Transfusion of Stored Autologous Blood Does Not Alter Reactive Hyperemia Index in Healthy Volunteers

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ABSTRACT

Background: Transfusion of human blood stored for more than 2 weeks is associated with increased mortality and morbidity. During storage, packed erythrocytes progressively release hemoglobin, which avidly binds nitric oxide. We hypothesized that the nitric oxide mediated hyperemic response after ischemia would be reduced after transfusion of packed erythrocytes stored for 40 days.

Methods and Results: We conducted a crossover randomized interventional study, enrolling 10 healthy adults. Nine volunteers completed the study. Each volunteer received one unit of 40-day and one of 3-day stored autologous leukoreduced packed erythrocytes, on different study days according to a randomization scheme. Blood withdrawal and reactive hyperemia index measurements were performed before and 10 min, 1 h, 2 h, and 4 h after transfusion. The reactive hyperemia index during the first 4 h after transfusion of 40-day compared with 3-day stored packed erythrocytes was unchanged. Plasma he-

What We Already Know about This Topic

- Storage time of packed erythrocytes appears to impact clinical outcomes
- Increased free oxyhemoglobin concentrations from infusion of stored packed erythrocytes might scavenge nitric oxide, resulting in morbidity

What This Article Tells Us That Is New

- Transfusion of one unit of autologous packed erythrocytes stored for 40 days (40DS) did not produce hemodynamic changes, systemic inflammation, or endothelial dysfunction (measured as reactive hyperemic index) in healthy volunteers
- Nitrite levels increased significantly after transfusion of 40DS packed erythrocytes compared with packed erythrocytes stored for 3 days
- The results suggest that increased nitric oxide production from endothelial nitric oxide synthase might serve as a compensatory mechanism for reduced nitric oxide bioavailability by plasma oxyhemoglobin scavenging of nitric oxide

moglobin and bilirubin concentrations were higher after transfusion of 40-day than after 3-day stored packed erythrocytes ($P = 0.02$, [95% CI difference 10–114 mg/l] and 0.001, [95% CI difference 0.6–1.5 mg/dl], respectively). Plasma concentrations of potassium, lactate dehydrogenase, haptoglobin, and cytokines, as well as blood pressure, did not differ between the two transfusions and remained within the normal range. Plasma nitrite concentrations increased after transfusion of 40-day stored packed erythrocytes, but not after transfusion of 3-day stored packed erythrocytes ($P = 0.01$, [95% CI difference 0.446–0.66 μM]).

Conclusions: Transfusion of autologous packed erythrocytes stored for 40 days is associated with increased hemolysis, an unchanged reactive hyperemia index, and increased concentrations of plasma nitrite.

⊕ Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

A PPROXIMATELY 15 million units of packed erythrocytes are transfused each year in the United States.[1] Blood transfusion represents one of the greatest advances of modern medicine and can be life saving; however, transfusion poses a risk of several infectious and noninfectious hazards. Recently, packed erythrocytes storage time has been related to adverse clinical outcomes. Evidence from retrospective investigations suggests that transfusing blood stored for a prolonged period of time (more than 14 days of storage) can increase mortality and morbidity after cardiac surgery and trauma,[2,3] but these findings are controversial[4] and the underlying mechanisms remain to be elucidated.

Many biochemical and physical changes in packed erythrocytes appear related to the duration of storage.[5] The increased fragility and hemolysis of packed erythrocytes stored for longer periods leading to increased plasma-free oxyhemoglobin concentrations and, consequently, enhanced scavenging of nitric oxide has been suggested as a central mechanism producing storage-related adverse effects after transfusion.[6] A widespread reduction of nitric oxide bioavailability due to enhanced removal of nitric oxide by circulating free oxyhemoglobin, as well as hemoglobin in lipid-enclosed microparticles of erythrocytes, could produce inflammatory, thrombotic, and vasoactive effects.[7,8]

Nitric oxide bioavailability within blood vessels can be assessed by the vasodilator response that follows 5 min of regional limb ischemia, a response entitled postischemic reactive hyperemia. This vasodilation response is usually assessed noninvasively by either ultrasonic measurement of brachial artery flow-mediated dilation or by calculating the reactive hyperemia index (RHI) after measurement of the pulse volume amplitude *via* peripheral arterial tonometry (PAT).[9] A reduction of flow-mediated dilation or RHI has been reported when hemoglobin is released into plasma, such as in sickle cell disease,[10] malaria,[11] or during hemodialysis.[12] A complementary approach to assess the fate of nitric oxide is by nitric oxide metabolite profiling in blood. Nitric oxide metabolism is complex and is influenced by many factors, including diet, orogastric bacterial colonization and metabolism, oxygenation status, antioxidant capacity, and hemolysis.[13] After its synthesis from arginine by endothelial nitric oxide synthase, luminally released nitric oxide is rapidly oxidized to nitrite and nitrate. A small fraction of nitric oxide reacts with proteins, generating nitrosation products, such as S-nitrosothiols (RSNO) *and N-nitrosamines* (RNNO).[14] The impairment of endothelial function, as assessed by flow-mediated dilation or RHI of patients with cardiovascular risk factors, is correlated with reduced plasma concentrations of nitrite[15] and nitrosation products,[16] suggesting that decreased concentrations of either species reflect reduced nitric oxide bioavailability.

We hypothesized that transfusion of 40-day stored (40DS) packed erythrocytes into healthy volunteers would result in impaired peripheral vascular function, as reflected by a reduced RHI, and would be associated with increased plasma hemoglobin compared with transfusion of 3-day stored (3DS) packed erythrocytes. We measured greater concentrations of cell-free hemoglobin in both stored packed erythrocytes supernatant and the plasma of volunteers transfused with 40DS packed erythrocytes. Circulating nitrite concentrations in plasma were higher in volunteers transfused with 40DS packed erythrocytes compared with 3DS packed erythrocytes, whereas RHI was unchanged and cytokines were not detectable after packed erythrocytes transfusion.

Materials and Methods

Study Design

All procedures were approved by the local Institutional Review Board (Partners Human Research Committee, Boston, Massachusetts) and enrolled participants provided written informed consent.

In this crossover study of healthy volunteers, biochemical and physiologic data were collected before and after receiving a 3DS or 40DS autologous packed erythrocytes transfusion. During the first phase of the study, volunteers donated one unit of blood that was leukoreduced and stored as packed cells in additive solution AS-1 (Adsol, Fenwal Inc., Lake Zurich, IL) following American Association of Blood Bank standards,[17] and then transfused over 1 h after either 3 or 40 days of storage at 4°C in the Massachusetts General Hospital Blood Bank. During the second phase of the study, one unit of packed erythrocytes was collected from the same volunteer and then transfused after either 3 or 40 days of storage. By the end of the study, each volunteer had received both a 3DS and a 40DS packed erythrocytes unit. There was an interim period of 2 weeks between the two phases of the study. The order of new and old blood administration was randomized. Block randomization was used with an allocation ratio of 1:1. Blood bank and laboratory personnel were blinded to the patient's treatment group. Volunteers fasted for at least 8 h before each transfusion. On the day of transfusion, they received one packed erythrocytes unit of more than 1 h. During the subsequent 4 h, they remained on bed rest, and hemodynamic measurements and venous blood sampling were performed.

Population

We recruited volunteers (five females and five males) through the Massachusetts General Hospital and Partners clinical studies website. Volunteers were screened over the phone for inclusion and exclusion criteria, and if eligible they were invited for a physical examination and blood testing. Our inclusion criteria were: age older than 18 yr and younger than 40 yr; body mass index between 18 and 25 kg/m^2; a normal physical examination; a total hemoglobin concentration greater than 12.5 g/dl before the two blood donations; blood tests within normal values (leukocyte and platelet count; electrolytes, urea, creatinine, glucose, and transaminase concentrations). Exclusion criteria were: psychiatric disturbances; systemic disease with or without any functional limitation;

57

Berra *et al.*

pregnancy, as determined by urine pregnancy test; active smoking or less than 1 yr of smoking cessation; alcohol use (consuming more than 0.5 l/day of wine or equivalent); any use of medications during the past 7 days; antibiotic use within 48 h before blood donation; having received or donated blood in the past 4 months; any type of cancer; or being enrolled in another research study. The study was conducted between July and November 2010 at the Massachusetts General Hospital Blood Bank, Boston, Massachusetts.

Reactive Hyperemia Index

We measured the RHI to assess digital vasodilatory capacity after 5 min of transitory arm ischemia. Patients were supine in a quiet, temperature-controlled room. Inflatable probes were placed on the index finger of each hand. Pulse volume amplitude was measured *via* PAT using the EndoPAT 2000 instrument (Itamar Medical Ltd., Caesarea, Israel). After 5 min of PAT recording (preischemia phase), the brachial blood pressure cuff was inflated for 5 min (ischemic phase) to a pressure of 250 mmHg producing transitory ischemia of one arm (verified by absence of PAT signal). The cuff was then released (postischemia phase), and the PAT signal recorded for another 5 min.

The PAT ratio (postischemia to preischemia PAT amplitude) was calculated for each 30-s interval of the postischemia phase in the test finger and then divided by the ratio of the control finger to account for any systemic hemodynamic changes.[18] RHI was defined as the mean PAT ratio during the 5 min of hyperemia after cuff release. RHI was assessed before transfusion and again at 10 min, 1 h, and 4 h after the end of the transfusion. Changes from baseline were considered for statistical analysis.

Blood Tests

Concentrations of leukocytes, platelets, and plasma bilirubin, lactate dehydrogenase, and potassium were measured before transfusion and 4 h after the completion of transfusion. The concentration of haptoglobin (GenWay Biotech, Inc., San Diego, CA) was measured before transfusion, and at 1 and 4 h after transfusion ended. The concentration of plasma hemoglobin (BioAssaySystems, Hayward, CA), as well as nitric oxide metabolites[19] (nitrite, nitrate, RNNO, and RSNO) in both plasma and erythrocytes, were measured before transfusion, and at 10 min, 1 h, 2 h, and 4 h after transfusion ended.

In addition, an aliquot of blood from each unit of packed erythrocytes was collected at the end of the assigned storage period to measure plasma hemoglobin and nitric oxide metabolite concentrations.

Plasma concentrations of interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12p70, and tumor necrosis factor were measured using a BD™ Cytometric Bead Array Human Inflammatory Cytokine Kit (BD Biosciences, San Jose, CA) with a minimum detectable concentration of cytokine of 20 pg/ml.

Further information is provided in Methods, Supplemental Digital Content 1, http://links.lww.com/ALN/A843, where measurements of haptoglobin, plasma hemoglobin, nitric oxide metabolites, and plasma cytokines are described in detail.

Vital Sign Parameters

Blood pressure and heart rate were recorded with a Propaq monitor (Welch Allyn Inc., Skaneateles Falls, NY) at 10-min intervals before and during transfusion, and over the 4 h after completion of the transfusion.

Statistical Analysis

The number of patients enrolled was determined as follows. We hypothesized a difference in RHI between the two transfusions of 0.4, and a SD of the difference in RHI for the same patient equal to 0.4. In the context of a crossover study, a total of 10 volunteers is adequate to provide a power greater than 80% to detect a treatment difference at a two-sided 0.05 significance level (primary endpoint). Continuous variables are expressed as mean (SD), unless otherwise specified. RHI, plasma nitrite, and plasma hemoglobin are expressed as mean difference between the 3DS and the 40DS blood transfusion; the 95% CI is provided. Differences in pretransfusion values between males and females were analyzed by an independent sample Student t test. The effects of storage time (3 *vs.* 40 days) were assessed by a paired Student t test examining only the 4-h time point when analyzing variables collected before transfusion and at 4 h; by a two-way analysis of variance (ANOVA) for repeated measures when analyzing variables collected before transfusion and repeatedly thereafter. The ANOVA for repeated measures analysis was performed using multiple timepoints (before transfusion and 10 min, 1 h, 2 h, and 4 h after) and the two transfusions (3DS and 40DS blood) as within-patients factors. Thus, this model took into account the fact that each patient underwent multiple measurements (both before and after transfusion) and received both transfusions (3DS and 40DS blood). We report P values of the two-way ANOVA comparing the effects of the two-storage periods (3DS *vs.* 40DS). A Bonferroni correction was used for *post hoc* tests.

Eight blood samples were grossly hemolyzed of a total of 108, and were excluded for analysis of plasma hemoglobin (further information is provided in Supplemental Digital Content 1, http://links.lww.com/ALN/A843). The SPSS EM algorithm was used for imputation of missing data (the plasma hemoglobin levels) in ANOVA analyses, after checking with Little Missing Completely At Random test.[20] Linear regressions were performed using a nonparametric test (Spearman correlation coefficient). RHI and nitric oxide metabolite data were natural-logarithm transformed and followed a normal distribution.[18] Statistical analyses were performed using SPSS software version 18.0 (Chicago, IL). Statistical significance was reached when $P < 0.05$.
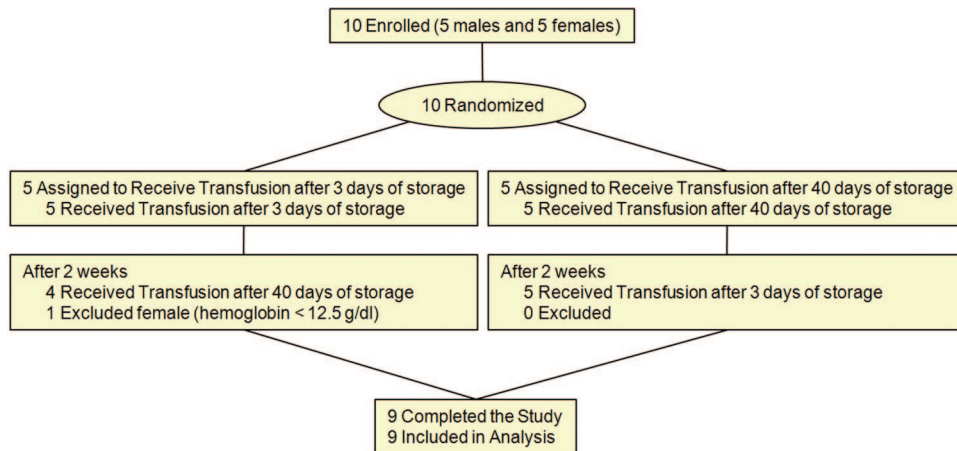
Fig. 1. Study protocol and randomization. Ten healthy volunteers were enrolled and received one unit of 3DS and one of 40DS packed erythrocytes in random order. One patient was excluded because of anemia and failure to meet criteria for the second blood donation. 3DS and 40DS = blood stored for 3 and 40 days, respectively.

Results

Of the 10 healthy volunteers that we recruited, 9 (5 males and 4 females) completed the study. One volunteer could not donate blood a second time because of a persistently low hemoglobin concentration (less than 12.5 g/dl) after the first blood donation. Because she did not meet our inclusion criteria, she was excluded from the analysis (fig. 1). Demographics and study population characteristics before autologous transfusion are shown in table 1. Reactive hyperemia index was recorded before and after blood transfusion and trace is shown in figure 2. Pretransfusion levels of total hemoglobin were higher before the transfusion of 40DS compared with the 3DS packed erythrocytes (table 2). All the other pretransfusion measurements did not differ.

No major adverse events (dyspnea, hypotension, skin rash, etc.) occurred after transfusion of either 3DS or 40DS packed erythrocytes.

Vital Signs
The difference in systolic blood pressure after transfusion of 40DS packed erythrocytes, compared with 3DS packed erythrocytes, was not statistically significant ($P = 0.37$, fig. 3A). Heart rates also did not differ.

Reactive Hyperemia Index
Due to a slight difference of RHI at baseline, changes from baseline were considered in the RHI statistical analysis. The vascular hyperemic response as assessed by RHI was unchanged after transfusion of 40DS packed erythrocytes compared with transfusion of 3DS packed erythrocytes (mean difference 0.110; 95% CI -0.477–0.677; $P = 0.67$, fig. 3B). Exemplary RHI recordings at 4 h after transfusion 3DS and 40DS packed erythrocytes are provided in figure 2.

Hemolysis
Transfusion of 40DS packed erythrocytes resulted in plasma hemoglobin levels higher than those after 3DS packed erythrocytes (mean difference 62 mg/l; 95% CI 10–114; $P = 0.02$, fig. 3C). The *post hoc* analysis showed an increase of plasma hemoglobin levels at 1 h, 2 h, and 4 h after transfusion of 40DS packed erythrocytes in comparison with pretransfusion plasma levels, and an increase at 10 min after transfusion of 3DS packed erythrocytes. The *post hoc* analysis showed a significant difference of plasma hemoglobin levels after 4 h, comparing 40DS and 3DS packed erythrocytes transfusions. Plasma haptoglobin levels did not differ after transfusion of stored packed erythrocytes ($P = 0.49$, fig. 3D).

Total bilirubin levels at 4 h after transfusion of 40DS packed erythrocytes correlated with the level of plasma hemoglobin (Spearman $\rho = 0.94$, $P < 0.001$) and were higher than after transfusion of 3DS packed erythrocytes ($P = 0.001$, [95% CI difference 0.6–1.5 mg/dl], table 2), whereas plasma lactate dehydrogenase and potassium concentrations did not differ.

Table 1. Study Population Characteristics before Autologous Transfusion

	Overall Population (n = 9)	Males (n = 5)	Females (n = 4)	P Value
Age, yr	26 ± 4	26 ± 2	27 ± 5	0.59
Height, m	1.75 ± 0.11	1.80 ± 0.09	1.68 ± 0.1	0.08
Weight, kg	72 ± 11	80 ± 7	63 ± 8	0.03*
Body mass index, m²/kg	23.6 ± 2	24.5 ± 0.8	22.5 ± 2.5	0.13
Heart rate, beats/min	66 ± 7	61 ± 4.9	71 ± 3.5	0.02*

* $P < 0.05$ by t test comparing males to females.
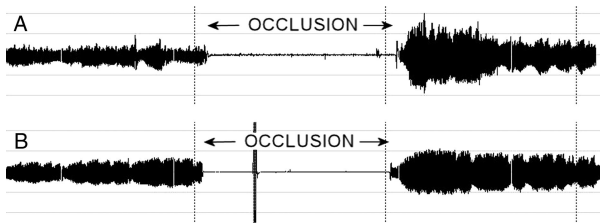
Fig. 2. Examples of peripheral arterial tonometry (PAT) measurements. PAT recording in one patient 4 h after transfusion of either (A) 3DS or (B) 40DS packed erythrocytes. The brachial blood pressure cuff was deflated to elicit the hyperemic response after 5 min of inflation to 250 mmHg (arrows). 3DS and 40DS = blood stored for 3 and 40 days, respectively.

Inflammation and Platelets

The leukocyte count did not differ after transfusion of 40DS compared with 3DS packed erythrocytes ($P = 0.06$, fig. 3E). Platelet count did not differ at 4 h after transfusion of 40DS or 3DS packed erythrocytes. Transfusion with either 40DS or 3DS did not alter plasma cytokine levels (IL-1β, IL-6, IL-8, IL-10, IL-12p70, and tumor necrosis factor), which remained below the detectable threshold at all times.

Nitric Oxide Metabolites

The plasma concentration of nitrite was higher after transfusion of 40DS packed erythrocytes compared with 3DS packed erythrocytes (mean difference 0.256 μM; 95% CI 0.446–0.66; $P = 0.01$, fig. 3F). The post hoc analysis showed a significant increase at 1, 2, and 4 h after the transfusion of 40DS packed erythrocytes. Concentrations of other nitric oxide metabolites (nitrate, RNNO, RSNO) did not significantly differ after the two transfusions both in plasma and erythrocytes (see figure, Supplemental Digital Content 2, http://links.lww.com/ALN/A844, which represents plasma concentrations of nitrate, RNNO, RSNO, and erythrocyte concentrations of nitrate, nitrite, RNNO, and RSNO). Plasma RNNO concentration measured 4 h after transfusion was correlated with the RHI after transfusion of 40DS packed erythrocytes, but not after transfusion of 3DS packed erythrocytes (Spearman $\rho = 0.7$ and $P = 0.03$ vs. $P = 0.86$, respectively).

Blood Sampled from Packed Erythrocytes Storage Bags

After 40 days of storage, plasma hemoglobin in the storage bag supernatant was higher than that observed after 3 days of
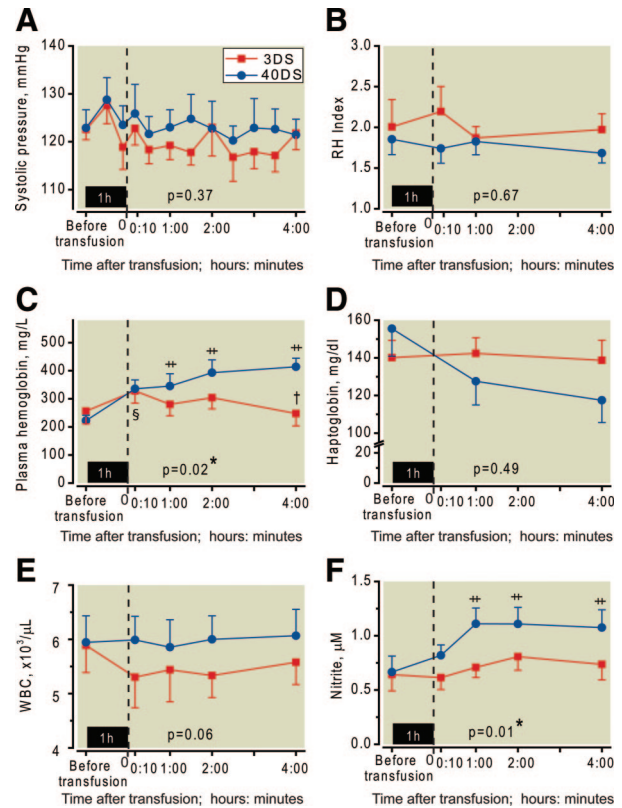
Fig. 3. Effects of transfusing 3 and 40 day stored autologous packed erythrocytes. Measurements of in vivo (A) systolic blood pressure, (B) reactive hyperemia (RH) index, (C) plasma hemoglobin, (D) haptoglobin, (E) leukocytes (white blood cells), and (F) nitrite before and after transfusions of either 40DS (blue) or 3DS (red). All data are represented as mean ± SE. * $P < 0.05$ by two-way ANOVA for repeated measures analysis; log-transformed data were used for statistical analysis of RHI and nitrite. † $P < 0.05$ for post hoc within timepoint comparison of the two treatments. ‡, § $P < 0.05$ for post hoc comparison with pretransfusion values (40DS and 3DS blood, respectively). 3DS and 40DS = blood stored for 3 and 40 days, respectively.

storage (1,120 ± 440 vs. 370 ± 150 mg/L, $P < 0.001$ by paired Student t test). Intracellular and extracellular concentrations of nitric oxide metabolites did not differ in packed erythrocytes stored for 3 or 40 days (see Supplemental Digital Content 3, http://links.lww.com/ALN/A845, which is a

Table 2. Differences between Transfusion of 3DS and 40DS Blood

	Before Transfusion			4 h after Transfusion		
	3DS	40DS	P Value	3DS	40DS	P Value
LDH, U/L	159 ± 27	166 ± 21	0.37	153 ± 22	163 ± 22	0.10
Potassium, mEq/l	3.6 ± 0.4	3.7 ± 0.4	0.72	3.7 ± 0.4	3.5 ± 0.3	0.05
Total bilirubin, mg/dl	0.5 ± 0.5	0.7 ± 0.8	0.06	0.6 ± 0.8	1.7 ± 1.1	0.001*
Indirect bilirubin, mg/dl	0.4 ± 0.4	0.6 ± 0.8	0.10	0.6 ± 0.8	1.6 ± 1.1	0.01*
Platelet count, ×10³/μl	263 ± 63	285 ± 71	0.05	249 ± 55	258 ± 53	0.29
Hemoglobin, g/dl	12.8 ± 1.4	13.6 ± 1.6	<0.001*	12.9 ± 1.4	13.7 ± 1.6	0.007*

* $P < 0.05$ by paired Student t test comparing transfusion of 3DS and 40DS blood.
LDH = lactate dehydrogenase; 3DS and 40DS = blood stored for 3 and 40 days, respectively.

Berra et al.

table listing cell-free hemoglobin and nitric oxide metabolite concentrations in the stored erythrocytes bags).

Discussion

This study focused on the hemodynamic, metabolic, and inflammatory effects of blood storage time. To eliminate confounding factors related to immune incompatibility, we transfused autologous leukoreduced packed erythrocytes. To exclude the effects of coexisting conditions or medication use, we enrolled young volunteers taking no medications. To magnify any possible deleterious effects of transfusing packed erythrocytes stored for a prolonged period of time, we maximized the difference in storage time between the two transfusions (3 days *vs.* 40 days, whereas current Food and Drug Administration regulations allow packed erythrocytes storage for up to 42 days before transfusion[17]) and used a crossover design (each patient receiving both autologous transfusions, with a 2-week rest period between each transfusion). To ensure the safety of our healthy volunteers, we transfused a relatively small amount of blood (one packed erythrocytes unit was approximately 6% of the circulating blood volume for males and 7.5% for females) over a relatively long period (1 h). In our study, systolic blood pressure and heart rate were unchanged after transfusing either 40DS or 3DS packed erythrocytes. These results suggest that the transfusion of one unit of packed erythrocytes stored for 40 days does not produce major hemodynamic changes in healthy volunteers.

In patients undergoing cardiac surgery, blood transfusion induces an inflammatory state, as reflected by induction of IL-6 and C-reactive protein.[21] In our study, the leukocyte concentration tended to be decreased after transfusion of 3DS but not 40DS packed erythrocytes, whereas plasma levels of IL-1β, IL-6, IL-8, IL-10, IL-12p70, and tumor necrosis factor did not differ. Recently Hod *et al.* reported a study comparing the autotransfusion of 3–7 day and 40–42 day stored autologous leukoreduced packed erythrocytes into healthy volunteers. Similar to our study, they report no increase of plasma IL-6 after transfusion.[22] Taken together, these similar results suggest that the transfusion of one unit of autologous packed erythrocytes stored for 40 days does not produce systemic inflammation in healthy volunteers. The absence of an inflammatory response to blood stored for a prolonged period of time may also be explained by several reasons. First, the use of autologous and leukoreduced packed erythrocytes avoids immune incompatibility; leukoreduction is commonly used to reduce transfusion-related febrile reactions,[23] cytomegalovirus transmission, and human leukocyte antigen alloimmunization in both the United States[24] and Europe.[25] Second, the absence of any diseases in our healthy volunteers may have reduced the susceptibility of the subjects to inflammation. Finally, the small amount of blood administered over a relatively long period may be more tolerable than transfusing a larger volume over a shorter time.

The vascular response following a brief period of arm ischemia, assessed by RHI, was not altered after transfusion of 40DS packed erythrocytes compared with 3DS packed erythrocytes (rejection of the primary endpoint of the study). No previous clinical trial has reported functional testing of endothelial function after stored blood transfusion. Previous studies have demonstrated that the magnitude of the hyperemic response following ischemia, expressed by RHI levels, is dependent upon nitric oxide bioavailability.[9] Reduction of postischemic reactive hyperemia is commonly associated with increased levels of plasma hemoglobin.[12,26] In our study the increase of levels of cell-free hemoglobin after 40DS packed erythrocytes transfusion were similar to results found by Meyer *et al.*,[12] who found a reduction of flow-mediated dilation after hemodialysis. However, their patients had endothelial dysfunction at baseline whereas our healthy volunteers likely had normal vascular endothelium, capable of producing more nitric oxide as nitric oxide was scavenged by plasma hemoglobin without reducing RHI.

The nitric oxide scavenging activity of plasma hemoglobin is one possible mechanism by which transfusion of 40DS packed erythrocytes could reduce nitric oxide bioavailability, as suggested by the nitric oxide scavenging properties of plasma obtained from patients affected by hemolytic diseases.[27,28] In our study, the plasma hemoglobin concentration returned to pretransfusion values by 1 h after 3DS packed erythrocytes transfusion, but remained significantly increased at 4 h after transfusion of 40DS packed erythrocytes (fig. 3C). The transfusion of 40DS packed erythrocytes delivered 140 mg supernatant hemoglobin to the recipients' circulation, whereas transfusion of 3DS packed erythrocytes delivered only 50 mg. Considering an average circulating plasma volume of 3.1 l for hemoglobin distribution in our volunteers (3.3 l for males and 2.8 l for females), we estimate the average plasma concentration of hemoglobin should have acutely increased by 15 and 45 mg/l after the transfusion of 3DS and 40DS packed erythrocytes, respectively. However, we measured a much greater increase in plasma hemoglobin at 10 min after transfusion of 3DS and 40DS packed erythrocytes (70 and 110 mg/l, respectively). Thus, the cell-free hemoglobin present in the packed erythrocytes storage bag is insufficient to account for these increments, and lysis of damaged erythrocytes after transfusion is likely to contribute to the elevation of plasma hemoglobin. The observation that plasma hemoglobin increased at 1 h, 2 h, and 4 h after the transfusion of 40DS packed erythrocytes, but not 3DS packed erythrocytes, suggests that erythrocytes stored for long periods have greater damage and are more susceptible to posttransfusion hemolysis. This finding is consistent with the fact that approximately 25% of transfused human radiolabeled erythrocytes stored for 42 days disappear from the circulation within 24 h.[29] Other evidence of increased hemolysis includes the higher levels of bilirubin and a trend to reduced haptoglobin levels after transfusion of 40DS but not 3DS packed erythrocytes. Hod *et al.* reported similar elevations of bilirubin after autologous transfusions and no changes of haptoglobin levels.[22]

Among other molecules that possibly influence local nitric oxide bioavailabilty, S-nitrosylated hemoglobin has been proposed as a possible mediator of the storage lesion effects. In our study, total erythrocytic RSNO levels (which include S-nitrosylated hemoglobin) were low and did not differ significantly in the packed erythrocytes storage bags after 3 or 40 days of storage, and in the recipient's blood after transfusion. A similar trend was seen for plasmatic RSNO levels after transfusion, which would include cell-free S-nitrosylated hemoglobin. Our findings are consistent with a previous report showing that S-nitrosylated hemoglobin levels in packed erythrocytes are quite low after 24 h of storage, and remain persistently low for the duration of storage.[30]

To further characterize the biochemical fate of nitric oxide after the transfusion of 40DS and 3DS packed erythrocytes in our healthy volunteers, we measured nitric oxide metabolites in plasma and erythrocytes. With the exception of nitrite, all of the nitric oxide metabolites we measured did not differ after the transfusion of either 40DS or 3DS packed erythrocytes. We measured a doubling of nitrite levels after transfusion of 40DS packed erythrocytes, levels peaking at 1 h and remaining increased for 4 h. There was no change in plasma nitrite after transfusing 3DS packed erythrocytes. There are three possible sources of the increased plasma nitrite after transfusion of 40DS packed erythrocytes: these are nitrate reduction, erythrocyte release of nitrite, and nitric oxide synthase activity. Bacterial reduction of dietary nitrate in the orogastric tract is a known source of nitrite.[31] In our volunteers, it is unlikely that diet and bacterial activity are relevant sources of nitrite after 8 h of fasting. However, we cannot exclude that nitrate-reductase activity is present elsewhere and contributes to the increase in plasma nitrite. Erythrocyte concentration of nitrite after storage was always similar to that of plasma (see Supplemental Digital Content 3, http://links.lww.com/ALN/A845), thus plasma concentration of nitrite should not change despite hemolysis. Increased plasma nitrite levels reflect increased nitric oxide synthesis after endothelial nitric oxide synthase stimulation by intraarterial infusion of acetylcholine.[32] Although we cannot exclude a possible contribution from the erythrocytes themselves,[33] we propose that after transfusing 40DS packed erythrocytes the healthy endothelium increases its nitric oxide production rate from endothelial nitric oxide synthase as a compensatory mechanism to sustain perfusion, because nitric oxide bioavailability is reduced by plasma oxyhemoglobin scavenging nitric oxide. Such a compensatory mechanism would explain why the normal RHI was maintained after 40DS transfusion in healthy volunteers.

Plasma RNNO levels, although not different after transfusion of 40DS or 3DS packed erythrocytes, correlated with RHI at 4 h after transfusion of 40DS packed erythrocytes. RNNO may act as a nitric oxide donor *in vivo*,[14] and therefore its plasma concentrations might also be influenced by decreased nitric oxide bioavailability after transfusion of 40DS packed erythrocytes, as suggested by reduced plasma levels of RNNO and RSNO in patients with endothelial dysfunction.[16]

We report that autologous transfusion of 40DS packed erythrocytes, compared with 3DS packed erythrocytes, was associated with greater hemolysis, as evidenced by increased plasma hemoglobin and bilirubin levels. We hypothesize that the effects of transfusing blood stored for a prolonged period of time might be more pronounced in certain patient populations. Yu *et al.* have recently reported that stored murine packed erythrocytes transfusion produces vasoconstriction and hypertension in diabetic mice with endothelial dysfunction but not in wild- type mice.[34]

In trauma resuscitation, many units of blood are often transfused rapidly, resulting in higher levels of hemolyzed erythrocytes than seen in this study and perhaps more significant hemodynamic effects. Some transfusion recipients may have comorbid conditions (*e.g.,* patients with sepsis or those who are critically ill) that may predispose them to significant inflammatory or thrombotic reactions. Comorbidities such as cardiovascular disease can impair endothelial nitric oxide synthesis, and therefore the ability to compensate for nitric oxide scavenging by plasma oxyhemoglobin may be reduced. Two randomized clinical trials are now underway examining whether the length of storage of packed erythrocytes is related to the incidence of adverse postoperative outcomes in patients after cardiac surgery.***

*** Clinical trial NCT00991341, http://clinicaltrials.gov/ct2/show/NCT00991341, Clinical trial NCT00991341, http://clinicaltrials.gov/ct2/show/NCT00458783. Accessed March 3, 2012.

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