autologous transfusion. Both Hod *et al.*[36] and Hendrickson *et al.*[47] described an increase of plasma IL-6 levels after transfusion of stored blood in an allogenic murine model of stored blood transfusion. Yu *et al.*[20] reported similar increases of plasma IL-6 after transfusion of stored blood in a syngeneic murine model of stored blood transfusion. Our sheep data suggest that autologous blood storage and transfusion for planned surgery might reduce complications related to an increased inflammatory state.

In summary, transfusion of stored PRBC increased plasma concentrations of cell-free hemoglobin and produced pulmonary vasoconstriction in lambs, without producing an inflammatory reaction. L-NAME sensitized the pulmonary circulation to the vasoconstriction produced by transfusion of stored PRBC, whereas concurrent inhalation of nitric oxide at 80 ppm prevented the pulmonary vasoconstrictor response to stored PRBC transfusion. Our data suggest that patients with disorders associated with endothelial dysfunction might benefit from transfusion of fresh PRBC. If fresh PRBC are not available, concurrent inhalation of nitric oxide might limit complications by preventing pulmonary vasoconstriction. Thus, further studies elucidating the effects of transfusion of PRBC stored for prolonged periods of time in high-risk populations with endothelial dysfunction are warranted.

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