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Physiologic effects of RBC storage in chronic transfusion recipients: vasoreactivity, exercise capacity, and oxygen consumption.

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SPECIFIC AIMS: Transfusion of red blood cells (RBCs) is a common intervention aimed at preventing mortality and morbidity in anemic and bleeding patients. However, some RBC units may have functional defects that impair their efficacy and could actually harm patients. For example, a growing body of evidence suggests that transfusion of older stored RBCs (termed storage-aged RBCs [saRBCs]) is associated with worse clinical outcomes including multi-organ failure and mortality.[1-4] A constellation of cellular changes, commonly termed 'the storage lesion', may underlie these outcomes. Among the saRBC storage changes of interest are those that reduce nitric oxide (NO) bioavailability.[5,6] NO is a crucial vasodilator in small arterioles and capillaries which actively adjust to increase blood flow (and O_2 delivery) to local tissues with high oxygen demands (hypoxic vasodilation).[7,8] Some common disorders, such as cardiovascular disease (CVD) and diabetes mellitus, are known to reduce NO bioavailability which leads to vascular dysfunction and related morbidities in affected patients[9,10]. We, and others have likewise demonstrated that saRBCs reduce NO bioavailability in vascular models, and that transfusion of saRBCs reduces NO-mediated vasodilation in animal and human transfusion recipients[11-13] (please see preliminary data). One important component of the proposed studies will be our investigations to determine whether the impairment of NO-mediated vasodilation seen in these previous studies produces significant physiologic consequences on exercise tolerance and oxygen consumption in transfused patients.

Up to now, prospective mechanistic investigations of the effects of saRBC transfusions on human recipients have been limited to either hospitalized subjects or healthy volunteers[14-17]_ENREF_14(preliminary data). The use of inpatient subjects is complicated by the fact that that are often critically ill with complex and dynamic medical conditions, their transfusion needs are unpredictable and heterogeneous. In contrast, with healthy volunteers, the timing and volume of study transfusions can be controlled. However, healthy volunteers cannot ethically have their hematocrits reduced to levels seen in anemic hospitalized patients, nor do they typically have underlying endothelial dysfunction seen in hospitalized patients, which may synergize with the effects of saRBC transfusions to further reduce NO bioavailability. Thus, while both groups have been used successfully to study the effects of fresh vs. saRBC transfusions, each study population has limitations. Ideally, investigations of the vasoinhibitory effects of saRBCs would best be performed in anemic patients who have predictable, repetitive transfusion requirements such that a cross-over transfusion design can be used to control for inter-individual heterogeneity. At Emory, we care for a large population of chronically anemic patients who require outpatient blood transfusions every 2 to 4 weeks for thalassemia, aplastic anemia, myelodysplastic syndrome and other disorders. Thus, the second important component of the proposed studies will be the recruitment of these patients as study subjects: they have relatively stable disease-related anemias and vascular dysfunctions, are transfusion-dependent and can be randomized into a cross-over study design to receive fresh transfusions during one visit, and saRBC transfusions during the next (or vice versa). The proposed studies will test our hypothesis that transfusion of saRBCs (day 21-42 of storage), but not fresh RBCs (< 10 days), will impair NO-mediated vasodilation and also reduce exercise tolerance and oxygen carrying capacity in an ambulatory transfusion-dependent adult population. **The Specific Aims are:**

Aim 1: *To assess the effects of fresh vs. saRBC transfusions on NO-dependent endothelial function in chronically anemic adults using: 1) flow mediated dilation (FMD) and 2) reactive hyperemic index (RHI) measured by pulsatile arterial tomography (PAT). The primary endpoint of this Aim is the change in FMD with fresh vs. saRBC transfusion. The secondary endpoint is change in RHI*.

Aim 2: *To assess the effects of fresh vs. saRBC transfusions on functional exercise capacity and oxygen carrying capacity by cardiopulmonary exercise testing. The primary endpoint is the change in peak oxygen consumption (VO_2Max) after transfusion.*

Secondary endpoints *for the above Aims are changes in metabolic markers before and after transfusion/exercise: inflammation (IL-2, IL-6, hsCRP), oxidative stress (glutathione/glutathione disulfide and cysteine/cystine redox potentials), and nitric oxide metabolites (nitrite, nitrate, and nitrosylated hemoglobin and thiols).*

These studies are a continuation of research I performed during my postdoctoral fellowship and represent an interdisciplinary approach with mentoring expertise in transfusion medicine and cardiovascular function. These novel studies will be crucial for my continued research training, and will provide data to support future independent NIH funding to further dissect the mechanisms of the adverse effects of saRBC transfusions and to develop approaches to abrogate those effects.

BACKGROUND AND SIGNIFICANCE

Clinical Significance: While more than 15 million units of RBCs are transfused annually in the U.S., RBC transfusion is in some cases associated with serious adverse outcomes.[18] Although optimal utilization of donated RBC units is dependent on the ability to store RBCs at refrigerated temperatures for up to 42 days prior to transfusion, multiple perturbations in RBC structure and function occur with prolonged storage. Notable alterations include: 1) increased free hemoglobin, extracellular potassium, and lactate levels, 2) accumulation of shed microparticles (shedisomes) and soluble biological response modifiers, and 3) decreased pH, 2,3-disphosphoglycerate (2,3-DPG) and S-nitrosyl hemoglobin (SNO-Hb).[1,2,5,19-21] When infused, saRBCs with these defects may alter recipient physiology and contribute to poor outcomes. Physiologic processes that may be perturbed include the disruption of nitric oxide (NO)-mediated vasodilation. In the INOBA (Insufficient Nitric Oxide Bio-Availability) hypothesis,[22] we postulated that an interplay of variables related to the RBC unit (e.g., storage age) and the transfusion recipient (e.g., endothelial dysfunction) together determined local NO levels in the vasculature and hence affected tissue blood flow. Thus, when NO bioavailability declines below a critical threshold (precipitated by saRBC transfusions, for example), local blood flow and O_2 delivery are insufficient to meet tissue demands, resulting in end-organ complications. We now present data that validates key tenets of the INOBA hypothesis (please see preliminary data). In particular, we have shown in both *in vitro* model systems[13] and in human transfusion studies that saRBCs (but not fresh RBCs) significantly disrupt NO-mediated vasodilation.

The role of RBC storage time in transfusion-associated mortality and morbidity: Even when the well-recognized non-infectious hazards of transfusion are excluded from analysis, transfusion (particularly of RBCs) remains an independent predictor of morbidity and mortality.[2,3,5,6,18,20,23-34] Tinmouth and Wang have performed systematic reviews of dozens of studies that investigated the relationship between blood storage interval and adverse transfusion events [4,18]. Meta-analyses showed worse recipient outcomes following transfusion of saRBC. Since the largest clinical studies included in these reviews were retrospective, further elucidation of the possible adverse effects of saRBCs may be provided by prospective randomized trials. The largest to be published to date, ARIPI [15], compared fresh blood (stored up to 7 days; median = 5 days) with standard of care (stored up to 42 days; median = 13 days) in low-birthweight neonates. Although the outcomes showed no difference between study arms, it is important to note that only 25% of the units transfused in the standard issue group were stored for > 20 days, and thus the study does not address the safety of RBC units near the end of the 42 day storage period. In fact, our results using *in vitro* aortic ring assays (see preliminary data) suggest that the vasoinhibitory effects of saRBCs first manifest after 21 days of storage[13]. The possibility of adverse clinical outcomes following transfusion of saRBCs near outdate (35-42 days) may be better addressed in the ongoing RECESS [35], ABLE [16], and Red Cell Storage Duration and Outcomes in Cardiac Surgery studies [17].

The role of endothelial- and RBC-derived nitric oxide (NO) in the control of vascular tone: The endothelium plays a crucial role in actively regulating vascular tone. This effect depends on tightly regulated release of vasodilators (NO, prostacycline, and endothelium-derived hyperpolarizing factor [EDHF]) and vasoconstrictors (endothelin and thromboxane).[36-38] NO is synthesized in the endothelium by NO synthases (NOS) and acts in a paracrine manner by diffusing to the underlying smooth muscle where it causes muscle relaxation, and thus increases vessel diameter and blood flow to local tissues.

Intrinsic RBC mechanisms that regulate NO levels have also been postulated to play a role in controlling local blood flow in order to preferentially perfuse, and thus provide O_2, for the most hypoxic tissues.[1] RBCs may participate in this process of hypoxic vasodilation in order to match blood flow (and O_2 delivery) with tissue needs.[39] Evidence suggests that this "endocrine" form of NO signaling by RBCs may affect NO bioavailability via at least three potential pathways: 1) S-nitrosylation of Hb (SNO-Hb), [40,41] in which NO is captured by the cys β-93 on the R (oxygenated) conformation of Hb creating a storage form of NO which can then be released as bioactive NO when pO_2 is reduced and the Hb transitions to the T-state (deoxygenated). Thus, RBCs may serve as a storage pool for NO, forming SNO-Hb under oxygenated conditions for subsequent downstream release of NO to promote vasodilation when O_2 tension is low [1,6,21,42,43] 2) Nitrite, present in high concentrations in plasma, can be converted to NO by Hb in hypoxic conditions.[44] In this model, deoxygenated Hb binds nitrite and a proton to generate NO and met-Hb. The NO can then leave the RBC to cause vasodilation or can bind a second deoxy-Hb molecule to form iron-nitrosyl-Hb ($HbFe^{2+}$-NO). With either mechanism, NO must escape from the RBC to act on smooth muscles and produce vasodilation, although the mechanisms of NO egress are unclear.[45,46] 3) By promoting O_2 release and stabilizing deoxy-Hb, 2,3-DPG likely facilitates the production of NO through this latter pathway. Thus, stored RBCs with depleted 2,3-DPG may be deficient in NO production. Interestingly, the time course over which 2,3-DPG is completely depleted (after ~ 14 days of storage) correlates with studies showing

that RBCs stored >14 days are more likely to cause morbidity and mortality.[3,5] With any of these three pathways, its reasonable to postulate that storage of RBCs prior to transfusion may disrupt these mechanisms of NO production/release, and thus transfusion of saRBCs may impair NO bioavailability in the transfusion recipient.

Alternatively, transfused saRBCs could produce an inhibitory factor that interrupts normal NO signaling between endogenous RBCs, endothelium and the underlying smooth muscle thereby allowing a small volume of transfused saRBCs to broadly impair hypoxic vasodilation. Disruption of NO signaling by saRBCs could be mediated by free hemoglobin (or other RBC constituents) released into the plasma as a consequence of RBC hemolysis, or by hemoglobin or other factors encapsulated in RBC-derived microparticles.[45-47] Under normal circumstances, Hb is isolated from the endothelium by encapsulation within RBCs that tend to flow in center of the blood stream and away from the endothelial wall.[45,46,48] Microparticles may be important because they can flow closer to the endothelium than intact RBCs, bringing hemoglobin close to the sites of NO synthesis, which may further accentuate NO scavenging after transfusion.[11] There also appear to be other mechanisms by which saRBCs can inhibit NO signaling, For example, our studies have indicated that plasma supernatant constituents, including free hemoglobin and microparticles, were not the only cause of vasoconstrictive effects in an aortic ring model[13] (see preliminary data section). Removal of the supernatant from saRBCs, either through volume reduction or washing, did not improve NO-mediated vasodilation. While addition of day 42 supernatant alone to the aortic rings did inhibit NO-mediated vasodilation, the effect was only significant when compared to aortic rings with no supernatant added; there was no difference in ring vasodilation when day 42 supernatant was compared to day 0 supernatant. Thus, while free Hb exerts some inhibitory effects on NO signaling in this model, it does not appear to account for the majority of the vasoinhibitory activity seen with stored RBC samples. Rather, the saRBCs themselves appear capable of inhibiting NO-mediated vasodilation, as others have recently suggested [12]. This effect may not occur through NO scavenging, but instead through suppression of endothelial NO production, a possibility we are currently investigating. Furthermore, we note that these *in vitro* vasoinhibitory effects are comparable to the inhibition of NO-mediated vasodilation we have observed in hospitalized patients transfused with saRBCs, but not fresh blood (manuscript in preparation; see preliminary data). Thus, there are several potential mechanisms by which saRBCs may impair endothelial NO signaling and the normal vasodilatory response to hypoxia.

Endothelial function and dysfunction: The endothelial dysfunction associated with chronic illnesses such as CVD is primarily due to reduced NO bioavailability and correlates with the traditional cardiovascular risk factor "burden", such as hypertension and hypercholesterolemia.[9,49,50] The presence and magnitude of endothelial dysfunction has significant clinical implications as it is an independent predictor of adverse long-term cardiovascular outcomes even in patients without overt atherosclerosis.[10,51-61] Furthermore, clinical interventions can alter endothelial dysfunction.[62,63] Many of the subjects of the clinical studies showing adverse effects of RBC storage had coronary artery disease risk factors, or were critically ill, and likely had some degree of endothelial dysfunction. Non-invasive methods for measurement of NO mediated vasodilation, and endothelial dysfunction, include brachial artery flow mediated dilation (FMD) and pulsatile arterial tomography (PAT). FMD represents a well-validated, non-invasive, ultrasound based approach to assess conduit vessel endothelial function.[64] PAT measures blood flow in the digital microvessels. The digital reactive hyperemia index (RHI) measured using PAT correlates with coronary and peripheral endothelial function, is largely NO-dependent, and is an independent predictor of cardiovascular events.[65] Used in concert, FMD and PAT provide a sensitive, non-invasive assessment of endothelial function and thus NO bioavailability in both the conductance and resistance vessels.

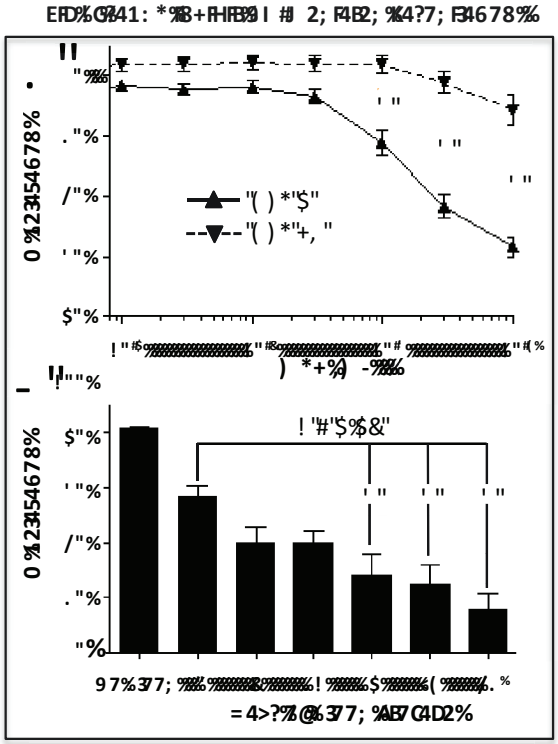
Physiologic effects of defective saRBCs on vascular function*:* In addition to our *in vitro* and *in vivo* studies described above and discussed in more detail in the preliminary results section, other studies have also investigated physiological interactions between saRBCs and blood vessels. Using aortic ring assays and animal transfusions, saRBCs have been shown to reduce NO-mediated vasodilation as compared to fresh RBCs.[5,6] In a recent study in patients undergoing hemodialysis, hyper-hemolysis was associated with impairment of FMD, suggesting a measurable role for NO scavenging by free hemoglobin.[66] However, no change in RHI using PAT was reported with transfusion of fresh (3 day) vs. stored (40 day) autologous transfusions in healthy adults.[14] It is quite possible, however, that healthy adults who lack pre-existing endothelial dysfunction are less susceptible to saRBC-induced reductions in NO bioavailability (as postulated in the INOBA hypothesis[67]). For example, we have seen impairments in NO-mediated vasodilation in hospitalized patients transfused with saRBCs, but not fresh RBCs (see preliminary data section).

Transfusion and Cardiopulmonary Exercise Testing: Hypoxic vasodilation results in matching O_2 supply with demand. At the level of the whole organism, cardiopulmonary exercise testing (CPX) measures

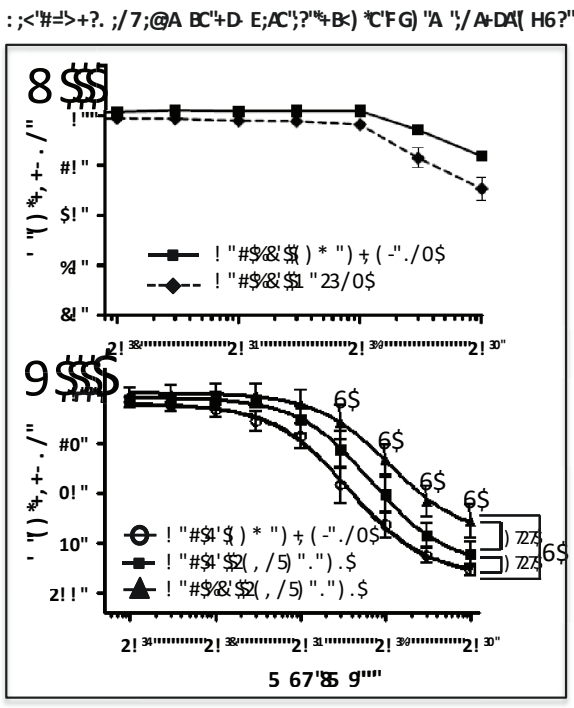
global oxygen consumption taking into account lung function, cardiac function, and O_2 carrying capacity.[68] CPX including determination of maximal aerobic capacity (peak VO_2 or VO_2Max) has long been used to evaluate performance in athletes and as a diagnostic and prognostic tool in cardiac and pulmonary disease.[68] VO_2Max represents an individual's physiologic limit and is calculated from the cardiac output and the arteriovenous oxygen difference during peak exercise. Factors limiting VO_2Max include ventilation, O_2 diffusion at the lung, O_2 transport in the circulation (central and regional), peripheral perfusion and mitochondrial function. Of these, in untrained humans at low altitude, circulatory O_2 transport accounts for 70% of VO_2Max.[69] Of particular relevance to the present proposal, in both trained athletes and subjects with chronic anemia, increasing O_2 carrying capacity via RBC transfusion improves both VO_2Max and endurance capacity.[70] [71] Thus, studying changes in VO_2Max following fresh vs saRBC transfusions represents a powerful non-invasive approach to gauge whether RBC storage time adversely affects the physiologic benefits of transfusion. To our knowledge, CPX has not previously been used as a physiologic measure to investigate the clinical effects of different RBC storage times on transfusion recipients, and is a major aim of this proposal. Our hypothesis is that impairments of NO-mediated vasodilation caused by saRBC transfusion may produce a decrease in (or less improvement in) exercise capacity as compared to the same patient when they received fresh RBC transfusions.

PRELIMINARY DATA:

In vitro vasoinhibitory effects of stored RBCs: We have demonstrated with *in vitro* aortic ring bioassays that saRBCs, but not fresh RBCs, significantly decrease NO bioavailability; this work has been accepted for publication[11-13] (section E). We collected, leukoreduced and stored RBC units up to 42 days according to standard protocols. To determine the effects of saRBCs on NO-mediated vasodilation, rat aortic rings were incubated with RBC samples and then exposed to vasodilatory stimuli. In the presence of fresh unstored blood (day 0), aortic rings exhibited 60% mean relaxation in response to 10^{-5} M methacholine (MCh). However, after incubation with saRBCs stored for 42 days, there was no significant vasodilation ($p<0.001$, Fig 1A). As compared to day 0 blood, blood stored 28-42 days produced significantly more inhibition of vasodilation ($p<0.01$, Fig 1B). Published data suggested that the vasoinhibitory activity would be found in the saRBC supernatant, as degenerating saRBCs release hemoglobin and arginase which can reduce NO bioavailability [11]. However, washing saRBC samples to remove the plasma/buffer supernatant did not reduce the inhibitory effect: maximal relaxation was not different between unmanipulated and washed day 42 saRBCs ($p>0.05$, Fig 2A). As an alternative approach, plasma supernatants removed from blood units on day 0 or day 42 were added directly to aortic rings prior to MCh (Fig 2B). Vasodilation in the presence of day 0 supernatant was not different from the responses seen without plasma supernatant ($p > 0.05$). Addition of day 42 supernatant to aortic rings did produce a small decrement in vasodilation (63.8% max) which was not different from day 0 plasma ($p > 0.05$) but was different from the no-plasma control ($p < 0.01$). This inhibitory activity was quite small, however, as compared to day 42 blood and washed RBCs. Thus, these *in vitro* bioassays demonstrate that saRBCs themselves exert significant inhibitory effects on NO-mediated vasodilation.

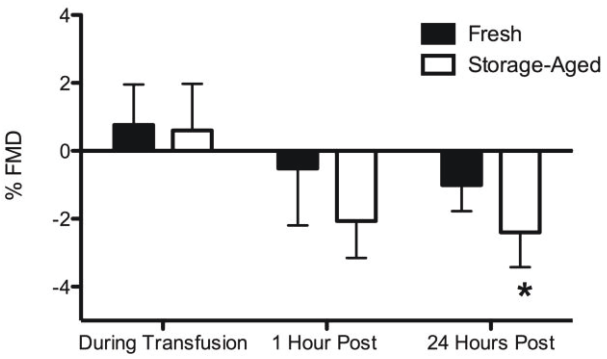


Impaired FMD responses in hospitalized patients transfused with saRBCs but not fresh RBCs. As a extension of the *in vitro* aortic ring assays, and as part of a larger NIH funded investigation into the adverse effects of saRBC transfusions, we have also successfully studied the vascular effects of fresh RBCs vs. saRBCs on inpatients receiving medically indicated blood transfusions for anemia. After informed consent, subjects were randomized to receive either a fresh (<10 days old) or aged (>21 days old) blood transfusion. Endothelial function was assessed by FMD of the brachial artery prior, during, 1 hour after, and 24 hours following transfusion. Data were analyzed using a linear mixed effects model.



The 45 subjects who have completed the study had a mean age of 58.5±13 (SD). 52% were male and 50% were white. There were no differences in demographics, CVD risk factors, known CVD, amount of RBCs transfused, or baseline FMD between groups. Mean age of RBC units was 9.1±3.1 days and 29.8±5.7 days in the fresh and saRBC groups, respectively. As compared to pretransfusion FMD results, patients receiving fresh RBCs had no significant change in FMD at any time point either during or after transfusion (p>0.05; Fig 3, black bars). In recipients transfused with saRBCs, FMD showed a marked decrease at 1 hour post-transfusion (which did not reach statistical significance, p>0.05); however, by 24 hours after transfusion the continued reduction in FMD was statistically significant (p=0.037; Figure 3, white bars; manuscript in preparation). These inhibitory effects of saRBCs, but not fresh RBCs, on NO-mediated vasodilation in hospitalized patients are consistent with our *in vitro* studies shown above (Figs 1 and 2), further supporting the INOBA hypothesis. Unlike recently published reports which found no vascular effects of either fresh or saRBC transfusions in healthy recipients,[14] we studied ill, anemic patients receiving standard allogeneic transfusions, thus underscoring the likely importance of recipient factors to adverse effects of saRBC transfusion.

Figure 3: Change in % FMD with pRBC Transfusion



* p<0.05 for change from baseline FMD

Cardiopulmonary exercise testing in peripheral arterial disease. As part of an unrelated NIH funded study in patients with peripheral arterial disease (PAD), our group has studied an intervention (GM-CSF injected twice weekly) to improve collateral blood vessel formation and lower extremity blood flow in patients with PAD and claudication symptoms. The primary outcome is peak treadmill walking time at 3 months. The hypothesis is that GM-CSF will stimulate increased numbers of endothelial progenitor cells (EPCs), which are stem cells destined to form endothelium, thus improving blood flow and oxygen delivery. As a secondary outcome in this study, cardiopulmonary exercise testing, including measurement of VO_2Max was completed on each participant at baseline and at 6 months. 124 of the recruited participants had VO_2Max results at baseline and 3 months available for review. The mean±SD VO_2Max was 1202±347 ml/min and 1236±369 ml/min at baseline and 3 month follow-up, respectively, demonstrating a significant 2.83% increase (p=0.021) in VO_2Max across all 124 participants. Because the study is ongoing, these results cannot yet be divided into treatment arms to determine whether GM-CSF was more effective than the control intervention. Nonetheless, these data demonstrate our ability to conduct serial CPX testing in a large study population with limited exercise capacity, supporting the use of CPX testing in Aim 2 of the proposed investigations.

EXPERIMENTAL DESIGN AND METHODS

Rationale:

In our preliminary studies, we demonstrated that saRBCs (but not fresh RBCs) impair NO-mediated vasodilation when studied using *in vitro* models as well as in hospitalized transfusion recipients. Some limitations of studying transfusion outcomes in hospitalized patients include the heterogeneity of this population, unpredictable times of transfusions, urgent/emergent circumstances, and a lack of standardization of inpatient covariates including time of day, diet and other associated therapies. Vascular tests (FMD and PAT) and measures of circulating NO, oxidative stress and inflammation are sensitive to co-morbidities, diet and medications. Finally, dynamic changes in patients' condition can also potentially confound experimental results. Conversely, transfusion studies can be performed in healthy volunteers. However, these subjects rarely have underlying endothelial dysfunction (which may synergize with saRBCs and magnify their effects on NO bioavailability) and for ethical reasons cannot be rendered anemic or transfused with allogeneic blood. Thus, transfusion effects in healthy volunteers may not necessarily be reflective of outcomes seen in sick patients.

In order to overcome these limitations, and to investigate the relationships between blood storage, reduced NO bioavailability, impaired vasodilation, and physiologic outcomes of transfusion, we propose to investigate the effects of saRBC transfusions in a chronically transfusion-dependent but ambulatory population. This approach has the following advantages: 1) these patients are stable over the course of days to weeks, 2) studies are performed under conditions of controlled diet, circadian rhythms, medications, etc., 3) the same subject receives both fresh and saRBCs in a randomized cross-over study design (see schema in Fig 4), and 4) because these patients are ambulatory, they can undergo cardiopulmonary exercise testing. Successful completion of the proposed studies will advance our understanding of mechanisms underlying the adverse events and functional consequences of saRBC transfusion and validate a model for subsequently evaluating potential treatments and storage techniques to reverse saRBC-mediated impairments (the latter objectives are beyond the scope of the present investigations).

Specific Aims:

Aim 1: To assess the effects of fresh vs. saRBC transfusions on NO-dependent endothelial function in chronically anemic adults using: 1) flow mediated dilation (FMD) and 2) reactive hyperemic index (RHI) measured by pulsatile arterial tomography (PAT). The primary endpoint of this Aim is the change in FMD with fresh vs. saRBC transfusion; The secondary endpoint is change in RHI.

Aim 2: To assess the effects of fresh vs. saRBC transfusions on functional exercise capacity and oxygen carrying capacity by cardiopulmonary exercise testing. The primary endpoint is the change in peak oxygen consumption (VO_2Max) after transfusion.

Secondary endpoints *for the above Aims are changes in metabolic markers before and after transfusion/exercise: inflammation (IL-2, IL-6, hsCRP), oxidative stress (glutathione/glutathione disulfide and cysteine/cystine redox potentials), and nitric oxide metabolites (nitrite, nitrate, SNO-Hb and SNO-thiols).*

Hypotheses: In patients with chronic transfusion-dependent anemia, transfusion of saRBCs will impair vascular reactivity compared to transfusion of fresh RBCs (Aim 1). Impairment of NO-mediated vasodilation will be associated with decreased exercise tolerance and peak oxygen consumption (VO_2Max) following saRBC transfusion as compared to fresh RBCs (Aim 2), and will be associated with increased systemic oxidative stress and inflammation, and decreased circulating NO metabolites (Secondary endpoints).

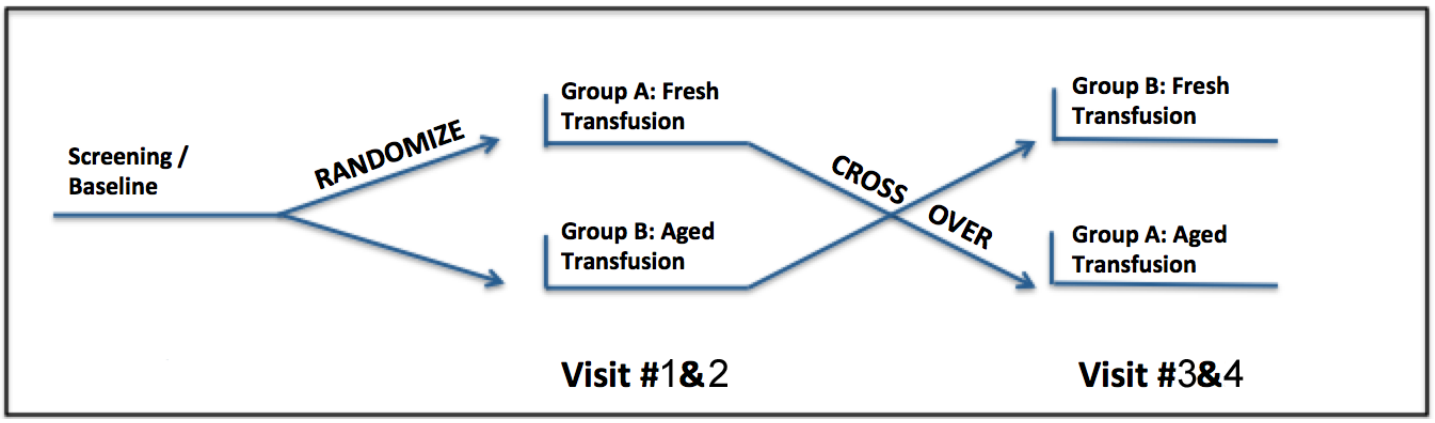
Subject Population: Twenty-four subjects diagnosed with stable transfusion dependent anemia will be recruited and undergo a screening health questionnaire and physical examination to ensure eligibility. All subjects will provide informed consent prior to entry into the study. We will recruit adult subjects without preference to any race, gender or socioeconomic status. Dr. Quyyumi's translational research group in the Department of Cardiology regularly enrolls patients into clinical studies, and has experience consenting patients, randomizing them to study arms, and performing study protocols including the use of these non-invasive assays.[65,72-74] This clinical research team oversaw execution of the clinical study comparing fresh vs. saRBC transfusions in hospitalized patients described in the preliminary results, Furthermore, we have also consulted with our Hematology colleagues, and have identified a large number of potential subjects who fit our criteria for this study. The proposed study has been approved by the Emory IRB.

Eligibility Criteria: Male or female volunteers age 21-80 with any condition resulting in RBC transfusion-dependent anemia will be eligible to participate. Participants may receive either 1- or 2-unit transfusions as required clinically so long as their transfusion requirement is stable throughout the study period.

Ineligibility Criteria: Age <21 or > 80 years, pregnancy, acute infection in previous 4 weeks, active substance abuse within the past year, inability to give informed consent, inability to return for follow-up or the presence of alloantibodies that would limit the blood bank's ability to obtain correctly aged RBC units. Participants with contraindications to cardiopulmonary exercise testing may be allowed to participate in the other study aims.

Study Design: Studies will be performed in a temperature-controlled vascular laboratory in the outpatient unit of the Clinical Research Network, ACTSI, at Emory University Hospital after an overnight fast. After providing informed consent, subjects will be randomized to receive their next clinically-indicated outpatient transfusion with fresh (<10 days old) blood or storage-aged (>21 day old) blood. Following their first transfusion, subjects will be crossed-over to receive the opposite aged product. Subjects and individuals conducting and interpreting experimental tests will be blinded to the age of RBC units. To maintain safety, clinical staff will not be blinded. All subjects will receive clinically indicated transfusions with cross-matched, leukoreduced AS1 units of packed RBCs from available blood bank inventory. The effects of RBC transfusion on blood flow and peripheral O_2 delivery will be directly tested in the 24 volunteers using non-invasive assessments of endothelial function including: brachial artery flow-mediated dilation (FMD) and pulsatile arterial tonometry (PAT). Symptoms of fatigue and anemia will be objectively assessed at every visit using the self-administered FACT-An questionnaire (Functional Assessment of Cancer Therapy – Anemia). Vascular function will be assessed prior to transfusion as well as 1 hour and 24 hours after blood transfusion. Concurrently, subjects will undergo cardiopulmonary following each transfusion. Blood samples will be obtained at baseline, prior to and following each transfusion and from each unit transfused to perform metabolic marker testing.

Study Schedule (Figure 4, below): Each subject will be studied over a total of 4 visits, as shown in the Figure and described below.



Screening: Subjects will be screened with history and physical for inclusion and exclusion criteria and provide written consent. Subjects to meet inclusion and exclusion criteria, and give consent, will be randomized into either Group A or Group B.

Visit #1 (Transfusion #1): Subjects will return fasting to receive a scheduled, clinically-indicated RBC transfusion. Depending on their randomization to Group A or B, they will receive either fresh or saRBCs; an infusion pump will be used to administer the blood over a 1 hour period. FMD and PAT and will be performed and blood samples will be obtained for measurement of oxidative stress and other metabolic markers prior to and 1 hour following RBC transfusion.

Visit #2 (Post-transfusion #1): Subjects will return fasting the day following transfusion. FMD and PAT will be performed and blood samples will be obtained. CPX will be performed. The visit #3 (and #5) time points are important to this study since our preliminary data showed that impairment of NO-mediated vasodilation by saRBC transfusion was greatest 24 hours after transfusion.

Visit #3 (Transfusion #2): Visit #3 will recapitulate Visit #1 except that subject will receive the opposite aged product, as shown in the cross-over study design schema (Fig 4).

Visit #4 (Post-transfusion #2): Visit #4 will recapitulate Visit #2.

Procedures:

Blood Transfusion: Participants will receive either 1 or 2 packed RBC units as ordered by their attending physician. Units will be infused intravenously via programmable electronic infusion pump (Baxter, Inc) over 1 hour per unit. The subjects will receive only cross-matched leukoreduced units. No pre-medication will be permitted. Subjects will be monitored closely for any reactions during transfusion following standard hospital policy. In the event that blood units of the prespecified age are not available, patient will receive their clinically indicated transfusion with blood units of any age per hospital protocol. This would be considered a protocol deviation and those patients will then be excluded from the study

Study Measurements:

Endothelial function assessment -- Brachial artery flow-mediated vasodilation (FMD): All studies will be performed in a temperature-controlled vascular laboratory after an overnight fast. The brachial artery of the non-dominant arm will be imaged using a high-resolution 13 MHz ultrasound transducer (Acuson) as previously described in detail and as used for the studies shown in Fig 3 of the preliminary results section.[10,75,76] Briefly, a blood pressure cuff on the forearm will be inflated to supra-systolic pressures to produce 5 minutes of ischemia. On cuff deflation, reactive hyperemia produces an acute increase in shear stress with an accompanying release of NO. Imaging of the brachial artery is performed continuously for the next 120 seconds, providing the data necessary to calculate flow-mediated dilation. After a 10 minute recovery interval, sublingual nitroglycerin will be given (0.4 mg) and the brachial artery will be re-imaged after 3 minutes. Image analysis will be performed by an experienced FMD investigator who is blinded to the treatment arm. Arterial diameter will be measured using a validated proprietary software program (Medical Instruments Inc.). Brachial artery FMD will be calculated as [(postischemia diameter - baseline diameter) / baseline diameter] X 100.

Reproducibility: FMD is well-accepted technique[64]. In our laboratory, the mean difference in FMD (%) between 2 consecutive assessments performed in 11 subjects an average of 8 days apart was 1.26% (±0.76%, r=0.75). The mean difference in the FMD (%) between 2 readings of the same 11 measurements was 0.82% (±0.48%, r=0.97). (unpublished data)

Endothelial function assessment – Reactive Hyperemia Index (RHI) as measured using Pulsatile Arterial Tonometry (PAT, Itamar Medical Ltd.): This assay uses a device that measures reactive hyperemia of the finger. The magnitude of hyperemia correlates with coronary microvascular endothelial function, peripheral vascular endothelial function measured as brachial reactivity, and can be inhibited by LN^G monomethyl arginine, indicating that it is NO-dependent. We are employing this technique currently in our catheterization lab population and have found it to be easy to use and reproducible.[65]

Technique: Subjects will be studied in the supine position in a temperature-controlled room. Baseline blood pressure of both hands is measured and PAT probes are placed one on each hand at the same finger (fingers 2, 3 or 4). Following a 10 min. equilibration period, the blood pressure cuff is inflated to 60 mmHg above systolic pressure for 5 minutes followed by deflation of the cuff while the pulsatile recordings from both study and control fingers are measured.

Cardiopulmonary Exercise Testing (CPX): Graded treadmill exercise testing will be performed following American Heart Association guidelines using the Modified Balke protocol.[68] A treadmill with full metabolic cart (Carefusion INC, USA) will be used for CPX. Testing measurements will include 1) Maximal oxygen uptake (VO_2Max): This is the Primary Endpoint of Aim 2 and is defined as the value achieved when VO_2 remains stable despite a progressive increase in the intensity of exercise. This is synonymous with peak aerobic capacity. Secondarily, CPX will report: 2) Respiratory exchange ratio (RER): Related but not equivalent to its cellular counterpart, the respiratory quotient, RER is defined as the ratio of VCO_2 to VO_2. 3) O_2 pulse: The amount of O_2 consumed from the volume of blood delivered to tissues by each heartbeat; this index is calculated as: O_2 pulse = VO_2 / heart rate. 4) Peak VO_2lean: the peak oxygen uptake adjusted for lean body mass. Reported as a lean body weight–adjustment parameter in mL/kg per minute. These data are then integrated with heart rate, blood pressure, work rate, electrocardiography findings, and the occurrence of any clinical symptoms for a comprehensive analysis of exercise tolerance before and after transfusion.

Blood Testing: Biomarkers of oxidative stress (glutathione/glutathione disulfide and cysteine/cystine redox potentials) are measured in an Emory University Core Lab directed by Dr. Dean Jones, and available for use by all Emory investigators. Dr. Jones is an internationally recognized expert in the area of glutathione (GSH) and cysteine metabolism, and has substantial experience with measurements of reactive oxygen species. He has optimized HPLC methods for quantitation of oxidative stress markers. NO metabolites (nitrate, nitrite, and nitrosylated hemoglobin and thiol) levels will be measured in Dr. David Lefer's laboratory at Emory (see support

letter). 2,3-DPG, plasma free hemoglobin and inflammatory marker levels will be measured in Dr. Roback's laboratory.

Expected outcomes:

Specific Aim 1: FMD and RHI at 24 hours hour post-transfusion with saRBCs will be lower than following transfusion of fresh RBCs in the same patient.

Specific Aim 2: Exercise tolerance and VO_2Max will increase following fresh RBC transfusions; in contrast, transfusion of saRBC will either lead to a lesser increase in these markers or will produce an actual decrease in these parameters.

Secondary Endpoints: Markers of oxidative stress (glutathione and cysteine redox ratios) and inflammatory markers will show a greater increase following saRBC transfusion than after fresh transfusions.

Potential Limitations and Alternative Techniques:

Since we have previously observed significant differences in FMD following transfusion of saRBCs, but not fresh RBCs, in hospitalized subjects, we do not anticipate difficulty measuring vascular reactivity in response to transfusion in this ambulatory chronically-transfused patient population. In fact, the proposed design using outpatients allows for improved adherence to optimal FMD and PAT technique including control of study timing relative to transfusions, medications, diet and circadian rhythms[64]. Furthermore, a cross-over design allows for control of inter-individual variation which was a significant limitation of our prior mechanistic studies. It is possible that some of these patients do not have the level of preexisting endothelial dysfunction we observed in the hospitalized population. If this is the case, and it turns out to limit our ability to measure the physiologic effects of saRBC transfusions, we can narrow the study population to transfusion dependent-individuals with baseline cardiovascular disease.

Given that this is a chronically ill population, we may have difficulty recruiting participants may who are able to complete the CPX procedure. However, as demonstrated by our preliminary data in patients with peripheral arterial disease, we have previously had success measuring exercise outcomes in other populations with functional physical limitations. We are also prepared to employ other exercise protocols, if necessary, including the modified Balke or Gardner protocols for use with treadmill testing, or by utilizing a seated cycle ergometer as opposed to treadmill.

Statistical considerations:

Sample size justification for Aim 1: Subject numbers are based on the primary objective (Aim 1) of measuring change in FMD (%) with transfusion. For sample size calculations, we use the paired t-test statistic and assume a two-sided test with α=0.05 and power = 0.8. Based on the FMD changes we observed in transfused hospitalized patients (see preliminary data) of 2.4% with saRBC transfusion and a standard deviation of FMD in our lab of 3.11%, with a sample size of 24 we have power to observe a 0.85% change in FMD with blood transfusion. This is a clinically meaningful change.[10]

Sample size justification for Aim 2: Subject numbers are based on the primary objective of measuring change in VO_2Max with transfusion of fresh vs saRBCs (Aim 2). A sample size of 24 achieves 86% power to detect a VO_2Max difference of 130 ml/min between fresh and saRBC transfusions. These calculations are based on a mean of 1100 and the alternative hypothesis mean of 1230 with an estimated standard deviation of 190.0 and with a significance level (alpha) of 0.05 using a two-sided Wilcoxon test assuming that the actual distribution is normal. This calculation is based on published VO_2Max responses to exercise in an anemic population.[71]

Statistical analysis will be performed in consultation with faculty statisticians within the Emory Clinical Cardiovascular Research Institute. Descriptive statistics will be performed initially by calculating means, standard deviation, range, descriptive plots, and box-plot histograms for each variable. This will assist in determining distributional properties of the outcome variable and the choice of appropriate subsequent analytical approaches (parametric vs non-parametric). For most analyses, we will use a generalized linear mixed model to analyze these data provided that outcomes are normally distributed. Otherwise we will use transformation methods. Given that there are multiple hypotheses with multiple outcomes it is necessary to adjust for multiple comparisons. With the proposed sample size, we will not adjust for multiple comparisons with different outcomes but will adjust for comparisons within each outcome. The sample size may be too small for certain outcomes given the heterogeneity of subjects: therefore we will interpret results carefully, particularly with secondary outcomes. In such cases we will report the confidence intervals.

Time table:

Subjects will be enrolled concurrently into Aims 1 and 2 over the first 18 months of the grant period. Data will be recorded continuously and analyzed over that same period with manuscript preparation in months 18-24.

	Year 1	Year 2
Subject enrollment -Aim 1 and 2		
Data analysis		

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