VO_2 Max: In Vivo Model for Functional Red Cell Testing. Can RECESS be Explained?

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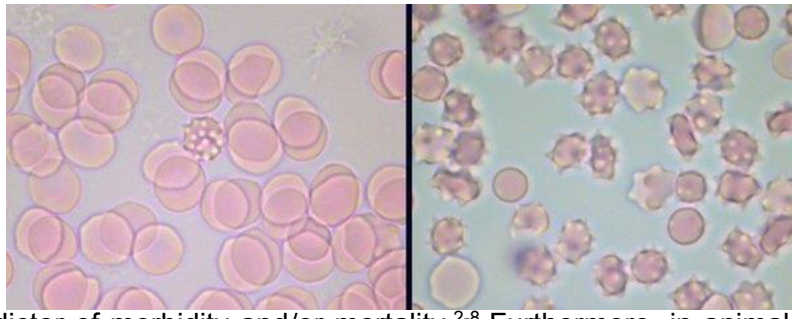
A. SPECIFIC AIMS

1) To confirm our pilot data in a larger sample size and show that 42-day RBCs do not deliver oxygen as effectively as 7-day RBCs, and
2) To explain the RECESS results, we hypothesize that 28-day RBCs are not inferior to 7-day RBCs in our model

B. BACKGROUND AND SIGNIFICANCE

RBCs progressively develop biochemical, immunologic, structural, and physiological abnormalities during storage, collectively referred to as the "storage lesion".[1] For example, morphologic differences in fresh vs older stored RBCs (photos right) are striking.



These changes may be clinically significant since several observational studies have reported that transfusion of older RBCs is an independent predictor of morbidity and/or mortality.[2-8] Furthermore, in animal lethality models, randomization to the oldest RBCs (42 days) results in higher mortality.[9,10] Recently completed randomized trials, i.e. ABLE and RECESS, essentially comparing "young" (median 7)[11] vs "middle-aged (median 28-day)[11] RBCs, show no difference in clinical outcome. These data are important, since most patients are exposed to RBCs in this spectrum of age. However, they do not inform us about the safety and effectiveness of old RBCs, which some patients receive. Due to pragmatic and ethical reasons a large clinical trial that randomizes patients to "old" blood may not be feasible. We have generated compelling preliminary data in a healthy volunteer model of strenuous exercise that strongly suggests that old RBCs (42 days) do not deliver oxygen as effectively as fresh (7 day) RBCs. The 3 arm (7-, 28-, 42-day RBCs) VO_2 max study we propose is

designed to address 2 important hypotheses: 1) to confirm our pilot data in a larger sample size and show that 42-day RBCs do not deliver oxygen as effectively as 7-day RBCs, and 2) to explain the RECESS results, we hypothesize that 28-day RBCs are not inferior to 7-day RBCs in our model.

The proposed study is significant for several reasons: 1) We believe that the proposed study will confirm our very encouraging preliminary data, showing that the oldest RBCs allowed by FDA regulations (42 days) are inferior to 7-day RBCs in a functional assay of oxygen delivery. This is important scientifically since, to our knowledge, no studies have definitively shown that storage duration can affect RBC function, i.e. oxygen delivery; 2) The proposed study is also significant because it is expected to shed light on why the RECESS and ABLE outcome trials, comparing young and "middle-aged" RBCs were negative. We hypothesize that 28-day RBCs are not inferior to 7 day RBCs, which are exactly the median ages of storage for the 2 study arms in RECESS.[11] 3) Finally, we suggest that this model holds promise as a rigorous and quantitative *in vivo* functional assay of RBC function, which can address the quality of the oldest red cells since a clinical trial is likely not feasible and also addresses an unmet need for future RBC transfusion research as identified by NHLBI and others.

Potential Importance of RBC Duration of Storage

The US Food and Drug Administration (FDA) regulations permit additive-containing RBCs (AS-1, AS-3, AS-5) to be stored for up to 42 days prior to transfusion. The efficacy of older stored blood, i.e., effective oxygen delivery, has not been established in rigorous controlled studies. It is well known that RBCs progressively develop biochemical and physiological abnormalities during storage, and our group has been responsible for generating some of these data.[1] Changes during storage may be of clinical significance since accumulating evidence has linked transfusion of RBCs stored for a longer period of time with increased morbidity and mortality compared with transfusion of fresher RBCs, although a number of studies have failed to find such an association.[12-16] These study designs have mostly been observational and retrospective; therefore, residual confounding is a possibility, and it may not be possible to establish causality between adverse outcome and transfusion of older blood.[17]

Recently completed randomized trials, e.g. ABLE and RECESS, essentially comparing "young" (median 7)[11] vs "middle-aged (median 28-day)[11] RBCs, show no difference in clinical outcome. These data are important, since most patients are exposed to RBCs in this spectrum of age. However, they do not inform us about the safety and effectiveness of older blood. Due to pragmatic and ethical reasons, a large clinical trial that randomizes patients to "old" blood may not be feasible.

Therefore, we have developed a model of autologous transfusion in conjunction with strenuous exercise that leverages an established quantitative and reproducible test of oxygen delivery (VO_2 max) to address important gaps in knowledge. Prior to describing our preliminary data, we summarize below previous efforts to use physiological tests to assess the impact of storage duration on various endpoints.

Limitations of Previous "Physiologic" Studies of RBC Storage Duration

Previous studies involving the impact of RBC storage duration on surrogates of tissue perfusion and oxygenation showed conflicting results, often due to limitations in the experimental techniques used. In a non-randomized study, Kiraly et al showed a significant decrease in peripheral tissue oxygenation (StO_2) of anemic, critically-ill, trauma patients following transfusion of older RBCs (≥ 21 days) compared to patients who received a transfusion of fresher RCBS (≤ 21 days).[18] In contrast, in a randomized study comparing transfusion of 2 units of fresh (≤ 5 days) vs. old (≥ 20 days) RBCs, Walsh et al found that transfusion of older RBCs to euvolemic, anemic, critically-ill patients had no clinically significant adverse effects on gastric tonometric or global indices of tissue oxygenation.[19] In their randomized study, Weiskopf et al found that RBCs stored for 3 weeks were as effective as RBCs stored for 3.5 hours in reversing a neurocognitive deficit induced by acute anemia (hemoglobin 5 g/dL) in healthy volunteers.[20] Two studies (Kor et al,[21] Weiskopf et al[22]) focused on the effect of stored blood on pulmonary function, and did not assess functional measures of oxygen delivery. Marik and Sibbald failed to demonstrate a beneficial effect of transfusing 3 units of RBCs on measured systemic oxygen uptake in patients with sepsis.[23] Patients in their study who received RBCs stored for more than 15 days developed evidence of splanchnic ischemia; however, this study was observational and not randomized.

C. PRELIMINARY STUDIES

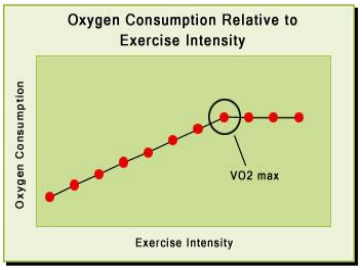
Preliminary Data of VO_2 Max After 7 vs 42 day Stored AS-3 RBCs

Limitations in these previous studies led us to propose strenuous exercise (VO_2 max testing) for studying potential differences in oxygen delivery attributable to duration of storage. We recently completed a pilot study in which 8 healthy subjects had 2 units of leukocyte-reduced RBCs collected (by apheresis) in AS-3 using standard methods. This was done at Rex Hospital (a blood collection facility near Durham) since Duke Transfusion Service does not collect RBCs. Subjects were randomized to receive both (2) units of their autologous RBCs at either 7 or 42 days following blood collection. VO_2 max testing was performed 2 days before (Monday) and 2 days after (Friday) the transfusion visit (Wednesday). This study design avoids the confounding effects on intravascular volume from the 2-unit blood transfusion if VO_2 max tests were performed before and after transfusion on a single day. In other words, we sought to assess the impact of transfused RBCs on exercise performance independent of its impact on intravascular volume. While our research group had experience collecting and administering autologous RBCs[24] as well as conducting VO_2 max tests,[25-35] we had never combined these interventions and wished to collect preliminary data that would inform the feasibility of conducting the study we propose in this grant submission.

Nine subjects were consented but 1 failed screening tests (abnormal pulmonary function testing) and 8 completed the study. There was strong interest from fit habitual exercise enthusiasts to participate in this type of study and enrollment was completed quickly. Subjects in both study arms, encouraged by the same individual (M Natoli), exercised to exhaustion with high peak heart rates, e.g. mean of 184 ± 5 vs 184 ± 11. Despite the small sample size (4 per study arm) we observed intriguing differences in VO_2 max (p=0.2) and exercise duration (p=0.02) between study arms (figures below). Indeed in the 42-day arm, in half of the subjects, VO_2 max and exercise duration actually decreased (was worse) after transfusion with 2 units of 42 day RBCs, whereas all 4 subjects who received 7 day RBCs exhibited increases in these endpoints. Of note, in order to avoid potential confounding from differences in 24-hour viability of 42- vs 7-day RBCs, we only administered 85% of the 7-day RBC units, compared with 100% of the 42 day units. Therefore, these preliminary data should reflect differences in RBC quality/function and not RBC mass. Indeed, peripheral blood hemoglobin increased similarly (p=0.6) after transfusion of 2 units of 7- or 42-day RBCs using this protocol. To our knowledge, no previous study has demonstrated functional differences in oxygen delivery/exercise performance due to changes in RBC storage duration. We believe these preliminary data demonstrate the feasibility of using rigorous VO_2 max testing as an *in vivo* model for RBC function, and are proposing a larger more rigorous study to confirm these observed differences. In addition, the proposed study will contain a 3rd study arm (28-day storage). This will add valuable information that may help explain the RECESS Trial results.[11] Our hypothesis is that there is a difference in VO_2 max between the oldest RBCs (42-day) and fresh blood (7-day), but that, consistent with RECESS results, there is no difference in oxygen delivery between 7- and 28-day RBCs, which were the median duration of storage from the 2 RECESS study arms.[11]

Before we describe our experimental approach we will present below general background information on VO_2 max testing and factors that affect it.

Maximal oxygen uptake (VO_2 max) testing: Background



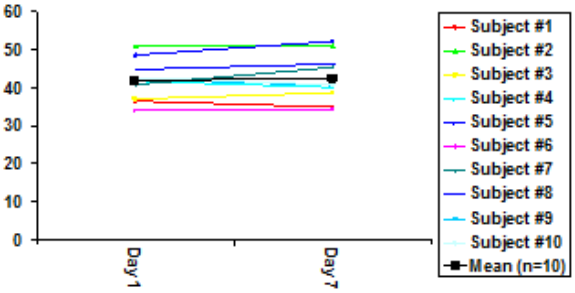
Maximal oxygen uptake (VO_2 max) is the maximum rate at which the body can consume and use oxygen during incremental to maximum whole-body exercise. Oxygen consumption is linearly related to energy expenditure; so, measuring oxygen consumption is an indirect way to measure a person's maximal capacity to do work aerobically. In order for oxygen to be used by the body, it must be extracted from the atmosphere and delivered to working cells. As shown in the Figure, oxygen consumption increases as exercise intensity increases until it plateaus, which represents the VO_2 max. This occurs approximately 5-20 minutes into a standardized exercise protocol, with the duration depending on the subject's age, level of fitness, and genetic factors.

Although indirect methods of calculating VO_2 max have been used, the gold standard is graded exercise to exhaustion on a treadmill or cycle ergometer with direct measurement of O_2 consumption using a metabolic cart to analyze inspired and expired gases. VO_2 max can be expressed as L/min but is more typically reported as mL/kg/min to adjust for differences in body weight. The photo shows a female subject on a cycle ergometer. Her nares are occluded to force all respiratory gases to pass through the mouthpiece that is connected to the metabolic cart. She is looking at a monitor that shows her cadence (revolutions per minute), which must be maintained at a certain rate (75 rpm in our lab), while the resistance to pedaling increases incrementally. In the photo (right), a coach provides encouragement to a subject in our laboratory.

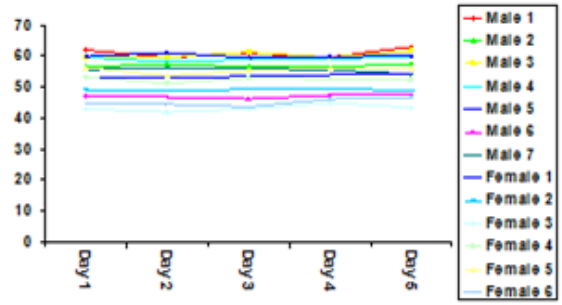
Factors Affecting VO_2 max

The 4 main mechanisms that determine VO_2 max are: absorption of O_2 from the lungs, O_2 transport by the circulation, O_2 diffusion from the capillary blood to the mitochondria, and mitochondrial capacity.[36] As reviewed by Pollock,[37] VO_2 max responds to changes in training. For example, 20 days of bed rest resulted in a decrease in VO_2 max from 43.0 to 31.8 mL/kg/min, which then increased to 51.1 mL/kg/min after 60 days of training.[38] Elite athletes can have a VO_2 max as high as 75-90 mL/kg/min.[39]

While VO_2 max can change with training and other interventions, it is remarkably constant over a period of days in the absence of any significant changes. For example, with a standardized protocol, i.e. absence of any interventions such as blood removal or transfusion, and use of the same "coach" to encourage the volunteers to exercise to exhaustion, VO_2 max test results show very little intra-subject variability over several days. Relevant data are below.[26,40]



Serial VO_2 max tests on days 1 and 7 in 10 healthy volunteers show minimal intra-subject variability (Moon et al).



Serial VO_2 max tests over 5 days in 13 healthy volunteers show minimal intra-subject variability (Jamnik et al).

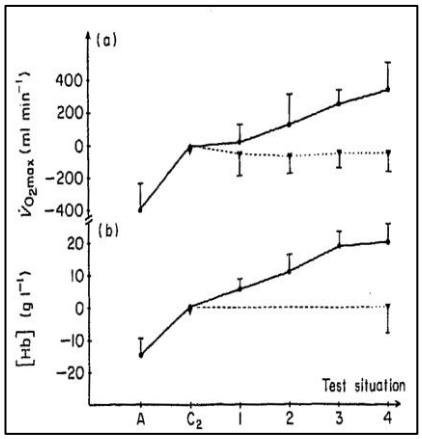
Therefore, the reproducible and quantitative nature of VO_2 max testing makes it ideal for studying interventions that may affect oxygen delivery.

VO_2 max: Previous Studies of Anemia/Hemoglobin Reduction and RBC Transfusion

As previously reviewed,[39,40] numerous studies regarding the effects of blood removal and transfusion on VO_2 max and/or exercise performance have been performed, some as early as 1940.[41] This research has been driven in part by an interest in "blood doping," a technique used to improve exercise performance.[39,40,42,43] Many studies report the effects of blood removal and transfusion on both exercise performance and VO_2 max; however, some report effects on only one of these outcomes.

Blood Removal: Gledhill and others have shown that a reduction in arterial oxygen content (specifically a reduction in hemoglobin) results in a reproducible decrease in VO_2 max.[40] This is attributable to decreased arterial oxygen content,[29,33,40] and not decreased cardiac output if VO_2 max is measured at least 48 hours after removal of blood in order to allow the total plasma volume to normalize.[40,44,45] Indeed, for hemoglobin levels ranging from 11-16 g/dL, each decrease in hemoglobin of 0.3 g/dL results in a 1% decrease in VO_2 max.[40]

Blood Transfusion: Early studies (1976, 1980) in healthy volunteers failed to show an improvement in VO_2 max after transfusing liquid RBCs, possibly due to the low quality of the stored RBCs.[46,47] In contrast, later studies that transfused at least 2 units of cryopreserved, deglycerolized autologous RBCs to healthy volunteers showed consistent increases in VO_2 max, even if pre-transfusion Hb was as high as 16 g/dL.[44,45,48-50] In other words, the increase in VO_2 max does not require anemia at baseline to show improvements in VO_2 max. For example, in 8 volunteers, Celsing et al removed 450 mL of whole blood 5 times, resulting in lower VO_2 max at A (anemia; figure right).[48] One unit of cryopreserved RBCs was transfused on visits 1, 2, 3, and 4 over several weeks, resulting in a stepwise increase in both hemoglobin and VO_2 max (L/min). As expected, control subjects (n=8, dotted line) showed no change in hemoglobin or VO_2 max.



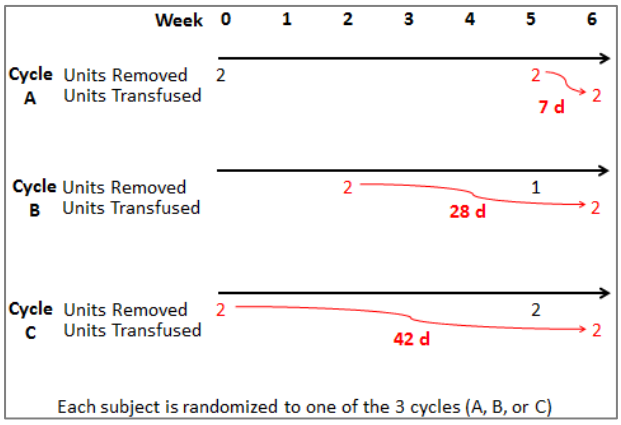
Our preliminary data are consistent with the above studies, since we showed a predictable increase in VO_2 max after transfusion with 2 units of 7-day RBCs. None of these previous studies, however, determined whether storage duration impairs the ability of the RBCs to deliver oxygen, which was the focus of our pilot study and will be characterized further in the proposed study.

D. RESEARCH DESIGN AND METHODS

This is a single-center, randomized, partially blinded study.

1) Overview

After consent and screening the study interventions last 6 weeks and consist of 2 phlebotomy visits at Stony Brook hospital and transfusion with 2 units of autologous AS-3 RBCs that are either 7-, 28-, or 42-days old. Subjects will be randomized (1:1:1) to either cycle A (7 day RBCs), cycle B (28 day RBCs, or cycle C (42 day RBCs).



During week 6 of each cycle, subjects will have 3 study visits consistent with the pilot study we just completed.

- On Monday subjects will have a VO_2 max test.
- On Wednesday, they will be transfused with 2 units of autologous RBCs (each over 1 hour), followed by a VO2 max test 2 hours later to assess the "acute" effects of transfusion (intravascular volume + RBC mass), which is a secondary endpoint of the proposed study.
- On Friday, they will have a VO_2 max test, which is compared with the baseline VO_2 max result (Monday) in order to calculate a delta VO_2 max related to transfusion (primary endpoint), avoiding potential confounding from acute intravascular volume effects.

Removal of waste unit(s): During each cycle, subjects will also have a second visit to Stony Brook For example, Cycle A has 2 units removed at Week 0, Cycle C has 2 units removed at Week 5. These units are removed for 2 reasons: 1) to maintain blinding for the 7- vs 42-day RBC comparison since subjects will not know whether they are receiving their 7- or 42- day RBCs; and 2) to induce mild anemia (projected Hb 11-12 g/dL), which, while still extremely safe, may enhance increases in VO_2 max from transfusion in order to detect more robust differences between study arms. We have numerous safeguards in place to protect human subjects (see Human Subjects section).

Blinding: We cannot fully blind Cycle B (28-day RBCs). However, we plan to partially blind the subject by informing them that they may receive either 2 units of 28-day RBCs or 1 unit of 28-day RBCs and 1 unit of 7-day RBCs. This "deception" should be allowed since it is inconsequential to the subjects, as they will already have agreed to receive 7- and 42-day RBCs in other cycles, and is merely done to improve blinding of the study. Notwithstanding the above, we believe this strategy is a reasonable compromise since VO_2 max is a quantitative measurement and subjects are motivated fit athletes who, in our experience, exercise to exhaustion.[25-35] Furthermore, subjects are not likely to have preconceived ideas/bias about the efficacy of

28-day RBCs. In cycles A and C, 2 units of blood will be removed 7 days before the transfusion week. However, for cycle B, only 1 unit of blood will be removed 7 days before the transfusion week because their initial 2 unit phlebotomy will be at day 28 and not at day zero. They will have had 14 fewer days to recover hemoglobin compared to cycles A and C. Based on our estimates of RBC mass removal and restoration (synthesis), we anticipate that this will result in a pre-transfusion Hb that is similar to cycles A (7-day) and C (42-day).

2) Research Site

All study procedures will be completed at a single center, Stony Brook University Hospital.

3) Study Sample

Consistent with our pilot data, qualified volunteers will be enrolled at Stony Brook University Hospital.

4) Screening

Subjects will be preliminarily screened via private phone call made by the study coordinator on a hospital phone line, in a private office. In-person screening of subjects will take place in a private office space in the Health Sciences Center at Stony Brook University Hospital. After subjects provide written informed consent, eligibility will be confirmed by a questionnaire and screening tests (below). We have used these methods before, and they are designed, in large part, to insure the safety of participants.

Inclusion Criteria:

i. Healthy male or female
ii. Age 18-40 (American College of Sports Medicine Guidelines for Exercise Testing defines this age group, with ≤ 1 coronary heart disease risk factor, as low-risk for VO_2 max testing.)
iii. Habitual exerciser defined as ≥ 30 minutes of at least moderate or high intensity exercise ≥ 3 times per week. After consent, and at the subsequent screening visit, a VO_2 max test will be performed, and subjects with a low value (< 35 mL/kg/min or unable to achieve at least 95% of maximum HR predicted from the standard formula 220 minus age) will be excluded (screen failure). Based on our previous experience, we anticipate that <10% of the subjects will fall into this category
iv. Calculated total blood volume (TBV) ≥4,500 mL using an established formula:
    a. Men: $(0.006012 \times H^3) + (14.6 \times W) + 604 = TBV$
    b. Women: $(0.005835 \times H^3) + (15 \times W) + 183 = TBV$
    [H=height in inches; W=weight in pounds]
ii. Has access to transportation to visit the blood collection facility and to return to Stony Brook for all study visits.

Exclusion Criteria:

i. Any significant acute or chronic medical illness or problem, including, but not limited to, diabetes, hypertension, cardiac disease, asthma, COPD
ii. Current or recent (last 60 days) tobacco or nicotine use
iii. History of sickle cell trait or disease or any other acquired or hereditary hematological abnormality
iv. History of fainting or other significant adverse reaction during phlebotomy or donation of blood
v. Known prolonged QTc (or evidence of such at screening) defined as QTc >470 ms
vi. Known or suspected illicit drug or alcohol abuse
vii. Known or suspected HIV, Hepatitis B, or Hepatitis C infection
viii. History of thrombophilia or anticoagulant therapy
ix. Pregnancy
x. Obesity defined as BMI >30 kg/m^2
xi. Recent history of blood donation: a) Single whole blood unit donation within the past 8 weeks; b) Double RBC donation by apheresis within the past 16 weeks; or c) Plasma donation by apheresis within the past 4 weeks
xii. Inadequate RBC mass based on TBV <4500 ml (above) or screening Hb <14.0 g/dL

Screening tests (performed after consent is obtained): The following will be performed to confirm eligibility and optimize the safety of subjects: 1) written questionnaires that provide written confirmation of eligibility for blood donation and confirm appropriateness for exercise testing (PAR Q+ questionnaire);[51] 2) hemoglobin must be ≥ 14.0 g/dL; 3) hemoglobin A1c to rule out diabetes; 4) Sickledex to rule out sickle trait or anemia; 5) ECG to rule out prolonged QTc; 6) height and weight to calculate and confirm required total blood volume (above); 7) spirometry to rule out undiagnosed pulmonary disease; and 8) serum hCG test if female

Subjects will be informed that during the study they may take a multivitamin but they should not take iron supplements.

5) Procedures

Blood Donation, Processing, and Storage (Visit B on Day 0, Visit C on Day 35)

We will use the same procedures successfully used in our recently completed pilot study and our TASER study.[24] Stony Brook's Transfusion Services is registered and licensed by the FDA and accredited by the American Association of Blood Banks. Dr. Galanakis, medical director of Transfusion Services, is a co-Investigator this study.

After passing a written screen at Stony Brook University Hospital's Blood Bank, 2 units of RBCs will be collected in CP2D via venous apheresis using a Trima Accel® Automated Blood Collection System: (http://www.caridianbct.com/location/emea/products-and-services/Pages/trima-accel-collection-system.aspx). Blood will be processed by technicians at this center using standard techniques. All RBCs will be leukoreduced (pre-storage), tested for all required infectious diseases, stored in AS-3 additive solution. AS-3 RBCs will be used since apheresis products collected by Trima Accel® have only been validated for AS-3. There are no data to suggest that our results would not be generalizable to AS-1 or AS-5 RBCs. Before every blood donation, each subject's hemoglobin will be measured, and only those with a value ≥ 12.5 g/dL will be allowed to donate.

Each RBC unit will be weighed, and its volume will be calculated according to the Donor Center's standard formula: red cell volume (mL) without AS-3 = product weight/1.08.

RBC units will be stored in the blood bank under standard conditions (1˚C -6˚C) and under the supervision of its medical director (Dr. Galanakis, Co-Investigator).

MONDAY of Week 6: VO_2 max Test (Baseline)

During week 6 of each cycle, the VO_2 max test on Monday will serve as the "baseline" for changes in VO_2 max measured on Friday (primary endpoint) and Wednesday (secondary endpoint) of that same week. Although we are targeting mild anemia for this visit (Hb 11-12 g/dL), it is probably not critical that we achieve a certain hemoglobin level before transfusion since previous studies have shown increases in VO_2 max for a very wide range of Hb levels, i.e. up to 16 g/dL.[44,45,48-50]

WEDNESDAY of Week 6: Blood Transfusion and VO_2 max Testing

We will use the same procedures for transfusing autologous stored RBCs that we used successfully in our recently completed pilot study and TASER study.[24] Procedures (details below) have been designed to optimize safety of research subjects.

Before the subject's visit, a sterile welding device will be used to remove a small aliquot from each RBC unit. This aliquot will be sent to Stony Brook Hospital's Microbiology Laboratory for culture to rule out potential bacterial contamination of a stored unit. Based on *in-vivo* viability at 24 hours after transfusion,[52] we anticipate that transfusion of 100% of 42-day RBCs, 95% of 28-day RBCs, and 85% of 7-day RBCs, will generally compensate for differences in post-transfusion 24-hour viability. In order to confirm that this adjustment is appropriate, we will measure the Hb levels for each subject before and after transfusion, and 2 days later (Friday visit) to ensure that there are no differences in RBC mass that could confound the results. In the unlikely event that this is an issue, our statistician can adjust for this potential interaction.

Subjects will arrive at Stony Brook Hospital at approximately 8 am for each visit. An intravenous (IV) cannula will be placed in an upper extremity vein. Before transfusion, Stony Brook Hospital Transfusion Services will confirm compatibility of the autologous units by an extended cross-match, comparing a fresh sample of the subject's blood and blood from the autologous units. Each unit of autologous RBCs will be transfused over 60 minutes using an Alaris® infusion pump (CareFusion, San Diego, CA). Thus, the 2 units of RBCs (7- or 28- or 42-day) will be administered over a 2- hour period. The blood will not be diluted or warmed before infusion. During the transfusion, vital signs will be monitored by the RN coordinator or physician every 15 minutes.

The subjects will be asked to rest for 2 hours after the end of the transfusion, and will be given a light snack. They will then undergo a VO_2 max test. Of note, this VO_2 max test (2 hours after transfusion) will reflect both acute hypervolemia (due to transfusion of the 2 RBC units) and increased hemoglobin mass from the transfused RBCs. This will inform our secondary outcome = Δ VO_2 max between Wednesday and Monday in the 2 study arms. However, since our goal is to focus on the oxygen delivery (not volume) effects of these RBCs, our prespecified primary endpoint is the change from Monday's VO_2 max to Friday's VO_2 max, when subjects will have equilibrated from the volume effects of the transfusions. [40,44,45] This allows us to rigorously assess the *in vivo* function of RBCs to deliver oxygen independent of intravascular volume effects from transfusion.

FRIDAY of Week 6: VO_2 max Test

This VO_2 max test will be used for analysis of the primary endpoint as described in Statistical Methods. Briefly, a Δ VO_2 max between Friday and Monday will be calculated for each subject, and statistical analysis will test for a proportional difference from baseline between study intervention groups (7- vs 42-day RBCs).

Detailed Description of VO_2 max

We are experienced in performing VO_2 max testing as evidenced by our pilot study. Procedures (details below) have been designed to optimize the safety of research subjects while obtaining valid reproducible data.

VO_2 max testing will be performed in Level 2 of the Health Sciences Center. Each subject will be instrumented with a 3-lead ECG to monitor cardiac rhythms and a pulse oximeter to record heart rate. Mixed expired oxygen, carbon dioxide, breathing frequency, tidal volume, minute ventilation, oxygen consumption, and carbon dioxide production will be measured with a metabolic cart. Data will be recorded every 30 seconds as 30-second averages. Subjects will breathe air through a two-way non-rebreathing valve connected to the metabolic cart.

Stage	Time (min)	RPM (rpm)	Total Workload (watts)	Leg Load (kp)
1	0 - 3	75	50	0.68
2	3 - 6	75	100	1.36
3	6 – 9	75	150	2.04
4	9 - 10	75	175	2.38
5	10 - 11	75	200	2.72
6	11 - 12	75	225	3.06
7	12 - 13	75	250	3.4
8	13 - 14	75	275	3.74
9	14 - 15	75	300	4.08
10	15 - 16	75	325	4.42
11	16 - 17	75	350	4.76
12	17 - 18	75	375	5.1
13	18 – 19	75	400	5.44
14	19 – 20	75	425	5.78
15	20 – 21	75	450	6.12

Subjects will be seated on a cycle ergometer. The seat height will be adjusted so that when sitting squarely on the seat with the pedal in the lowest position, a slight bend of approximately 10 degrees will be maintained at the knee. Digital displays of rpm will be visible to the subjects.

Three minutes of initial resting baseline measures will be collected before the start of exercise. The subject will then begin to pedal the cycle ergometer at a cadence of 75 rpm. Resistance will be manually set according to the progressive protocol shown in the Table (right). A rating of perceived exertion (RPE) will be obtained at the end of each exercise stage. The VO_2 max test will be terminated when the subject reaches volitional fatigue, or, in the very unlikely event, achieves one of the other test termination criteria as defined (see table in Human Subjects: Adequacy of Protection Against Risk).

The screening VO_2 max test (Visit A) will allow each subject to become familiar with this testing protocol.

VO_2 max is determined as the highest oxygen consumption averaged over 2 30-second periods, which typically occurs in the last stage of the progressive maximal exercise test. Peak heart rate is considered to be heart rate at VO_2 max. Maximum heart rate (HR_{max}) is considered to be heart rate at or near VO_2 max.

To minimize variability in VO_2 max testing procedures, the same facilitator (Sabeen Rizwan- Study Coordinator) will be present at every test. To further minimize variability, subjects will be required to avoid moderate or heavy exercise the weekend before week 6 and during the week of testing (week 6), which will eliminate the risk for confounding from recent strenuous exercise, e.g. a 10-mile run the day before testing.

Additional Measurements During VO_2 max Testing

On 2 of the visits for VO_2 max testing (Monday and Friday for our primary endpoint), peripheral blood Hb will be measured before each VO_2 max test to confirm that our strategy for ensuring similar RBC mass between

study arms (7-, 28-. 42-days) has been effective. If necessary our statistician can correct for a possible interaction.

Subject Payment
Subjects will be paid for each study visit that they complete: $50 for completing screening visit, $50 for completing each of the two blood donation visits, $50 for completing the day 40 exercise test visit, $150 for completing the transfusion and exercise test visit and $350 after completing the final visit of exercise testing

E. STATISTICS

Randomization and Allocation Concealment: The pattern randomization scheme will be generated using a computer algorithm. Transfusion Services personnel will assign each subject to an administration sequence (above) using a sealed envelope technique. Subjects will be randomized to receive 7-, 28- or 42-day-old blood at the time of their enrollment.

Statistical Analyses: All analyses will be conducted by the team's biostatistician (J.R.). Summary statistics, including 95% confidence intervals, will be reported on all demographic and outcome data collected, including all multiple hemoglobin and VO_2 max measures.

- The **primary outcome** of this study is the proportional change from baseline in VO_2 max from Monday to Friday of week 6, after receiving 7-day or 42-day old blood on the Wednesday of Week 6. We hypothesize that transfusion of RBCs stored for 42 days are less effective at increasing oxygen delivery than RBCs stored for 7 days, which would confirm results from our pilot study where we observed an 82% difference in proportional change between 7- and 42-day VO_2 max study arms. We will test the change scores for normal distribution, and will compare the 42- to 7-day groups using either a student's t-test or Wilcoxon rank sum test in a one sided superiority hypothesis test at the 0.05 significance level. Subjects who withdraw or are excluded before randomization will simply be dropped from analysis. We will further examine the treatment effect by adjusting for potential effects of hemoglobin, and BMI in a linear regression model.
- **Secondary outcomes**: Our secondary outcomes include mean proportional change between 7- and 28-day VO_2 max study arms, and change in duration of exercise between all three groups. Of note, in contrast to the primary endpoint, this secondary analysis is expected to show increases in VO_2 max attributable to intravascular volume expansion and increased hemoglobin mass. Nevertheless, these changes are of interest and it is possible that storage duration may affect VO_2 max acutely after transfusion.

Sample Size: Sample size and power are estimated for a superiority study design using a t-test with a power of 80%, and a one-sided significance level of 0.05. Based on the collected pilot data, we expect the mean percent change VO_2 max for subjects who receive 7-day blood to be 8.7% (SD = 6%), and the mean percent change VO_2 max for subjects who receive 42-day blood to be 1.6% (SD = 6%), providing an effect size of 1.183. Thus, the sample size needed for each group is 10 participants, for a study total of 30. While this calculated sample size may not allow us enough power to test a non-inferiority margin of 20% between the 7 day and 28 day groups, it will provide exploratory evidence for a future trial by providing a more accurate assessment in the variation of the data.

F. FUNDING STATUS, DETAILS

This study is funded by a grant from the National Institutes of Health/NHLBI.

G. HUMAN SUBJECTS RESEARCH PROTECTION FROM RISK

Risks to Subjects
Numerous aspects of the study procedures are designed to minimize potential risk to subjects. Most of these procedures have been used by us in our pilot study and in our TASER study of blood donation/transfusion[24] and maximal exercise testing.[25,26] All potential risks will be included in the consent form:

1. Risks associated with venipuncture and intravenous catheter insertion: Venipuncture and insertion of a peripheral intravenous catheter results in momentary discomfort. Infection, excess bleeding, clotting, or

fainting is also possible, although unlikely. Very rare complications include hematoma, nerve injury, thrombophlebitis, arterial puncture, and compartment syndrome.

2. Risks associated with blood donation at Stony Brook Hospital Transfusion Services: Approximately 3.5% of all donations report an adverse reaction, the majority of which are mild and self-limiting, with less than 1:3,400 blood donors seeking medical care post-donation. Vasovagal reactions (faintness or loss of consciousness) are the most common reactions, with less than 0.01% of reported reactions resulting in fainting with injury. There is a 2% risk of adverse reactions to citrate, the anticoagulant used to maintain the blood in liquid state (tingling sensation, dizziness, muscle cramping) when undergoing apheresis double red cell collections. Nausea, vomiting, and post-donation fatigue are also reported. To minimize the chances of this occurring, per standard procedures, all volunteers will be offered some food and liquid after donation and will be encouraged to sit for 30 minutes after donating. It should be noted that fewer donor adverse events are seen with the double red cell collection we will be using due to the saline blood volume replacement they receive. The subject will be informed that they will not be able to donate platelets or RBCs for clinical use for 1 year following this study. The subject will also be informed that if any of the screening tests for HIV, hepatitis B, or syphilis are positive, these results must be reported to the state. This required disclosure could breach the subject's confidentiality.

   For this research study subjects will donate blood sooner than would be done in routine clinical practice. Volunteers, however, should not exhibit deleterious levels of anemia during the study since a hemoglobin of at least 12.5 g/dL will be required before donating blood at any time. Based on previous studies we expect the lowest hemoglobin levels to be approximately 11-12 g/dL (hematocrit of 33-36%) after donation. Volunteers will be told that while anemia of this level is not dangerous, they may feel fatigued, be more likely to experience dyspnea on exertion, and have decreased exercise performance until their hemoglobin level is increased on the transfusion day. In order to minimize these potential effects they will be advised to refrain from moderate or heavy exercise during these times when they may have mild anemia.

   Subjects will be informed that during the study they may take a multivitamin but they should not take iron supplements.

3. Risks of blood transfusion (Wednesday visit during week 6 of each cycle):
   a. During blood donation bacteria may be introduced into the donated unit because it is impossible to completely sterilize the skin through which venipuncture occurs. Also, donors may have asymptomatic infection of bacteria in the blood stream due to recent dental procedures or developing illness. The reported rates for allogeneic RBC unit bacterial contamination range from 0.01% to 0.27%. As the subjects in this study have allogeneic donor criteria applied to them, this would be a fair estimation of bacterial contamination risk. If the subject experiences fever within 2 days after donating their unit of whole blood, they will be asked to call Dr. Bennett-Guerrero at 919- 812-3975. The subject may be excluded from continued participation in the study. If the blood is stored longer than 14 days prior to transfusion, a sample of the stored blood will be tested for the presence of bacteria 7 days before re-infusion. If tests are positive for the presence of bacteria, the subject will be notified and their participation in the study will not continue.
   b. Additional transfusion reactions, such as chills, fever, drop in blood pressure, transfusion associated circulatory overload (TACO) or pulmonary edema, and shock may also occur. There may also be allergic reactions such as hives, rashes, or (very rarely) anaphylactic reactions that could result in death.
   c. The subject may experience pain around the IV site or in the arm with the IV. Investigators will select the largest available vein in the forearm or antecubital fossa to minimize pain during blood infusion.
   d. Mistransfusion, arising from errors in unit labeling, component preparation or other clerical errors occurs in 1:16,000 - 1:25,000 autologous donations. To minimize the risk of mistransfusion, which in the worst possible case may trigger a fatal or serious acute reaction, multiple safeguards to prevent this are written into our study protocol and include the following:
      i. multiple sample and bag identification checks
      ii. 2 persons will confirm the subject's identity with the stored RBCs, using established hospital approved standard Stony Brook Hospital transfusion procedures
      iii. a standard crossmatch will be performed between the subject's stored donated unit and a fresh sample of the subject's blood (if an antibody is found, an extended crossmatch will be performed)
      iv. only one subject will be transfused on a given day to minimize risks

v. the research subject will be asked to review their signature on the labels for their autologous units to confirm they are receiving their own blood

4. Risks associated with exercise (VO_2 max) testing: The most common complaint from those performing the exercise test on a bicycle is thigh pain during the test. In healthy volunteers, the likelihood of death or a serious complication, e.g. sustained arrhythmia, is low, but not impossible. The following is language from a recent publication:
   Risks of Physical Activity, Exercise Training, and Exercise Testing
   Recommendation 1: Maximal exercise stress testing is associated with a very low risk of fatal and nonfatal cardiac events in either healthy asymptomatic or clinical populations. In healthy, asymptomatic individuals, the respective incidences of fatal and nonfatal events are approximately 0.3-0.8/10,000 tests and 1.4/10,000 tests (Level 3, Grade B).[51]
5. Risks associated with loss of protected health information: As with any participation in a research study there is a small chance that a loss of confidentiality could occur, but our use of standard risk management procedures will minimize this risk.

Adequacy of Protection Against Risks

Numerous aspects of the study procedures are designed to minimize potential risk to subjects. Most of these procedures have been used by us in previous studies of blood donation/transfusion including our pilot study and our TASER study[24] and maximal exercise testing.[25,26]

1. Safety Monitoring Plan: We will record and analyze both expected and unexpected adverse events in order to monitor for and minimize potential risks to human subjects. Adverse events that occur during any study procedure will be collected and reported to the IRB, NIH, and FDA as applicable. The Principal Investigator will review serious and non-serious AEs on a rolling basis as well as in aggregate on a monthly basis. Given the long standing safety of VO_2 max testing as well as blood collection and transfusion studies (many removing far larger volumes of RBCs) we do not expect to see a significant number of adverse events. In our recent pilot study and TASER study[24] no adverse events were observed.
2. Protection from Loss of Confidentiality: Study records that identify the subject will be kept confidential as required by law. All participants will be given a unique code. The key to the code will be kept in a locked file in the Study Coordinator's or Dr. Bennett-Guerrero's office. All computer records will be secured with password protection and appropriate firewalls will ensure confidentiality and safety of all patient materials and data. Study documents will be kept in a locked file cabinet in either the Study Coordinator's or Dr. Bennett-Guerrero's office. Source documents sent to the NIH and FDA as applicable will have all direct identifiers masked with opaque black ink. Except when required by law, the subject will not be identified by name, social security number, address, telephone number, or any other direct personal identifier in study records disclosed outside of Stony Brook University. The results of this research study may also be presented at meetings or in publications; however, identity will never be disclosed in those presentations. In no way will any subjects' name be used in any published form. Medical records that identify the subject and the consent form may be inspected by the FDA, other regulatory agencies, and the Institutional Review Board (IRB), in order to meet federal and state regulations. If the volunteer's research record is reviewed by any of these groups, they may need to review the entire medical record. The primary purpose of such review is to ensure the protection of the rights and welfare of the human subjects.
3. Protection from Risks associated with blood donation: Our eligibility criteria are designed in part to minimize the likelihood of individuals who may be at higher risk for blood donation. For example, individuals with anemia, medical problems, or total blood volume less than 4,500 will not be eligible. In addition, we will exclude individuals with a history of fainting or similar adverse reactions during blood donation. Subjects will only be allowed to donate blood (2 unit apheresis collection) if their pre-donation hemoglobin is at least 12.5 g/dL measured using SBUH Blood Bank's standard FDA approved device. A hemoglobin of 12.5 is consistent with the following guideline published by AABB: "*The minimum acceptable hemoglobin for both men and women is defined by the FDA as 12.5 g/dL, with the essentially equivalent hematocrit requirement of 38%."* Per standard SBUH Blood Bank procedures, all volunteers will be offered some food and liquid after donation and will be encouraged to sit for 30 minutes after donating. The SBUH Blood Bank has routine emergency resuscitation equipment in the collection area, which further minimizes risk. In addition, volunteers will be told that while the mild anemia they are expected to have after blood collection is not dangerous for fit healthy volunteers, they may feel fatigued, be more likely to experience dyspnea with exertion, and have decreased exercise performance until their

hemoglobin level is increased on the transfusion visit in week 6 of each cycle. They will be advised to refrain from moderate or heavy exercise during the weekend prior to week 6 and during week 6 of each cycle. Since subjects will agree to this exercise restriction at the time they provide informed consent, we believe few to none of them will drop out due to anemia symptoms or restricted exercise during this short (9 days) period of time each cycle. Subjects are unlikely to have significant symptoms of anemia during light exercise, e.g. walking.

4. Protection from Risks associated with blood transfusion: To minimize risks, the following study procedures will be used:
   a. If the subject experiences fever within 2 days after donating their unit of whole blood, they will be asked to call Dr. Bennett-Guerrero at 919-812-3975. The subject may be excluded from continued participation in the study.
   b. If the blood is stored longer than 14 days prior to transfusion, a sample of the stored blood will be tested for the presence of bacteria 7 days before re-infusion. If tests are positive for the presence of bacteria, the subject will be notified and their participation in the study will not continue.
   c. During blood transfusion (which will occur in a Health Sciences Center lab or the SBUH Blood Bank), subjects will be monitored by an experienced RN study coordinator and one of the physician investigators will be either at the bedside or immediately available. We will monitor temperature, blood pressure, ECG, and pulse oximetry in order to detect possible transfusion reactions. In addition, consistent with our previous pilot study, each RBC unit will be administered over 60 minutes to minimize the likelihood of transfusion associated circulatory overload (TACO) or pulmonary edema, which is unlikely in healthy, fit subjects.
   d. Investigators will select the largest available vein in the forearm or antecubital fossa to minimize pain during blood infusion.
   e. To minimize the risk of mistransfusion, which in the worst possible case may trigger a fatal or serious acute reaction, multiple safeguards to prevent this are written into our study protocol and include the following:
      i. multiple sample and bag identification checks
      ii. 2 persons will confirm the subject's identity with the stored RBCs, using standard Stony Brook Hospital transfusion procedures
      iii. a standard crossmatch will be performed between the subject's stored donated unit and a fresh sample of the subject's blood (if an antibody is found, an extended crossmatch will be performed)
      iv. only one subject will be transfused on a given day to minimize risks
      v. the research subject will be asked to review their signature on the labels for their autologous units to confirm they are receiving their own blood

5. Protection from Risks associated with exercise (VO_2 max) testing: To minimize risks of VO_2 max testing, we will only enroll healthy, habitually exercising, non-obese subjects between 18-40 years of age. This represents a group at very low risk for adverse events. Nevertheless, during testing all subjects will be monitored by a physician with appropriate monitors and emergency resuscitation, e.g. defibrillator, will be immediately available. All exercise tests will be performed in a co-investigator's lab in the Health Sciences Center of Stony Brook University Hospital. Additional safeguards include a screening ECG to rule out long QT interval, and spirometry to rule out occult pulmonary disease. In addition, we will use standard criteria to terminate the VO_2 max test.

Termination Criteria for VO_2 max Test

1. Subject fatigue or subject request for any reason
2. Inability to maintain the prescribed cadence (75-80 rpm) at the current work rate
3. Onset of chest pain or equivalent angina symptoms, such as unusual or extreme shortness of breath, or signs of poor perfusion, as noted by the subject or determined by the monitoring physician
4. Sequential ventricular beats of two or more (i.e., 2 beats of ventricular tachycardia)
5. New onset of sustained (>15 seconds) atrial fibrillation, supraventricular tachycardia (SVT), or other supraventricular tachyarrhythmias
6. Development of new left bundle branch block (LBBB)
7. Systolic blood pressure >240 mm Hg, or diastolic blood pressure >130 mm Hg
8. Progressive decrease in heart rate or systolic blood pressure during increasing exercise intensity accompanied by clinically significant signs or symptoms
9. Second-degree Type II or third-degree heart block.

Potential Benefits of the Proposed Research to the Subject and Others

There is no benefit to the subject for participating in this study. There may be a benefit to future patients based on results from this study.

Importance of the Knowledge to be Gained

The proposed study is significant for several reasons: 1) We believe that the proposed study is likely to confirm our very encouraging preliminary data, showing that the oldest RBCs allowed by FDA regulations (42 days) are inferior to 7 day RBCs in a functional assay of oxygen delivery. This is important scientifically since, to our knowledge, no studies have definitively shown that storage duration can affect RBC function, i.e. oxygen delivery; 2) The proposed study is also significant because it may shed light on why the RECESS and ABLE outcome trials, comparing young and "middle-aged" RBCs were negative. We hypothesize that 28 day RBCs are not inferior to 7 day RBCs, which are exactly the median ages of storage for the two study arms in RECESS.[11] 3) Finally, we suggest that this model holds promise as a rigorous and quantitative in vivo functional assay of RBC function, which is an unmet need for future RBC transfusion research as identified by NHLBI and others.

Risk Benefit Assessment

We believe that the information to be gained from this study is reasonable compared with the small risks of transfusion of autologous blood to healthy volunteers and VO_2 max testing in healthy volunteers.

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